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FUNCTIONAL CHARACTERIZATION OF VP1 OF INFECTIOUS BURSAL DISEASE VIRUS

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Masters of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Vinay K. Dhama

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ABSTRACT

VP1 is an internal polypeptide encoded by segment B of Infectious Bursal Disease Virus (IBDV), which is believed to have RNA dependent RNA polymerase and guanylyl-transferase activities. In order to characterize its functions, experiments were conducted towards expression of VP1 *in vitro* in a cell-free system. Recombinant plasmids, containing the complete open reading frame of VP1 of IBDV strain OH were transcribed using "Ambion MEGAscript *in vitro* Transcription Kit". Time course experiments, nucleotide concentration and plasmid DNA concentration optimizations were done to obtain the maximum transcription yield. Recombinant VP1 was expressed *in vitro* by using the rabbit reticulocyte lysate system. Transcripts concentration optimization reactions were also done to obtain maximum translation yield.

The recombinant VP1 obtained in cell-free system was further used for assaying the functional activities of VP1. However, no activity could be demonstrated in the recombinant VP1, even after various optimization reactions. Further experiments for enzymatic assays were performed utilizing purified IBDV particles. For guanylylation of IBDV VP1, salt and temperature optimization experiments were conducted. To determine whether VP1 has a guanylyl-transferase activity "competition and replacement reactions" with cold and labeled GTP were performed. It was found that even though there was guanylylation of IBDV VP1, this VP1-GMP complex was not reversible and therefore VP1 could not transfer the labeled GMP to an acceptor molecule. Therefore, IBDV VP1 is not a guanylyl-transferase/capping enzyme.

In conclusion, the results of this study indicate that maximum transcription yield is obtained after 4 hours of incubation at 37.5mM nucleotide concentration. Recombinant VP1 of approximately 80 kDa was obtained on analysing by SDS-PAGE followed by autoradiography. Efforts to detect for VP1 enzymatic activities utilizing recombinant VP1 were not successful. However, guanylylation activity associated with IBDV VP1 was characterized in detail by utilizing purified IBDV particles. It has been demonstrated that VP1 of IBDV is not a guanylyl-transferase/capping enzyme. Thus, contrary to previous reports, VP1 of IBDV may not be a multifunctional enzyme.

DEDICATION

To my Parents: with much love and deep respect

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COMMONLY USED ABBREVIATIONS

Term	Abbreviation
Standard units of measurement	
base pair	bp
degree Celsius	°C
centrifugal force	g
gram per milliliter	gm/ml
Kilodalton	kDa
kilobase	kb
mililiter	ml
milimolar	mM
molecular weight	MW
microliter	µl
microgram	µg
nanometer	nm
Others	
hydrogen ion activity (negative logarithm)	pH
percent	%
Metal ions and Compounds	
Calcium ion	Ca^{++}
Cesium chloride	CsCl
diethylpyrocarbonate	DEPC
dimethyl sulfoxide	DMSO
ethylenediaminetetraacetate	EDTA
Hydrochloric acid	HCl
Magnesium ion	Mg^{++}
Magnesium chloride	$MgCl_2$
Manganese ion	Mn^{++}
Sodium hydroxide	NaOH
Sodium chloride	NaCl
Polyethylene glycol	PEG
Sodium dodecyl sulphate	SDS
Trichloroacetic acid	TCA

Term	Abbreviation
Greek alphabet	
alpha	α
beta	ß
lambda	λ
Other abbreviations	
adenosine triphosphate	ATP
complementary DNA	cDNA
cytosine triphosphate	CTP
deoxyribonucleic acid	DNA
double-stranded	ds
Escherichia coli	E. coli
guanosine monophosphate	GMP
guanosine triphosphate	GTP
messenger RNA	mRNA
open reading frame	ORF
ribonucleotide triphosphate	rNTP
ribonucleic acid	RNA
single-stranded	ss
three prime	3'
five prime	5'
chicken embryo fibroblast	CEF
chicken embryo kidney	CEK
cytopathic effect	CPE
Drosophila X virus	DXV
human immunodeficiency virus	HIV
human T-leukemia virus	HTLV
infectious bursal disease	IBD
infectious bursal disease virus	IBDV
infectious pancreatic necrosis virus	IPNV
oyster virus	OV
polyacrilamide gel electrophoresis	PAGE
polymerase chain reaction	PCR
RNA dependent RNA polymerase	RdRp
tellina virus	TV

CHAPTER 1. REVIEW OF LITERATURE

1.1 Introduction

Infectious bursal disease (IBD) is a highly contagious viral disease of young chickens which is characterized by destruction of the lymphoid cells in the bursa of Fabricius (Cheville, 1967), leading to severe immunosuppression. This increases susceptibility to other diseases, and also interferes with effective vaccination against Newcastle disease (Allan *et al.*, 1972), Marek's disease (Jen and Cho, 1980) and infectious bronchitis (Winterfield *et al.*, 1978). Therefore, IBDV is one of the most important viral pathogens of commercial poultry.

The aetiological agent of IBD, IBD virus (IBDV) belongs to the family Birnaviridae (Brown, 1986). Members of the family are characterized by a double stranded (ds) RNA genome consisting of two segments (A and B) within a single-shelled icosahedral capsid of 60 nm in diameter (Dobos *et al.*, 1979). IBDV exists worldwide with at least two distinct serotypes (1 and 2). However, only strains of serotype 1 are known to cause disease (McFerran *et al.*, 1980). In recent years very virulent strains of IBDV with (Snyder, 1990) or without changes in their antigenicity (Van Den Berg *et al.*, 1991), have caused severe diseases in several countries, including France (Eterradossi *et al.*, 1992), U.K. (Chettle *et al.*, 1989), the Netherlands (Box, 1989), and Belgium (Van Den Berg *et al.*, 1991). Therefore

IBDV remains a major problem in the poultry industry, and the need for understanding the mechanisms causing these antigenic and pathogenic variations have become critical.

It is now agreed that IBDV codes for five mature viral proteins, VP1, VP2, VP3, VP4, and VP5 (Mundt *et al.*, 1995). Sequence analysis of genome segment A of IBDV showed a long open reading frame (ORF) coding for a polyprotein that is cleaved into the major structural proteins, VP2 and VP3, and a minor protein, VP4, considered to have a protease function (Azad *et al.*, 1987; Jagadish *et al.*, 1988). In addition, a small ORF overlapping the polyprotein reading frame encodes a minor protein, VP5 (Mundt *et al.*, 1995), of unknown function.

Our laboratory is interested in understanding the functions of VP1, the only gene product of genome segment B. The two reported functions of VP1, RNA dependent RNA polymerase (RdRp) and guanylyl-transferase activities (Spies *et al.*, 1987; Spies and Müller, 1990), may be important in determining virulence. Also, these functions make VP1 an attractive target for development of anti-viral drugs for IBD prophylaxis. It has also been shown that VP1 is a genome linked protein in IBDV and other birnaviruses (Müller and Nitschke, 1987b; Calvert *et al.*, 1991) which supports the idea that VP1 may act as a primer in initiation of the viral replication (Müller and Nitschke, 1987b; Salas, 1991). Recently, VP1 primed *in vitro* RNA

transcription has been demonstrated in IPNV particles (Dobos, 1995). While it is tempting to speculate that these activities may link VP1 with a viral replication function, no confirmation of this role exists. Because IBDV virions potentially contain other non-structural polypeptides, direct evidence implicating VP1 in the suggested functions is lacking. Appropriate evidence would arise from molecular genetic studies with VP1 mutants, *in vitro* reconstitution with isolated VP1 or experiments utilizing cloned VP1 (Macreadie and Azad, 1993).

1.2 Classification and Morphology of Infectious Bursal Disease Virus (IBDV)

IBDV is a member of the birnavirus genus, family Birnaviridae (Brown, 1986). The family name describes animal viruses with two segments of dsRNA (Dobos *et al*, 1979). Other members of the family include IPNV of fish, tellina virus (TV) and oyster virus (OV) of bivalve molluscs, and drosophila X virus (DXV) of the fruit fly Drosophila melanogaster (Dobos *et al*, 1979). Until 1976 these viruses were classified in the family Reoviridae (Nick *et al.*, 1976). However, the birnaviruses morphologically (Nick *et al.*, 1976), biophysically and biochemically are distinguishable (Dobos *et al*, 1979; Nick *et al.*, 1976) from reoviruses, hence the establishment of a new virus family Birnaviridae (Brown, 1986). Most recently, the International Committee on Taxanomy of Viruses approved the subdivision of the members of family Birnaviridae into 3 genera, genus Avibirnavirus for IBDV,

Aquabirnavirus for IPNV, TV and OV, and Entomobirnavirus for DXV (Dr. Peter Dobos, personal communication).

Virus grown in tissue culture can be concentrated by polyethylene glycol precipitation and purified by density equilibrium banding on CsCl gradients (Kibenge *et al.*, 1988b). The buoyant density of mature IBDV particles in CsCl gradient is 1.32 g/ml (Müller and Becht, 1982; Nick *et al.*, 1976) whereas defective interfering particles have a buoyant density of 1.29 g/ml (Müller and Becht, 1982). Electron microscopic observations of purified virus revealed that the virion has a single-shelled icosahedral capsid of 60 nm in diameter (Dobos *et al.*, 1979). The capsid of IBDV consists of 92 capsomers (Kelly and Loh, 1972) which are made of four to five polypeptides (Nick *et al.*, 1976). The sedimentation coefficient of IBDV is 460S and the average molecular weight of the virion is 67.2 x 10^6, of which the virus genome constitutes approximately 9%, the remaining 91% being viral proteins (Dobos *et al.*, 1979).

IBDV is a very stable virus. The virus is unaffected at pH 2; however, it is inhibited at pH 12. IBDV remains viable after exposure to 56° C for 5 hours, and is resistant to choloroform and ether (Lukert and Davis, 1974; Benton *et al.*, 1967). Isolates of IBDV from turkeys have been reported to be essentially identical to the chicken strains, but the turkey isolates were not pathogenic and could be

distinguished by the virus neutralization test (McNulty *et al.*, 1979; Jackwood *et al.*, 1982). A second serotype (serotype 2) of IBDV was therefore established (McFerran *et al.*, 1980).

1.3 IBDV Genome

IBDV replicates in the cytoplasm, and is unaffected by 5-iodo-2-deoxyuridine (Lukert and Davis, 1974). This suggested that the nucleic acid of IBDV was RNA. Until 1979 it was not known whether the IBDV genome consisted of 2 segments of single-stranded (ss)RNA (Nick *et al.*, 1976) or it was partially double-stranded (Todd and McNulty, 1979). However, it was then observed that IBDV RNA was RNase resistant and had a melting temperature of 95.5°C (Müller *et al.*, 1979). Also the purine:pyrimidine ratio of 1 and the guanine plus cytosine content of 55.3% were observed, which led to the conclusion that the IBDV genome consisted of two segments of dsRNA that sedimented as 14S components in sucrose gradients (Müller *et al.*, 1979).

The nucleic acid of IBDV can be studied by agarose gel or polyacrylamide gel electrophoresis (PAGE). The migration rate of the two genomic segments of various strains of IBDV in PAGE was studied by Jackwood *et al.* (1984). It was observed that the segment B of the SAL strain migrated faster in the gel than the corresponding bands of the MO and OH strains (Jackwood *et al.*, 1984). Because

the SAL strain belonged to serotype 1 and the MO and OH strains to serotype 2 of IBDV, the differences between the migration patterns of segment B are associated with the serotype of IBDV. The migration rate of segment A of the serotype 2 MO strain and serotype 1 SAL strain is similar but that of the OH strain of serotype 2 is different (Jackwood *et al.*, 1984). The presence of urea in the polyacrylamide gels may also influence the migration rate of the IBDV genome segments. The segments migrate more slowly in the gels containing urea (Nick *et al.*, 1976) than in gels without urea (Müller *et al.*, 1979).

The sizes of the two segments of dsRNA from strain 002/73, both under non-denaturing and denaturing conditions, were observed to be approximately 3,400 (molecular weight, MW, 2.06 X 10^6) and 2,900 (MW, 1.76 X 10^6) base pairs (bp) long (Azad *et al.*, 1985). Similar MW values of the two dsRNA segments were observed in Cu-1 strain of IBDV (Müller and Nitschke, 1987a). The larger RNA segment with 3,400 bp was designated segment A and the smaller segment having 2,900 bp was designated segment B (MacDonald and Dobos, 1981). Electron microscopic studies of heat denatured virus particles have shown a dsRNA-protein complex (Müller and Nitschke, 1987b). It has been proposed that the attachment of the protein considered to be VP1, leads to circularization of the individual genome segments (Müller and Nitschke, 1987b). See also Section 1.6.1.

IBDV genome segment A contains one large ORF which encodes a 110 kDa primary protein in which the viral polypeptides are present in the following order: N-VP2-VP4-VP3-C (Azad *et al.*, 1987). It has been established that VP2, VP3, and VP4 arise by co-translational proteolytic cleavage of the precursor polyprotein (Azad *et al.*, 1987; Jagadish *et al.*, 1988). Deletion mapping studies have indicated the possible involvement of VP4 in this proteolytic cleavage (Azad *et al.*, 1987; Jagadish *et al.*, 1988). The coding assignment of the large RNA segment was confirmed by peptide sequence analysis (Hudson *et al.*, 1986). The absence of any precursor molecule initially suggested that the large segment of IPNV was polycistronic (Mertens and Dobos, 1982). However, the nucleotide sequence data of IBDV (Hudson *et al.*, 1986; Kibenge *et al.*, 1990) clearly showed that the large segment has only one long ORF with a single translation termination codon at the 3' end of the plus RNA strand. This suggests that the viral proteins encoded by the large segment arise by specific proteolytic cleavage of a large precursor polyprotein (Azad *et al.*, 1987).

Sequence analysis of genome segment A of serotype 1 of various IBDV strains revealed the existence of a second, albeit small ORF (Kibenge *et al.*, 1990; 1991; Spies *et al.*, 1989). This ORF precedes and partially overlaps the 110 kDa polyprotein ORF (Spies *et al.* 1989). Recently, a small protein has been expressed from this ORF in E. coli and has been identified by immunoprecipitation (Mundt

et al., 1995). This protein designated VP5, exhibited an apparent molecular mass of 21 kDa by SDS-PAGE analysis, which is larger than the calculated size of 16.5 kDa from the deduced amino acid sequence (Mundt *et al.*, 1995). This difference could be due to extensive high order structuring leading to abarrent migration in SDS-PAGE (Mundt *et al.*, 1995).

The smaller dsRNA segment (segment B) of the IBDV genome encodes a single polypeptide of approximately 90 kDa (VP1) (Spies *et al.*, 1987). This has been confirmed by the consensus nucleotide sequence derived from independent and overlapping cDNA clones of strain 002/73, which showed the presence of a single ORF beginning with an exact Kozak sequence and encoding a polyprotein with 878 amino acid residues (Morgan *et al.*, 1988). Similar observations have been made with VP1 of strain OH of IBDV which has 876 amino acid residues that are 97% homologous to strain 002/73 (Dybing, 1992).

1.4 IBDV Proteins

Recent confirmation of the presence of a novel protein in IBDV (Mundt *et al.*, 1995) has led to the conclusion that IBDV has VP1 to VP5 (Mundt *et al.*, 1995). Of these, VP2 and VP3 are the major proteins forming about 51% and 40% of the total IBDV proteins, respectively, and VP1 (3%) and VP4 (6%) are the minor proteins (Dobos *et al.*, 1979). The relative proportion of VP5 is not known (Mundt *et al.*,

1995), and purified virions always contain the VP2 precursor, VPX, as a minor protein (Kibenge *et al.*, 1988a).

Although the molecular weights of the individual viral proteins of IBDV show variations that appear to be dependent on different parameters in the methodologies used by different laboratories (Kibenge *et al.*, 1988b), there is enough evidence that some genuine differences exist, especially between the two serotypes of IBDV (Becht *et al.*, 1988; Jackwood *et al.*, 1984) and between the European (Cu-1) and Australian (002/73) serotype 1 isolates (Fahey *et al.*, 1985a). Another possible explanation for these MW variations is variable cleavage of the precursor protein especially when different host systems are used to grow the viruses (Kibenge *et al.*, 1988b). Also, because variations are observed in the migration of RNA segments of the two serotypes (Jackwood *et al.*, 1984), it is possible that differences in the sizes of their ORFs exist (Kibenge *et al.*, 1991). The distinct differences among the structural proteins of serotype 1 and serotype 2 viruses may be useful in distinguishing the two serotypes (Ture and Saif, 1992).

Due to prominance of VP2 and VP3, Becht (1980) speculated that one or both make up the viral capsid. Now it is known that both VP2 and VP3 form the major portion of capsid proteins. VP3 was originally thought to be the major viral immunogen, particularly during the early phase of the antibody response (Fahey *et al.*, 1985b).

