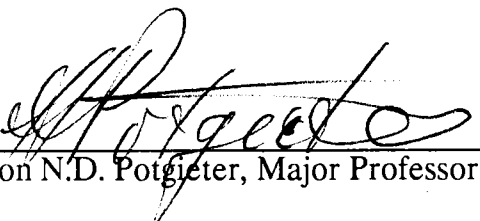
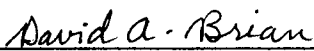


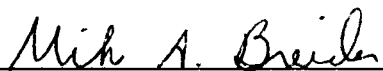
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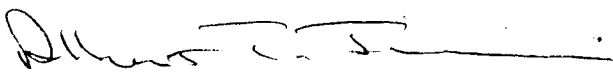
I am submitting herewith a dissertation written by Melissa A. Kennedy entitled "The Amplification, Cloning, Sequencing, and Expression of the Putative Gene Encoding the Major Envelope Protein of a Noncytopathic Strain of Bovine Viral Diarrhea Virus." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.


Leon N.D. Potgieter, Major Professor

We have read this dissertation
and recommend its acceptance:







Accepted for the Council:


Associate Vice Chancellor
and Dean of The Graduate School

**AMPLIFICATION, CLONING, SEQUENCING, AND EXPRESSION OF THE
PUTATIVE GENE ENCODING THE MAJOR ENVELOPE PROTEIN OF A
NONCYTOPATHIC STRAIN OF BOVINE VIRAL DIARRHEA VIRUS**

**A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

Melissa Anne Kennedy

December 1991

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This dissertation is dedicated to my family, and, in particular, to my parents: to my mother, Barbara Benton Kennedy, for her boundless support and understanding, guidance wise beyond measure, and constant provision of inspiration in all I do; to my father, the late Thomas J. Kennedy, Jr., who got me started on this road so long ago - I think he would be proud.

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ABSTRACT

An investigation was undertaken to clone, analyze, and express the gene encoding the major envelope protein of Bovine Viral Diarrhea Virus. Polymerase chain reaction was used to generate cDNA encoding the major envelope protein of noncytopathic BVDV strain 2724 for cloning. Primers for cDNA synthesis and amplification were based on published sequence data. The 5' ends of the primers were modified by the addition of restriction sites to allow for ligation into a plasmid vector. Recombinant plasmids were sequenced by dideoxy chain termination. The insert was 1323 nucleotides in length capable of encoding a protein of 441 amino acids. Sequence analysis furthered the characterization of this genomic region of BVDV. Comparisons of this region to flaviviruses and to other pestiviruses revealed a high degree of homology between BVDV strain 2724 and the pestiviruses, but not with the flaviviruses. This construct was expressed in a cell-free translation system and data suggested that this protein was translocated and glycosylated. Results from the immunoprecipitation of the translation product indicated it was an authentic BVDV-specific protein. Identification of the putative transmembrane domain was also accomplished. It was concluded that the putative major envelope protein of BVDV strain 2724 is highly homologous to the major envelope proteins of the pestiviruses whose genomes have been cloned and sequenced. Additionally, this protein of BVDV strain 2724 was determined to be translocated, glycosylated, and anchored. The results of this study may have implications for the control and prevention of BVDV infection through improved diagnostics and immunoprophylaxis.

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CHAPTER 1

LITERATURE REVIEW

PATHOGENESIS

The pathogenesis of bovine viral diarrhea is multi-faceted. The consequences of infection are dependent upon several factors, some involving the host, some the agent itself. Host-related factors that affect the disease course include the occurrence of viremia, the degree and duration of immune dysfunction, and the gestational status of the infected cow (Radostits et al., 1988). Viral determinants of the course of infection include the strain and biotype of the pathogen (Radostits et al., 1988; Bolin, 1990b). Three clinical syndromes resulting from bovine viral diarrhea virus (BVDV) infection have been recognized: bovine viral diarrhea, fetal disease, and mucosal disease (Duffell et al., 1985; Ames et al., 1986).

Bovine viral diarrhea results from infection of nonimmune immunocompetent cattle. Normally, the majority of primary postnatal BVDV infections are easily managed by the animal with the development of neutralizing antibodies followed by elimination of the virus (Radostits et al., 1988). When disease does occur following BVDV infection of the animal, symptoms usually are mild and transient (Ames et al., 1986; Radostits et al., 1988). The latter include fever, depression, inappetance, oculonasal discharge, oral erosions, and diarrhea, and are observed most often in cattle 6 months to 2 years of age (Olafson et al., 1946; Brownlie, 1985; Radostits et al., 1988). BVDV infection can result in severe enteric disease in neonatal calves (Lambert et al., 1968; Lambert et al., 1974; Ames et al., 1986; Baker, 1987; Radostits et al., 1988).

A more significant consequence of postnatal BVDV infection in cattle is transitory immunosuppression that is induced by the virus (Baker, 1987; Potgieter, 1988; Radostits et al., 1988; Castrucci et al., 1990). BVDV targets epithelial cells of the digestive tract and immunocompetent cells (Truitt et al., 1973; Baker, 1987; Ohmann et al., 1987; Potgieter, 1988; Bolin, 1990b). This immunosuppression results, not only from direct destruction of lymphocytes and monocytes infected with

BVDV, but also, and perhaps more importantly, from functional impairment of these cells and neutrophils (Truitt et al., 1973; Johnson et al., 1973; Muscoplat et al., 1973a; Muscoplat et al., 1973b; Reggiardo et al., 1981; Roth et al., 1986; Potgieter, 1988). The result of this immune dysfunction is precipitation or potentiation of disease caused by coinfecting opportunistic pathogens (Tyler et al., 1965; Potgieter, 1977; Bohac et al., 1980; Ames et al., 1986; Baker, 1987; Potgieter et al., 1988). The development and outcome of these opportunistic or secondary infections are determined in part by environmental and management conditions such as stress, exposure to pathogens, and pre-existing disease (Baker, 1987; Potgieter, 1988). The precise mechanism of the immune dysfunction associated with BVDV infection is unclear.

Fetal disease may occur after BVDV infection of the nonimmune pregnant cow. The virus crosses the placental barrier efficiently (Kahrs, 1973; Done et al., 1980; Duffell et al., 1985; Potgieter, 1988). Manifestation of fetal infection depends upon the developmental stage of the fetus (Kendrick, 1971; Roeder, 1986a; Baker, 1987; Potgieter, 1988). Infection in the first trimester may result in fetal death, followed by abortion or mummification (Kahrs, 1968; Casaro et al., 1971; Kendrick, 1971; Kahrs, 1973; Done et al., 1980; Stober, 1984; Brownlie, 1985; Duffell et al., 1985). Fetal infection during the second trimester can result in a multitude of congenital defects primarily affecting the immune, musculoskeletal, and nervous systems (Duffell et al., 1985; Baker, 1987; Potgieter, 1988). Thymic aplasia, alopecia, growth retardation, cerebellar hypoplasia, hydranencephaly, retinal degeneration, optic neuritis, defective spinal cord myelination, and pulmonary hypoplasia may be caused by BVDV infection (Kahrs, 1973; Done et al., 1980; Ohmann, 1984; Duffell et al., 1985; Baker, 1987; Radostits et al., 1988). By the third trimester, immunological maturity in the unborn calf is sufficient to mount an adequate immune response to eliminate the virus (Casaro et al., 1971; Muscoplat et al. 1973a; Baker, 1987). These calves are usually clinically normal, and are seropositive for BVDV at birth (Casaro et al., 1971; Muscoplat et al., 1973a; Baker, 1987).

Fetal infection by a noncytopathic strain of BVDV prior to development of immunocompetence by the fetus may result in specific immunotolerance to the infecting strain of BVDV and a lifelong, persistent viremia (Coria et al., 1978; Cutlip et al., 1980; Martin et al., 1982; Brownlie, 1985; Duffell et al., 1985; McClurkin et al., 1985; Bolin et al., 1985d; Baker, 1987; Radostits et al., 1988).

Though unable to respond immunologically to homologous strains of BVDV, the calf develops immunocompetence to other viral antigens and to heterologous strains of BVDV (McClurkin et al., 1984; Bolin et al., 1985a; Bolin et al., 1985c; Radostits et al., 1988). The animal will continually shed the virus to which it is immunotolerant and thereby constitute a major source of this pathogen to other cattle within the herd (McClurkin et al., 1984; Roeder et al., 1984; Duffell et al., 1985; Roeder et al., 1986b; Baker, 1987; Radostits et al., 1988). The exact mechanism for establishment of viral persistence in calves, and the role of viral biotype in development of immunotolerance remain unclear (Radostits et al. 1988).

The persistently infected bovine is at high risk for development of mucosal disease (Radostits et al., 1988). Mucosal disease occurs only in cattle with persistent BVDV infection, and may result from superinfection of these animals by a cytopathic strain of the virus, as this syndrome has been reproduced experimentally by inoculating persistently infected animals with a cytopathic strain of the virus (Brownlie et al., 1984; Bolin et al., 1985a; McClurkin et al., 1985; Baker, 1987; Ohmann, 1988; Radostits et al., 1988). Furthermore, recent studies suggest that the superinfecting virus precipitating mucosal disease is antigenically identical or closely related to the persisting noncytopathic strain (Corapi et al., 1988). The origin of superinfecting virus precipitating mucosal disease is uncertain, but mutation of the pre-existing noncytopathic strain converting the latter to a cytopathic biotype has been suggested (Corapi et al., 1988; Radostits et al., 1988; Brownlie, 1990). The origin of superinfecting virus, the exact role of biotype and antigenic relatedness in the development of mucosal disease, and the identity of other factors involved remain to be elucidated.

Mucosal disease occurs sporadically and the mortality rate is high (McClurkin et al., 1985; Radostits et al., 1988; Potgieter, 1988). The disease is characterized by depression, anorexia, fever, and weakness. Also, oral lesions; oculonasal discharge; lameness resulting from skin lesions at the interdigital cleft, laminitis, and coronitis; profuse watery diarrhea; and severe leukopenia may occur (Baker, 1987; Radostits et al., 1988). Death usually ensues within 5-10 days after infection (Baker, 1987; Radostits et al., 1988). Occasionally, cattle may become chronically ill, surviving up to 18 months (Baker, 1987; Radostits et al., 1988). The pattern of lesions observed with mucosal disease reflects the propensity for BVDV to infect epithelial cells (Ruth, 1986; Baker, 1987). Additionally, antigen-antibody

complexes may lead to glomerulonephritis and other lesions suggestive of immune complex disease (Cutlip et al., 1980; Winter et al., 1984; Hewicker et al., 1987; Radostits et al., 1988). The cytopathic and noncytopathic BVDV present in animals with mucosal disease have in common the serotype-specific antigen, such as serum virus neutralizing epitopes (Radostits et al., 1988). However, some antigens may differ between the two viruses and antibodies to heterologous antigens of the superinfecting virus may cause immune complex disease in the animal (Radostits et al., 1988).

IMMUNOSUPPRESSION

BVDV-induced immunosuppression is perhaps a more serious consequence of primary postnatal BVDV infection than the primary disease syndromes, as measured by the financial losses sustained from the consequences secondary to this impairment. Suppression of the immune system is broad in scope, transitory, and may go unrecognized because typical signs of BVD may be absent (Reggiardo, 1979; Greig et al., 1981; Baker, 1987; Potgieter, 1988; Radostits et al., 1988). Pulmonary immunity is affected also, resulting in potentiation of respiratory disease (Potgieter, 1977; Yates, 1982; Turk et al., 1985; Potgieter, 1988; Richer et al., 1988).

