VP5, the Nonstructural Polypeptide of Infectious Bursal Disease Virus, Accumulates within the Host Plasma Membrane and Induces Cell Lysis

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Infectious bursal disease virus (IBDV) encodes a 17-kDa nonstructural polypeptide known as VP5. This polypeptide is not essential for virus replication in vitro but it plays an important role in in vivo dissemination and pathogenesis. We have characterized the expression of VP5 in three eukaryotic systems: (i) IBDV-infected chicken embryo fibroblasts; (ii) BSC-1 cells infected with a recombinant vaccinia virus vector; and (iii) Cos-1 cells transiently transfected with a plasmid vector. Immunofluorescence analyses showed that upon expression VP5 accumulates within the plasma membrane. This finding was consistent with sequence-based topology predictions, indicating that VP5 is a class II membrane protein with a cytoplasmic N-terminus and an extracellular C-terminal domain. Brefeldin A treatment of VP5-expressing cells prevented the accumulation of this polypeptide in the plasma membrane, thus showing the requirement of an active exocytic pathway to reach that compartment. Expression of VP5 was shown to be highly cytotoxic. Induction of VP5 expression resulted in the alteration of cell morphology, the disruption of the plasma membrane, and a drastic reduction of cell viability. VP5-induced cytotoxicity was prevented by blocking its transport to the membrane with Brefeldin A. Our findings suggest that VP5 plays an important role in the release of the IBDV progeny. © 2000 Academic Press

INTRODUCTION

Infectious bursal disease virus (IBDV), a member of the Birnaviridae family, infects young chickens causing a disease (IBD) characterized by the destruction of B-lymphocyte precursors found within the bursa of Fabricius. This causes a severe immunodeficiency that increases the susceptibility to infections with opportunistic pathogens and reduces the growth rate of surviving animals. IBDV is one of the most important pathogens affecting the poultry industry worldwide (Müller et al., 1992; Nagarajan and Kibenge, 1995; Saif, 1998). IBDV consists of a nonenveloped capsid of 60 nm (Bötcher et al., 1997; Hirai and Shimakura, 1974; Özel and Gelderblom, 1985) that contains two segments of double-stranded RNA (dsRNA) of 3.2 kb (segment A) and 2.8 kb (segment B) (Hudson et al., 1986). Segment B contains one open reading frame (ORF) that encodes VP1, a protein of 97 kDa considered to be an RNA-dependent RNA polymerase (RdRp) responsible for the replication and transcription of the viral genome (Macreadie and Azad, 1993). Segment A contains two overlapping ORFs. The largest one encodes a 109-kDa polyprotein that is proteolytically cleaved, rendering three polypeptides: VPX, VP3, and VP4 (Dobos et al., 1995). VPX is further processed into VP2 (Müller and Becht, 1982). VPX, VP2, and VP3 are structural proteins forming the virus capsid and VP4 is responsible for the processing of the polyprotein (Fahey et al., 1985; Hudson et al., 1986; Kibenge et al., 1997). The smallest ORF encodes VP5, also known as NS, a nonstructural protein of 17 kDa, highly basic, cysteine-rich, and conserved among all pathogenic strains of IBDV. It has been shown that VP5 is expressed in IBDV-infected cells but it is not incorporated into the virion (Mundt et al., 1995).

The use of a reverse genetics system allowed the generation of a VP5 knockout mutant. Work with the VP5− mutant has shown that VP5 is dispensable for virus replication in vitro (Mundt et al., 1997). Interestingly, this mutant showed a highly attenuated phenotype and did not cause bursal lesions after experimental infection of susceptible chickens, demonstrating that VP5 plays a key role in IBDV pathogenesis and dissemination (Yao et al., 1998). Consequently, VP5 has attracted attention as a potential target for the development of strategies to control IBDV. Despite strong interest in VP5, very little was known about it. We have sought to gather new information that might provide clues about the functional role of this protein and its contribution to the virus life cycle. The Birnavirus life cycle has not been characterized in detail. Hence key steps such as entry, genome transcription and replication, morphogenesis, and release of the viral progeny are poorly understood. Birnaviruses are cytolytic viruses, but the molecular

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mechanism(s) employed for virus egress is, as yet, unknown. Most of the knowledge on virus release mechanisms derives from studies on enveloped viruses that bud from the plasma membrane (Garoff et al., 1998). In contrast, nonenveloped viruses have long been thought to be released following cell lysis. It is thought that either the viral gene expression or the formation and accumulation of virus particles induce changes in membrane permeability, eventually leading to cell lysis. However, data from different virus–cell systems suggest that expression of a single viral protein may be responsible for cell lysis (Carrasco, 1995). Several such proteins have been identified, i.e., the 2B proteins of poliovirus and coxsackievirus, the rotavirus NSP4, and the adenovirus E3-11.6K. Death proteins have been implicated in the alteration and eventual disruption of the host cell plasma membrane permeability (Aldabe et al., 1996; Newton et al., 1997; Tollefson et al., 1996; Van Kuppeveld et al., 1997).