However, it was later demonstrated that VP2 contains the antigenic region responsible for the production of virus neutralizing antibodies (Becht *et al.*, 1988). VP2 also has epitopes which do not induce neutralizing antibodies and also there is evidence that the antigenic region responsible for the production of neutralizing antibodies is highly conformation-dependent (Becht *et al.*, 1988; Azad *et al.*, 1987). VP2 is a major host protective immunogen of IBDV (Azad *et al.*, 1987) and neutralizing antibodies directed against VP2 differentiate between the two serotypes of IBDV (Becht *et al.*, 1988).

VP3 is recognised by monoclonal antibodies directed against it in strains from both serotype 1 and serotype 2. It is therefore considered to be a group-specific antigen (Becht *et al.*, 1988). Also the basic C-terminal region of this protein may be involved in either packaging or stabilizing the RNA genome within the interior of the viral capsid (Hudson *et al.*, 1986).

The deletion expression studies of segment A of IBDV cDNA suggest that VP4 contributes to the processing of a precursor polyprotein of VP2, VP3 and VP4 (Azad *et al.*, 1987; Jagadish *et al.*, 1988). Similar data have been reported for IPNV (Duncan *et al.*, 1987; Manning *et al.*, 1990). However the exact way in which this occurs is unclear (Manning *et al.*, 1990).

The function of the VP5 is still unclear, as are the functional aspects of other proteins of similar size encoded by other RNA viruses (Mundt *et al.*, 1995). These proteins presumably carry out regulatory functions during virus replication. Whether VP5 is involved in the viral replicative cycle remains to be investigated (Mundt *et al.*, 1995).

The only gene product of genome segment B is a minor internal polypeptide, VP1. VP1 is present in the virion in two forms; as a free polypeptide and as a genome linked protein (VPg). Based on its size, low copy number in virions, and the presence of several conserved domains associated with RNA dependent RNA polymerase (RdRp) of other RNA viruses, VP1 is the putative virion-associated RdRp (Duncan *et al.*, 1991). Furthermore, VP1 was also suggested to possess a guanylyl-transferase activity in IBDV (Spies and Muller, 1990), suggesting its role as a multifunctional enzyme (See also Section 1.6).

1.5 Replication of IBDV

IBDV has been adapted to replicate and produce cytopathic effect (CPE) in primary cell cultures such as chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) (Lukert and Davis, 1974; McNulty *et al.*, 1979). Cell culture adapted IBDV can also be grown in several mammalian continuous cell lines such as monkey Vero cells (Lukert *et al.*, 1975), and ovine kidney cells (Kibenge and McKenna, 1992).

A single cycle of replication of IBDV in CEF cells takes 10 to 16 hours at 37°C (Nick *et al.*, 1976). However, it has been observed that, even at high multiplicity of infection, only a limited number of cells infected *in vitro* support virus replication (Hirai and Calnek, 1979). This has hampered the study of IBDV replication. Also, because of the sensitivity of this virus to actinomycin D (Petek *et al.*, 1973), which is normally used to selectively inhibit cellular RNA synthesis, difficulties are encountered in studying IBDV replication.

1.5.1 Viral RNA synthesis

IBDV replicates in the cytoplasm without generally depressing cellular RNA or protein synthesis. IBDV replication involves a virion-associated RNA polymerase which transcribes ssRNA from viral dsRNA (Spies *et al.*, 1987). In IPNV, it has been demonstrated that *in vitro* transcription by the virion RdRp is primed by VP1 (Dobos, 1995). The viral RNA polymerase of IBDV is thought to be VP1 (Spies *et al.*, 1987). The enzyme activity could be demonstrated without any special pretreatment of virus particles (Spies *et al.*, 1987). Its activity requires the presence of Mg^{++} ions and the removal of Ca^{++} ions from the reaction mixture and is optimum at 40°C and pH 8.5. This implies that transcription and replication of birnaviruses can be initiated when the virus has penetrated into the host cell without the need of uncoating or any form of degradation of the capsid (Spies *et al.*, 1987).

The mechanism of synthesis of both virus-specific ssRNA and dsRNA during infection with IBDV has not been clearly determined. A genome-linked protein has clearly been demonstrated in IBDV (Müller and Nitschke, 1987b; Spies *et al.*, 1987). IPNV replicates its nucleic acid by a strand displacement (semi-conservative) mechanism (Mertens *et al.*, 1982; Spies *et al.*, 1987; Dobos, 1995). IBDV RNA synthesized *in vitro* are 24S ssRNA and 14S dsRNA (Spies *et al.*, 1987). The 14S dsRNA component is indistinguishable from the 14S virion dsRNA described previously by Müller *et al.* (1979). The 24S ssRNA component of IPNV contains no polyadenylic acid tracts and is believed to be the viral RNA that serves as the template for the synthesis of complementary strands to form dsRNA (Somogyi and Dobos, 1980). Birnaviruses synthesize two genomic length and a small size mRNA transcript that are translated into different viral proteins.

1.5.2 Viral protein synthesis

Five mature viral proteins designated VP1, VP2, VP3, VP4, and VP5 are synthesized in IBDV infected cells (Mundt *et al.*, 1995). The pattern of newly synthesized cellular proteins does not differ between uninfected and IBDV infected cells analysed by SDS-PAGE, indicating that IBDV does not shut off the synthesis of cellular proteins (Becht, 1980). A VP2 precursor designated VPX was demonstrated in IPNV infected cells by Dobos *et al.* (1979). Similar observations were made for IBDV by Müller and Becht (1982). VP2 could not be found in

lysates of infected cells, however VPX was demonstrated inside the cells in relatively large quantities (Müller and Becht, 1982). This indicated that post-translational modification of the 50K polypeptide (VPX) to 40K polypeptide (VP2) probably goes on during virus maturation or assembly (Müller and Becht, 1982). Precursor-product relationships also seem to exist in the biosynthesis of the other viral polypeptides. Precursor-product relationships were reported for VP3 and VP4 of the Australian IBDV isolate 002/73 (Azad *et al.*, 1986; Hudson *et al.*, 1986). See also Section 1.3.

1.6 IBDV VP1 Functions

The only gene product of genome segment B (approx. 2,900 bp) is a minor internal polypeptide VP1. VP1 is present in the virion in two forms: as a free polypeptide and as a genome linked protein (VPg). In IPNV, the genome-linked form of VP1 is linked to the 5' end of both genome segments by a serine-5' GMP phosphodiester bond (Calvert *et al.*, 1991). Birnaviruses are the only dsRNA viruses with a VPg, the size of which is the largest of the VPgs of RNA viruses (Calvert *et al.*, 1991). VPgs are thought to play a fundamental role in the replication of viral genomes and might also be important during viral morphogenesis (Calvert *et al.*, 1991).

The first indication that birnaviruses might contain a genome linked protein came from the work of Persson and MacDonald (1982), who isolated an RNA-protein

complex from disrupted IPNV particles. When viewed under the electron microscope, the RNA-protein complex appeared as circular structures which became linear after proteinase K treatment. When the radioiodinated RNA-protein complex was treated with RNase A, the released radioactive polypeptide comigrated with VP1 on polyacrylamide gels. Müller and Nitschke (1987b) made similar observations using purified IBDV. Nevertheless it cannot be ruled out that the genome segment ends are circularized after extraction, and there is no conclusive evidence that the genome segments may be circular in the virus particle (Müller and Nitschke, 1987b). Also, when highly concentrated IBDV preparations were used as sources of dsRNA, or when after heating, specimens were allowed to stand for several hours, flower like forms of IBDV RNA were observed (Müller and Nitschke, 1987b). This indicates the tendency of VP1 to aggregate the viral genome which in turn suggests that it is present at the 5' end of both plus and minus strands, and that when these terminal proteins stick to each other they circularize the RNA (Calvert *et al.*, 1991). Thus it was postulated that VPg and VP1 are the same, and that both the free polypeptide and its genome linked form can be found in the purified virus.

1.6.1 VP1 as a primer in genome replication

The physiological function of genome-linked protein is unknown. Involvement in RNA replication as well as assembly may be possible. The genome-linked protein

in poliovirus and in adenovirus suggest a role of the protein in genome replication, possibly at the level of the initiation (Wimmer, 1982). Precursor proteins are used as primers in viral replication, and these precursors are ultimately cleaved into an encapsidated terminal protein (Wimmer, 1982). Because of these precedents, it has been suggested that the IBDV genome-linked protein, VP1, as well may be a primer for replication (Persson and MacDonald, 1982). Studies in the case of IPNV have shown that the protein is present in all detectable IPNV intracellular ssRNA (Persson and MacDonald, 1982). These single-stranded molecules act both as intermediates in viral genome replication and as mRNA (Somogyi and Dobos, 1980). Recent evidence favors a mechanism of strand displacement for the synthesis of these ssRNAs in purified virions (Dobos, 1995). Moreover, strand displacement is a feature that birnaviruses have in common with poliovirus and adenovirus, both of which have VPgs. Wimmer (1982) has speculated that all viruses that replicate their nucleic acid by strand displacement may have a common ancestral replication system which utilizes a protein primer. If the genome-linked protein is truly a primer, and if IBDV RNA synthesis is indeed by strand displacement, the hypothesis made by Wimmer (1982) is further supported. In a recent report on IPNV, it has been demonstrated that *in vitro* transcription by the virion RdRp is primed by VP1 and then proceeds via an asymmetric, semiconservative, strand-displacement mechanism (Dobos, 1995).

1.6.2 VP1 as RNA dependent RNA polymerase (RdRp)

Eukaryotic cells do not produce an RdRp enzyme, and since the genome of IBDV is not infectious, this virus must carry its own RdRp enzyme. An RdRp activity of IBDV could be detected without any special treatment of the virus particle (Spies *et al.*, 1987) indicating that transcription and translation can be initiated without the need for uncoating, unlike reovirus (Skehel *et al.*, 1969). The virus can be made transcriptionally active by altering salt conditions; presence of Mg^{++} ions is essential while Ca^{++} ions have to be removed completely from the reaction (Spies *et al.*, 1987). The RNA synthesized *in vitro* is 14S dsRNA and 24S ssRNA. The 24S ssRNA does not self anneal, hybridizes with virion RNA, and induces *in vitro* translation of virus specific polypeptides; therefore, it is considered to be the newly transcribed mRNA (Spies *et al.*, 1987).

Five properties of VP1 are consistent with the polymerase function : (1) it is similar in size to other known viral polymerases, (2) only about 22 molecules are present per virion (Dobos *et al.*, 1979), (3) it is an internal protein (Dobos and Rowe, 1977), (4) temperature sensitive mutants are most readily isolated in the RNA segment encoding VP1, suggesting that it has an activity very sensitive to conformational changes (MacDonald and Dobos, 1981), (5) the high conservation of the genome segment B nucleotide sequences of several IBDV strains, examined by polymerase

chain reaction (PCR) amplification of cDNA fragments and restriction enzyme (RE) analysis (Kibenge and Qian, 1994).

Sequence comparison of IBDV and IPNV genome segments B revealed only 41% homology (Duncan *et al.*, 1991). Despite the low overall homology between these two birnaviruses, homologous regions were detected within the central portion of the proteins (Duncan *et al.*, 1991). Three consensus sequences associated with RNA dependent RNA polymerases were also detected in VP1 (Duncan *et al.*, 1991). A comparison of the amino acid sequences of IBDV VP1 with those of other DNA-dependent and ssRNA-dependent RNA polymerases had initially failed to reveal any homology between VP1 and the conserved regions in enzymes (Morgan *et al.*, 1988). It had been suggested that the IBDV VP1 may represent a new class of polymerases which are involved in the replication of dsRNA genomes (Morgan *et al.*, 1988). However, Duncan *et al.* (1991) suggested that the function of the birnavirus viral polymerase depended upon the presence of similar, rather than identical amino acid sequences which resulted in the same secondary and tertiary structure of the protein.

To prove that only VP1 is involved in the RdRp activity, the VP1 gene of IBDV strain 002/73 was expressed in E. coli and in Saccharomyces cerevisiae and tested for RdRp activity (Macreadie and Azad, 1993). No activity could be conclusively

demonstrated in the VP1 obtained from either of the expression systems. Thus, so far, there is no conclusive evidence that could prove VP1's exclusive role as RNA dependent RNA polymerase.

1.6.3 VP1 as a guanylyl transferase capping enzyme

Guanylyl transferase is a key enzyme in the cap formation of mRNA (Furuichi *et al.*, 1975). The existence and function of the 7-methylguanosine (m^7G) cap has been demonstrated by Furuichi *et al.* (1975). It enhances maturation of mRNAs, including splicing and translatability (Shatkin, 1985). Animal viruses that replicate in the cytoplasm routinely encode their own capping enzymes (Kozak, 1986). This property has been shown in poxviruses (Moss *et al.*, 1976), reoviruses (Furuichi *et al.*, 1976), vesicular stomatitis virus (Abraham *et al.*, 1975), and alphaviruses (Cross, 1983).

Among dsRNA viruses, the mechanism of formation of reovirus mRNA 5'-terminal cap structures has been studied in detail (Furuichi *et al.*, 1975; Fausnaugh and Shatkin, 1990). The guanylylation reaction is a two step reversible reaction which involves an enzyme-guanylate (VP1-GMP) intermediate followed by nucleotide transfer to an acceptor molecule and recycling of guanylyl-transferase (Fausnaugh and Shatkin, 1990).

$$E + GTP \rightleftharpoons E\text{-}pG + PPi \qquad (1)$$

$$E\text{-}pG + ppRNA \rightleftharpoons GpppRNA + E \qquad (2)$$

Spies and Müller (1990) demonstrated the formation of the enzyme-guanylate complex (VP1-GMP). The sequence GXXXXGKS/T is a constant motif present in ras-typing GTP binding proteins (Argos and Leberman, 1985). This constant motif is found in several viral proteins with a tentative role in RNA replication (Duncan *et al.*, 1991). The same sequence is present in both IBDV and IPNV VP1 (Duncan *et al.*, 1991). As such, this region represents a potential GTP binding site in the VP1 of birnaviruses. Therefore, the presence of this region and formation of the IBDV VP1-guanylate complex (Spies and Müller, 1990) are consistent with the idea that this polypeptide acts as guanylyl-transferase capping enzyme.

However, recent studies done in the case of IPNV VP1 revealed that in contrast to reovirus guanylyl-transferase, the formation of IPNV VP1-GMP complex is not reversible and VP1 is not able to transfer the GMP to the mRNA (Dobos, 1993). This indicated that birnavirus VP1 is not a capping enzyme. Furthermore, the VP1-GMP of IBDV was shown not to be linked by a phosphoamine bond which is characteristic of capping enzymes, but rather by a phosphodiester bond (Spies and Müller, 1990; Dobos, 1993), a characteristic of genome linked proteins. Time course experiments revealed that after the initial guanylylation of VP1 to form VP1-

pG, a second GMP is added to form VP1-pGpG, the formation of which is template dependent (Dobos, 1993). The formation of VP1-pGpG complex, is more compatible with VP1 acting as a primer during initiation of RNA synthesis. The fact that genome-linked proteins have been shown to act as primers in viral genome replication (Salas, 1991) and that IBDV contains such a protein add further weight to this arguement. However, there is no report on guanylylation experiments utilizing birnavirus recombinant VP1.

1.7 Control of Infectious Bursal Disease

Because of the stability of IBDV and the fact that it is ubiquitous among poultry flocks, disease control through sanitation and isolation is difficult. IBD is normally controlled in young chickens by passively transferred immunity from breeder flocks vaccinated with inactivated oil emulsion vaccines containing serotype 1 virus (Wood *et al.*, 1981; Wyeth and Cullen, 1979). Recent developments in the production of bio-engineered vaccines have promised better disease control. Macreadie *et al.* (1990) demonstrated that when microgram quantities of yeast-derived IBDV antigens were injected into chickens, high titres of virus neutralizing antibodies were detected that were capable of protecting young chickens against IBDV infection. To demonstrate the active protection against IBDV infection, Bayliss *et al.* (1991) expressed the VP2 antigen of IBDV in a recombinant fowlpox virus and showed that it could protect chickens against mortality, but not against bursal atrophy. Recently,

it was demonstrated that the recombinant baculovirus-expressed IBDV antigens are highly immunogenic and capable of actively and passively protecting chickens against IBDV infection (Vakharia *et al.*, 1994).

Despite extensive vaccination, viruses are maintained in the environment, by evolving various mechanisms such as antigenic shift and antigenic drift (Lasher and Shane, 1994). In the recent past, numerous IBD outbreaks have been reported (Chettle *et al.*, 1989; Eterradossi *et al.*, 1992), making investigation of other means of disease control critical. One of these, the use of antiviral drug therapy could prove beneficial. The antiviral agents such as Zidovudine, a synthetic pyrimidine analog that inhibits a number of animal and human retroviruses including HTLV-1, HIV-1, and HIV-2 (Furman and Barry, 1988; Hirsch, 1988) act on viral enzymes which are involved in the viral replication. Since the consensus sequences involved in the viral gene expression are highly conserved, once means to alter these sequences are identified then the problem of control of viral infections could be permanently solved. Until now the success in development and use of anti-viral drugs has been limited and mostly confined to human medicine but this field holds great potential for veterinary medicine as well. In the case of IBDV, the polymerase and guanylyl transferase activities, which are thought to be associated with VP1 (Spies and Muller, 1990; Spies *et al.*, 1987), make it an attractive target for the development of anti-viral drugs for prophylaxis of IBD. Once the active sites

involved in these activities are determined, it would be possible to design the drug that would act to block the replication of this virus.