The mechanism of the immunosuppression involves both direct destruction and diminished function of immunocompetent cells (Potgieter, 1977; Potgieter, 1988). Both lymphocytes and macrophages support replication of BVDV (Truitt et al., 1973; Ohmann et al., 1987; Potgieter, 1988). The reduced capacity of infected cells to combat invading pathogens renders the animal susceptible to a multitude of disease agents (Potgieter, 1988; Richer et al., 1988). Current data indicate that BVDV may play a critical role in multiple-etiology diseases (Baker, 1987; Potgieter, 1988; Radostits et al., 1988; Richer et al., 1988). It is commonly isolated as a coinfecting agent along with other pathogens such as IBRV, PI-3, rotavirus, coronavirus, *Pasteurella* spp., *Salmonella* spp., and actinomycoses spp., and may act synergistically with these agents to produce disease (Thomas et al., 1977; Yates, 1982; Baker, 1987; Wray et al., 1987; Potgieter, 1988; Radostits et al., 1988; Richer et al., 1988). The virus affects several elements of the immune system, including lymphocytes, macrophages, neutrophils, platelets, cytokine production and cytokine responses (Roth et al., 1986; Baker, 1987; Bolin et al., 1987; Potgieter, 1988;

Radostits et al., 1988; Rebhun et al., 1989; Atluru et al., 1990; Woldehiwet et al., 1990).

Following infection of the bovine with BVDV, lesions can be found in lymph nodes, spleen, Peyer's patches, and other lymphoid tissue of cattle (Pritchard, 1963; Prier, 1966; Ruth, 1986; Baker, 1987). Distribution of lesions reflects the predilection of BVDV for lymphocytic cells (Prier, 1966; Ruth, 1986; Baker, 1987; Potgieter, 1988). Direct cytolysis of lymphocytes results in a decrease in the absolute numbers of circulating B and T cells, and the percentage of T cells (Bolin et al., 1985b; Baker, 1987; Potgieter, 1988; Radostits et al., 1988; Ellis et al., 1988). Furthermore, lymphocytes present are less responsive to external mitogens such as PHA (Markham et al., 1985; Roth et al., 1986; Baker, 1987; Potgieter, 1988; Radostits, 1988; Brown et al., 1991). Lymphocytes obtained from persistently infected cattle, and lymphocytes infected with BVDV in vitro may have impaired function (Johnson et al., 1973; Muscoplat et al., 1973a; Muscoplat et al., 1973b; Ketelson et al., 1979; Roth et al., 1986). B lymphocytes have diminished humoral antibody production due to a metabolic defect (Atluru et al., 1979; Steck et al., 1980; Potgieter, 1988; Radostits et al., 1988). Additionally, the mobility of these cells in response to chemotactic stimuli is reduced (Potgieter, 1988).

Macrophages are another target of BVDV infection. Some of these cells are destroyed by the virus, and the surviving cells' capacity for proliferation, locomotion, and chemotaxis may be significantly reduced (Markham et al., 1985; Roth et al., 1986; Baker, 1987; Potgieter, 1988). BVDV inhibited IL-1 and IL-2 production, and reduced proliferation of bovine mononuclear cells. Furthermore, this suppression was not reversed by the addition of exogenous interleukins (Atluru et al., 1990). In a study by Markham et al., supernatant fluid from BVDV-infected cell cultures inhibited proliferation of bovine mononuclear cells. This suppression may be due to prostaglandin production by infected cells (Markham et al., 1985).

Neutrophils from BVDV-infected cattle also have several abnormalities. They include decreased bactericidal, fungicidal, and virucidal activities due to impaired myeloperoxidase reaction. Antibody-dependent, cell-mediated cytotoxicity (ADCC) of neutrophils is inhibited as well (Potgieter, 1988; Brown et al., 1991). The mechanism of this dysfunction is unknown, but degranulation of PMN lysosomes may be impaired by BVDV which in turn may block or reduce ADCC of BVDV-infected cells by PMN leukocytes (Roth et al., 1981). Recent studies indicate

that BVDV infection affects platelet number. Rebhun et al. found that a small percentage of cattle infected with a noncytopathic strain of BVDV had thrombocytopenia. The etiology was not determined, but may represent a direct effect of BVDV on bone marrow elements (Rebhun et al., 1989). Interferon production and antiviral capability of interferon may be reduced in BVDV-infected cell cultures, but it is not known whether this occurs in vivo (Diderholm et al., 1966; Rossi et al., 1980; Potgieter, 1988).

Pulmonary immunity of cattle may also be affected by BVDV infection. Numbers of pulmonary lymphocytes and neutrophils were reduced in bronchoalveolar lavage fluids from BVDV-infected calves (Turk et al., 1985; Potgieter, 1988). Bacterial clearance in BVDV-infected calves was inhibited promoting dissemination of bacteria and enhanced disease within the respiratory tract (Potgieter et al., 1984a; Turk et al., 1985; Potgieter, 1988).

Cattle with mucosal disease may have immune dysfunction, though it's character differs somewhat from that seen in animals with primary postnatal infection. Lymphocyte function and mitogenic responsiveness were decreased whereas the actual numbers of lymphocytes were not affected in cattle with mucosal disease (Johnson et al., 1973; Bohac et al., 1980; Steck et al., 1980; Bolin et al., 1985a; Larsson et al., 1988a; Larsson et al., 1988b; Potgieter, 1988). Neutrophils from these animals have impaired iodination, cellular cytotoxicity, and bacterial phagocytosis (Roth et al., 1986; Potgieter, 1988). The basis for immune dysfunction in persistently-infected cattle is not clear, but may be due to immune complex interference with PMN receptor function (Roth et al., 1986).

It is not known whether the degree of BVDV-induced immune dysfunction varies with the strain of infecting virus, but research suggests that some strains are more immunosuppressive than others (Itoh et al., 1984a; Potgieter et al., 1985; Potgieter, 1988; Castrucci et al., 1990).

EPIDEMIOLOGY

Bovine viral diarrhea virus occurs worldwide, and has been isolated in most cattle-producing countries (Baker et al., 1954; Gillespie et al., 1959; Pritchard, 1963; Porta, 1985; Dow et al., 1986; Radostits et al., 1988). Prevalence of serum antibodies in cattle ranges from 50% - 90%, and in some herds with endemic viral infection,

the level may approach 100% (Howard et al., 1985; Radostits et al., 1988). About 0.5-1% of cattle in the general population are persistently infected and therefore persistently viremic (Howard et al., 1986; Radostits et al., 1988). A higher proportion of persistently-infected animals may exist on individual farms (Brownlie et al., 1987). In a study by Bolin et al., 89% of serum samples from 3,157 cattle in 66 herds in the U. S. were seropositive for BVDV. Of these, 1.7% were persistently infected (Bolin et al., 1985d). In Great Britain, the frequency of seroconversion indicated that over 70% of cattle had been infected during their first year of life (Harkness et al., 1978). In Denmark, 1 % of cattle going to slaughter were viremic (Radostits et al., 1988). A study done in the United States indicated 9% of the herds tested had persistently viremic cattle and 1.7% of all cattle tested were viremic (Bolin et al., 1985d; Baker, 1987). Additionally, antibody to BVDV has been found in captive exotic ruminants, sheep, goats, and deer (Nettleton, 1987; Harkness, 1987; Radostits et al., 1988).

The duration of viremia and of viral shedding in urine following primary postnatal infection lasts from 4 to 10 days in experimentally infected calves (Mills et al., 1968; Brownlie et al., 1987). Virus was isolated from nasal secretion, bronchial nodes, and lungs of these calves for much longer periods suggesting respiratory spread of the virus may be the most important mechanism of transmission (Mills et al., 1968; Brownlie et al., 1987). Persistently-infected animals, shed the virus constantly, and are the major source of virus in nature (Mills et al., 1968; Brownlie et al., 1987; Radostits et al., 1988). Transmission of BVDV to susceptible animals usually results from contact with carrier animals (Roeder et al., 1984; Radostits et al., 1988). Persistently infected cattle shed virus in urine, feces, saliva, oculonasal discharge, semen, milk, uterine secretions, and placental tissue (Coria, 1978; Stober, 1984; Duffell et al., 1985; Baker, 1987; Radostits et al., 1988). The virus can persist in the environment for up to 14 days (Duffell et al., 1985; Baker, 1987).

Infection of susceptible cattle generally occurs by ingestion or inhalation of virus, but management practices such as the reuse of hypodermic needles or the use of nose grips may also promote spread of the virus from infected cattle to susceptible animals (Duffell et al., 1985; Roeder et al., 1986; Baker, 1987; Radostits et al., 1988). Transplacental infection of the fetus occurs with relative ease when the dam is infected (Malmquist et al., 1968; Kendrick, 1971; Done et al., 1980; Harkness, 1987; Potgieter, 1988). Carrier cows usually have persistently infected

calves that then act as carriers, thus maintaining the virus within the herd (Radostits et al., 1988). Finally, vaccines and other biologicals, such as reagents used in embryo transfer may be contaminated with BVDV, constituting another source of infection (Lohr et al., 1983; Radostits et al., 1988; Kirkland et al., 1990).

The costs attributed to BVDV infection in cattle are large, and are generally related to the immunosuppression and consequences of transplacental infection (McClurkin et al., 1979; Grahn et al., 1984; Duffell et al., 1985; Baker, 1987; Potgieter, 1988; Radostits et al., 1988). The dimension of the losses that occur in cattle as a consequence of BVDV infection depend also on management practices (Radostits et al., 1988). Three separate studies reviewed by Harkness determined that over three fourths of the financial losses suffered by farmers as a result of BVDV infection were due to prenatal infection (1987). These included such consequences as fetal death, congenital defects, and neonatal death. Even when death due to opportunistic infections and respiratory disease following primary postnatal infection were considered, the proportion of total financial losses due to prenatal infection remained over 75% (Harkness, 1987). Additionally, outbreaks of mucosal disease can result in herd losses of over 25% in economic devastation to the cattle farmer (Nagele, 1984; Radostits et al., 1988). In Great Britain, the cost to cattle farmers is over \$60 million annually (Harkness, 1987).

MOLECULAR BIOLOGY OF BOVINE VIRAL DIARRHEA VIRUS

Molecular biological data on Bovine Viral Diarrhea Virus has accumulated slowly. The primary obstacle to analysis of BVDV at the molecular level is the difficulty in growing and manipulating the virus (Renard et al., 1985). However, recent investigations have given insight into the molecular virology of BVDV.