Here we describe the characterization of the expression of VP5 in several cell systems. Topological predictions indicating that VP5 is a class II transmembrane protein were confirmed by the results of immunofluorescence (IF) analyses. Expression of VP5 in different cell systems caused severe cytotoxic effects characterized by extensive alterations in cell morphology, the disruption of the plasma membrane, and the eventual induction of cell lysis. Based on the results of this study and previous reports, we propose that VP5 acts as a death protein, modifying the cell membrane and promoting the egress of the newly formed IBDV virions.

RESULTS

VP5 topology

As shown in Fig. 1A, VP5 is formed by 145 amino acid residues and has a predicted molecular mass of 16.7 kDa. Analyses of the secondary structure of VP5 using the TMPred and TopPred programs showed the presence of a putative transmembrane hydrophobic helix within the central region of the polypeptide (aa 69 to 88) (Fig. 1A). According to these results, VP5 would belong to the class II transmembrane protein group. VP5 would possess an N-terminal cytoplasmic tail and a C-terminal...
extracellular domain. Extensive searches of protein data banks did not detect significant homologies to known polypeptides other than VP5 counterparts from different Birnaviruses.

VP5 expression in IBDV-infected cells

As a first step in our analysis we characterized the expression of VP5 during the virus life cycle. For this, IBDV-infected chicken embryo fibroblasts (CEF) were analyzed by IF using antisera against the five virus-encoded mature polypeptides including VP5. The anti-VP5 antiserum used in this report was raised against a 16-amino-acid-long synthetic oligopeptide containing the VP5 C-terminus (see Fig. 1A). As shown in Fig. 2, the structural polypeptides VP1, VPX/2, and VP3 are associated with large viroplasm-like aggregates within the cell cytoplasm. The VP4 antiserum yielded a characteristic needle-like signal, most likely corresponding to the described IBDV type II tubules (Granzow et al., 1997). Pre-immune sera, used as control for these experiments, gave dull negative signals (data not shown). Noteworthy, the anti-VP5 antiserum gave a distinctive plasma membrane staining. This result was in agreement with topology predictions indicating that VP5 is a transmembrane polypeptide.

Expression of recombinant VP5 in mammalian cells

To carry out a systematic characterization of VP5 it was important to develop vectors to achieve the expression of this polypeptide in different cell systems. A cDNA corresponding to the VP5 coding region was obtained and cloned into the VV transfer inducible vector pVOTE.2 (Ward et al., 1995) (see Materials and Methods). The resulting plasmid, pVOTE/VP5, was utilized to generate recombinant VV (rVV) VT7/VP5, which was then used to follow the expression of VP5 in mammalian cells.

Monolayers of BSC-1 cells were infected with VT7/VP5. After infection, cells were supplemented or not with the specific inducer isopropyl β-D-thiogalactoside (IPTG). At 10 h postinfection (p.i.) cells were harvested and analyzed by SDS–PAGE and autoradiography. As expected, addition of IPTG specifically triggered the expression of a 17-kDa polypeptide that was recognized by the VP5-specific antiserum (Figs. 3A and 3B, lanes 4). The electrophoretic mobility of the IPTG-induced product was similar to that of the original VP5 expressed in baby Grivet monkey kidney (BGM70) cells infected with IBDV (Figs. 3A and 3B, lane 6). Interestingly, in cells infected with VT7/VP5, a reduction in the total protein synthesis was consistently observed after induction of VP5 expression (Fig. 3B, lanes 3 and 4), indicating that VP5 might possess cytotoxic properties.