1.8 Research Objectives

VP1 has been suggested to be a multifunctional enzyme (Spies *et al.*, 1987; Spies and Muller, 1990). Because birnavirus virions potentially contain non-structural proteins such as VP4 and VP5, the functions of VP1 have not been unequivocally identified. In our laboratory, reversed genetics studies have been initiated to construct virions from molecularly cloned DNA copies of IBDV genome sequences. It is considered that this would provide a means to define the molecular determinants of viral virulence, antigenicity and functional characterization of mutations in IBDV. As part of these studies, the complete genomic segment B ORF cDNA of OH-IBDV was constructed in a plasmid under the control of the T7 RNA polymerase promoter for *in vitro* expression.

The objectives of the research described in this thesis were:

1. To express the recombinant VP1 in a cell-free system.
2. To assay for VP1 activities of the *in vitro* translated protein.

All previous studies that have successfully demonstrated enzyme activities of the birnavirus VP1 multifunctional enzyme have utilized virion-associated VP1.

Moreover, there is no report on guanylylation experiments utilizing birnavirus recombinant VP1.

The purpose of the work reported here was to characterize the functional activities of IBDV VP1 expressed in a cell-free system and compare them to the virion-associated VP1.

CHAPTER 2: EXPRESSION OF THE OH-IBDV SEGMENT B cDNA IN A CELL-FREE SYSTEM

2.1 Introduction

The genome of IBDV consists of two segments, A and B. Segment A encodes information for two major (VP2, VP3) and two minor (VP4, VP5) polypeptides, whereas segment B of IBDV encodes information for a minor internal polypeptide, VP1, which is believed to have polymerase and guanylyl-transferase activities (reviewed in Chapter 1).

Expression of the proteins in large quantities is required for their assay and characterization as well as for production of vaccines. Various expression systems such as prokaryotic, eukaryotic and *in vitro* cell-free systems have been successfully used to express IBDV proteins.

Among these, the prokaryotic expression system has been most extensively used. Expression in bacteria is a simple and efficient means of synthesizing proteins encoded by foreign genes. Azad *et al.* (1987) successfully expressed genome segment A of 002-73-IBDV in E. coli. Jagadish *et al.* (1988) confirmed that VP4 is the viral protease by expressing cDNA fragments of genome segment A of 002/73 IBDV in E. coli. Expression of VP5 of segment A of IBDV strain P2 in E. coli has

recently been reported by Mundt *et al.* (1995). The cloned cDNA of segment B of 002/73 IBDV has also recently been expressed in E. coli to produce VP1 (Macreadie and Azad, 1993). However, the bacterial (prokaryotic) expression systems are limited in their ability to modify expressed eukaryotic proteins post-translationally (Sambrook *et al.*, 1989; Ellis, 1989). Consequently, they are incapable of folding proteins correctly and efficiently. A difference in secondary and tertiary structures of a protein can affect its immunogenicity, function, and biological activity (Ellis, 1989).

The limitations associated with prokaryotic expression systems could be overcome by using eukaryotic expression systems. Post-translational modifications such as glycosylation and disulphide bridging, necessary for correct folding, occur in yeast cells (Vlak and Keus, 1990). An almost full length 002/73 IBDV cDNA fragment encoding the precursor polyprotein was expressed in various yeast expression systems such as S. cerevisiae and S. pombe (Jagadish *et al.*, 1990). Macreadie and Azad (1993) expressed the IBDV segment B clone in the S. cerevisiae expression system. However, only a limited number of promoters and vectors are available for yeast expression systems. Also the yield of expressed proteins is relatively low (Vlak and Keus, 1990).

Baculoviruses are being used as vectors in insect expression systems for production of IBDV proteins. These systems are capable of synthesizing large quantities of recombinant proteins that are antigenically and immunogenically indistinguishable from their authentic counterparts (Nagy *et al.*, 1990). Vakharia *et al.* (1994) expressed the structural protein genes of a variant IBDV strain (GLS) in the insect cells using a baculovirus expression system.

In addition to the prokaryotic and eukaryotic expression systems, *in vitro* transcription and translation systems are available for synthesis of ssRNA and protein, respectively. *In vitro* transcription is a simple and efficient method for synthesizing pure and biologically active RNA (Melton *et al.*, 1984; Gurevich *et al.*, 1991). This *in vitro* transcribed RNA could be used for protein production in a cell-free translation system. The cell-free translation systems like rabbit reticulocyte lysate and wheat germ extract have the advantages of being easy and quick, and usually provide an efficient method of *in vitro* protein production. During the past few years *in vitro* translation has been used to produce several functionally active proteins for protein engineering studies (Olate *et al.*, 1988; Boissel *et al.*, 1988) and has even been proposed as a method for large-scale industrial polypeptide production (Spirin *et al.*, 1988). Azad *et al.* (1985) identified VP1 - VP4 and a 16.6 kDa polypeptide by *in vitro* translation of purified dsRNA of 002-73-IBDV. IPNV proteins from both segment A and B have also been expressed by *in vitro*

transcription and translation (Huang *et al.*, 1986; Duncan *et al.*, 1987; Manning *et al.*, 1990). However, unlike IPNV, so far there is no report on expression of viral proteins from viral cDNA clones of the IBDV genome by *in vitro* transcription and translation.

Experiments were conducted towards the expression of the OH-IBDV genomic segment B cDNA clone in a cell-free system. The cDNA of the complete ORF of VP1 of IBDV strain OH was constructed in a plasmid under the control of the T7 RNA polymerase promoter for *in vitro* expression (Qian and Kibenge, 1995). The main purpose of this study was to express the VP1 in a cell-free system i.e., to obtain ssRNA transcript that was active in a cell-free translation system. For this, experiments were conducted towards optimization of the conditions for *in vitro* transcription and translation. The *in vitro* expressed VP1 from this study was subsequently characterized to prove its direct role in polymerase and guanylyl-transferase activities (Chapter 3).

2.2 Materials and Methods

2.2.1 Virus and plasmids

IBDV strain OH of serotype 2 (Jackwood *et al.*, 1982) was kindly supplied by Dr. Y. M. Saif, The Ohio State University. Plasmids pOHB 27-1 and pOHB 27-2,

containing the cDNA of the complete ORF of segment B of OH-IBDV, constructed in our laboratory (Qian and Kibenge, 1995), were used (Figure 1). The orientation of the IBDV cDNA insert in both plasmids is identified by the 5' and 3' ends. Thus, the DNA templates linearized by *Xho*1 and transcribed with T7 RNA polymerase would generate a positive sense transcript from pOHB 27-1 and a negative sense transcript from pOHB 27-2.

2.2.2 Virus propagation and purification

OH-IBDV was grown in Vero cells (American Type Culture Collection, Rockville, Maryland, USA) as previously described (Kibenge *et al.*, 1988a). Briefly, confluent Vero cells grown in 150 cm^2 tissue culture flasks were infected with 0.01 plaque forming units of virus per cell. After adsorption for one hour at 37°C, the inoculum was replaced with maintenance medium [Minimum Essential Medium (MEM) with 2% of fetal bovine serum (FBS)]. The flasks were incubated at 37°C in a humidified 5% CO_2-in-air-atmosphere until complete cytopathic effect developed at 5-6 days post-inoculation. The cells were disrupted by freezing and thawing three times. Virus was purified by a modification of the procedure described by Kibenge *et al.* (1988a). Briefly, cell-culture virus was clarified by precipitation of the cellular material by centrifugation at 2,060 x g for 20 minutes. The virus was concentrated by overnight precipitation with 10% polyethylene glycol 8000 (PEG) and 2.332% of NaCl at 4°C by continuous stirring. The precipitate collected after

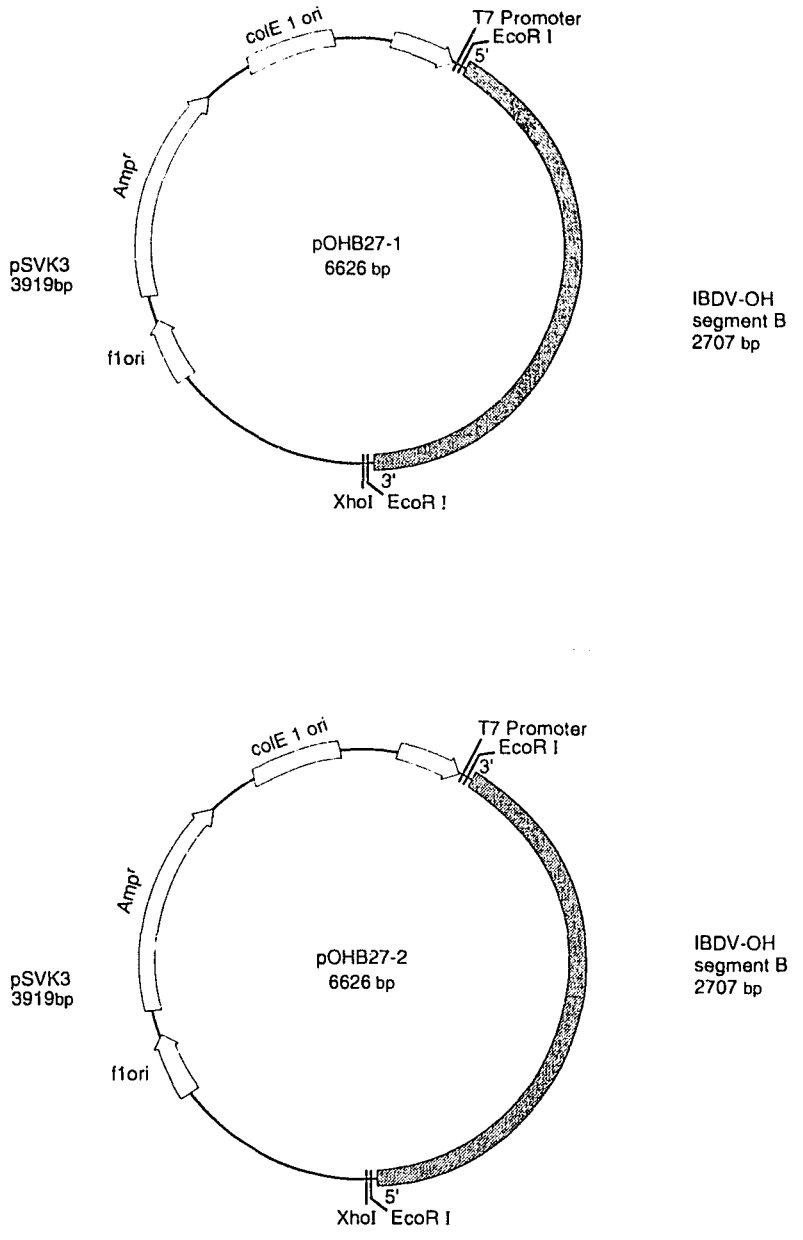

Figure 1. Plasmid maps of pOHB 27-1 and pOHB 27-2. IBDV VP1 cDNA insert in plasmid pSVK3 (Pharmacia, Bare d'Urfe, PQ). The orientation of the IBDV cDNA insert in both plasmids is identified by the 5' and 3' ends. Amp^r denotes the βlactamase gene which confers ampicillin resistance, f1 ori denotes the origin of replication and *coliE* 1 ori denotes origin for efficient replication in E. coli.

centrifugation at 17,600 x g for 2 hours in Beckman JA 14 rotor at 4°C was resuspended in TNE buffer (0.01M Tris-HCl, 0.1M NaCl and 0.001M EDTA, pH 7.9). Undissolved solid material was removed by centrifugation at 3,000 x g for 10 minutes. Concentrated virus was placed on a 2 ml CsCl cushion (density = 1.40 g/ml) in TNE buffer and centrifuged at 130,000 x g for 75 minutes using a Beckman SW 40 Ti rotor at 4°C. Virus collected from CsCl cushions was pooled, adjusted to a buoyant density of 1.32 g/ml and isopycnically banded at 145,000 x g for 18 hours using a Beckman SW 40 Ti rotor at 4°C. Tubes were illuminated in a dark room to locate the bluish virus bands, which were withdrawn by side puncture. The virus sample was dialysed against TNE buffer for 12 hours to remove the CsCl. The dialysed virus was again adjusted to a buoyant density of 1.32 g/ml and isopycnically banded as described above. This procedure of isopycnic centrifugation followed by dialysis was repeated 4 times. The final dialysis was against 100 mM Tris-HCl, pH 8.0. The virus was concentrated by centrifugation in centricon-10 microconcentrator (Amicon Canada Ltd., Oakville, Ont.) and stored at -20°C. The purity of the virus was checked by running it on a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) (section 2.2.7) followed by silver staining using a commercially available silver stain kit (BIO-RAD, Mississuaga, Ont.).

2.2.3 Isolation of plasmid DNA

Plasmid DNAs of pOHB 27-1 and pOHB 27-2 were prepared by the alkaline-lysis method of Birnboim and Doly (1979) with modifications as described by Kibenge *et al.* (1990). Briefly, 250 ml of 2X YT broth (16% bactotryptone, 10% bactoyeast ext., 5% NaCl) containing 100 µg/ml ampicillin (Boehringer Mannheim, Laval, PQ) was inoculated with E. coli containing recombinant plasmid (pOHB 27-1 and pOHB 27-2). The culture was incubated at 37°C overnight with constant shaking at 250 rpm. Bacterial cells were harvested by centrifugation at 9,630 x g for 10 minutes at 4°C. Plasmid DNA was released from bacterial cells by weakening cell walls with 0.005 g/ml lysozyme in lysozyme buffer (50 mM Glucose, 10 mM EDTA, 25 mM Tris base, pH 8.0), for five minutes at room temperature and lysis of cell walls for five minutes on ice with 1% SDS in 0.2 M NaOH, pH 12. RNA was digested with 0.12 mg/ml RNase A (Pharmacia, Baie d'Urfe, PQ) for 20 minutes in ice. Chromosomal DNA and bacterial proteins were precipitated by the addition of 7.5 M ammonium acetate for 10 minutes on ice and then collected by centrifugation at 16,300 x g for 15 minutes at 4°C. The plasmid DNA was precipitated from the supernatant with 2 volumes of absolute ethanol and was harvested by centrifugation at 17,640 x g for 30 minutes. The pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and precipitated with 0.4 volume of 30% PEG in 1.8 M NaCl, overnight at 4°C. The PEG was removed by phenol-chloroform-isoamyl alcohol extraction. Plasmid DNA was again precipitated with cold absolute

ethanol in high salt. It was then harvested by centrifugation at maximum speed in a microcentrifuge, washed with 1 ml 70% ethanol, and dried in a vacuum dryer (Savant SpeedVac Concentrator, Farmingdale, New York, USA). The DNA pellet was resuspended in 200 µl TE buffer and 10 µl was diluted 40-fold and quantitated in a Gene Quant RNA/DNA calculator (Pharmacia, Cambridge, England).

2.2.4 Restriction enzyme digestion

Plasmids pOHB 27-1 and pOHB 27-2 were characterized to confirm the size of the cDNA inserts, and the presence of a unique *Xho*1 restriction site for linearizing the plasmids. Two enzymes, *Eco*R1 (Bio-Can, Mississuaga, Ont.) and *Xho*1 (GIBCO/BRL, Burlington, Ont.) were used for this purpose. *Eco*R1 digested plasmids were used to check the size of the inserts while *Xho*1 digested templates were used for *in vitro* transcription. The basic protocol used was the same for both enzymes, however, *Eco*R1 digestion was done in a 20 µl reaction-volume with 1 - 2 µg of the plasmid DNA while *Xho*1 digestion was done in a 100 µl reaction-volume with 10 - 20 µg of the DNA. Other components of the reaction were enzyme buffer and restriction enzyme concentration of 1-2 U/µg of DNA template. Reaction components were properly mixed and briefly centrifuged before incubation at 37°C for 2 hours. After incubation, *Eco*R1 treated DNA was directly analyzed by 1% agarose gel electrophoresis. *Xho*1 treated samples were subjected to proteinase K digestion; 5 µl of 10% SDS in the presence of 2 µl of 20 mg/ml of

proteinase K were added and further incubated at 37°C for 1 hour. Proteinase K treatment was followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation in the presence of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of cold absolute ethanol. Plasmid DNA was pelleted by centrifugation for 15 minutes in a microcentrifuge, and washed with cold 70% ethanol, dried, and resuspended in 40 µl of diethylpyrocarbonate treated water (DEPC-water). The DNA template was quantitated and later analyzed by agarose gel electrophoresis.