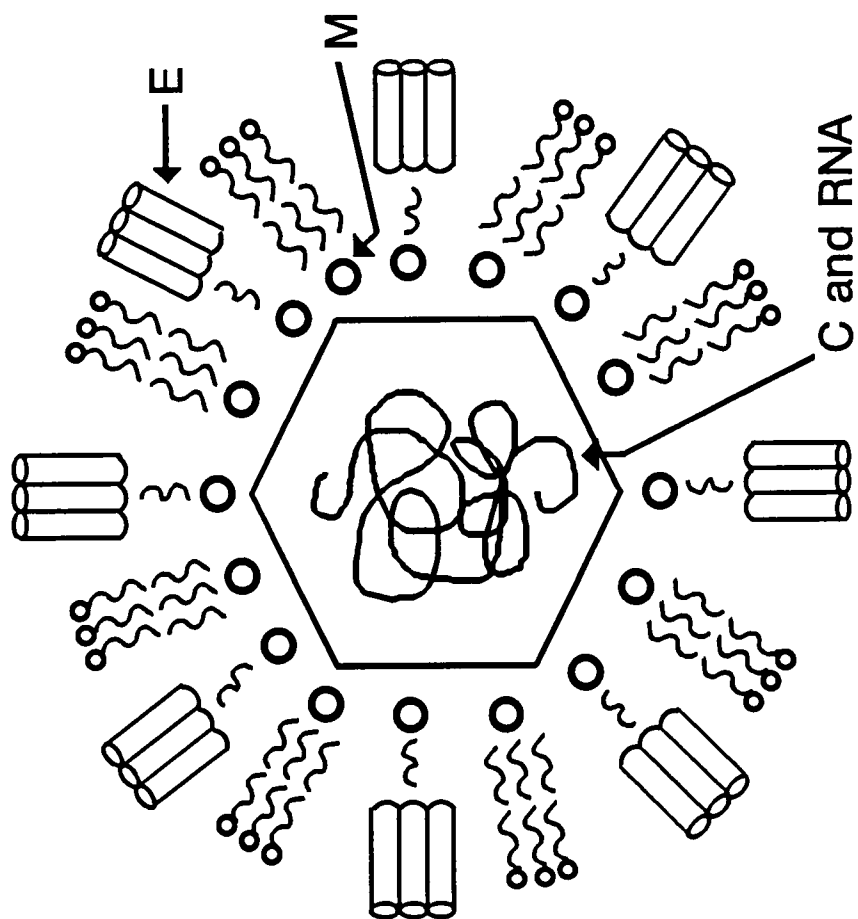
The agent causing BVD is a RNA virus classified within the genus Pestivirus along with the causative agents of Border Disease and Hog Cholera, as all share a similar viral genomic organization, structure, and pathogenesis (Moennig, 1990; Westaway et al., 1985). Currently, this genus is included within the family Togaviridae, but its reclassification into the Flaviviridae family is imminent (Collett, personal communication). This reclassification is based on recently acquired data that indicated a greater similarity of Pestiviruses to Flaviviruses (Horzinek et al., 1987; Collett et al., 1988c; Collett et al., 1989).

The virus is enveloped, and is one of the smallest animal viruses, with a diameter of 45-55 nm and a density of 1.12 g/ml (Hermodsson et al., 1962; Gillespie et al., 1963; Renard et al., 1985). The nucleocapsid is not helical, and may have icosahedral symmetry (figure 1.1) (Horzinek et al., 1971). The BVDV genome is single-stranded RNA, and is sensitive to destruction by RNase and alkali treatments, but is resistant to DNase and proteinase K (Horzinek, 1981; Renard et al., 1985; Renard et al., 1987b). The genetic material of the virus is infectious, indicating the RNA has positive polarity (Horzinek, 1973; Collett et al., 1989). The size of the genome is controversial, but most recent data indicated a length of approximately 12.5 kilobases (kb) corresponding to a molecular weight of 4.3 MDa (Renard et al., 1985; Collett et al., 1988a). Subgenomic species of viral RNA have not been detected in infected cells (Purchio et al., 1983; Renard et al., 1987).

Genomic RNA of BVDV has substantial secondary structure (Purchio et al., 1983; Purchio et al., 1984b; Collett et al., 1989). In a study by Purchio et al., the sedimentation rate of the viral RNA depended on sedimentation conditions (Purchio et al., 1983). The viral RNA sedimented at 21S in neutral sucrose gradients, but sedimented at 33S in sucrose gradients containing formaldehyde. This indicated the existence of significant secondary structure in neutral sucrose gradients which was disrupted in the presence of formaldehyde. Also in this study, the genomic RNA resisted low concentrations of RNase, but was hydrolyzed at higher concentrations. This indicated the molecule is not a true duplex, as double-stranded RNA is resistant to high concentrations of RNase. The genome was soluble in 2 M LiCl and bound to cellulose in the presence of 15% ethanol which suggested also the existence of double-stranded regions (Purchio et al., 1984b; Collett et al., 1988a; Collett et al., 1989). Additionally, Purchio et al., discovered that efficient translation of the genomic message in a reticulocyte cell-free translation system required denaturation immediately before translation (1984b).

The genome of BVDV does not have a polyadenylate tail as indicated by the determination that it is not retained by oligo (dT)-cellulose columns (Purchio et al., 1983; Renard et al., 1985). The precise character of the 3' end of the genome remains unclear. It can be modified by T4 RNA ligase-mediated addition of cytidine 3',5'-bisphosphate (pCp), but not by poly(A) polymerase-mediated addition of AMP residues (Renard et al., 1985; Collett et al., 1988a). Sequencing of this region of the genome should clarify the reason for this apparent inconsistency (Collett et al.,

Figure 1.1. Schematic diagram of bovine viral diarrhea virion. E denotes the major envelope protein; M denotes the membrane protein; C denotes the nucleopcapsid protein bound to the genomic RNA.



1989). Virtually nothing is known about the 5' end of the genome. Collett et al. were unable to add ^{32}P -radiolabelled ATP to the 5' terminus using polynucleotide kinase, and noted that the interpretation of this data as it pertains to the presence of a cap must await further testing (Collett et al., 1989).

Bovine Viral Diarrhea Virus Replication

The details of Pestiviruses' replication remain essentially unknown (Horzinek et al., 1987). The virus may enter susceptible cells via a cell-surface receptor (Collett et al., 1989). Studies with monoclonal antibodies against specific surface proteins on bovine cells interfered with BVDV infectivity (Teyssedou et al., 1987; Collett et al., 1989). The identity of these proteins is as yet unknown, but all pestiviruses may not use the same host cell receptor (Collett et al., 1989; Moennig, 1990). Viral inhibition by cell-surface-specific monoclonal antibody was not always complete, indicating either that more than one receptor for viral entry may exist or an alternative mechanism for entry can be utilized by the virus (Collett et al., 1989; Moennig, 1990). How this information helps to explain the histotropism of BVDV remains to be elucidated, but the variation in host range specificity of different strains of pestiviruses may reside in the mechanism of viral entry (Collett et al., 1989; Moennig, 1990).

The sequence of events that follow entry into the susceptible cells also remain unclear (Horzinek et al., 1987). Only a single genomic length RNA molecule has been identified within infected cells; neither replicative intermediates (RI) nor replicative forms (RF) have been detected (Collett et al., 1989). Actinomycin D does not inhibit BVDV replication under conditions that completely arrest host cell RNA synthesis (Horzinek, 1981; Purchio et al., 1983). The implication is that BVDV does not require host cell enzymes for replication of its genome, and instead, may utilize its own RNA polymerase (Collett et al., 1989). Collett et al. hypothesized that the production of pestivirus proteins occurs via translation of the large open reading frame (ORF) in the genome into a large polyprotein (Collett et al., 1988a; Collett et al., 1989). A significant ORF in the negative sense has not been found, which supports the conclusion that BVDV RNA has positive polarity (Collett et al., 1989). Mature viral proteins may be produced by proteolytic cleavage and processing of the polyprotein (Collett et al., 1989). Assembly and maturation of

BVDV occurs at the perinuclear endoplasmic reticulum and Golgi membranes in a manner similar to that of picornaviruses and flaviviruses (Horzinek, 1973).

Bovine Viral Diarrhea Virus Biotype

Cellular response to growth of different BVDV isolates in cell culture vary, but the significance of this phenomenon is unclear (Radostits et al., 1988). Certain strains caused marked cellular damage and cell death in infected cell cultures, while other strains resulted in few or no detectable changes (Underdahl et al., 1957; Gillespie et al., 1959; Omori et al., 1967; Itoh et al., 1984a; Radostits et al., 1988). Underdahl et al. first isolated a cytopathic strain of BVDV from outbreaks of mucosal disease in Nebraska and Iowa (1957). Lee et al. successfully cultivated a noncytopathic strain of virus in 1957. Additionally, these authors showed that this virus caused typical disease, and that virulence was not linked to viral growth characteristics in cell cultures (Lee et al., 1957). In 1959, Gillespie et al. isolated and propagated a cytopathic strain of BVDV from a calf with signs of viral diarrhea. These authors determined also that the virus caused the disease (Gillespie, 1959).

The biotypic property of the virus depends not only on the genetics of the virus, but also on the type of cell culture and culture conditions (Horzinek, 1981; Johnson et al., 1990). Bovine testicle and bovine embryonic spleen cells develop distinct CPE after infection with cytopathic BVDV, whereas bovine kidney cells do not; additionally subconfluent cultures are recommended for optimal viral CPE (Horzinek, 1981).

The nature of the disease in the fetal calf after transplacental BVDV infection may be determined by the particular biotype of the virus (Brownlie et al., 1984; Brownlie et al., 1989; Brownlie, 1990). Persistently viremic cattle, a consequence of in utero infection, were infected with noncytopathic BVDV strains only (Brownlie et al., 1984). The ability of the cytopathic BVDV biotype to cross the placenta efficiently has been demonstrated (Kendrick et al., 1971; Casaro et al., 1971; McClurkin et al., 1984). However, the cytopathic strain may be unable to induce a persistent state, as this biotype has not been associated with the production of persistently infected cattle (Brownlie et al., 1989). Done et al. recovered noncytopathic BVDV from liveborn calves after experimental inoculation of

pregnant dams during gestation with a cytopathic strain (1980). McClurkin et al. successfully produced persistent infection in calves by inoculation of pregnant cows with noncytopathic BVDV prior to day 125 of gestation, whereas inoculation of a cytopathic strain crossed the placenta but did not produce a viremic state in calves (1984). Brownlie et al. hypothesized that the preferred cell(s) for cytopathic infection are not present, or are not differentiated, in the fetus during early gestation when persistence is established (1989).

Experimental production of mucosal disease indicated that development of this syndrome requires superinfection of persistently infected cattle with a cytopathic strain of the virus, or, perhaps, mutation of the persistent noncytopathic strain to a cytopathic biotype (Brownlie et al., 1984; Bolin et al., 1985a). Brownlie et al. successfully reproduced clinical mucosal disease by superinfecting persistently infected cattle with a cytopathic strain of virus (1984). Bolin et al. also induced severe to fatal disease by inoculating persistently infected cattle with a cytopathic strain of BVDV (1985a). Evidence indicated that the superinfecting strain in natural mucosal disease is closely related antigenically to the BVDV strain present in viremic cattle (Gillespie et al., 1961; Corapi et al., 1988). The cytopathic virus may originate by mutation of the noncytopathic virus present in the persistently infected animal (Corapi et al., 1988). Epidemiological evidence from field outbreaks of BVD suggested that introduction of infected cattle was not a possible source of virus (Brownlie, 1990). Recent data indicated that both heterogeneity and homogeneity at the genomic and antigenic level in noncytopathic and cytopathic pairs of viruses isolated from individual animals with mucosal disease may exist (Ridpath et al., 1991). Hybridization to conserved viral genomic regions by DNA probes and binding of monoclonal antibodies to the viruses established that these biotype pairs were not identical to one another. Furthermore, genomic and antigenic similarities between viruses occur independently from one another, suggesting these viral properties do not correlate with one another. The relationship between virus pairs based on the results of this study could not be determined. Conclusive evidence about the source of superinfecting virus in mucosal disease will depend on further molecular characterization of both virus biotypes (Brownlie, 1990).