Interestingly, when the samples were subjected to nonreducing SDS–PAGE, the VP5-specific band was no longer detectable. A Western blot analysis of the corresponding gels showed the presence of several VP5-specific faint bands of over 200 kDa as well as a diffuse accumulation within the upper edge of the gel (data not shown). The dramatic reduction of the VP5-specific signal detected in Western blots performed after nonreducing SDS–PAGE might also be due to the masking of the epitope(s) recognized by the anti-peptide VP5 serum. It has been recently described that VP5 is able to self-aggregate (Tacken et al., 2000). Therefore, it seems likely that the high-molecular-weight anti-VP5 reactive bands correspond to VP5 aggregates.

VT7/VP5-infected BSC-1 cells were videorecorded by time-lapse phase-contrast microscopy. A set of selected images is shown in Fig. 3C. Uninduced VT7/VP5-infected cells showed cytopathic effects characteristic of hemagglutinin negative VV, i.e., cell rounding and fusion (Seki et al., 1990). Insertion of the heterologous genes into the
Subcellular distribution of VP5

BSC-1 cells were infected with VT7/VP5 and analyzed by IF using the anti-VP5 rabbit antiserum followed by incubation with FITC-conjugated goat anti-rabbit Ig. The samples were also stained with Texas red-conjugated (TXRD) wheat germ agglutinin (WGA) to determine the position of the plasma membrane and Golgi complex (Tartakoff and Vassalli, 1983). In TX-100 permeabilized cells, VP5 was detected as a large spot within the perinuclear region (Fig. 4A, panel viii), suggesting the presence of this polypeptide within the Golgi system. This was confirmed by the matching WGA signal (Fig. 4A, panel vii).

In nonpermeabilized IPTG-induced VT7/VP5-infected cells, both the anti-VP5 antiserum and the TXRD-WGA conjugate specifically stained the cell surface (Fig. 4A, panels v and vi). The membrane of IPTG-induced cells was shown to contain abundant VP5-specific fluorescent ring-like patches, suggesting the presence of large VP5 aggregates within this compartment (Fig. 4A, panel iv). Unexpectedly, incubation of nonpermeabilized VP5-expressing cells with either WGA or anti-VP5 antiserum gave a weak Golgi complex staining (Fig. 4A, panels v and vi). This finding suggested that VP5 might alter the cell membrane, thus allowing the access of macromolecules to the cell cytoplasm.

The subcellular distribution of VP5 was also analyzed in Cos-1 cells transfected with the plasmid expression vector pcINeo/VP5. Similarly to what had been observed in IBDV- and VT7/VP5-infected cells, in transfected Cos-1 cells the VP5 signal was associated with both the plasma membrane and the Golgi system (Fig. 4B, panels ii and iv).

Taken together these results demonstrate that, regardless of the expression system used for the analysis, VP5 is found within the plasma membrane and the Golgi system. The anti-VP5 serum used for this work was raised against a synthetic peptide containing the sequence corresponding to the 16 aa from the C-terminal VP5 region (see Fig. 1A). The strong reactivity detected with this antiserum in nonpermeabilized VP5-expressing cells strongly supports the prediction that the C-terminal region of VP5 is exposed on the external cell surface.

VP5 transport to the plasma membrane is blocked by Brefeldin A (BFA)

The results of the IF analysis described above demonstrated that VP5 accumulates within the plasma membrane and suggested that to reach this compartment it might require the exocytic pathway. To assess this hypothesis we studied the effect of the blockage of this pathway on the subcellular distribution of VP5. BSC-1 cells were infected with VT7/VP5 and, after adsorption, expression of VP5 was induced by adding IPTG to the cell medium. Cultures were then maintained either in the presence or the absence of BFA (5 \( \mu \)g/ml), an agent known to rapidly disassemble the Golgi complex (Misumi et al., 1986). At 16 h p.i. cells were fixed and analyzed by IF using anti-VP5 serum and TXRD-conjugated WGA.

The BFA treatment caused a strong reduction of membrane-associated VP5 signal (Fig. 5, panels ii and iv), indicating that the presence of BFA inhibits the transport of VP5 to the cell membrane. A dramatic effect of BFA on the subcellular distribution of VP5 was detected in T-X100-permeabilized cells. The large perinuclear fluorescent spots detected in untreated cells (Fig. 5, panel vi) were no longer found after the BFA treatment; instead a fine punctuated VP5 fluorescence was observed (Fig. 5, panel vii). This pattern was consistent with the accumulation of VP5 in vesicles from the cis/medial Golgi compartments. Similar results were obtained in pcINEO/VP5-transfected Cos-1 cells (data not shown). These results clearly show that VP5 transits to the plasma membrane is dependent upon the presence of a functional Golgi complex.
VP5 expression induces cell death

It was consistently noticed, in both VT7/VP5-infected and pcINEO/VP5-transfected cells, that VP5 expression caused a swift alteration in cell morphology. In addition to this, we had observed that expression of VP5 caused a reduction in the total protein synthesis (Fig. 3). These data suggested that accumulation of VP5 is toxic to the host cell.