2.2.5 *In vitro* transcription with T7 RNA Polymerase

To minimize ribonuclease contamination, all pipette tips and microcentrifuge tubes were soaked overnight in DEPC-water and then drained, dried, and autoclaved before use in transcription assays (Sambrook *et al.*, 1989). Transcription reactions were performed using the MEGAscript™ *in vitro* Transcription Kit for large scale synthesis of RNA (Ambion Inc., Austin, Texas, USA). Unless otherwise stated, transcription reactions were performed in 20 µl volumes containing 2 µl of 10X transcription buffer, 2 µl of NTPs to a final concentration of 37.5 mM, 1-2 µg linearized DNA template (from section 2.2.4) 2 µl enzyme mix with T7 polymerase. The reaction mixtures were incubated at 37°C for 4 hours. The DNA template was removed by digestion with 2 units/µl of RNase-free DNase I for 1 hour at 37°C. Thirty microlitres of RNase-free water were added in the reaction and transcripts were precipitated with 25 µl of lithium chloride overnight at -20°C. The RNA

transcripts were then recovered by centrifugation for 30 minutes in a microcentrifuge, quantitated and examined by both 1% agarose gel electrophoresis and formaldehyde denaturing gel electrophoresis.

Time course experiments at 2, 4, and 6 hours, and optimization of nucleotide concentration at 18.5 mM, 37.5 mM, 50 mM, and 75 mM as well as DNA template concentration optimization at 0.5X, 2X, 4X of the specified amount were done in order to obtain the maximum transcription yield. The RNA was quantitated spectrophotometrically in a Gene Quant RNA/DNA calculator (Pharmacia) and by trichloroacetic acid (TCA) precipitation.

For TCA precipitation 10μCi of ^{32}P-UTP was also included in the reaction. After termination of the reaction 1 μl was removed and mixed with 400 μl of 1mg/ml salmon sperm DNA and nucleic acid was precipitated by 2 ml of 10% TCA. The precipitated RNA was collected by vacuum filtration through a glass fibre filter. Finally, the quantitation was done by scintillation counting.

2.2.6 *In vitro* translation reactions

In vitro translation reactions used the Rabbit Reticulocyte Lysate System (Promega Corp., Madison, Wisconsin, USA). This system contains phosphocreatine kinase plus phosphocreatine, a mixture of transfer RNAs (tRNA), hemin, potassium acetate,

and magnesium acetate in addition to nuclease-treated rabbit reticulocyte lysate. The following reaction mixture was mixed gently with a pipet as each component was added to a final volume of 50 µl: 35 µl rabbit reticulocyte lysate, 7 µl nuclease-free water, 1 µl 40u/µl RNasin ribonuclease inhibitor, 1 µl 1 mM amino acid mixture without methionine, 2 µg RNA substrate in nuclease-treated water and 40µCi [^{35}S] methionine (1,200Ci/mmol) (Amersham Canada Limited, Oakville, Ont). The translation mixture was incubated for 60 minutes at 30°C. Protein translation products were mixed with an equal volume of 2X Laemmli sample buffer, pH 6.8. 2-β-Mercaptoethanol (2-ME) was added to the sample treatment buffer immediately prior to use to minimize evaporation of 2-ME (Dobos and Rowe, 1977). Experiments were also conducted to check the effect of different concentrations of RNA substrate on the translation product yield. The proteins were analyzed directly by SDS-PAGE and autoradiography. The protein samples were stored at -20°C.

The "ECL™ *In vitro* translation system" (Amersham Canada Limited, Oakville, Ont.), a Rabbit Reticulocyte Lysate System which utilized biotinylated amino-acid instead of the one labeled with [^{35}S], was also tried. The protocol for the reaction was followed exactly as specified by the manufacturer, which was almost similar to the one stated above. However, after the SDS-PAGE the proteins were transferred to nitrocellulose membrane by electroblotting. The nitrocellulose membrane was then exposed with Kodak X-Omat AR film (Eastman Kodak Company, Rochester,

New York, USA). The detection of biotinylated proteins on nitrocellulose membrane was by a chemiluminescent reaction of luminol/iodophenol with streptavidin-coupled horseradish peroxidase.

2.2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins from *in vitro* translation were analyzed by SDS-PAGE using a discontinuous gel system (Laemmli, 1970). Molecular weight markers; phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soyabean trypsin inhibitor (20,000), and lysozyme (14,400) (BIO-RAD Laboratories, Richmond, California, USA), were used. The molecular weight markers were diluted 1:20 in 1X Laemmli sample treatment buffer. Both protein samples and molecular weight markers were denatured for 2 minutes at 100°C, immediately chilled on ice, then loaded onto a gel (12.5% resolving gel and 5% stacking gel). Electrophoresis was conducted in Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3), at 15 mA until the samples entered the resolving gel (approximately 30 minutes). Power was then increased to 30 mA and the gel was electrophoresed for an additional 2-3 hours at which time the dye front had reached the bottom of the gel.

Following electrophoresis, proteins and molecular weight markers in the gel were visualized by staining with Coomassie Brilliant Blue R-250 (BIO-RAD Laboratories,

Canada Ltd, Mississiauga, Ont). The gel was then dried with vacuum at 80°C in a slab gel dryer (Hoeffer Scientific, San Francisco, California, USA) and subjected to direct autoradiography with Kodak X-Omat AR film (Eastman Kodak Company, Rochester, New York, USA) for 48 hours.

2.3 Results

2.3.1 Characterization of plasmids pOHB 27-1 and pOHB 27-2

After the initial isolation and purification of plasmids pOHB 27-1 and pOHB 27-2, the size of the segment B cDNA insert was checked by digestion with *Eco*R1 enzyme followed by electrophoresis in 1% agarose gel. Inserts of approximately 2,800 bp long were obtained (Figure 2), which is approximately the same size as genome segment B of OH-IBDV.

To linearize the plasmid DNA for *in vitro* transcription, digestion with *Xho*1 enzyme was done. The site of *Xho*1 digestion is close to the termination codon of the insert in plasmid pOHB 27-1 and close to the initiation codon of the insert in plasmid pOHB 27-2 (Figure 1). The restriction digests were analysed by electrophoresis in 1% agarose gel (Figure 2).

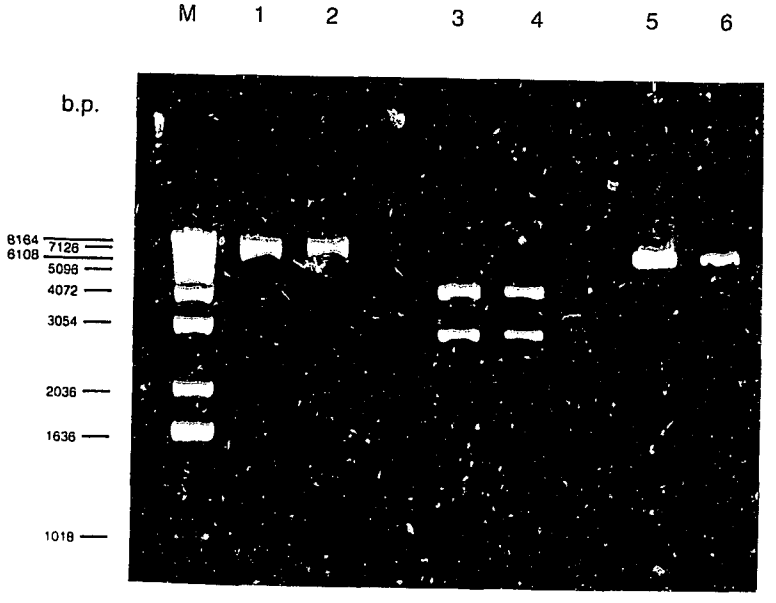

Figure 2. Characterization of plasmids pOHB 27-1 and pOHB 27-2. Lane M contains DNA markers. The size of the markers in base pairs (bp) are given on the left side; lanes 1 and 2 contain plasmids pOHB 27-1 and pOHB 27-2, respectively; lanes 3 and 4 contain *Eco*R1 digested pOHB 27-1 and pOHB 27-2, respectively; lanes 5 and 6 contain *Xho*1 digested pOHB 27-1 and pOHB 27-2, respectively.

2.3.2 Optimization of *in vitro* transcription reaction

In vitro transcription optimization reactions were performed in order to obtain the maximum transcription yield possible. For this, plasmid pOHB 27-1 which contains the segment B cDNA insert in the positive orientation was used.

2.3.2.1 Nucleotide concentration optimization

Optimization reactions with variable nucleotide concentrations were conducted. The reactions containing 18.5 mM, 37.5 mM, 50 mM or 75 mM of nucleotides were incubated for 4 hours at 37°C with the rest of the reaction components as described in Materials and Methods (Section 2.2.5). The reactions were repeated three times and the final RNA transcript yield was calculated by taking the mean of the three measurements. The mean RNA transcript yields at 18.5 mM, 37.5 mM, 50 mM, and 75 mM nucleotide concentration were 133.6 μg/ml, 296 μg/ml, 264 μg/ml, 168.3 μg/ml, respectively (Figure 3). The nucleotide concentration of 37.5 mM which gave the highest transcript yield was used in all subsequent experiments.

Quantitation by TCA precipitation was attempted but the background counts of the TCA control were greater than the radioactive counts of the RNA samples. Consequently, all the quantitations were done spectrophotometrically. The concentration of RNA was determined by measuring the OD_{260} of an aliquot of the final preparation. The spectrophotometric quantitation could be significantly

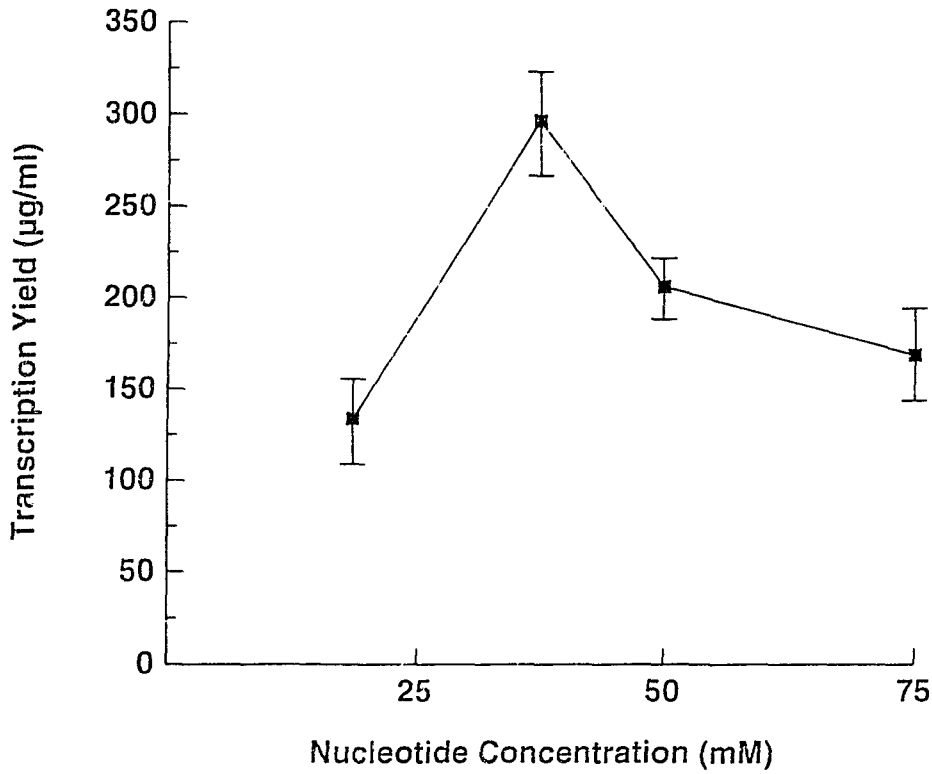

Figure 3. Nucleotide concentration optimization. Transcription yield was calculated by using different concentrations of nucleotides. Each reaction was performed three times and the mean of the RNA transcript yield was plotted.

affected if the sample is contaminated by prot ıs, phenol or other nucleic acid. All possible precautions were taken to avoid such contaminations. The ratio between reading at 260 nm and 280 nm (OD_{260}/OD_{280}) provided an estimate of the purity of the transcripts.

2.3.2.2 Optimization of duration of incubation

The time course experiments at 2, 4, and 6 hours of incubation with 37.5 mM of nucleotide concentration were conducted to determine the incubation period for the maximum transcription yield. The reactions were repeated three times and the final RNA transcript yield was calculated by taking the mean of the three observations. The mean transcription yields at 2, 4, and 6 hours were 269.86 µg/ml, 580.8 µg/ml, 550.93 µg/ml, respectively (Figure 4).

2.3.2.3 *In vitro* transcription by using T7 RNA polymerase

The final transcription reactions utilizing pOHB 27-1 and pOHB 27-2 were conducted with 37.5 mM nucleotide concentration and incubated for 4 hours which were determined to be optimal conditions for *in vitro* transcription of the IBDV segment B cDNA. In each case, only one size class of RNA was produced which corresponded to the expected size for run off synthesis by T7 RNA polymerase at its promoter. Based on the orientation of the cDNA insert, one transcript was plus-sense (from pOHB 27-1) while the other was minus-sense (from pOHB 27-2).

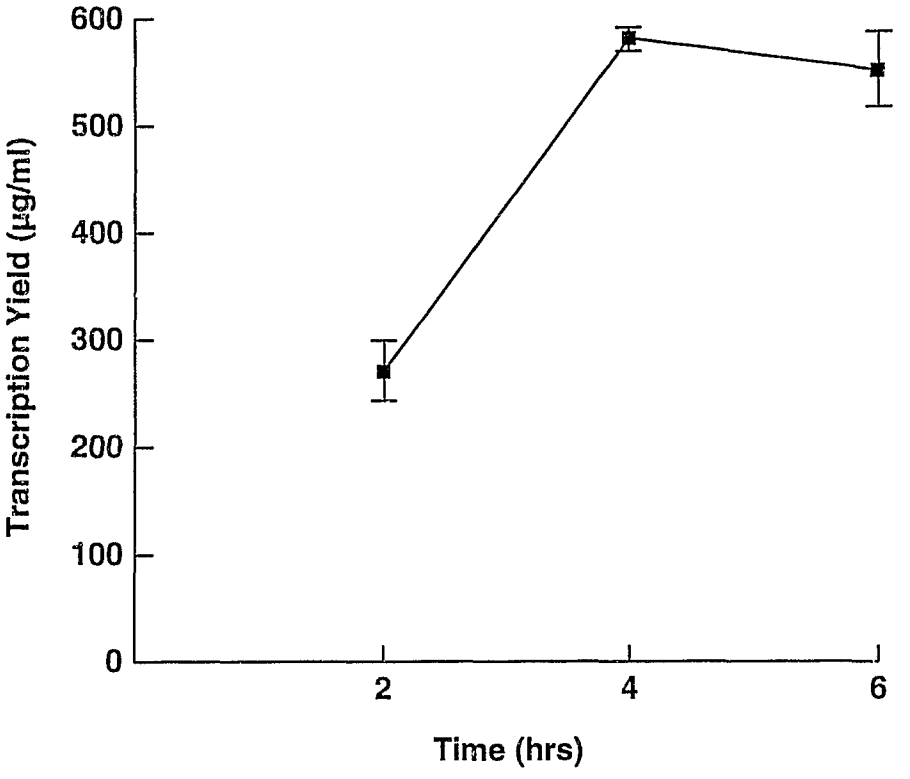

Figure 4. Time course experiment. RNA transcript yield was calculated from different time periods of incubation. Each reaction was performed three times and the mean of the RNA transcript yield was plotted.

Transcripts were first checked on 1% agarose gels (Figure 5) and then to confirm the size, they were checked by electrophoresis through gel containing denaturing formaldehyde. The transcript size of approximately 2.8 kb was obtained from pOHB 27-1 and pOHB 27-2 plasmids, which is approximately the size of segment B of IBDV (Figure 6).

2.3.3 *In vitro* translation product

Analysis of the *in vitro* translation reaction (in Rabbit Reticulocyte Lysate System) by SDS-PAGE followed by autoradiography indicated the presence of a protein approximately 80 kDa in reactions primed with the pOHB 27-1 transcript. The size of the protein is comparable to that of VP1 of IBDV (Kibenge *et al.*, 1988a). Also a number of smaller polypeptides were observed. As expected, no translation product was observed from the reaction primed with the minus-sense pOHB 27-2 transcript or the reaction with endogenous RNA only (Figure 7A).

The yield of VP1 as determined by SDS-PAGE was very low compared to the Luciferase control (Figure 7A). It was considered that the poor VP1 yield was either because of low mRNA concentration or inhibition by excess mRNA in the reaction. Therefore, *in vitro* translation reactions were done using different concentrations of the pOHB 27-1 transcript. No change in the translation yield of VP1 was observed when 1 μg, 2 μg, and 4 μg of transcripts were used (Figure 8).