The viral property responsible for variation in biotype is not known. Nuttall determined that noncytopathic and cytopathic BVDV have similar replication kinetics, but differ from one another in yield of virus in infected cell cultures (1980). Cytopathic BVDV strains produced a much greater yield of virus in cell cultures than noncytopathic viruses do (Nuttall, 1980). Certain BVDV strains induce "exaltation of Newcastle disease virus (NDV)" in cell cultures, which is the uninhibited replication of NDV due to BVDV-induced suppression of cellular interferon production, but it is not common to all BVDV strains (Gillespie et al., 1962; Omori et al., 1967; Itoh et al., 1984a). The explanation for this phenomenon is unknown, but it may indicate basic differences in the biology of the two biotypes (Gillespie et al., 1962; Omori et al., 1967; Itoh et al., 1984a). The viral polypeptide profile in cells infected with cytopathic BVDV biotypes differs from that of noncytopathic biotypes (Donis et al., 1987a; Pocock et al., 1987; Magar et al., 1988; Ridpath et al., 1990). Additionally, certain cellular RNA sequences are inserted within the nonstructural protein-encoding genomic region of cytopathic BVDV strains but this phenomenon has not been identified in noncytopathic virus strain (Meyers et al., 1990a; Meyers et al., 1990b; Meyers et al., 1991).

Bovine Viral Diarrhea Virus Strain Variation

Strain variation among Bovine Viral Diarrhea Viruses is known to exist (Collett et al., 1989). Early studies indicated antigenic homology among BVD viruses. Castrucci et al. demonstrated that infection of calves with one of three strains of virus generated antibodies not only to the infecting virus, but to two heterologous strains of virus as well (1975). The authors documented that reciprocal protective immunity exists between BVDV strains (Castrucci et al., 1975). Gillespie et al. compared six strains of BVDV by cross neutralization and all were antigenically related to one another (1960).

Many researchers agree that BVDV strains are related to one other as well as to other pestiviruses, but it is now widely accepted that a significant degree of antigenic variability among BVDV strains exists. Virus neutralization studies have suggested that defined groups occur. Fernelius et al. proposed that three distinct serotypes exist - NADL, C24V, and noncytopathic strains (1971). The authors noted

variation in cytopathic effects produced by each group. Heuschele et al. also noted differences in the character of cytopathogenicity in cell culture of different BVDV strains (1975). Additionally, the authors described variation among strains in the intensity of fluorescence in fluorescent antibody assay and suggested the existence of three serotypes of BVDV. Itoh et al. found antigenic variation by cross neutralization when comparing strains isolated in Japan to strains isolated in the United States (1984b).

In another investigation of antigenic relatedness of 11 pestivirus strains, four "clusters" were identified (Wensvoort et al., 1989a). One consisted entirely of hog cholera viruses (HCV), while the other three contained BVDV and border disease viruses (BDV). This illustrates the ability to distinguish BVDV from HCV but not from BDV by neutralization studies (Collett et al., 1989). Monoclonal antibody studies support these findings. Monoclonal antibodies to hog cholera virus did not react with BVDV or BDV strains indicating an unequivocal differentiation between the pestiviruses of ruminants and those of swine (Peters et al., 1986; Wensvoort et al., 1989b; Cay et al., 1989). Serological discrimination of BVDV and BDV was not possible in this study.

The use of monoclonal antibodies in the investigation of relatedness among BVDV strains has verified the presence of several distinct groups of viruses. Monoclonal antibodies generated against one noncytopathic and two cytopathic strains of BVDV allowed classification of 10 strains into four groups (Bolin et al., 1988a). Variation in antigenicity was unrelated to the biotype, as members of both biotypes were identified within groups. Edwards et al., using monoclonal antibodies generated against two strains of BVDV and one strain of HCV, identified two distinct antigenic groups among ruminant pestiviruses in Great Britain (1988). Corapi et al., using 22 monoclonal antibodies to 10 distinct antigenic determinants, determined that a high degree of homogeneity existed between cytopathic and noncytopathic BVD viruses present in the same animal with mucosal disease (1988). Unrelated biotypes varied to some extent at all antigenic sites examined (Corapi et al., 1988). Further evidence that antigenic dissimilarity exists among strains of BVDV is the strain specificity of the immunotolerance that persistently infected cattle have (Brownlie et al., 1987). Finally, cattle vaccinated with killed virus followed by exposure to an unrelated noncytopathic strain during early gestation, give birth to persistently infected calves indicating antibodies induced by the vaccine

virus failed to protect against the infecting noncytopathic virus (Bolin, 1990b). Additionally, antibodies produced in response to killed virus vaccines do not neutralize certain BVDV strains (Bolin, 1990b).

Variation in BVDV antigenicity does not necessarily indicate that discrete serotypes exist, but rather that it is a single serotype in which serologically distinguishable viruses are found (Howard et al., 1987). Fewer antigenic differences among BVDV strains may occur than is required to designate viruses as distinct serotypes (Howard et al., 1987; Cay et al., 1989).

Molecular Virology of Bovine Viral Diarrhea Virus

Nucleotide base sequence data of the genome of BVDV indicate open reading frames (ORF) in the positive polarity only (Renard et al., 1987a; Renard et al., 1987b; Collett et al., 1988a). However, disagreement exists on the number of ORF's. Renard et al. identified two long nonoverlapping open reading frames within the genome of the Osloss strain of BVDV, while Collett et al. found only one in the sequence of the NADL strain (Renard et al., 1987b; Collett et al., 1988a). However, evidence for the existence of only one ORF in the pestivirus genome is supported by the sequence of hog cholera virus which also has one long open reading frame only (Moorman et al., 1990a). It has been concluded that a mistake was made in determining the nucleotide sequence of the Osloss strain RNA rather than evidence for the existence of differences in the genomic organization between the two BVDV strains (Collett et al., 1989).

The initiation codon of the NADL strain genome may be at nucleotides 386-388 (Collett et al., 1988a). The open reading frame extends from the latter throughout the entire length of the genome to nucleotide residue 12,349, where a stop codon occurs (Collett et al., 1988a). This suggests that the genome encodes 3988 amino acids of a 449kD polyprotein (Collett et al., 1988a). The structural proteins may be encoded at the 5' end of the genome based on the hydrophobicity profile of, and location of glycosylation sites of the polyproteins encoded by the genome (Renard et al., 1987b; Collett et al., 1988b). The open reading frame likely is translated as a single polyprotein precursor that is then proteolytically cleaved and processed to produce mature proteins (Collett et al., 1988b). This hypothesis was supported by the absence of subgenomic RNA in infected cells (Purchio et al., 1983;

Renard et al., 1985; Collett et al., 1989). Additionally, genomic-length RNA was associated with ribosomes in infected cells suggesting the genome served as mRNA for viral protein synthesis (Purchio et al., 1984b).

The number and identity of BVD viral proteins produced by infected cells remains unclear. Number and molecular weight of the virus-specific polypeptides reported from different studies vary, due, at least in part, to the difficulty in purifying virus components and the difference in methods utilized. A compilation of data acquired by various investigators from BVDV protein analyses is shown in table 1.1. In an early investigation, Pritchett et al. identified four major proteins of molecular weight (MW) 93-110,000, 70,000, 50-59,000, and 23,000 Daltons in BVDV-infected cells (1975). In 1979, three virus-specific proteins with MW of 34,000, 44,000, and 54,000 Daltons were described (Matthaeus). The latter two were glycosylated. In analyzing the Singer strain of BVDV, Coria et al. identified four major proteins of MW 75,000, 66,000, 54,000, and 26,000 Daltons (1983). Two of these, the 75,000 and 54,000 Dalton proteins were glycoproteins. Purchio et al. described three major virus-specific proteins of MW 115,000, 80,000, and 55,000 Daltons, and two minor species 38,000 and 45,000 Daltons (1984a). The 55,000 Dalton protein was glycosylated.

More recently, Pocock et al. identified eight virus associated polypeptides from each of three cytopathic strains and two noncytopathic strains in infected cell culture (1987). These eight proteins ranged in size from 120,000 to 23,000 Daltons, and though a greater number were identified than in previous studies, their size range was in general agreement with data of others. Differences in electrophoretic gel mobility of some of the proteins from several of the isolates were noted. Most notably, noncytopathic BVDV strains lacked two of the proteins of molecular weight 87,000 and 69,000 Daltons present in cytopathic BVDV isolates. However, the absence of the latter protein may have been of questionable significance because the concentration of other proteins from noncytopathic BVDV-infected cell cultures were low making them difficult to detect. Four polypeptides were glycosylated. The relationship among all of these proteins to one another was not specifically characterized, though it was determined that the 120,000 Dalton and 87,000 Dalton polypeptides were structurally related to one another by tryptic peptide mapping.

Table 1.1 Intracellular polypeptides of BVDV as characterized by various investigators^a.

Pritchett (1975)	Matthaeus (1979)	Coria (1983)	Purchio (1984a)	Pocock (1987)	Donis (1987b)	Collett (1988b)	Akkina (1991)
93-110			115 80	120 87	165 135 gp118 80	133 125 80	172 168 135 175 130 80 96 76 72 59 50
70		gp75 66			gp75 gp65		
50-59	gp54 gp44 34	gp54	gp55 45 38	gp69 gp57 gp49 37	gp57 gp48 37	gp62 gp53 gp48	
23		26		33 gp23	32 gp25 19	gp25 20	

^aValues are MW in kilodaltons; gp denotes glycosylated proteins; protein analogies between investigators implied by similar columnar position within the table are speculative.

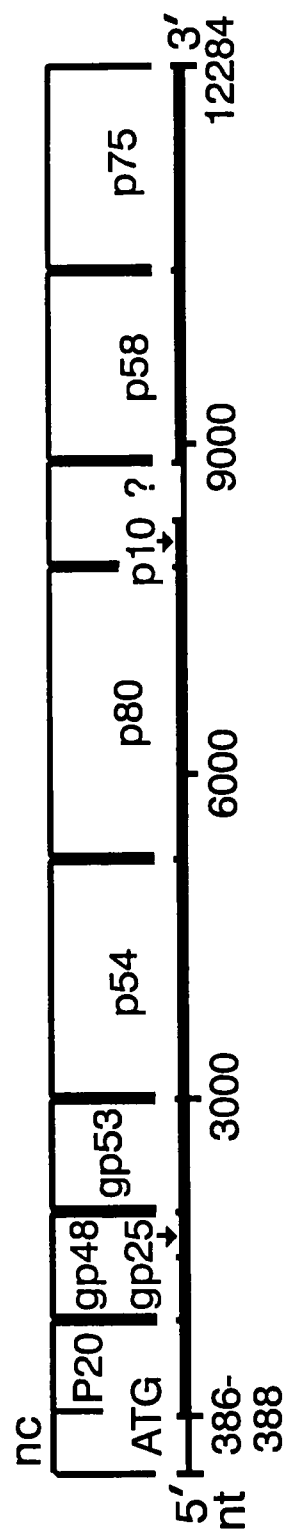
Donis et al. used two different methods for viral protein characterization (1987b). One method was virus protein radiolabelling under conditions that inhibit cellular initiation of protein translation, while the other was radioimmunoprecipitation of infected cell lysate for viral protein analysis. These methods allowed the identification of twelve virus-specific polypeptides ranging in size from 165,000 to 19,000 Daltons. Several of these proteins had not been identified in previous studies, and six of the twelve proteins were glycosylated. As in Pocock's study, the relatedness of the proteins to one another was not determined.