To further assess this possibility a series of experiments was carried out. First, we studied the effect of VP5 expression on cell viability using the trypan blue dye exclusion method (see Materials and Methods). BSC-1 cell cultures were infected with VT7/VP5 and maintained in medium supplemented with IPTG. The percentage of viable cells in the different cultures was determined at different times p.i. As controls, infections were carried out with the parental virus VT7LacOl and with VT7/VP3, a rVV expressing the IBDV VP3 protein (Fernández-Arias et al., 1997). As shown in Fig. 6A, a rapid decrease in cell viability was observed in IPTG-induced VT7/VP5-infected cultures. In these cultures, at 12 h.p.i. less than 20% of the total cell population remained viable by this criterion. In contrast, at the same time postinfection, IPTG-induced control cultures maintained viability levels of over 70%.

The effect of VP5 on cell viability was also monitored using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. As shown in Fig. 6B, the mitochondrial activity levels of VT7LacOl- and VT7/VP3-infected cells were not strongly affected by the infection. However, in VT7/VP5 cells a rapid decrease of MTT activity was specifically observed.

The trypan-blue and MTT assays showed that expression of VP5 specifically reduced cell viability. To further confirm these observations, a method to assess cell death was used. BSC-1 cell cultures were infected with VT7/VP5 and maintained either in the presence or in the absence of IPTG. At 16 h.p.i. cells were fixed, permeabilized or not with Triton X-100, and immunostained with rabbit anti-VP6 followed by incubation with FITC-conjugated goat anti-rabbit Ig. Thereafter, samples were incubated with TXRD-conjugated WGA. Samples were viewed by epifluorescence. (B) Cos-1 cells were transfected with the plasmid pcINEO/VP5. At 48 h.p.i. cells were fixed and processed for IF as described above. Arrows indicate the position of VP5-expressing cells.

FIG. 4. Subcellular distribution of VP5 expressed in mammalian cells. (A) BSC-1 cells were infected with VT7/VP5 at 5 PFU/cell and maintained either in the presence or in the absence of IPTG. At 16 h.p.i. cells were fixed, permeabilized or not with Triton X-100, and immunostained with rabbit anti-VP5 followed by incubation with FITC-conjugated goat anti-rabbit Ig. Thereafter, samples were incubated with TXRD-conjugated WGA. Samples were viewed by epifluorescence. (B) Cos-1 cells were transfected with the plasmid pcINEO/VP5. At 48 h.p.i. cells were fixed and processed for IF as described above. Arrows indicate the position of VP5-expressing cells.
absence of IPTG. At 18 h p.i., cultures were fixed and subjected to a double fluorescence staining using the cell death detection FITC-TUNEL reagent and anti-VP5 antiserum followed by incubation with TXRD-conjugated anti-rabbit IgG antiserum. As shown in Fig. 6C, a large proportion (over 80%) of the VP5-expressing cells were positive for TUNEL staining. The rather low proportion (less than 5%) of TUNEL-positive cells detected in uninduced VT7/VP5-infected cultures confirmed the specificity of this assay.

A direct correlation between VP5 expression and the induction of cell death was also documented in pcINEO/VP5-transfected Cos-1 cells (data not shown).

Results obtained with three independent methods demonstrated that expression of VP5 specifically induces a cell death response in mammalian cells.

BFA blocks the induction of cell death in VP5-expressing cells

We consistently observed that addition of BFA to the medium resulted in a marked delay in the onset of VP5-induced morphological changes. This suggested that the VP5-mediated cytotoxicity might require the accumulation of the polypeptide in the cell membrane. To analyze this possibility, IPTG-induced VT7/VP5-infected cells were maintained either in the presence or in the absence of BFA. At 18 h p.i., cells were fixed and stained with anti-VP5 and visualized by fluorescence microscopy. As shown in Fig. 7, approximately 85–90% of the untreated VP5-expressing cells were also positive for both VP5 antisera and TUNEL staining (Fig. 7, panels i and ii). In BFA-treated cultures the fraction of double-positive cells was less than 20% (Fig. 7, panels iii and iv). These results showed that the VP5-induced cytotoxicity is efficiently prevented by an agent that blocks the accumulation of VP5 within the plasma membrane.