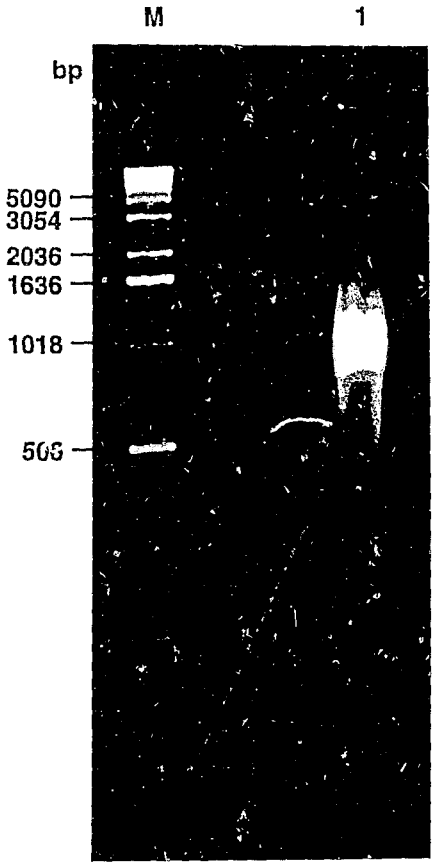

Figure 5. Agarose gel electrophoresis of RNA transcripts. Lane M contains DNA markers; lane 1 contains RNA transcript obtained from pOHB 27-1.

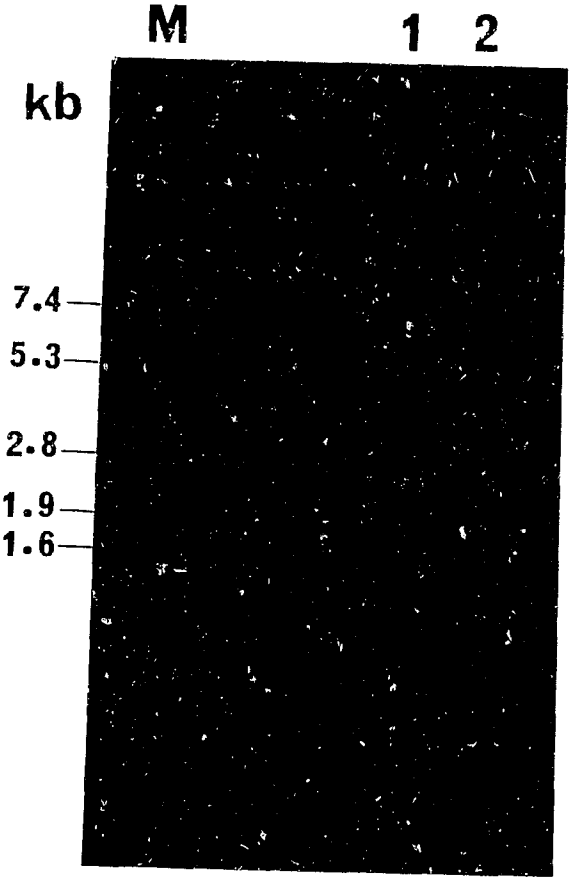

Figure 6. Electrophoresis of RNA transcripts through gel containing formaldehyde. Lane M contains RNA markers (kilobases (kb) are given on the left side); lane 1 contains plus-sense transcript from pOHB 27-1; lane 2 contains minus-sense transcripts obtained from pOHB 27-2.

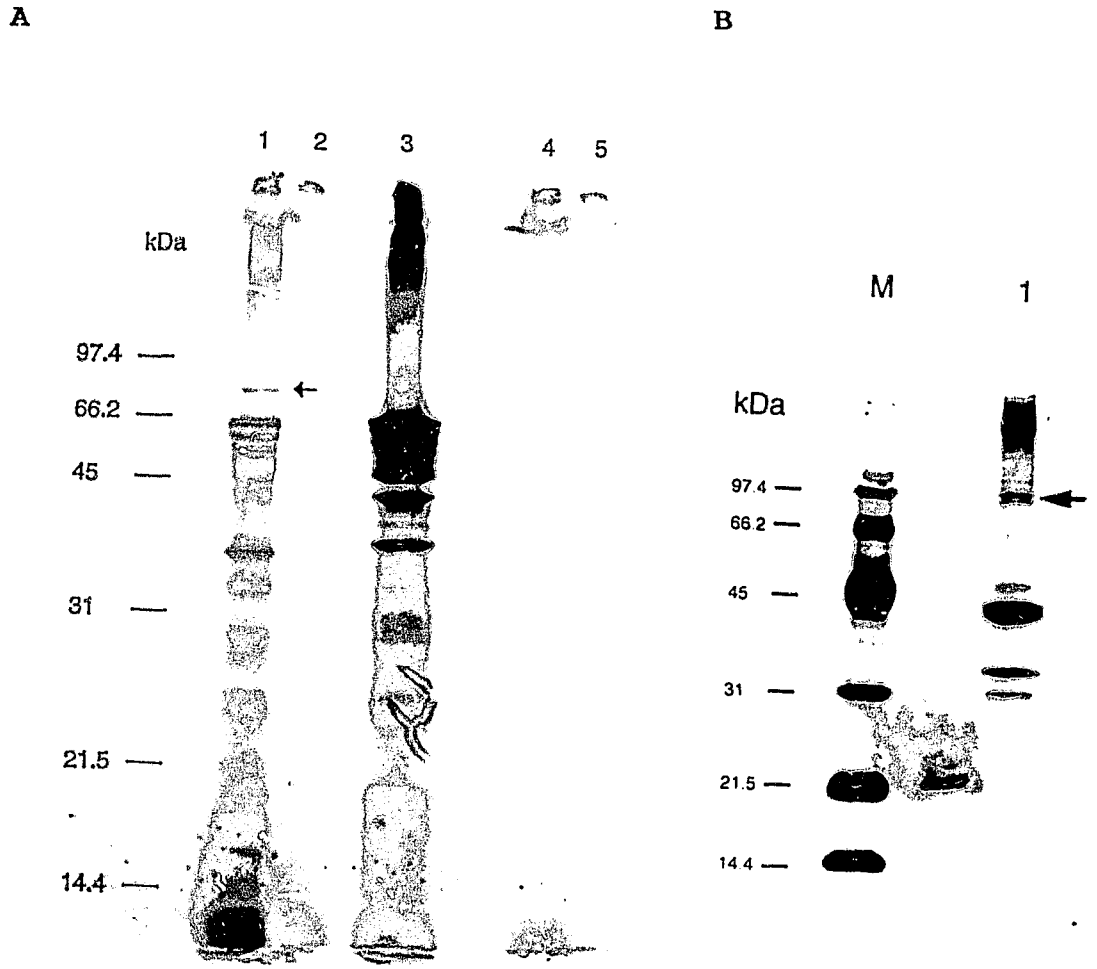

Figure 7. A) Autoradiograph of translation products after SDS-PAGE. Lane 1 contains translation product, VP1 (arrowed), obtained from the pOHB 27-1 transcript; lane 2 contains negative control, translation reaction with minus sense transcript from pOHB 27-2; lane 3 contains positive control, translation product (66kDa) obtained from luciferase transcript provided with the kit; lanes 4 and 5 contain only endogenous RNA i.e. "water only" negative control. Molecular weights are given on the left side. **B)** Purified IBDV analysed by SDS-PAGE and silver staining. Lane M contains molecular weight markers. The size of the markers are given on the left side; lane 1 contains purified IBDV proteins (VP1 arrowed).

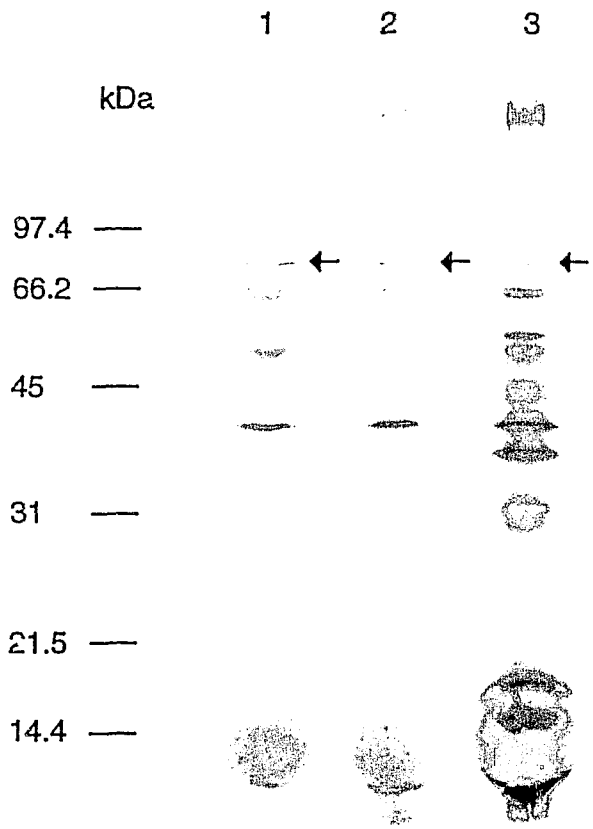

Figure 8. Autoradiograph of translation products with different concentrations of pOHB 27-1 transcript. Lane 1 contains translation reaction with 1 μg of RNA transcript of pOHB 27-1; lane 2 contains translation reaction with 2 μg of RNA transcript of pOHB 27-1; lane 3 contains translation reaction with 4 μg of RNA transcript of pOHB 27-1. VP1 is arrowed. Molecular weights are given on the left side.

2.4 Discussion

The experiments in this study examined the expression of the OH-IBDV segment B clone in a cell-free system by *in vitro* transcription and translation. Recombinant plasmids containing the complete ORF of VP1 of OH-IBDV (Qian and Kibenge, 1995) were used for the study.

After initial characterization of these plasmids by restriction enzyme digestion, linearized DNA templates were used for *in vitro* transcription. For this purpose, a commercially available transcription kit was used. Poor transcription yields were observed when the recommended protocol was followed. Melton *et al.* (1984) examined the optimal transcription conditions for linearized SP6 plasmid DNA, as a transcription template. The nucleotide concentration, enzyme and DNA concentration, incubation period and temperature were optimized in order to establish conditions for synthesizing large amounts of RNAs and for synthesizing RNAs at a high specific activity from DNAs cloned into the SP6 plasmid vectors (Melton *et al.*, 1984). Thus, based on that study, several factors can affect the RNA yield of *in vitro* transcription reaction. In the present study, the quality and quantity of the DNA template, nucleotide concentration, and the incubation period of the reaction were optimized. All possible efforts were also made to avoid any contamination of the template with RNase, and the purity and integrity of the DNA template were checked by spectrophotometric quantitation and electrophoresis,

respectively. Different concentrations of DNA template other than that specified did not have a notable effect on the transcription yield (data not shown). However, nucleotides when used in different concentrations showed a notable difference in the transcription yield. The maximum yield was achieved at 37.5 mM concentration of nucleotides as opposed to 75 mM recommended by the manufacturer. Time course experiments revealed that the maximum yield was achieved at 4 hours incubation as opposed to 2 hours recommended by the manufacturer. Two ssRNA, one plus-sense from plasmid pOHB 27-1 and the other minus-sense from pOHB 27-2 were obtained. The minus-sense transcript was used as a negative control in the *in vitro* translation reactions. Before *in vitro* translation the transcripts were checked for any degradation in a denaturing formaldehyde gel.

In vitro translation reactions were done in a rabbit reticulocyte lysate system. A protein product of approximately 80 kDa was obtained from the transcripts of plasmid pOHB 27-1, which is in accordance with the size of VP1 in strains OH, MO and SAL of IBDV (Kibenge *et al.*, 1988a). However, the widely accepted molecular weight of VP1 of IBDV is approximately 90 kDa (Morgan *et al.*, 1988; Macreadie and Azad, 1993). The difference in the molecular weights of the *in vitro* expressed VP1 and the VP1 obtained from the purified IBDV could be due to the absence of some extra molecules/ amino acids in the *in vitro* expressed VP1 which remains bound to VP1 obtained from purified IBDV.

There were a number of smaller polypeptides observed upon analysis in SDS-PAGE followed by autoradiography. Since there were no such polypeptides seen in translation of the minus-sense pOHB 27-2 transcript, the possibility of these being products of endogenous RNA was ruled out. The smaller proteins, some of comparable size to those in the pOHB 27-1 reaction, were also observed in the Luciferase control. Duncan *et al.* (1987) reported the production of several non-specific polypeptides when they expressed the IPNV segment A in a cell-free system. It was suggested that there could be certain factors present at suboptimal levels in reticulocyte lysates and that ribosome initiation can occur at multiple sites leading to appearance of several polypeptides in *in vitro* translations (Duncan *et al.*, 1987). Kozak (1989) reported that internal initiation events are common artifacts when translating viral mRNAs in cell-free lysate systems utilizing eukaryotic ribosomes.

Poor yields of VP1 and smaller proteins were previously reported in *in vitro* translation of the segment B clone of IPNV (Huang *et al.*, 1986); the smaller proteins were presumed to be premature translation products. This could be one of the reasons for the poor yields of VP1 observed in IPNV (Huang *et al.*, 1986) and in case of IBDV in the present study.

It has been shown that the relative amounts of the various translation products vary with the source of the RNA, the translation kit, and the salt concentration (Duncan *et al.*, 1987). Although the translation kit used was optimized for the Luciferase control, it is possible that the various conditions of this system were not suitable for translation of the pOHB 27-1 transcripts. This may explain why different concentrations of the pOHB 27-1 transcript had no effect on the yield of VP1 in the present study. It was considered that the weaker signal for VP1 compared to that of the Luciferase control on SDS-PAGE could be due to differences in the number of methionines in the 2 proteins since the labeling used [^{35}S] methionine. However, a rabbit reticulocyte lysate system using biotinylated lysine did not show any improved detection of VP1 (data not shown). Moreover, both, OH-IBDV VP1 (Dybing, 1992) and Luciferase have about 15 methionines each. Relatively poor expression of a polymerase gene compared to other viral protein genes in both *in vitro* and baculovirus expression systems was reported in case of rotaviruses (Liu *et al.*, 1992). This was suggested to be due to presence of a complex secondary structure at the 5'-terminus of the polymerase gene where the strong initiation codon of the gene is located and therefore its expression is regulated (Liu *et al.*, 1992). Perhaps the VP1 gene of IBDV is similarly regulated and therefore poorly expressed *in vitro*.

In conclusion, it has been established that the maximum transcription yield is obtained when 37.5 mM nucleotide concentration is used and the reaction is incubated for 4 hours at 37°C. Translation of the pOHB 27-1 transcript allowed the demonstration of VP1 of approximately 80 kDa by SDS-PAGE followed by autoradiography. However, the yield of VP1 in comparison to the Luciferase control was low and could not be increased by using different amounts of RNA transcript.

CHAPTER 3: ASSAY FOR ENZYMATIC ACTIVITIES ASSOCIATED WITH VP1 OF IBDV

3.1 Introduction

Segment B of IBDV encodes a minor internal polypeptide, VP1, which is present in the virions both in free as well as genome linked form. The genome linked form of VP1 may act as primer in initiation of the viral replication (reviewed in Chapter 1), demonstrated recently in IPNV (Dobos, 1995). VP1 of IBDV has been associated with RNA dependent RNA polymerase (RdRp) and guanylyl-transferase activities (reviewed in Chapter 1), therefore Spies and Müller (1990) suggested it to be a multifunctional enzyme.

A study of RdRp activity associated with IBDV particles has been reported (Spies *et al.*, 1987). Spies and Müller (1990) demonstrated the formation of the enzyme-guanylylate complex (VP1-GMP) in IBDV and proposed that VP1 could also act as guanylyl-transferase/capping enzyme. The VP1 of IPNV is not a capping enzyme (Dobos, 1993). However, this remains to be confirmed with IBDV associated VP1.

There are several other viral proteins which could be important in regulatory viral functions (reviewed in Chapter 1). VP4 has been shown to be a viral protease (Jagadish *et al.*, 1988). The basic C-terminal region of VP3 may be involved in

either packaging or stabilizing the RNA genome within the interior of the viral capsid. The functions of a recently reported minor polypeptide, VP5 (Mundt *et al.*, 1995), have not been characterized but it is speculated to have some regulatory functions. Because IBDV potentially contains other non-structural polypeptides, direct evidence implicating VP1 in the polymerase and guanylyl-transferase activities is lacking. Appropriate evidence would arise from molecular genetic studies with VP1 mutants, *in vitro* reconstitution with isolated VP1 or experiments utilizing cloned VP1 (Macreadie and Azad, 1993).

Various expression systems such as prokaryotic, eukaryotic, and *in vitro* cell-free systems could be used to produce large quantities of biologically active recombinant proteins (reviewed in Chapter 2). The VP1 gene of IBDV strain 002-73 was expressed in E. coli and S. cerevisae and tested for RdRp activity (Macreadie and Azad, 1993). However, no activity could be demonstrated in the VP1 obtained from either of the expression systems. Thus, so far, there is no conclusive data that could establish VP1's exclusive role as RNA dependent RNA polymerase. Also, there is no report of a guanylylation assay utilizing recombinant VP1 of birnaviruses.