Collett et al. generated antisera to the protein products expressed from cloned BVDV cDNA sequences (1988b). These proteins were expressed as fusion proteins with *E. coli* β -galactosidase gene product. The regions of the BVDV genome expressed were characterized enabling not only the identification of BVDV-specific proteins, but the mapping of the gene products as well. This genomic map has become the prototype in describing the genomic organization and identity of BVDV polypeptides (figure 1.2).

The structural proteins of BVDV may be encoded at the 5' end of the genome. This conclusion was made because the positioning of the glycoproteins (gp) occurs within this area (Collett et al., 1989). Collett postulated that a 116kD gp is the precursor for the structural glycoproteins (1988b). It is cleaved into a 62kD precursor and a 53kD structural glycoprotein. The former then is processed to gp48 and gp25, two structural glycoproteins. Based on the predicted amino acid sequence determined from the nucleotide sequence, this region possesses 14 glycosylation sites, eight occurring in gp48, two in gp25, and four in gp53. A coding region for a 20kD protein occurs upstream of the glycoprotein-encoding region. This protein may be the nucleocapsid protein based on the argument that the 5' region of the genome encodes the structural proteins (Collett et al., 1989).

The remainder of the genome may encode the nonstructural proteins, but the specific identities of the gene products produced from the genomic region downstream from the area defined above have not been determined. Several precursor-product relationships of these proteins may occur (Collett et al., 1988b). Collett et al. reported that the glycoprotein-encoding region may be followed by a region encoding a 30kD protein, but this protein had not been identified; however, recent data suggested that this protein-encoding region does not exist (Wiskerchen et al., 1991). Therefore, the glycoprotein-encoding region may be followed on the 3'

Figure 1.2. Proposed genomic organization of BVDV. Bracketed areas above the genome represent the proteins encoded by the genome. NC denotes the 5' noncoding region. ATG denotes the codon which may be used by the virus for translation initiation. Numbers below the genome represent nucleotide (nt) position. Data for this diagram was taken from Collett et al. (1988b).



end by the region encoding a 125kD protein (p125). In cytopathic strains, this protein may be cleaved further to produce 54kD and 80kD proteins (p54 and p80). Downstream from the p125 region is an area which could encode a 38kD protein (p38), but this protein has not been identified. Recent data indicates that a 10kD protein cleavage product (p10) encoded by this area does exist (unpublished results, M. Collett). The rest of the genomic open reading frame encodes a 133kD protein (p133) that is processed to produce 58kD and 75kD proteins (p58 and p75). The proposed protein cleavage scheme of Collett et al. is shown in figure 1.3 (1988b).

Akkina's research has prompted the proposal of an alternative cleavage pathway for the p125, p38, and p133 coding regions (figure 1.4) (Akkina, 1991). Two new large proteins of 175kD and 172kD were identified as possible precursors of p125, and of p38 and p133 respectively. Furthermore, several additional proteins (168kD, 96kD, and 72kD) were found by immunoprecipitation with antisera to p133. The author theorized that the 175kD protein is cleaved to produce p125 and a 45kD protein (p45) which may be the previously unrecognized p30 of Collett's original map. The existence of this latter protein awaits confirmation.

The 172kD protein may follow two possible cleavage pathways, cleavage to 37kD and 135kD products with the latter then undergoing further processing to p58 and p75 proteins identified by Collett et al., and processing to a 168kD protein, which is then cleaved into 96kD and 72kD proteins; the 96kD protein may be processed to produce the p58 and p38 proteins identified by Collett et al., while the latter would contain the majority of the amino acid sequence of p75 defined by Collett (1988b).

The function of the BVDV proteins remains speculative (table 1.2). As alluded to earlier, p20 may be the nucleocapsid protein. Wiskerchen et al. determined that the p20 protein has autoproteolytic activity and is responsible for cleavage of p20 from the polyprotein (1991). Site-directed mutagenesis data suggested that the histidine residues at amino acid positions 40 and especially at amino acid position 49 were required for this activity. Additionally, BVDV recombinant constructs encoding the putative capsid genomic region but lacking the nucleotide sequence encoding amino acids residues 111-124 were unable to express a protein with this proteolytic activity. The deleted region of the protein included a serine and threonine that may play a role in catalysis. Finally, the cleavage site for p20 may be between amino acid residues 163-173, as deletion of the nucleotides

Figure 1.3. Schematic illustration of the predicted polyprotein cleavage pattern of the structural and nonstructural proteins of BVDV (Collett et al., 1988b). Numbers indicate molecular weight in kilodaltons.

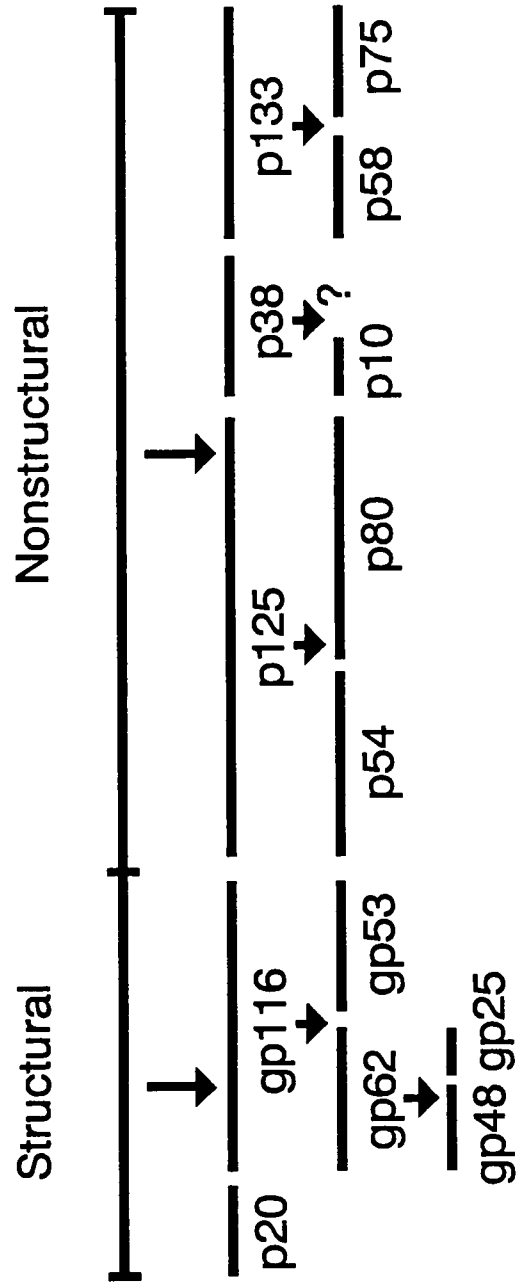


Figure 1.4. Proposed alternative cleavage pathways of the polyprotein containing the nonstructural proteins of BVDV (Akkina et al., 1991). Numbers indicate molecular weight in kilodaltons. Protein designations enclosed in parentheses denote analogous proteins characterized by Collett et al. (1988b).

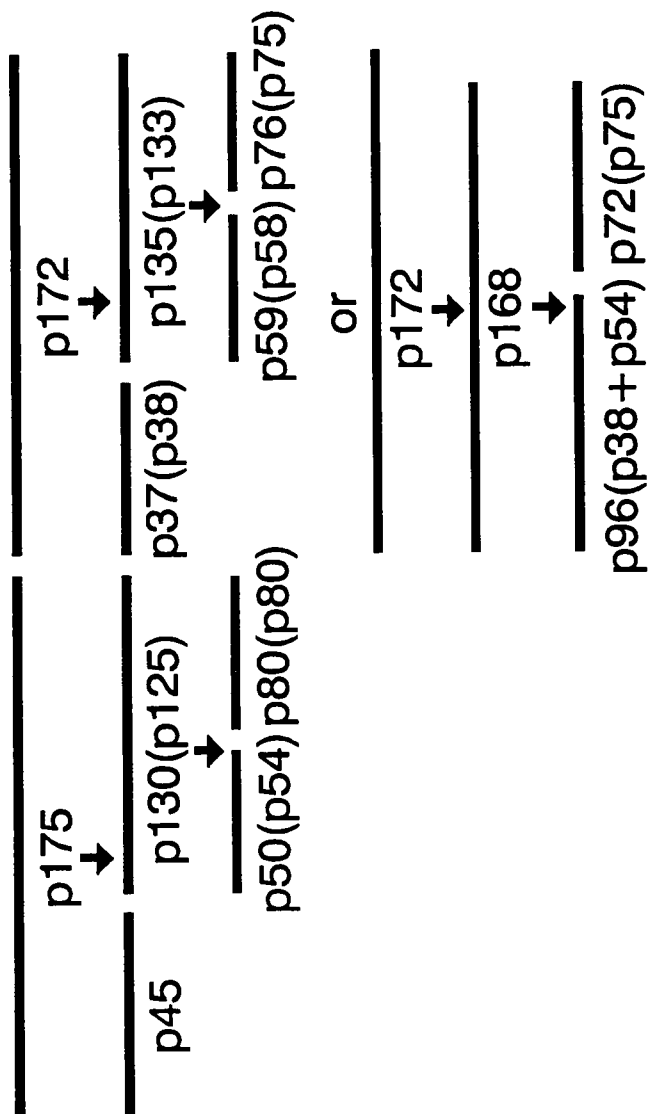


Table 1.2 Predicted function of BVDV proteins^a.

Protein	Function
p20	nucleocapsid
gp48 gp25 gp53	envelope and/or host cell membrane associated
p54/p125	RNA replication?, cellular RNA insertion locus, cytopathogenicity?
p80/p125	viral protease, helicase, RNA replication?
p10	?
p32?	?
p58	RNA replication
p75	RNA dependent RNA polymerase

^aCollett (personal communication).

encoding these residues resulted in abrogation of cleavage. Tryptophan at amino acid position 164 may be a likely candidate for the cleavage site, as mutation of this residue to alanine also inhibited cleavage.

A recent study by Thiel et al. prompted the investigators to propose the existence of a previously unidentified protein encoded immediately downstream of the p20 in pestiviruses (1991). Using antibodies specific for a fusion protein that included the carboxy-terminal half of p23 of hog cholera virus (which corresponds to the p20 of BVDV), a protein of 14kD molecular weight as well as p23 was consistently precipitated from hog cholera virus (HCV)-infected cells. Antibodies generated to small peptides from the putative carboxy terminus of p23 precipitated only the p14 from HCV-infected cells and from isolated virions, suggesting that this protein was not a cleavage product of p23, but a separate protein encoded downstream from p23. Its identification in purified virion preparations, and the absence of p23 from these preparations, led the investigators to conclude that p14 rather than p23 was the actual nucleocapsid protein, and that the latter was a nonstructural protein. These investigators obtained similar results from experiments using BVDV strains NADL and Osloss, and postulated that p20 was a nonstructural protein, and p14 was the authentic capsid protein. Autoproteolytic activity was proposed by these authors for the cleavage between p23 and p14 of HCV.

The next three proteins encoded by the BVDV genome, gp48, gp25, and gp53 are glycosylated, and they may function as envelope proteins. Gp48 may be the minor envelope protein of BVDV (Donis et al., 1987c). Dubovi has characterized a monoclonal antibody to gp48 which reacts with this protein from all strains of pestiviruses tested, indicating perhaps a degree of conservation among pestiviruses (Collett et al., 1989). Wiskerchen et al. have identified a stretch of highly hydrophobic amino acid residues at the amino terminus of gp48 which may function as the signal sequence for the BVDV glycoproteins (1991). A similar stretch of hydrophobic amino acids has been identified in the analogous glycoprotein of HCV, gp44/48, and is postulated to serve as the signal sequence for the structural glycoproteins of HCV (Stark et al., 1990; Rumenapf et al., 1991; Thiel et al., 1991). Additionally, Thiel et al. have demonstrated the formation of homodimers of gp44/48 in HCV virions (1991).