VP5 expression induces cell lysis

As described above, IF analyses indicated that expression of VP5 might alter the permeability of the plasma membrane, allowing the access of macromolecules to the cytoplasm of nonpermeabilized paraformaldehyde-fixed cells. These findings suggested that VP5 might induce gross changes in the cell membrane. Accordingly, the possibility existed that the plasma membrane of VP5-expressing cells might allow the release of cytosolic proteins to the extracellular medium.

To test this hypothesis, BSC-1 cells were metabolically labeled with $[^{35}]$S]methionine. After labeling, cells were infected with VT7LacO1, VT7/VP3, or VT7/VP5. The cultures were then maintained in either normal or IPTG-containing medium. The presence of radioactive proteins in the extracellular medium was assessed at different times postinfection. Samples collected from control cultures did not contain significant levels of $[^{35}]$S]methionine-labeled proteins (Fig. 8A). In contrast, the presence of radioactive proteins in samples from IPTG-induced VT7/VP5-infected cultures was detected as early as 8 h p.i. At 24 h p.i., the amount of acid-insoluble radioactivity found in samples from VP5-expressing cultures was approximately ninefold higher than that in control cell samples (Fig. 8A). The medium samples were also analyzed by SDS–PAGE followed by autoradiography. As expected, medium collected from IPTG-induced VT7/VP5-infected
cells contained proteins normally found as cell-associated polypeptides (data not shown).

We have documented that the presence of BFA blocks the accumulation of VP5 in the plasma membrane and prevents the induction of cell death. It was therefore important to assess the effect of BFA on the alteration of cell membrane permeability. Monolayers of BSC-1 cells were labeled with $[^{35}]$S)methionine, infected with VT7/VP5, induced with IPTG, and maintained either in the presence or in the absence of BFA. As shown in Fig. 8B, BFA treatment causes an inhibition of approximately 80% of the release of radioactive polypeptides to the extracellular medium.

These results demonstrate that, in addition to the in-

![Cell viability assays for VP5-expressing cells.](image)
induction of cell death, expression of VP5 strongly alters the plasma membrane permeability. Disrupting the exocytic pathway and thus preventing the accumulation of VP5 in the plasma membrane efficiently inhibited this effect.

DISCUSSION

The subcellular distribution of VP5 was studied using three expression systems: (i) IBDV-infected CEF; (ii) inducible rVV-infected BSC-1 cells; and (iii) pcINEO/VP5-transfected Cos-1 cells. In all cases, we detected the presence of VP5 within the Golgi system and the plasma membrane. The extensive VP5/WGA colocalization, observed in double-staining experiments, and the changes in VP5 subcellular distribution induced by treatments with BFA further confirmed these findings, indicating that VP5 associates with the plasma membrane. Furthermore, the anti-VP5 antisera used in this study, raised against the C-terminal VP5 domain, rendered a strong surface staining in nonpermeabilized cells. Our experimental data are in agreement with the topology predictions obtained with two independent algorithms, TMpred and TopPred. In light of these results, we propose that VP5 is a class II membrane protein with an intracellular N-terminal tail and an extracellular C-terminal region.

A previous report, using an IBDV VP5 knockout mutant, described the relationship between the inactivation of the VP5 coding sequence and the reduction of the IBDV-induced cytopathic effect (Yao et al., 1998). Hence, our observation that VP5 expression induced a potent cytotoxic effect on different cell systems was not unexpected. The results described in this report demonstrate that, in the absence of other IBDV-encoded polypeptides, expression of VP5 induces cell death. Expression of VP5 causes a rapid alteration of the cell morphology accompanied by a fast decline in cell viability as measured by trypan blue dye exclusion and MTT and TUNEL assays. The observation that the plasma membrane of VP5-expressing cells is not only permeable to small molecules, such as trypan blue, but also to macromolecules, such as lectin and IgG molecules, strongly supports the hypothesis that accumulation of VP5 alters the plasma membrane. This alteration might trigger cell death. In agreement with this hypothesis, BFA treatment, which

FIG. 7. BFA inhibits the induction of cell death caused by expression of VP5. BSC-1 cells were infected with VT7/VP5 and induced with IPTG. The cultures were treated or not with BFA. At 18 h p.i. coverslips were collected and processed for IF with anti-VP5 antisera using a secondary antibody coupled to TXRD. Thereafter, coverslips were stained with the In Situ Cell Death Detection kit to identify nicked nuclear DNA. Arrowheads indicate the position of apoptotic VP5-expressing cells.