In order to investigate VP1's direct role in the above mentioned activities, a recombinant clone of genome segment B of OH-IBDV was expressed in a cell-free system and examined for functions associated with VP1. The main aim of this

study was to assay for RNA dependent RNA polymerase and guanylyl-transferase activities in the *in vitro* expressed VP1 and to compare these activities with the virion-associated VP1. In addition, experiments were conducted to characterize the guanylyl-transferase activity in the IBDV-associated VP1.

3.2 Materials and Methods

3.2.1 Virus propagation and purification

Please see Section 2.2.2.

3.2.2 Viral genome extraction

The double-stranded (ds) RNA genome of IBDV was extracted and purified following the procedure described by Jackwood *et al.* (1989) with modifications. Briefly, OH-IBDV-infected Vero cells were disrupted by freezing and thawing three times. Cell-culture virus was clarified by pelleting of the cellular material by centrifugation at 2,060 x g for 20 minutes. The virus was then concentrated by ultracentrifugation at 140,000 x g for 4 hours using a Beckman SW 28 Ti rotor at 4°C. The pelleted virus was then resuspended in 300 µl TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Viral RNA was released from the virions by incubating with 2.0 mg/ml Proteinase K and 0.5% sodium dodecyl sulfate (SDS) for

2 hours at 37°C. RNA was extracted with phenol-chloroform and then ethanol precipitated. Cellular DNA was removed by RNase-free DNase I at a concentration of 100 units/mg of total nucleic acid for 1 hour at 37°C. The phenol-chloroform extraction and ethanol precipitation of RNA was repeated and the RNA pellet was resuspended in TSE buffer [50 mM Tris-HCl, pH 6.9, 100 mM NaCl, 1 mM EDTA, 35% (v/v) ethanol]. The dsRNA was passed through a CF-11 cellulose column to remove contaminating cellular ssRNA (Jackwood *et al.*, 1989). For this, a 5 ml syringe was plugged with silicone-treated glass wool and filled to 3 ml with sterile CF-11 cellulose powder (Whatman BioSystems Ltd, Maidstone, England). The column was hydrated with 5 ml of TSE buffer and the sample was loaded on the top. The ssRNA was eluted by two 5 ml washes with TSE buffer containing 15% (v/v) ethanol, after which the dsRNA was eluted in 4 ml of TSE buffer and precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of cold absolute ethanol. The dsRNA was quantitated by UV spectrophotometry and its purity was confirmed by 1% agarose gel electrophoresis.

3.2.3 *In vitro* translation reaction

In vitro translation reactions used the Rabbit Reticulocyte Lysate System (Promega). The following reaction mixture was mixed gently with a pipet as each component was added to a final volume of 50 µl: 35 µl rabbit reticulocyte lysate, 7 µl nuclease-free water, 1 µl 40u/µl RNasin ribonuclease inhibitor, 1 µl 1 mM amino acid

mixture containing cold methionine (instead of [^{35}S]-methionine so as to avoid any false positives during the enzymatic assays), 2 µg RNA substrate in nuclease-treated water. The translation mixture was incubated for 60 minutes at 30°C and stored at -20°C.

3.2.4 *In vitro* guanylylation assay

The *in vitro* guanylyl-transferase assays were done as previously described by Dobos (1993). Briefly, a 25 µl reaction in 10 mM Tris-HCl, pH 8.0, contained 10-15 µl of *in vitro* translation reaction mixture containing VP1 or 10-15 µg of purified IBDV particles, and 20µCi [α^{32}P]-GTP (sp.act. 3000 Ci/mmol; Amersham Canada Limited, Oakville, Ont). After incubation in a 37°C water bath for 10 minutes, the reaction was stopped by the addition of an equal volume of 2X electrophoresis sample buffer (ESB; 1X ESB: 50 mM Tris-HCl, pH 6.7, 2% SDS, 5% 2-ME, 10% glycerol, and a trace of bromophenol blue) followed by immersion in a boiling water bath for 5 minutes. Analysis of guanylylation of VP1 was by SDS-PAGE followed by autoradiography.

3.2.4.1 GTP-binding competition and replacement assays

These assays followed the general strategy developed by Dobos (1993) for VP1 of IPNV. The GTP-binding competition assay was performed exactly as the *in vitro* guanylyl-transferase assay (Section 3.2.5) except that 1 volume and 4 volumes of

unlabeled GTP were premixed with [α^{32}P]-GTP before adding to the reaction mixture. In the replacement assay, 1 volume and 4 volumes of unlabeled GTP were added to the [α^{32}P]-GTP-virus after an initial 15 minutes at 37°C. This preparation was incubated for an additional 15 minutes at 37°C before analysis by SDS-PAGE and autoradiography.

3.2.5 RNA dependent RNA polymerase (RdRp) assay

The RNA dependent RNA polymerase assay was performed as previously described by Spies *et al.* (1987) with the exception that [^{32}P]-UTP was utilised (Macreadie and Azad, 1993) instead of [^{3}H]-UTP. Briefly, transcription reactions were performed in 100 µl volumes containing 100 mM Tris-HCl (pH 8.5), 100 mM NaCl, 4 mM $MgCl_2$, 0.01 mM EDTA, 1 mM each ATP, GTP, CTP, 0.02 mM UTP (Boehringer Mannheim), 10µCi [^{32}P]-UTP (Amersham Canada), 1% Triton X-100, 50 U placental ribonuclease inhibitor (Bio-Can, Mississuaga, Ont), and 30-40 µg of purified IBDV particles or 35 µl of *in vitro* translation reaction mixture containing VP1 plus 10-12 µg of the IBDV dsRNA. The reaction mixtures were incubated at 40°C for 90 minutes. Analysis of incorporation of [^{32}P]-UTP into RNA was by electrophoresis in 1% agarose gels and autoradiography.

3.3 Results

3.3.1 Characterization of guanylylation activity associated with purified IBDV particles

3.3.1.1 Effect of Mg^{++} ions and temperature

Previous studies done on guanylylation activity associated with IBDV had utilized different concentration of Mg^{++} ions and incubation temperatures (Spies and Müller, 1990; Dobos, 1993). Spies and Müller (1990) performed the reactions at 42°C with 5 mM $MgCl_2$ after preincubation of the virus particle at 37°C for 5 minutes whereas Dobos (1993) did guanylylation reactions at 37°C with either 7 mM of $MgCl_2$ or without any $MgCl_2$. In the present study, experiments were conducted to determine and optimize the reaction conditions for Mg^{++} ions and incubation temperature.

Initially, the guanylylation reaction with purified IBDV particles was incubated at 42°C and 37°C in the presence of 5 mM and 7 mM $MgCl_2$. The analysis of the guanylylation reaction in SDS-PAGE revealed that radioactivity was incorporated into a high-molecular-weight protein of similar migration as VP1. To confirm that the incorporated protein was VP1, purified IBDV was run in a parallel lane of the same gel (Figure 9). Part of the gel containing purified IBDV and protein molecular weight markers was separated by cutting from the rest of gel and the proteins were

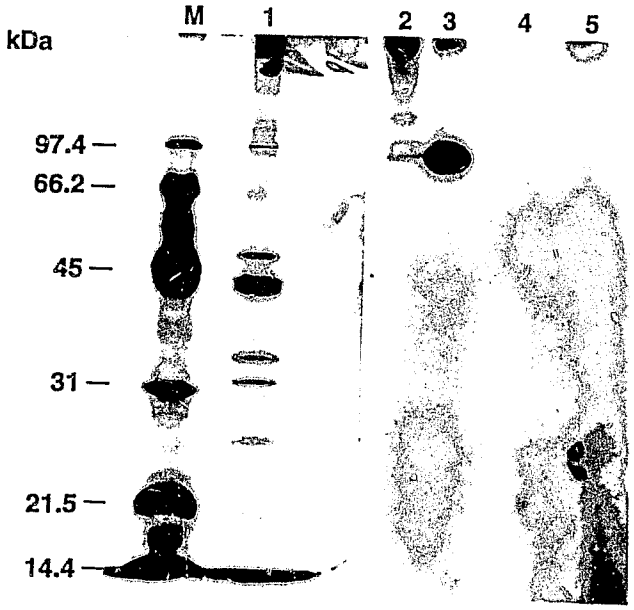

Figure 9. Autoradiograph of *in vitro* guanylylation product in the presence of Mg^{++} ions. Lane M contains molecular weight markers; lane 1 contains purified IBDV; lane 2 contains guanylylation product utilizing virus-associated VP1 at 37°C; lane 3 contains guanylylation product utilizing virus-associated VP1 at 42°C; lane 4 contains guanylylation product utilizing *in vitro* expressed VP1 at 37°C; lane 5 contains guanylylation product utilizing *in vitro* expressed VP1 at 42°C.

detected by silver staining. The silver stained gel was again realigned with the rest of gel containing the guanylylation samples and dried. The autoradiograph of the dried gel indicated a good incorporation of radioactivity into VP1 in reactions incubated at 42°C in the presence of 5 mM $MgCl_2$. The incorporation of the radioactivity into VP1 was very low at 37°C in the presence of 7 mM $MgCl_2$ (Figure 9). In addition, a "ladder" of bands was observed in samples from both reactions. Radioactive material was also trapped at the top of the gels and this varied between experiments although it was more commonly seen in reactions incubated at 37°C (Figure 9 and 10).

Guanylylation reactions were also conducted in the absence of $MgCl_2$ at 37°C and 42°C. A good incorporation of radioactivity into VP1 was observed in both reactions (Figure 10A). A "ladder" of bands was also detected in both reactions. However, when autoradiography was done for 4 hours instead of overnight, the "ladder" of radioactivity was not detected (Figure 10B). Subsequent guanylylation reactions were performed without $MgCl_2$ at 37°C and autoradiography was done for 4 hours.

3.3.1.2 Effect of boiling

It was considered that disruption of the virion capsid by boiling would free VP1, and therefore result in increased incorporation of radioactivity into VP1. Thus,

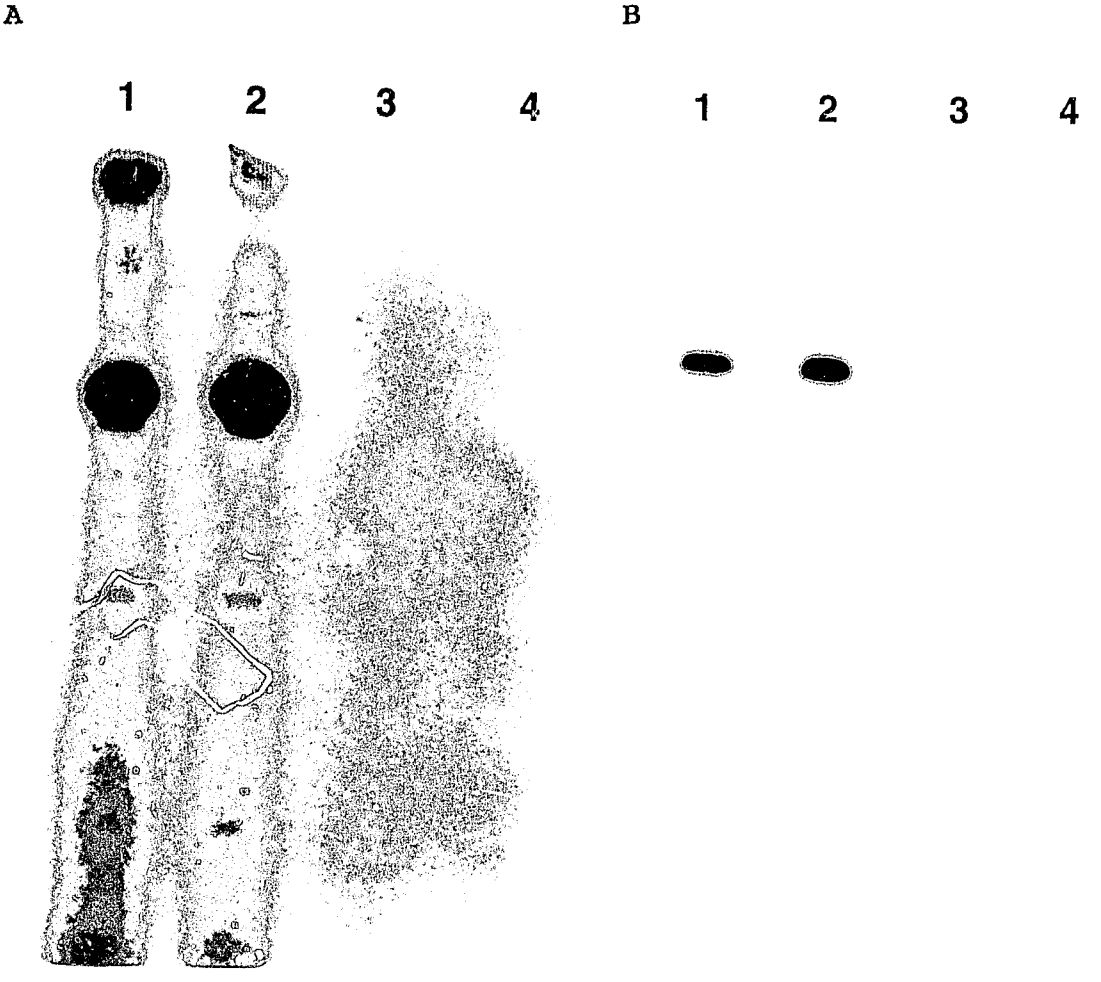

Figure 10. Autoradiographs of *in vitro* guanylylation product in the absence of Mg^{++} ions. Lane 1 contains guanylylation product utilizing virus-associated VP1 at 37°C; lane 2 contains guanylylation product utilizing virus-associated VP1 at 42°C; lane 3 contains guanylylation product utilizing *in vitro* expressed VP1 at 37°C; lane 4 contains guanylylation product utilizing *in vitro* expressed VP1 at 42°C. **A)** overnight exposure; **B)** four hours exposure. Unlabeled molecular weight markers were used and therefore could not be detected in the autoradiograph.

efficient GTP binding would occur even at low virus concentrations. However, preincubation of the virus at 100°C for 5 minutes resulted in loss of guanylylation activity (Figure 11).

3.3.1.3 Competition and replacement reactions assay

For VP1 to function as a guanylyl-transferase, it must be able to transfer the GMP moiety of VP1-GMP complex to an acceptor molecule. To study the nature of the guanylylation activity of the VP1 of IBDV, competition and replacement reactions were carried out. When increasing amounts of cold GTP (unlabeled GTP) were added to the guanylylation reactions, there was a dramatic decrease in the radioactivity associated with VP1 (Figure 12A) suggesting that there was competition between radioactive and cold GTP for binding with VP1. However, when the increasing amount of cold GTP was added after the preincubation of guanylylation reaction for 10 minutes, the radiolabel was not replaced from IBDV VP1-GMP complex (Figure 12B), suggesting a lack of transferase activity associated with VP1.

3.3.2 Guanylylation assay of *in vitro* expressed VP1

No radiolabeled product was detected when *in vitro* expressed VP1 was incubated in the presence of [α^{32}P]-GTP at 37°C for 10 minutes, followed by PAGE and

Figure 11. Autoradiograph showing the effect of boiling on *in vitro* guanylylation. Lane 1 contains guanylylation product utilizing IBDV associated VP1; lane 2 contains guanylylation product utilizing boiled IBDV. Unlabeled molecular weight markers were used and therefore could not be detected in the autoradiograph.