Gp25 is hydrophobic and may be a transmembrane protein (Collett et al., 1988b). It forms a heterodimer, by disulfide bridges, with the primary envelope protein, gp53, in infected cells (Weiland et al., 1990). The authors postulated that this dimerization occurs also in the virion.

Gp53 may be the major envelope protein (Donis et al., 1987b; Donis et al., 1987c). It is the immunodominant antigen of the virus, and antibodies to it neutralize the virus (Magar et al., 1988; Bolin et al., 1988a; Bolin et al., 1988b; Donis et al., 1988; Bolin et al., 1989; Bolin et al., 1990a). Bolin et al. identified three antigenic domains on this protein involved in virus neutralization (1988a). Moennig et al. identified 10 epitopes on gp53 to which neutralizing antibodies develop (1990). The mechanism by which BVDV is neutralized when antibody binds to gp53 is unknown, but antibody may interfere with attachment of the virus to host cell receptors or with fusion with the endosomal membrane (Donis et al., 1988). The molecular weight of gp53 varies by several kilodaltons among various BVDV strains (Donis et al., 1987a). This may be a result of evolutionary changes in the protein of certain viruses due to selective pressure from the immune response elicited by this protein (Donis et al., 1987a). Gp53 forms homodimers, and heterodimers with gp25 in BVDV-infected cells, and is postulated to do so in virions as well (Weiland et al., 1990; Thiel et al., 1991).

The protein encoded by BVDV immediately downstream from gp53 is the first of the nonstructural proteins, p125 (Collett et al., 1988b). This protein is highly conserved among pestiviruses, and may be the soluble antigen reactive in agar gel immunodiffusion tests with antisera to hog cholera virus and BVDV (Collett et al., 1989). The portion of the p125-encoding genomic region which encodes the p80 derivative may be the area of homogeneity among pestiviruses (Lecomte et al., 1990). P125 is cleaved to form p54 and p80 in cytopathic viruses only (Donis et al., 1987a; Pocock et al., 1987; Magar et al., 1988). The precursor-product relationship between these three proteins was firmly established by Collett et al. (1988b). The significance of the heterogeneity of peptides encoded by the p125 genomic region of the two biotypes is unclear (Brownlie, 1990).

Interestingly, the p125-encoding genomic region of cytopathic biotypes contains RNA insertions of cellular mRNA sequences (Meyers et al., 1990a; Meyers et al., 1990b). Cellular RNA sequences encoding ubiquitin was identified in one strain (Osloss) (Meyers et al., 1990a). This RNA insertion into the viral genome may

occur by recombination of RNA, and may be responsible for the alteration in biotype character (Meyers et al., 1990a; Meyers et al., 1990b). Meyers et al. identified a larger ubiquitin-coding sequence inserted in another cytopathic BVDV strain CP1 (1991). The nucleotide sequence at the 3' terminus of this RNA insertion was highly homologous to the flanking sequences of the Osloss strain, but genomic sequences at the 5' end of the insert were not homologous. These sites of homology may be areas for site-specific recombination. These investigators determined also that the cytopathogenicity was neither due to processing of p125, nor expression of p54, and that the only consistent indicator of cytopathogenicity was p80 expression. They postulated that the expression of an ubiquitin fusion protein from mutated ubiquitin sequences may be responsible for cytopathic effects. These mutated sequences existed in those viruses with cellular ubiquitin RNA sequences.

Additional properties of products encoded by the p125 region are suggested by amino acid sequence homology findings. A zinc finger domain, found in nucleic acid binding proteins, has been identified in the carboxy-terminal region of the p54 derivative of p125 (Moerlooze et al., 1990). The authors speculate that this domain may not function in noncytopathic biotypes in nucleic acid binding because the p125 remains uncleaved and the domain corresponding to p54 is different conformationally from the cytopathic virus p54 cleavage product. They also postulated that p54 may function in RNA binding. Nucleotide sequence comparison of pestivirus genomes to flavivirus genomes has revealed also homology of the p125-encoding region to known protease and helicase protein-encoding regions (Gorbalenya et al., 1989). Protease activity may exist at the amino terminus of the p80 portion of p125, while helicase activity may occur in the carboxy-terminal area (Gorbalenya et al., 1989). Furthermore, the protease may be similar to a serine protease of the trypsin family (Bazan et al., 1989). The flavivirus protease that processes the precursor polyprotein into the mature proteins is a serine protease and p125 or p80 may function in this capacity in pestiviruses (Bazan et al., 1989).

The genomic region between that coding for p125 and p133 could encode a protein of 38kD (Collett et al., 1988b). A 10kD protein has been identified as a product of this region, but its function remains unknown (Collett, personal communication). The remaining area between the p125- and p133-encoding regions

could encode a protein of 32kD, but this protein has not been identified (Collett, personal communication).

The remaining gene product encoded by the BVDV genome is a 133kD protein (p133) which is cleaved to form a 58kD protein (p58) and a 75kD protein (p75) (Collett et al., 1988b). These proteins may function in RNA replication (Collett et al., 1988c; Collett, personal communication). The RNA sequence encoding an amino acid sequence Gly-Asp-Asp, which is common to all flavivirus RNA polymerases, has been found only in this region of the BVDV genome (Collett et al., 1988c).

OTHER PESTIVIRUSES

The genus Pestivirus includes two additional viruses, border disease virus and hog cholera virus. Border disease virus (BDV) of sheep is more closely related to BVDV than hog cholera virus (HCV) is (Acland et al., 1972; Hamilton et al., 1972). Support for this conclusion is that BVDV and BDV cannot be distinguished serologically (Collett et al., 1989; Cay et al., 1989; Wensvoort et al., 1989a). Also, the disease syndromes caused by BDV in sheep bear a striking resemblance to those resulting from BVDV infection in cattle. Infection by either BDV or BVDV of the immunocompetent sheep or bovine results in only mild clinical disease (Nettleton, 1987). Much more serious disease occurs following BDV infections of pregnant ewes. Consequences of BDV infection of lamb fetuses include abortion, stillbirth, and severe fetal disease (Nettleton, 1987; Loken et al., 1991). Severity of disease in fetuses depends upon the stage of gestation of the ewe at the time of infection (Nettleton, 1987). Lambs infected with BDV after day 80 of gestation are not affected (Nettleton, 1987). Infection around 50-63 days of gestation results in the classical "hairy shaker" disease syndrome (Nettleton, 1987). This syndrome develops from destruction of oligodendrocyte precursors leading to myelin deficiency during central nervous system (CNS) development (Nettleton, 1987). A significant reduction both in size and density of the spinal cord, principally of the white matter, occurs in affected lambs (Done et al., 1985). Fetal resorption, mummification, abortion, or stillbirth of lambs can occur if infection occurs in immunologically immature fetuses (Nettleton, 1987). Fetal infection of lambs prior to 80 days of gestation can result in severe abnormalities, especially of the CNS, in lambs

surviving to term (Nettleton, 1987). These abnormalities include hydranencephaly, porencephaly, and cerebellar hypoplasia (Nettleton, 1987). Infection of the fetus prior to development of immunocompetence can result also in persistent viremia as a result of specific immunotolerance to the infecting virus (Nettleton, 1987).

Several factors affect the outcome of infection of a pregnant ewe: BDV strain virulence; the immune status of the ewe; and breed of sheep (Nettleton, 1987). Superinfection of persistently-infected sheep with certain immunologically homologous cytopathic BDV strains resulted in serious disease analogous to mucosal disease of cattle (Nettleton, 1987). Infection with a heterologous BDV strain did not produce disease (Nettleton, 1987). Virus recovered from sheep with mucosal disease-like syndrome was of cytopathic biotype similar to virus recovered from cattle with BVDV-induced mucosal disease (Nettleton, 1987). The origin of the cytopathic virus precipitating mucosal disease-like syndrome is unknown (Nettleton, 1987). Additionally, the immune system of sheep is affected by the virus since impaired immunoglobulin synthesis and reduced lymphocyte response to mitogens may occur in BDV-infected lambs (Terpstra, 1981; Salsbury, 1985; Lamontagne et al., 1989).

Sheep to sheep contact is the primary mode of spread of BDV, and persistently-infected sheep are the most important source of virus (Barlow et al., 1980; Terpstra, 1981; Nettleton, 1987). The virus is shed in most body excretions, including nasal excretions, saliva, feces, and urine (Terpstra, 1981).

Akkina et al. identified several viral peptides in BDV-infected cells (1990). Molecular weights of these peptides were 220kD, 165kD, 118kD, 84kD, 66kD, 58kD, 55kD, 53kD, 45kD, 37kD, and 31kD. The glycosylated proteins were the 165kD, 118kD, 84kD, 66kD, 58kD, 55kD, 53kD, 45kD, and 31kD proteins. All BDV proteins in this study resembled those of noncytopathic BVDV strains in that an 80kD protein was lacking but a larger 220kD protein was present. A protein of similar molecular weight to the latter protein was also identified in noncytopathic BVDV-infected cells by these investigators. Additionally, antisera to either BDV or BVDV precipitated similar proteins from BDV and BVDV-infected cell cultures. The researchers concluded that this indicated sharing of antigenic epitopes. The authors also suggested that because the smallest glycoprotein identified in all three pestiviruses varied in size (31, 33, and 25kD for BDV, HCV, and BVDV respectively) this protein may be a critical distinguishing factor among the three

viruses. In another study, by Dutia et al., using 10 monoclonal antibodies to BDV-infected cell lysate, an 80kD protein in addition to a 130kD protein, the probable precursor of the former, was precipitated from cytopathic BDV-infected cells, while only a 120kD protein was precipitated from noncytopathic BDV-infected cells (1990). The authors could not explain the discrepancy in size between the high molecular weight proteins from the two biotypes.

Hog cholera virus (HCV) has been analyzed in more depth than Border Disease virus has been. As with BVDV and BDV, the outcome of infection HCV is dependent upon several factors related to virus and host (Liess, 1987). Strains of HCV differ in virulence (Liess, 1987). The syndromes of HCV-induced disease also depend upon the age and immunocompetence of the infected animal (Liess, 1987). Fetal infection results in many of the same consequences that occur with BVDV and BDV; abortion, stillbirth, congenital malformations, and specific immunotolerance resulting in viral persistence (Liess, 1987; Vannier et al., 1988). The occurrence of the latter depend upon the stage of gestation at the time of fetal infection (Liess, 1987). Acute and/or chronic disease follow primary postnatal infection (Liess, 1987). Endothelial cells of capillaries and lymphatic vessels may be destroyed resulting in the classical acute disease of hog cholera, while chronic disease is a consequence of a slower course, and single organ systems only may be affected (Liess, 1987). Factors such as HCV strain virulence, occurrence of secondary infections in infected animals, and immune status of the animal determine the course of disease (Liess, 1987).

HCV replicates in cells of the immune system of infected pigs (Laude, 1987). Effects of viral infection on immunocompetent cells are not clear, but interferon production is impaired (Laude, 1987). Viral transmission among swine occurs primarily by the oronasal route (Liess, 1987). The infection can also be spread during coitus (Liess, 1987). Virus is shed in high concentrations in both oronasal and intestinal excretions of infected pigs (Liess, 1987).