FIG. 8. Effect of VP5 expression on the integrity of the plasma membrane. (A) BSC-1 cells were metabolically labeled with [3S]methionine and then infected with VT7/VP5. Cultures were supplemented or not with IPTG. At the indicated times the cell media from the different cultures were collected and used to determine the amount of acid-precipitable radioactivity. (B) BSC-1 monolayers were metabolically labeled with [3S]methionine, infected with VT7/VP5, induced with IPTG, and treated or not with BFA. At the indicated times the cell supernatants were collected and used to determine the amount of acid-precipitable radioactivity.
prevents the accumulation of VP5 in the plasma membrane, efficiently blocked the induction of cell death.

It has been shown that viral proteins involved in cell lysis modify the permeability of the cell membrane either by forming pore structures or by increasing the susceptibility of the membrane to phospholipases (Shai, 1995). Most of these proteins disrupt the intracellular ionic gradients by increasing the Ca\(^{2+}\) concentration. At this point we cannot speculate about the mechanism(s) responsible for the alteration of the plasma membrane induced by VP5. Work to determine the effect of VP5 on intracellular Ca\(^{2+}\) concentration as well as to assess the role of different VP5 domains, i.e., the membrane-spanning region and the highly basic C-terminus, in its biological activity is currently ongoing.

Results obtained with mutants lacking a functional VP5 gene demonstrated that this polypeptide is nonessential for virus replication. VP5 inactivation did not affect the rate of synthesis of other IBDV-encoded polypeptides (Mundt et al., 1997). The comparison of the one-step growth curves of wild-type and VP5\(^{-}\) mutant viruses revealed that VP5 inactivation caused a considerable delay in the production of extracellular infectious virus. The BFA treatment of IBDV-infected cells apparently mimicked the effect of the VP5 inactivation, causing a delay in the production of extracellular virus. These results completely fit with the hypothesis that accumulation of VP5 in the PM leads to a progressive alteration of the plasma membrane that facilitates the release of the virus progeny. Unfortunately, studies with the VP5\(^{-}\) mutants did not consider the effect of the mutation on the accumulation of intracellular virus. In light of our results it would be interesting to examine this aspect.

In this context it would be expected that the progeny of VP5\(^{-}\) mutants would remain cell-associated longer than the wild-type virus. In cell culture, infected cells would eventually die, thus discharging their contents, including the virus progeny, to the extracellular milieu. The in vivo situation would be rather different. A delay in the release of the virus progeny would provide more time for the clearance of the infected cells by the phagocyte. Indeed, this would be a critical factor in reducing the spread of the virus and, concomitantly, decreasing its pathogenicity.

Information about the IBDV replication cycle is, as yet, scarce. Very little is known about crucial steps such as penetration, transcription, replication, assembly, and the release of the virus progeny. As is the case with other naked viruses, it is expected that the release of IBDV might require the alteration of the cell membrane. Available information is consistent with the hypothesis that VP6 plays a role in virus release. Further characterization of the role of VP6 in virus egress would significantly contribute to the understanding of the molecular biology of birnaviruses. These studies might provide clues for the development of new strategies to control the spread of IBDV.

**MATERIALS AND METHODS**

**Cells, viruses, and antisera**

The IBDV Soroa strain, a pathogenic serotype I virus isolate, was propagated in CEF as previously described (Lombardo et al., 1999). The serotype I classic subtype IBDV Sal 1 strain was grown and titrated in BGM70 cells. Dr. Y. M. Saif (Ohio State University) kindly provided IBDV Sal 1 strain and BGM70 cells. The rVV VT7/POLY and VT7/VP3 have been previously described (Fernández-Arias et al., 1997, 1998). rVV were propagated and titrated in African green monkey kidney epithelial BSC-1 cells (American Type Culture Collection) as described (Earl and Moss, 1993). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) containing 10% newborn calf serum (NCS) at 37°C in a humidified 5% CO\(_2\) incubator.