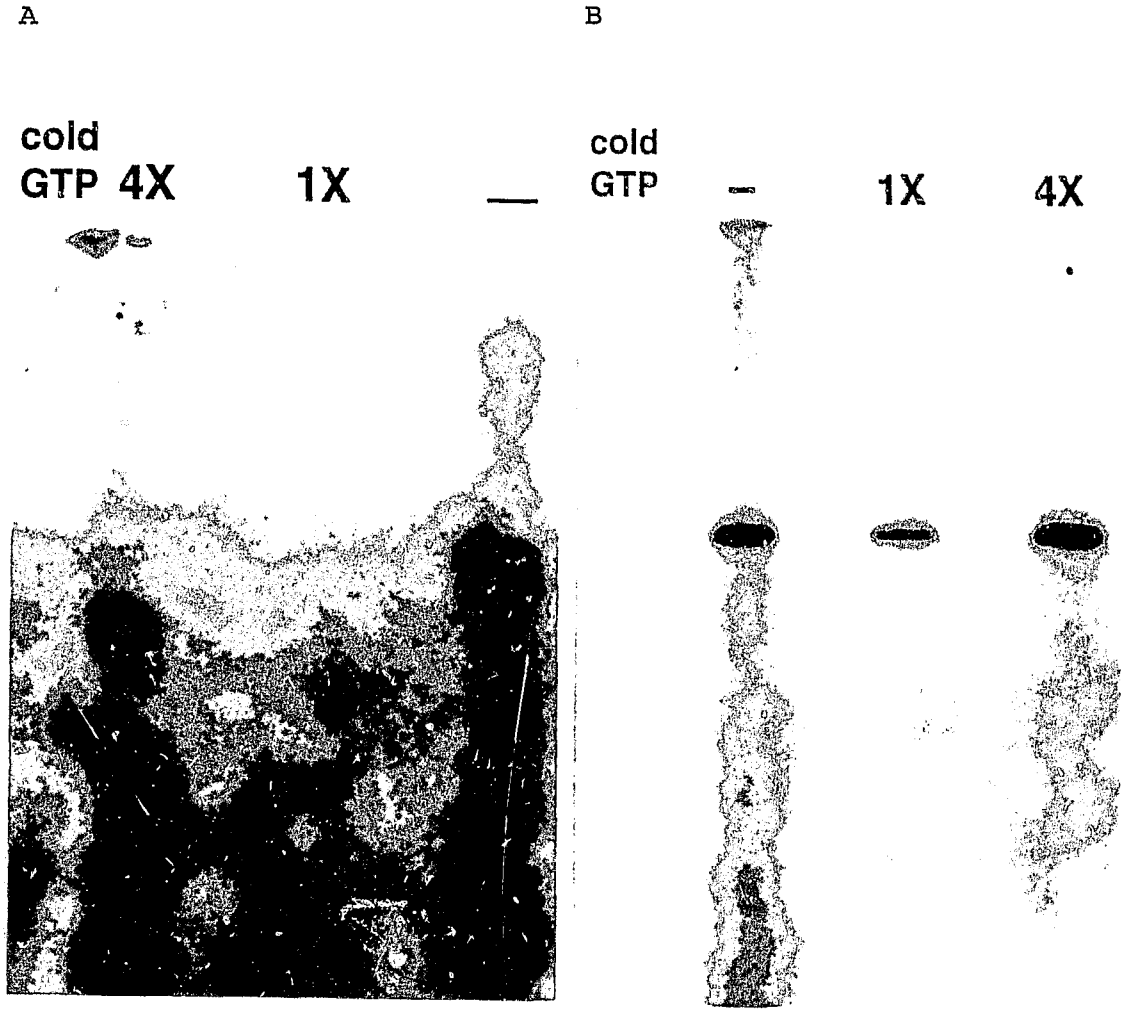

Figure 12. Autoradiographs of competition and replacement assay products. A) Effect of competition on guanylylation product with 1X and 4X amounts of cold GTP, respectively. B) The failure to replace the bound labeled GMP by added cold GTP in 1X and 4X amounts, respectively. The "-" lanes in both A and B are the controls containing labeled GTP only.

autoradiography. Incubation at 37°C or 42°C in presence of 7 mM or 5 mM $MgCl_2$ and in absence of $MgCl_2$ had no effect on the reactions (Figures 9 and 10).

It was considered that it may be possible to demonstrate the guanylylation of *in vitro* expressed VP1 in the presence of other core viral proteins. However, incubation of the *in vitro* expressed VP1 with boiled IBDV particles in the guanylylation reaction resulted in no observable guanylylation activity (Figure 13).

Experiments were also conducted to determine if the viral genome was essential for guanylylation of VP1, and to see if the reaction with *in vitro* expressed VP1 contained factors that inhibited with the guanylylation reaction. Double stranded RNA of the IBDV genome purified by CF-11 cellulose column chromatography (Figure 14) was incubated with the *in vitro* expressed VP1 in the guanylylation reaction, however no guanylylation was observed (Figure 13). Incubation of the *in vitro* expressed VP1 in the presence of purified IBDV particles was found to significantly inhibit guanylylation of virion-associated VP1 (Figure 13).

3.3.3 Assay of RdRp activity of VP1

To assay for RNA dependent RNA polymerase activity, the *in vitro* expressed VP1 and dsRNA genome of IBDV were incubated in the presence of rNTPs and [^{32}P]-UTP at 40°C for 90 minutes, followed by agarose gel electrophoresis and

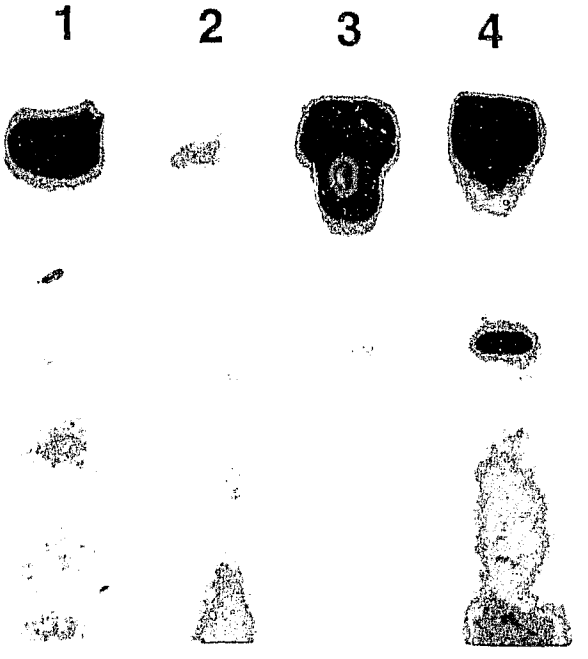

Figure 13. Autoradiograph showing the effect of *in vitro* synthesized VP1. Lane 1 contains guanylylation product utilizing *in vitro* synthesized VP1 and boiled IBDV; lane 2 contains guanylylation product utilizing *in vitro* synthesized VP1 and dsRNA; lane 3 contains the guanylylation product utilizing *in vitro* synthesized VP1 and purified IBDV particles; lane 4 contains guanylylation product utilizing virus associated VP1. Unlabeled molecular weight markers were used and therefore could not be detected in the autoradiograph.

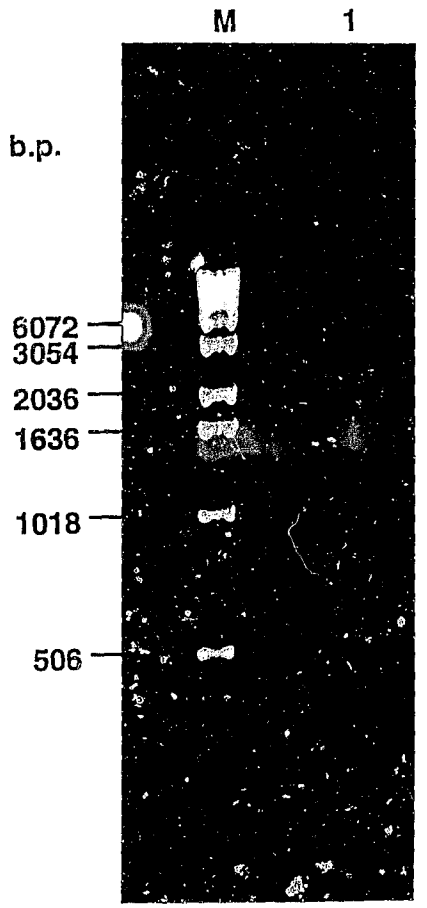

Figure 14. dsRNA genome of IBDV. Lane M contains the DNA markers; lane 1 contains the two segments of the IBDV genome.

autoradiography. No incorporation of radioactivity was observed suggesting that there was no new transcript synthesized (Figure 15). The purified IBDV particles by themselves showed a good incorporation of radioactivity indicating newly synthesized transcripts (Figure 15).

Newly synthesized birnavirus RNA has previously been shown to be attached to the virus and can be released by treatment with proteinase K (Spies *et al.*, 1987). In the present study, this phenomenon was confirmed by both proteinase K treatment as well as boiling post-incubation. In both cases, increased migration of the newly synthesised RNA in the gel was observed (Figure 16).

Failure to demonstrate the polymerase activity in the *in vitro* expressed VP1 may have been due to presence of inhibitory factors in the *in vitro* translation mix as demonstrated for the guanylylation reaction. Thus, IBDV particles were mixed with the *in vitro* expressed VP1 and assayed for RdRp activity. It was found that polymerase activity of virion-associated VP1 was almost completely lost in the presence of the *in vitro* expressed VP1 reaction mixture (Figure 17).

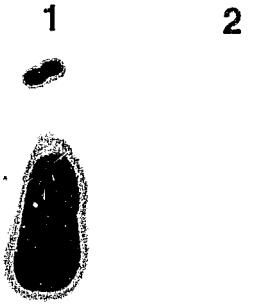

Figure 15. Autoradiograph of *in vitro* polymerase assay product. Lane 1 contains polymerase assay product utilizing purified IBDV; lane 2 contains polymerase assay product utilizing *in vitro* expressed VP1. Unlabeled molecular weight markers were used and therefore could not be detected in the autoradiograph.

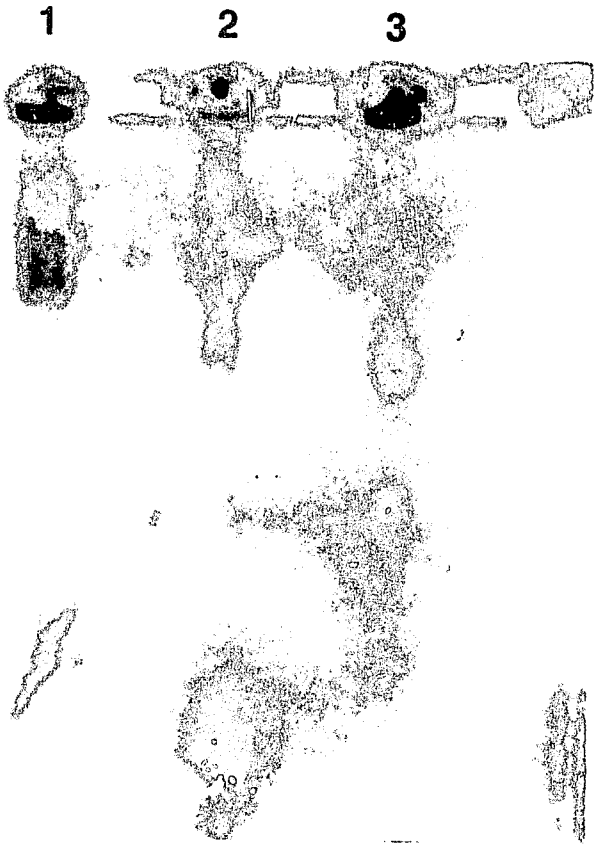

Figure 16. Autoradiograph showing the effect of boiling and proteinase K treatment. Polymerase reactions utilizing IBDV were either proteinase K treated or boiled after the completion of the reaction. Lane 1 contains polymerase assay product, positive control; lane 2 contains boiled polymerase assay product; lane 3 contains proteinase K treated polymerase assay product. Unlabeled molecular weight markers were used and therefore could not be detected in the autoradiograph.

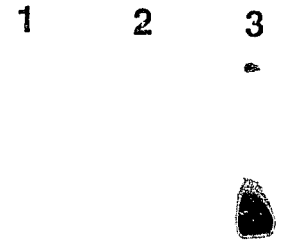

Figure 17. Autoradiograph of RdRp assay product utilizing *in vitro* expressed VP1. Lane 1 contains polymerase assay product utilizing *in vitro* expressed VP1 and IBDV particles; lanes 2 and 3 contain polymerase assay products utilizing *in vitro* synthesised VP1 and IBDV particles, respectively. Unlabeled molecular weight markers were used and therefore could not be detected in the autoradiograph.

3.4 Discussion

In this study the guanylyl-transferase and polymerase activities of VP1 of IBDV were examined. Both purified virus and *in vitro* expressed VP1 were used for the study in order to implicate VP1 to guanylyl-transferase and RdRp activities (see Chapter 2).

There are reports on guanylylation of a recombinant viral protein of African swine fever virus expressed in E. coli (Pena *et al.*, 1993), rotavirus VP3 expressed in baculovirus expression system (Liu *et al.*, 1992) and reovirus λ2 expressed in vaccinia virus expression vector (Moa and Joklik, 1991). For the first time efforts were made to demonstrate a guanylylation reaction utilizing a recombinant VP1 of a birnavirus. A clone of IBDV genome segment B was expressed *in vitro* and used for the guanylylation assay. However, no GTP binding activity of VP1 could be demonstrated in the *in vitro* expressed product even when temperature and salt optimization reactions were performed. To see if the presence of viral genome or core viral proteins could have any effect, guanylylation reactions in the presence of dsRNA and boiled IBDV, respectively, were carried out. Again, the binding of GTP to the recombinant VP1 was not seen. The *in vitro* expressed VP1 was examined for the presence of factors that may inhibit the guanylylation reaction. A significant decrease in the guanylylation of purified IBDV was observed when the *in vitro*

expressed VP1 was included in the reaction, suggesting the presence of inhibitory factors in the reaction mixture.

There could be several other factors which might be involved in failure of the guanylylation of *in vitro* expressed VP1. As indicated in Chapter 2, high levels of VP1 could not be obtained in the cell-free system used. Sub-optimal amounts of VP1 could cause failure of the experiment. One of the possibility for this could be due to the unstability of transcripts in the *in vitro* translation reaction mixture. Freezing might lead to the loss of enzyme activity, however, the activity could not be demonstrated even when the assays were done immediately after the *in vitro* translation reactions. Liu *et al.* (1992) expressed the GTP binding protein of rotaviruses *in vitro* in a cell-free system along with expression in a baculovirus expression system. These authors did not mention any guanylylation activity using the *in vitro* expressed product although guanylylation of the product expressed in the insect cells was successful. The lack of post-translation modifications during *in vitro* VP1 synthesis could be another reason for lack of guanylylation activity. The relatively smaller size of the *in vitro* expressed VP1 suggests that probably there is some extra molecule/ amino acids missing which could have some role in this activity. It is also possible that formation of VP1-GMP linkage in birnaviruses may require the presence of intact virions. The requirement for intact virions for guanylylation activity may explain the observation made in this study that the

guanylylation could not be demonstrated in the boiled IBDV particles. To date, there is no report on guanylylation activity associated with any recombinant viral protein expressed in a cell-free system.

Although there is a detailed report on characterization of guanylylation activity in the case of VP1 of IPNV (Dobos, 1993), this remains to be characterized in detail in VP1 of IBDV. In the past, guanylylation reactions for IBDV VP1 were carried out at different temperatures and varying salt conditions in different labs (Dobos, 1993; Spies and Müller, 1990). In the present study, several guanylylation reactions were done at different temperatures and salt conditions. Guanylylation was carried out at 37°C and 42°C, respectively, and the effect of Mg^{++} at both temperatures was studied. When Mg^{++} was used, guanylyation was very weak at 37°C compared to guanylylation at 42°C. Whether the GTP binding occured specifically with VP1 was confirmed by running SDS-PAGE of purified virus in a parallel lane. It was observed that the enzyme-GMP complex migrated to exactly the same level as VP1 of IBDV (Figure 9). Thus, VP1 is the protein that binds to the GTP. These results are in agreement with previous studies (Dobos, 1993; Spies and Müller, 1990). In addition, other bands were observed. Dobos (1993) also reported the appearance of such bands ("ladder") in both IPNV and IBDV. These bands were presumed to be VP1-associated genomic dsRNA or products of RdRp activity of virion-associated VP1 since they were degradable by RNase treatment (Dobos, 1993). In the present

study, guanylylation reactions were also performed both at 37°C and 42°C in the absence of Mg^{++} ions. Good VP1-GMP linkage was observed at both 37°C and 42°C indicating that, unlike rotaviruses where the presence of divalent ions (Mn^{++} or Mg^{++}) is a prerequisite for formation of an enzyme guanylate complex (Liu *et al.*, 1992; Pizzaro *et al.*, 1991), no divalent ions are required for guanylylation of birnavirus VP1. This was previously reported for both IBDV and IPNV at 37°C (Dobos, 1993); the present study shows that it is also true for guanylylation assay carried out at 42°C. Dobos (1993) also reported that the extra bands were not observed in the absence of salt. He suggested that these extra bands were due to Mg^{++} ions which allowed for virion-associated RdRp activity during the 10 minute labeling period, in the presence of residual precursors which remained in purified virions. However, in the present study, the extra bands were present even in the absence of Mg^{++} ions both at 37°C and 42°C. Therefore, the exact reason for the presence of extra bands in guanylylation reactions remains to be determined. In summary, good incorporation of radioactivity into VP1 was obtained both at 37°C and 42°C without the Mg^{++} ions and at 42°C with Mg^{++} ions. However, guanylylation was poor at 37°C in the presence of Mg^{++} ions.

The effect of boiling on the guanylylation reaction was also studied. Boiling could disrupt the virus capsid and it was considered that perhaps there could be increased guanylylation with free VP1. Boiling alone was preferred over boiling in the

presence of SDS because SDS could lead to the permanent denaturation of the proteins. However, no VP1-GMP linkage was observed. Perhaps this could be due to alterations in VP1's secondary and tertiary structure by boiling. It is also possible that the GTP binding to VP1 in birnaviruses requires intact virus particles.