Few HCV strains are cytopathic for infected cell cultures, and only two cytopathic strains have been identified thus far (Laude, 1987). Only two HCV serological subgroups have been identified, indicating less heterogeneity among HCV strains than exists with the genome of BVDV (Laude, 1987). The genome of two strains of HCV have been sequenced and analyzed; the Alfort strain and the Brescia strain (Meyers et al., 1989; Moorman et al., 1990a; respectively). Both

strains are noncytopathic for cell cultures (Meyers et al., 1989; Moorman et al., 1990a).

The polypeptide profile of HCV appears to be very similar to that of BVDV. In a study by Rumenapf et al., specific antisera to HCV precipitated proteins of 115-125kD, 80kD, and 53kD, 48kd, 44kD, and 33kD from HCV-infected cells (1989). The latter four were glycosylated. Also, the genomic RNA of HCV was similar in size to that of BVDV, and no subgenomic species of RNA were identified.

The genome of the Alfort HCV strain was 12,284 nucleotides long with one long open reading frame (Meyers et al., 1989). It encoded a protein containing 3898 amino acids and with a molecular weight of 435kD (Meyers et al., 1989). The nucleotide homology of HCV RNA with NADL BVDV strain was 66%, but the amino acid homology between the two viruses was 85% (Collett et al., 1988a; Meyers et al., 1989). In contrast to some cytopathic BVDV genomic sequences, no cellular RNA insertion was evident in noncytopathic HCV; whether this absence of a cellular RNA insertion in the genome of HCV Alfort is causally related to the observed noncytopathic biotype of the virus has not been determined (Meyers et al., 1989). Basic amino acids encoded at the 5' end of HCV may indicate the core virion protein is a product of this genomic region (Meyers et al., 1989). Additionally, glycoproteins may be encoded immediately downstream of this basic region as may be the case with the BVDV genome (Meyers et al., 1989). The genomic organization of the glycoproteins was determined to be gp44/48-gp33-gp55 from N to C terminus (Stark et al., 1990). This was determined by immunoprecipitation of HCV viral proteins with polyclonal antisera generated against fusion proteins expressed from cloned viral sequences (Stark et al., 1990). The HCV genomic area encoding the protein analogous to p133 of BVDV is not highly conserved between HCV and BVDV, but the region encoding p115-125 may be well conserved between these two viruses (Meyers et al., 1989).

The genome of the Brescia HCV strain is 12,283 nucleotides long (Moorman et al., 1990a). Nucleotide sequence analysis revealed one long open reading frame spanning the genome, starting from the codon at nucleotide residues 361-363, and stopping at codon at residues 12,055-12,057. This open reading frame codes for a protein of 3898 amino acid residues with molecular weight of 438kD. Sequence analysis of the predicted amino acid sequence suggested the existence of a signalase cleavage site at amino acid residue 689, a clustering of potential glycosylation sites

between amino acid residues 150 and 950, and basic residues between amino acids 175 and 250. Based on this data, it was concluded that the structural proteins are encoded at the 5' end as they are with BVDV. Antisera to synthetic peptides with amino acid sequences predicted by the nucleotide sequence in this area of the genome reacted with a 51-54kD protein from HCV-infected cell lysates. This protein may be encoded by genomic region corresponding to the amino acid residues 690 to 1060. This genomic region includes five glycosylation sites and a large hydrophobic stretch at the C-terminus that may serve as the membrane anchor.

No cellular RNA sequence insertion was found in the genomic region encoding p125 of the Brescia HCV strain (Moorman et al., 1990a). Nucleotide sequence conservation occurred between this area in HCV Brescia strains and that of BVDV NADL was noted, and included those areas encoding peptides predicted to function as helicases and serine proteases. Finally, conservation of the nucleotide sequence encoding a three amino acid sequence common to RNA polymerases, Gly-Asp-Asp, was also identified within the gene product closest to the 3' end.

Analysis of the epitopes on the major envelope protein of the Brescia HCV strain identified four distinct antigenic domains, A, B, C, and D (Wensvoort, 1989c). Domain A was subdivided further into three subdomains, A1, A2, and A3. Analysis with monoclonal antibodies indicated that domains A1 and A2 were conserved among all HCV strains tested, and antibody to A1 was capable of neutralizing all 94 HCV strains of the study. In another study using eight monoclonal antibodies, a 56kD and 60kD protein doublet was precipitated from HCV-infected cell lysates (Wilke et al., 1990). The authors concluded that this protein represents the major HCV glycoprotein, and suggested that its epitopes were unique and highly conserved. Few viruses were neutralized by these monoclonal antibodies. Two antigenic domains A and B were detected on the major glycoprotein in contrast to the three domains identified by Wensvoort (Wensvoort, 1989c). Additionally, five subdomains of antigenic subdomain A were identified in contrast to the three subdomains detected by Wensvoort.

As with BVDV, the smallest HCV glycoprotein, gp33, formed a heterodimer with the major HCV glycoprotein (Weiland et al., 1990; Thiel et al., 1991). In one study, monoclonal antibodies specific for gp55 also precipitated gp33 (Weiland et al., 1990). Under nonreducing conditions, antibodies to both gp55 and gp33

precipitated one protein of 68,000 Daltons which may have been a dimer of the two proteins. The mobility of this protein was greater than the sum of its two component parts (68,000 vs. 53,000 + 33,000) which suggested the presence of intramolecular disulfide linkages. Another study, using monoclonal antibodies to gp55 and gp44/48 of HCV, indicated, not only that gp55 formed heterodimers with gp33 in the virion, but that gp55 and gp44/48 formed homodimers with themselves in the virion as well (Thiel et al., 1991). Comparison of the glycoproteins of HCV to those of BVDV revealed that the hydrophobicity profiles were almost identical (Weiland et al., 1990). Cysteine residues are conserved between gp33 of HCV and gp25 of BVDV. BVDV gp53 possessed two more cysteines than gp55 of HCV. The conservation of cysteines among Flaviviruses is evidently important (Weiland et al., 1990).

Recent investigation of a previously uncharacterized disease agent of people, hepatitis C virus, has revealed some similarities to Pestiviruses. Though hepatitis C virus is not classified within this genus, it may be related to pestiviruses (Miller et al., 1990). This virus causes non-A, non-B hepatitis in people after blood transfusion and the disease can progress to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Kato et al., 1990).

The genome of hepatitis C virus is single stranded RNA of positive polarity and is approximately 10 kilobases in length (Kato et al., 1990). Data suggested that the virus has one long open reading frame encoding 3,010 amino acids (Takamizawa et al., 1991). Sequence analysis of the genome indicated some homology to the flavi- and pestiviruses (Takamizawa et al., 1991). Most amino acids encoded at the 5' end of the genome were very basic in nature which is similar to the core proteins of both pesti- and flaviviruses (Kato et al., 1990). Additionally, a clustering of glycosylation sites of proteins encoded at the 5' end of the genome similar to that observed with the envelope proteins of the pesti- and flaviviruses has been identified (Kato et al., 1990).

The nucleotide sequence of the hepatitis C virus genomic region analogous to that of the nonstructural proteins of the pesti- and flaviviruses were similar, especially within the regions encoding p125/NS3 proteins of the latter two genera (Miller et al., 1990). The region encoding the putative replicase in pestiviruses, however, does not have significant homology with hepatitis C virus, although the latter does encode the three amino acid sequence common to RNA polymerases of flaviviruses (Miller et al., 1990; Takamizawa et al., 1991). The hydrophobicity

profile of predicted proteins encoded by the hepatitis C virus genome is similar to that of flavi- and pestiviruses (Miller et al., 1990; Takamizawa et al., 1991; Kato et al., 1991). Despite the apparent resemblance of hepatitis C virus to pesti- and flaviviruses, its exact relationship of these viruses will require further investigation.

FLAVIVIRUSES

The pestiviruses are currently being reclassified within the family Flaviviridae (M. Collett, personal communication). Interest in flaviviruses, some of which cause serious human diseases, has led to more extensive characterization of these viruses than has been done with pestiviruses. This family, consisting of more than seventy pathogens of veterinary and human importance, includes the agents of yellow fever, dengue, Japanese encephalitis, louping ill, Wesselsbron, and West Nile encephalitis (Rice et al., 1985; Monath, 1986). The important Flaviviridae members are listed in table 1.3.

Flaviviruses have an RNA genome which is single stranded and of positive polarity (Westaway, 1987). The genomic length is about 11 kilobases and encodes one long open reading frame spanning the genome (Rice et al., 1985; Brinton, 1986). The RNA genome possesses a cap at the 5' terminus, but no 3' polyadenylate tail (Brinton, 1986). The virus is enveloped, and has a diameter of approximately 45 nm (Brinton, 1986; Westaway, 1987). The capsid has icosahedral symmetry (Rice et al., 1985). Structural proteins, designated capsid (C), membrane (M), and envelope (E), are encoded at the 5' end, while the nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5, are encoded by 75% of the genome at the 3' end (Rice et al., 1985; Rice et al., 1986b). The genomic organization has been determined from nucleotide sequencing of several flaviviruses (Rice et al., 1985; Rice et al., 1986b; Hahn et al., 1988). The sequence of proteins encoded from the 5' end are C, prM/M, E, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (figure 1.5; Rice et al., 1985).

The capsid protein of flaviviruses, as with pestiviruses, has a high basic amino acid content (Rice et al., 1985; Westaway, 1987). These positively charged amino acids may function in neutralization of negative charges of the RNA genome (Westaway, 1987). PrM may be the precursor of a structural protein, M (Rice et al., 1985; Rice et al., 1986b). The precursor is glycosylated, while the mature protein,