Sera anti-VP5 was raised against the oligopeptide CTKRHHKRRDLPRKPE, corresponding to residues 130–145 of the VP5 sequence, coupled to keyhole limpet hemocyanin (KLH, Pierce). The anti-VP4 serum was generated by immunization with KRFPHNPRDWRDLPYC coupled to KLH. Oligopeptide synthesis, coupling, and immunization were carried out as described (Fernández-Arias et al., 1998; Harlow and Lane, 1988). Anti-VP1, -VP2/X, and -VP3 sera have been previously described (Lombardo et al., 1999; Sánchez and Rodríguez, 1999).

**Construction of plasmids and recombinant viruses**

Genomic IBDV dsRNA segments were isolated from purified virus as previously described (Sambrook et al., 1989). A cDNA corresponding to the IBDV ORF A1 was generated by RT-PCR using IBDV genomic RNA as template and the primers 5'-CGGCTATGTTAGTAGAGATCAGACAAAC and 5'-GACTCTGGGCTGTCACTGC following a previously described protocol (Heine et al., 1991). The resulting DNA product was subjected to digestion with *NdeI*. The resulting 551-bp fragment containing the complete VP5 ORF was inserted into the *NdeI* site of the pBSK\(^{+}\) vector generating the derivative plasmid pBSK/VP5. The nucleotide sequence of the cloned fragment was analyzed and shown to be correct. Thereafter, the VP5 fragment was isolated from pBSK/VP5 by restriction with *NdeI*. This fragment was cloned into the VV insertion/expression vector pVOTE.2 (Ward et al., 1995) that had been digested with *NdeI*. The resulting plasmid, pVOTE/VP5, was purified and used to obtain the recombinant virus VT7/VP5. For this, BSC-1 cells were infected with the rVV VT7LacO1 (Ward et al., 1995) and transfected with pVOTE/VP5. Selection and amplification of VT7/VP5 were carried out as previously described (Earl and Moss, 1993). Dr. B. Moss (NIH, Bethesda, MD)
kindly provided the plasmid vector pVOTE.2 and the rVV VT7LacO1.

For the construction of pclNeo/VP5, a DNA fragment containing the VP5 ORF was generated using the primers 5′-GGCTCAGATGTTAGTAGAGATCAGAC and 5′-CCCGGCgggcgctCACTCGCGCCCTTCTTGAAAG and the plasmid pBSK/VP5 as template. The PCR product was digested with XbaI and NotI and cloned into the multiple cloning site of the eukaryotic expression vector pclNeo.

### Analysis of protein expression

Cultures of BSC-1 cells were infected with VT7LacO1 or with VT7/VP5 at a multiplicity of infection of 5 PFU/cell and maintained in the presence or absence of the inducer IPTG (4 mM final concentration). At 10 h p.i., cells were washed twice with methionine-free DMEM and metabolically labeled for 1 h with 100 µCi/ml of [35S]methionine. After being labeled, cells were washed twice with PBS and resuspended in sample buffer (62.5 mM Tris–HCl, pH 6.8; 2% SDS; 0.25% bromophenol blue; 5% glycerol; and 5% β-mercaptoethanol). Protein samples (5–10 µg/sample) were subjected to 15% SDS–PAGE and then transferred to nitrocellulose filters by electroblotting. Filters were incubated for 16 h at 5°C in blocking buffer (PBS containing 5% nonfat dry milk). Thereafter, filters were maintained for 2 h at RT with anti-VP5 antiserum diluted in blocking buffer and then incubated for 1 h at RT with goat anti-rabbit IgG conjugated with horseradish peroxidase (ICN). The membranes were developed in PBS containing 0.02% 1-chloro-4-naphthol and 0.006% hydrogen peroxide. After the Western blot results were recorded, filters were air-dried and subjected to autoradiography. IBDV infections were carried out on BGM70 cells at an m.o.i. of 5 PFU/cell using the Sal1 strain. At 18 h p.i. cells were metabolically labeled with 100 µCi/ml of [35S]methionine. Samples were prepared and analyzed by SDS–PAGE and Western blotting as described above.