Is VP1 of IBDV a capping enzyme? Spies and Müller (1990) reported the formation of IBDV VP1-guanylylate complex, which indicated that perhaps VP1 has guanylyl-transferase activity. It was also reported that this GTP linkage to the VP1 is by a phosphodiester bond which is in contrast to phosphoamide bonds formed by reovirus guanylyl-transferase. Further studies on characterization of this activity in IPNV VP1 proved that VP1 is not a capping enzyme (Dobos, 1993). This was confirmed in the present study with IBDV by carrying out competition and replacement assays. As Dobos (1993) indicated, it was found that when increasing amounts of cold GTP were added to the guanylylation reactions, there was a dramatic decrease in the radioactivity associated with VP1. The high concentration of cold GTP outcompeted the labeled GTP in the reaction yielding almost exclusively unlabeled VP1-GMP complexes. Another interpretation of this competition assay may be that the guanylylation of VP1 is reversible (Dobos, 1993). This was tested by allowing the labeled guanylylation reaction to take place for 15 minutes followed by the addition of increasing concentrations of cold GTP to each reaction and incubating for an additional 15 minutes before boiling and processing

for PAGE and autoradiography. However, the radiolabel was not removed from the IBDV VP1-GMP even after incubation with excess of cold GTP. This result indicates that VP1 of IBDV like VP1 of IPNV cannot transfer the labeled GMP to an acceptor molecule such as GTP. Therefore VP1 of IBDV does not appear to function as guanylyl-transferase enzyme. It is possible that the failure to demonstrate a reversible guanylylation of VP1 may be related to the reaction system and the absence of a required acceptor molecule (ie mRNA).

Another VP1 associated activity studied was RNA dependent RNA polymerase. A comprehensive study was previously done on polymerase activity associated with purified IBDV (Spies *et al.*, 1987). In the present study the RdRp activity was successfully demonstrated in the purified IBDV. Earlier studies indicated the attachment of newly synthesized RNA to the protein (Macreadie and Azad, 1993). This was confirmed by both boiling and proteinase K treatment of polymerase reactions before gel electrophoresis. Transcripts from both boiled and proteinase K treated samples migrated faster in the gel than the untreated one, indicating that indeed the newly synthesized RNA was attached to the protein,

Although there are several properties of VP1 that link it to the polymerase activity (reviewed in Chapter 1), there is no direct evidence that can implicate VP1 to this activity. Macreadie and Azad (1993) produced the recombinant VP1 of IBDV in

E. coli and S. cerevisiae and tried to demonstrate the polymerase activity in these preparations. However, no conclusive evidence could be demonstrated in either of the expression systems. In the present study, efforts were made to demonstrate the polymerase activity with the *in vitro* translated VP1 but without success. When polymerase reactions utilizing purified IBDV were carried out in the presence of *in vitro* translated VP1, the polymerase activity associated with IBDV was almost completely lost. Therefore, the presence of inhibitory factor may explain why the polymerase activity could not be demonstrated in the *in vitro* expressed VP1. In addition, insufficient amounts of the protein or improper folding or lack of some additional amino acids/ molecules to the protein expressed *in vitro* could also be other important factors. Pizarro *et al.* (1991) reported that rotavirus transcription involves other associated enzymatic activities present in the viral core, in addition to the RNA polymerase activity. In IBDV, a recent report confirmed the presence of a minor polypeptide, VP5, whose functions are not known although these small size proteins are thought to possess some regulatory functions in viral replication (Mundt *et al.*, 1995). It is possible that the polymerase activity in IBDV and other birnaviruses may require the presence of other viral proteins such as VP5. In IPNV a protein-primed RNA synthesis has been demonstrated (Dobos, 1995) which suggests the necessity of a genome linked VP1 in the formation of transcripts. There is a possibility that the *in vitro* expressed VP1 might not have attached to dsRNA IBDV genome during the *in vitro* polymerase assays, particularly if the 5' ends of

the viral RNA are blocked by residual amino acids of VP1, and therefore it is not surprising that the RdRp activity could not be demonstrated for the *in vitro* translated VP1. Liu *et al.* (1992) reported that in rotaviruses the precise roles of various proteins in RNA transcription have been difficult to prove because single shelled particles must be structurally intact to possess RNA polymerase activity. This could also be true for birnaviruses. However, this remains to be investigated, particularly when it has been shown that unlike rotaviruses and reoviruses there is no guanylyl-transferase activity associated with IBDV or other birnaviruses even though they are both dsRNA viruses. Also a recent confirmation that birnaviruses are the only dsRNA viruses exhibiting protein-primed RNA synthesis (Dobos, 1995) suggests that there are some fundamental differences involved in their replication.

In conclusion, the guanylyl-transferase activity associated with IBDV has been characterized in detail using the purified virus. It was confirmed that VP1 of IBDV, like that of IPNV, does not possess guanylyl-transferase activity. Attempts to demonstrate the guanylylation and polymerase activities with *in vitro* expressed VP1 of IBDV were not successful. The possible reasons for this have been discussed. Since it has been demonstrated that VP1 of IBDV is not a guanylyl-transferase/capping enzyme therefore it may not be a multifunctional enzyme as previously suggested by Spies and Müller (1990).

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

In brief restatement of the purpose of the research, our laboratory has initiated studies to define the molecular determinants of the viral virulence, antigenicity, and functional characterization of the mutations in IBDV. As a part of these studies, the complete cDNA of segment B ORF of OH-IBDV was constructed in an *in vitro* transcription vector. The purpose of the work reported in this thesis was to compare the functional activities, RdRp and guanylyl-transferase, of IBDV VP1 expressed in a cell-free system with the virion-associated VP1.

The experiments were conducted towards the expression of the OH-IBDV segment B clone in a cell-free system by *in vitro* transcription and translation. Recombinant plasmids containing the complete ORF of VP1 of OH-IBDV were constructed in our laboratory (Qian and Kibenge, 1995). After initial characterization of these plasmid by restriction enzyme digestion, linearized DNA templates were used for *in vitro* transcription. Initially, the yield of the transcripts was poor. However, upon optimization of nucleotide concentration and incubation temperature of the transcription reaction, optimum yield of full length transcripts were obtained. These transcripts were further used for *in vitro* translation using the Rabbit Reticulocyte Lysate System.

Upon *in vitro* translation a protein of approximately 80 kDa was obtained from the transcripts of plasmid pOHB 27-1, which is in accordance with the size of VP1 in strains OH, MO and SAL of IBDV (Kibenge *et al.*, 1988a). However, the yield of VP1 compared to the Luciferase control was low. As expected, no translation product was observed in the reactions utilizing pOHB 27-2 obtained transcripts, since the transcripts were of minus-sense.

Besides VP1, there were a number of smaller polypeptides observed upon analysis in SDS-PAGE followed by autoradiography. Poor yields of VP1 and smaller proteins were also previously reported in *in vitro* translation of the segment B clone of IPNV (Huang *et al.*, 1986), the smaller proteins were presumed to be premature translation products. This could be one of the reasons for the poor yields of VP1 observed in IPNV (Huang *et al.*, 1986) and in case of IBDV in the present study. Alternatively, the poor yield of VP1 could be due to the Rabbit Reticulocyte Lysate Systems used; perhaps the reaction conditions were not optimum for the large scale translation from plasmid pOHB 27-1. In the present study two types of rabbit reticulocyte systems were used, one utilized [^{35}S] labeled amino acid and the other utilized biotinylated amino acid. However, in both systems a very weak VP1 band was observed. Transcript concentration did not have any effect on the protein yield. Relatively poor expression of a polymerase gene compared to other viral protein genes in both *in vitro* and baculovirus expression systems was reported in the case

of rotaviruses (Liu *et al.*, 1992). This was suggested to be due to the presence of a complex secondary structure at the 5'-terminus of the polymerase gene where the strong initiation codon of the gene is located and therefore its expression is regulated (Liu *et al.*, 1992). Perhaps the VP1 gene of IBDV is similarly regulated and therefore poorly expressed *in vitro*.

The second objective of the present study was to examine the guanylyl-transferase and RdRp activities of VP1 of IBDV. Both purified virus and *in vitro* expressed VP1 were used for the study in order to implicate VP1 to guanylyl-transferase and RdRp activities.

The report on characterization of guanylylation activity in case of VP1 of IPNV (Dobos, 1993) did not characterize VP1 of IBDV in detail. Attempts were made to optimize the temperature and salt conditions for the guanylylation reaction. Guanylylation was carried out at 37°C and 42°C, respectively, and the effect of Mg^{++} ions at both temperatures was studied. When Mg^{++} ions were used, guanylylation was very weak at 37°C compared to guanylylation at 42°C. Guanylylation reactions were also performed both at 37°C and 42°C in the absence of Mg^{++} ions. Good VP1-GMP linkage was observed at both 37°C and 42°C indicating that, unlike rotaviruses where the presence of divalent ions (Mn^{++} or Mg^{++}) is a prerequisite for formation of an enzyme guanylylate complex (Liu *et al.*, 1992; Pizzaro *et al.*, 1991), no

divalent ions are required for guanylylation of birnavirus VP1. Also the effect of boiling on the guanylylation reaction was studied. Boiling could disrupt the virus capsid and it was considered that perhaps there could be increased guanylylation with free VP1. However, no VP1-GMP linkage was observed.

The question, whether VP1 of IBDV is a capping enzyme was answered by the same strategy as was adopted by Dobos (1993) for VP1 of IPNV. Competition and replacement assays were carried out using different concentrations of cold GTP. It was found that when increasing amounts of cold GTP were added to the guanylylation reactions, there was a dramatic decrease in the radioactivity associated with VP1. The high concentration of cold GTP outcompeted the labeled GTP in the reaction yielding almost exclusively unlabeled VP1-GMP complexes. Another interpretation of this competition assay may be that the guanylylation of VP1 is reversible (Dobos, 1993). This was tested by allowing the labeled guanylylation reaction to take place for 15 minutes followed by the addition of increasing concentrations of cold GTP to each reaction and incubating for an additional 15 minutes before boiling and processing for PAGE and autoradiography. However, the radiolabel was not removed for IBDV VP1-GMP even after incubation with an excess of cold GTP. This result indicates that VP1 of IBDV like VP1 of IPNV cannot transfer the labeled GMP to an acceptor molecule such as GTP. Therefore IBDV is not a capping or guanylyl-transferase enzyme.

For the first time efforts were made to demonstrate a guanylylation reaction utilizing a recombinant VP1 of a birnavirus. A clone of IBDV genome segment B expressed *in vitro* was used for the guanylylation assay. However, no GTP binding activity of VP1 could be demonstrated in the *in vitro* expressed product even when temperature and salt optimization reactions were performed. To see if the presence of viral genome or other viral proteins could have any effect, guanylylation reactions in the presence of dsRNA and boiled IBDV, respectively, were carried out. Again, the binding of GTP to the recombinant VP1 was not seen. The *in vitro* expressed VP1 was examined for the presence of factors that may inhibit the guanylylation reaction. A significant decrease in the guanylylation of purified IBDV was observed when the *in vitro* expressed VP1 was included in the reaction, suggesting the presence of inhibitory factors in the reaction mixture.

There could be several other factors which might be involved in the failure of the *in vitro* expressed VP1 to perform guanylylation reactions. As indicated in Chapter 2, high amounts of VP1 could not be obtained in the cell-free system used. Sub-optimal amounts of VP1 could cause the failure of the experiment. One of the possibilities for this could be unstability of the transcripts in the *in vitro* translation reaction mixture. Freezing might also lead to the loss of enzyme activity, however, no activity could be demonstrated even when the assays were done immediately after the *in vitro* translation reactions. The lack of post-translational modifications

during *in vitro* VP1 synthesis could be another reason for lack of guanylylation activity. The relatively smaller size of the in vitro expressed VP1 suggests that there may be some extra molecule/ amino acid missing which could have role in this activity. It is also possible that formation of VP1-GMP linkage in birnaviruses may require presence of intact virions. The requirement for intact virions for guanylylation activity may explain the observation made in this study that the guanylylation could not be demonstrated in the boiled IBDV particles. To date, there is no report on guanylylation activity associated with any recombinant viral protein expressed in a cell-free system.

Another VP1 associated activity studied was RNA dependent RNA polymerase. A comprehensive study was previously done on polymerase activity associated with purified IBDV (Spies *et al.*, 1987). In the present study the RdRp activity was successfully demonstrated in the purified IBDV. The newly synthesized transcripts were found to be attached to the viral protein.

Although there are several properties of VP1 that link it to the polymerase activity (reviewed in Chapter 1), there is no direct evidence that can implicate VP1 to this activity. Macreadie and Azad (1993) produced recombinant VP1 of IBDV in E. coli and S. cerevisiae and tried to demonstrate the polymerase activity of this protein. However, no conclusive evidence could be demonstrated in either of the expression

systems. In the present study, efforts were made to demonstrate the polymerase activity with the *in vitro* expressed VP1 but without success. When polymerase reactions utilizing purified IBDV were carried out in the presence of *in vitro* expressed VP1, the polymerase activity associated with IBDV was almost completely lost. Therefore, the presence of inhibitory factors may explain why the polymerase activity could not be demonstrated in the *in vitro* expressed VP1. In addition, insufficient amounts of *in vitro* expressed VP1 or improper folding or lack of some additional amino acids/ molecules to the protein expressed *in vitro* could also be important factors. Pizarro *et al.* (1991) reported that rotavirus transcription involves other associated enzymatic activities present in the viral core, in addition to the RNA polymerase activity. In IBDV, a recent report confirmed the presence of a minor polypeptide, VP5, whose functions are not known although these small size proteins are thought to possess some regulatory functions in viral replication (Mundt *et al.*, 1995). There is a possibility that the polymerase activity in IBDV and other birnaviruses may require the presence of other viral proteins such as VP5. In IPNV a protein-primed RNA synthesis has been demonstrated which suggests the necessity of a genome linked VP1 in the formation of transcripts (Dobos, 1995). There is a possibility that the *in vitro* expressed VP1 might not have attached to dsRNA IBDV genome during *in vitro* polymerase assays and therefore it is not surprising that the RdRp activity could not be demonstrated in the *in vitro* expressed VP1. Liu *et al.* (1992) reported that in rotaviruses precise roles of various proteins

in RNA transcription have been difficult to prove because single shelled particles must be structurally intact to possess RNA polymerase activity. This could also be true for birnaviruses. However, this remains to be investigated particularly when it has been shown that unlike rotaviruses there is no guanylyl-transferase activity associated with IBDV or other birnaviruses even though they are both dsRNA viruses. Also a recent confirmation that birnaviruses are the only dsRNA viruses exhibiting protein-primed RNA synthesis suggests that there are some fundamental differences involved in their replication.

In conclusion, the experiments in this study examined the expression of the OH-IBDV segment B clone in a cell-free system by *in vitro* transcription and translation. Full size optimum quantity *in vitro* transcripts were obtained upon optimizing several conditions for the reaction. It has been established that the maximum transcription yield is obtained when 37.5mM nucleotide concentration is used and the reaction is incubated for 4 hours at 37°C. Translation of the pOHB 27-1 transcript allowed the demonstration of VP1 of approximately 80 kDa by SDS-PAGE followed by autoradiography. However, the yield of VP1 in comparison to the Luciferase control was low and could not be increased by using different amounts of RNA transcript. The guanylyl-transferase activity associated with IBDV has been characterized in detail using the purified virus. It was confirmed that VP1 of IBDV, like that of IPNV, does not possess guanylyl-transferase activity.

Attempts to demonstrate the guanylylation and polymerase activity with *in vitro* expressed VP1 of IBDV were not successful. The possible reasons for this have been discussed. Since it has been proved that VP1 of IBDV is not a guanylyl-transferase/capping enzyme, therefore, it may not be a multifunctional enzyme as suggested earlier by Spies and Müller (1990).

For future studies, dialysis of the *in vitro* translation mixture, before the enzymatic assays, could be tried. However, the purification of the *in vitro* expressed VP1 from the rabbit reticulocyte lysates would be the best approach to avoid the inhibitory factors affecting enzymatic assays. The purification could be achieved by electroelution of the protein from the SDS-PAGE gel or by affinity chromatography. The isolated protein could be checked by immunoprecipitation. However, the disadvantage of this cell-free system (at least in this case) is that the VP1 yield was poor. Therefore, even if one isolates and purifies the VP1 from the rabbit reticulocyte lysate translation system, there is still the possibility of failure of experiments because of the low yield. The low yield of VP1 could be due to the unstability of transcripts in the *in vitro* translation reaction mixture, this could be checked by electrophoresis after the translation reaction to see if the RNA transcript is still present. A coupled transcription and translation system which utilizes recombinant plasmid DNA could also be tried to determine whether the poor yield of VP1 was due to degradation of RNA in the *in vitro* translation system.

Alternatively, VP1 could be expressed in the baculovirus expression vector system. The baculovirus expression vector system is superior to most other eukaryotic expression vector systems to date in terms of amount of protein produced (Vlak and Keus, 1990). The fact that the recombinant proteins are very similar, if not identical, to their authentic counterparts and that they are usually functionally active adds to the burgeoning interest in this expression vector system (Vlak and Keus, 1990). The further continuation of this study is important because VP1 associated RdRp activity and VP1 acting as a primer in viral replication make it an attractive target for development of anti-viral drugs. This could be achieved by deletion mapping studies to identify the active sites involved in these activities in a recombinant VP1 and then designing the drugs to permanently block these sites. This method of IBDV control could prove very effective and might help in permanently eradicating IBD in poultry.

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