### Immunofluorescence

BSC-1 cells were plated on coverslips and infected with VT7/VP5 at an m.o.i. of 5 PFU/cell in the presence or absence of IPTG. At 16 h p.i. cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at RT. Cells were permeabilized or not with 0.1% Triton X-100 for 10 min at RT. Coverslips were blocked in PBS containing 20% NCS for 20 min and incubated with anti-VP5 diluted 1:100 in PBS containing 5% NCS for 45 min at 37°C followed by incubation with TXRD anti-rabbit IgG sera (Jackson ImmunoResearch Laboratories Inc.). Coverslips were mounted on glass slides and examined by epifluorescence using a Zeiss Axiophot microscope. CEF cells were infected at an m.o.i. of 5 PFU/cell. At 18 h p.i. cells were fixed, permeabilized, incubated with antisera against VP1, VPX, VP3, VP4, or VP5, and visualized by epifluorescence as described above. Control IF was carried out using the corresponding preimmune sera. IF Images were captured with a MicroMAX digital camera (Princeton Instruments Inc.) using the IPLab Spectrum software (Signal Analytics Corp.).

Cos-1 cells grown in coverslips were transfected with 200 ng of plasmid DNA per 10^6 cells using lipofectamine (Gibco BRL), following the instructions of the manufacturer. To minimize the potential toxic effects arising from an excess of plasmid DNA or transfecting agents, transfections were performed using suboptimal conditions. Under these conditions only 10–15% of the cells were shown to express VP5. At 48 h posttransfection cells were incubated with selected antisera and analyzed by IF as described above.

### MTT assay

BSC-1 cells were infected with the appropriate rVV at an m.o.i. of 5 PFU/cell (3 × 10^3 cells/well in a 24-well multwell plate) and maintained in the presence or absence of IPTG. At different times p.i., MTT (Sigma Chemical Co.) in PBS (0.5 mg/ml) was added to a final volume of 220 µl/well. Thereafter, cultures were further incubated at 37°C for 1 h. The reaction was stopped by replacing 150 µl of medium by an equal volume of lysis buffer (10% Triton X-100, 0.5% isopropanol, 0.5% HCl). After incubating at 37°C for 10 min, plates were read on a multiscan microplate reader at 540 nm. Determinations were carried out in triplicate.

### Trypan blue exclusion assay

BSC-1 cells were infected in triplicate with the appropriate rVV at an m.o.i. of 5 PFU/cell and maintained in the presence or absence of IPTG. At the corresponding times p.i., the medium was removed and cells were trypsinized. The medium and cell fractions were combined and trypan blue (Gibco BRL) added to a final concentration of 0.02%. After 3 min, the percentage of colored cells was determined by direct counting under the microscope (at least 500 cells were counted at each time point).

### 35S-release assay

BSC-1 cells were plated at a density of 1 × 10^5 cells in 24-well multwell plates and maintained at 37°C and 5% CO₂ overnight. Thereafter, cultures were incubated for 2 h with methionine-free DMEM supplemented with 20 µCi/ml of [35S]methionine. Labeled cultures were washed three times with DMEM and then infected with the corresponding rVV at an m.o.i. of 5 PFU/cell. Cultures were maintained in the presence or absence of IPTG. At different times p.i., the medium from selected wells was recovered and spun down to remove cell debris. These samples were used to determine the amount of acid-
precipitable radioactivity released from the cells as previously described (Lombardo et al., 1999).

TUNEL assay

BSC-1 or Cos-1 cells grown on coverslips were either infected or transfected as described above. At 24 h.p.i. or 48 posttransfection, respectively, cells were fixed with 4% paraformaldehyde for 30 min at RT. Coverslips were then washed twice with PBS and then incubated for 10 min with PBS containing 100 mM glycine to block reactive aldehyde groups. After two washes with PBS, cells were permeabilized by incubation with 0.1% Triton X-100 supplemented with 0.1% sodium citrate for 10 min at RT. Coverslips were washed twice with PBS and subjected to RNase A treatment (10 μg/ml in 10 mM Tris, pH 8.0; 1 mM EDTA) for 1 h at 37°C. Thereafter, coverslips were processed for IF with anti-VP5 antisera as described above. Subsequently, cells were stained using the In Situ Cell Death Detection (TUNEL) Kit (Boehringer Mannheim) following the instructions of the manufacturer. Coverslips were examined by epifluorescence using the FITC or the TXRD filters to detect the TUNEL and the VP5 signals, respectively. Images were captured and processed as described above.

Primary sequence analysis

The topological analysis of VP5 was carried out using the TMpred and TopPred 2 programs. TMpred makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. The prediction is made using a combination of several weight matrices for scoring (Hofmann and Stoffel, 1993). TopPred 2 predicts the topology of membrane proteins on the basis of hydrophobicity analysis, automatic generation of a set of possible topologies, and ranking of these according to the positive-inside rule (Von Heine, 1992).

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