Table 1.3 Flaviviridae causing important diseases.^a

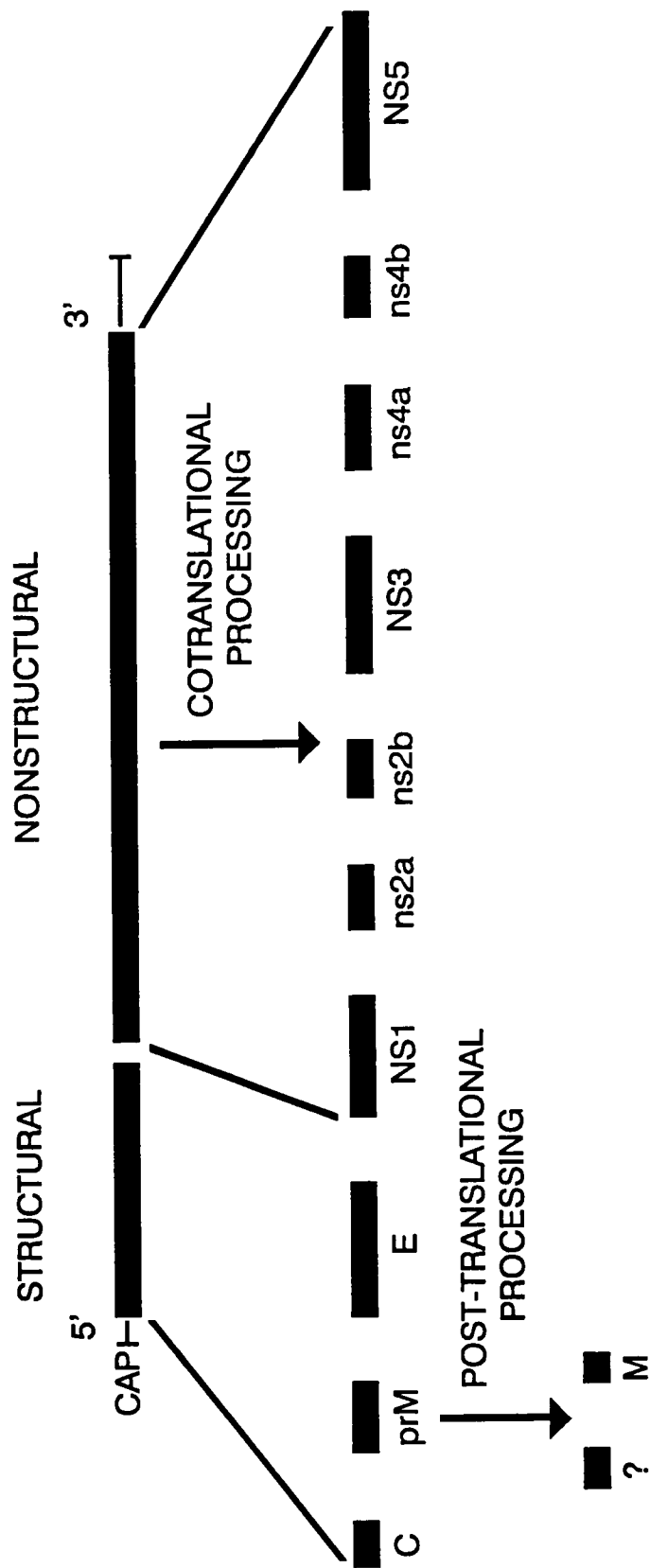
Genus	Disease	Species affected
Flavivirus	Yellow Fever	man
	Dengue	man
	Japanese Encephalitis	man, horses, pigs
	Murray Valley Encephalitis	man, horses
	West Nile Encephalitis	man, horses
	Tick-Borne Encephalitis	man
	St. Louis Encephalitis	man
	Wesselsbron	sheep, cattle
	Louping Ill	sheep, cattle, pigs, horses, deer, captive red grouse
Pestivirus	Bovine Viral Diarrhea	cattle, sheep, goats, deer, captive exotic ruminants, man(?) ^b
	Hog cholera	pigs
	Border Disease	sheep, pigs, cattle, goats
	Hepatitis C(?) ^c	man

^aMonath et al. (1986).

^bNot proven to cause disease in man.

^cClassification is speculative.

Figure 1.5. Genomic organization of the flaviviruses. The noncoding regions are shown as a solid line. The translated region is shown as a large shaded line, with demarcation between the structural and nonstructural protein-encoding regions indicated by a line break. The structural and nonstructural proteins encoded by the genome are shown below the genome as solid boxes. The protein designations are described in the text (Rice et al., 1985).



encoded at the C-terminal portion of prM, is not (Rice et al., 1986b). Additionally, prM possesses a stretch of hydrophobic amino acid residues at its amino terminus that may function as a signal sequence, while M contains two long segments of uncharged amino acids that may serve as a membrane anchor (Rice et al., 1985). This entire region encoding the capsid and prM proteins may be analogous to the p20 of BVDV based on hydrophobicity profiles (Collett et al., 1988c).

The next gene product is the envelope protein which hemagglutinates and induces neutralizing antibodies (Rice et al., 1986b). This protein may or may not have two possible glycosylation sites depending on the viral strain (Hahn et al., 1988). It also has a hydrophobic stretch at its carboxy terminus that may function in membrane anchorage (Rice et al., 1985). This region may be comparable to gp62 of BVDV, but the latter contains many more sites of glycosylation (Collett et al., 1988c).

Following the E protein gene is the gene encoding NS1 protein (Rice et al., 1985; Rice et al., 1986b). Interestingly, this nonstructural protein is glycosylated, and occurs on the surface of infected cells (Rice et al., 1985). Antibodies to it are capable of inducing complement-mediated lysis of infected cells (Rice et al., 1985). It possesses a carboxy-terminal hydrophobic stretch that may serve as a transmembrane domain, and may function in virus assembly (Rice et al., 1985). This region may be equivalent to gp53 of BVDV (Collett et al. 1988c).

Flaviviruses have two major nonstructural proteins, NS3 and NS5 (Rice et al., 1985; Rice et al., 1986b; Collett et al., 1988c). These proteins may function in RNA replication, and are analogous to p125 and p133 of BVDV respectively (Collett et al., 1988c). Nucleotide sequence analysis of the NS3- and NS5-encoding region of flaviviruses and the p125- and p133-encoding regions of pestiviruses has revealed stretches of amino acids encoded by these areas that are conserved among the respective proteins of the two virus genera (Collett et al., 1988c). Five expanses within p125 align with NS3 of yellow fever virus, while three such regions are found in p133 comparable to NS5 (Collett et al., 1988c).

The other nonstructural proteins of flaviviruses, NS2a, NS2b, NS4a, and NS4b, have not been well characterized (Rice et al., 1985). NS2a may function in the processing and/or transport of NS1, as epitopes of both have been identified within one polyprotein (Chambers et al., 1989). NS4b may be associated with the cell membrane, and may assist in forming a membrane-bound replicase complex

(Chambers et al., 1989). Analogous proteins have not been identified in BVDV-infected cells (Collett et al., 1988c).

Viral entry of flaviviruses into cells may occur via endocytosis to intracellular vesicles and lysosomes (Brinton, 1986). Viral replication occurs in the perinuclear region of cells (Brinton, 1986). Following uncoating, the genome is translated producing the viral replicase (Brinton, 1986). A minus strand copy of genomic RNA then is produced from the positive template which in turn serves as the template for positive strand production (Brinton, 1986). Newly formed positive strand RNA serves as messenger RNA or becomes encapsidated to form the virion genomic material (Brinton, 1986). It is not known how RNA synthesis is initiated, or how the positive strand is selected for translation (Brinton, 1986). The single open reading frame is translated to produce a polyprotein that is co- and post-translationally processed to form the mature proteins (Westaway, 1987). Cleavages occur after the first methionine residue, after dibasic residues, and, for the glycoproteins, after amino acids with short side chains which are preceded by signal sequences (Westaway, 1987). It is unclear whether viral and/or cellular proteases affect cleavage of the polyprotein (Westaway, 1987). The locale of virion assembly within infected cells is unknown, though budding is thought to occur within intracellular vesicles (Westaway, 1987).

PREVENTION OF BOVINE VIRAL DIARRHEA VIRUS INFECTION

One of the primary means of controlling BVDV infections is prevention, especially by vaccination (Radostits et al., 1988). Two types of vaccines are available against BVDV, killed (inactivated), and modified live virus (Baker, 1987; Radostits et al., 1988; Ames et al., 1990). Controversy surrounds the use of both (Ernst et al., 1983).

The modified live vaccine was the first vaccine to be developed to protect against BVDV infection (Neaton, 1986). Though these vaccines have the advantage of inducing a higher level of and longer-lasting immunity than killed virus vaccines, several drawbacks exist that diminish their usefulness. It is well documented that the vaccine virus readily crosses the placenta when administered to pregnant cattle (Orban et al., 1983). Though some reports indicate that a degree of protection of the calf in the postnatal period following vaccination of the pregnant cow occurs

(Sanders et al., 1983), many of the sequelae seen following fetal infection with wild type BVDV may occur also after vaccination (Baker, 1987; Radostits et al., 1988; Ames et al., 1990). These include abortion, stillbirth, congenital abnormalities, and persistent viremia (Stober, 1984; Baker, 1987; Radostits et al., 1988; Ames et al., 1990).

Outbreaks of BVDV-induced disease have occurred following vaccination with modified live BVDV vaccines (Peter et al., 1967; Bittle et al., 1973; Edwards et al., 1986; Baker, 1987; Ames et al., 1990). Several factors, such as the immune status of the animal, the presence of pre-existing BVDV infection, or contamination of the vaccine with a virulent virus, may be responsible for BVD disease after vaccination (Rosner et al., 1968; Bittle et al., 1973; Ernst et al., 1983). In one study, reviewed by Lambert, 178 cattle out of 92,621 developed clinical mucosal disease following vaccination (1973). In some smaller herds the incidence was higher. Mucosal disease storms may be the result of vaccination of persistently infected cattle (McKercher et al., 1968; Baker, 1987; Radostits et al., 1988). The vaccine virus may cause immunosuppression following its administration to immunocompetent cattle (Roth et al., 1983; Baker, 1987; Ames et al., 1990). Studies in the Bruce County beef cattle project indicated that vaccination of feeder calves with modified lived vaccine resulted in increased incidence and severity of respiratory disease (Martin et al., 1980; Martin et al., 1981; Martin et al., 1982; Martin et al., 1983).

A temperature-sensitive safe live virus vaccine was developed in 1984 (Lobmann et al., 1984; Lobmann et al., 1986). It did not cross the placenta, and was not immunosuppressive, but induced disease after its administration to stressed animals (Lobmann et al., 1984; Lobmann et al., 1986). Killed virus vaccines also are safe, do not cross the placenta, do not cause immunosuppression, and induce antibody production (Lambert, 1973; Baker, 1987). However, these vaccines are of dubious efficacy, as they neither induce high antibody titers nor cell-mediated immunity, and revaccination must be frequent because duration of immunity is much shorter (Bittle et al., 1973; Neaton, 1986).

An additional concern with both modified live and killed BVDV vaccines is the spectrum of protection produced after vaccination (Ames et al., 1990). Studies indicated incomplete or no protection in vaccinates against infection with heterologous strains (Ames et al., 1990).

An effective vaccine must fulfill several requirements: it must be safe to administer, it must protect the fetus, prevent clinical disease in vaccinates, and it protect vaccinates against heterologous strains of BVDV (Duffell, 1984; Perdriest et al., 1987; Harkness, 1987; Potgieter et al., 1987). Genetic engineering holds great promise in satisfying these requirements (Harkness, 1987; Potgieter et al. 1987).

A key target for a genetically engineered vaccine is the gp53 protein, as it is the only virally-induced polypeptide that is consistently precipitated with BVDV antisera, and is the target in viral neutralization by specific antibodies (Bolin et al., 1990a). Precedence exists for this approach. Immunization of cattle with recombinants of a togavirus and vaccinia virus expressing capsid and envelope proteins of Sindbis virus resulted in high levels of neutralizing antibodies to the virus (Franke et al., 1985). Administration of detergent-solubilized viral proteins to calves resulted in antibody levels similar to vaccination with inactivated BVDV suggesting recombinant vaccines producing viral peptides or vaccines containing synthetic viral proteins may provide effective protection against BVDV-induced disease (Mohanty et al., 1989).

Perhaps the most convincing data in support of recombinant vaccines for BVDV control comes from work with hog cholera virus. A recombinant vaccinia virus with the genes for all four structural HCV genes provided complete protective immunity from lethal challenge inoculation in pigs (Rumenapf et al., 1991). Administration of the same recombinant lacking the genetic sequence of most of gp55 provided partial protective immunity in pigs, even though neutralizing antibodies were not induced. The latter observation indicated that cellular immunity may play a vital role in protection. A pseudorabies recombinant possessing the gene for gp55 induced neutralizing antibodies to both pseudorabies and hog cholera in pigs (Zijl et al., 1991). Pigs given this recombinant were protected against lethal challenge by inoculation of both viruses. The authors concluded that the envelope protein was sufficiently immunogenic for generation of protective immunity. Gp53 of BVDV may well prove to be the key to protection against BVDV.

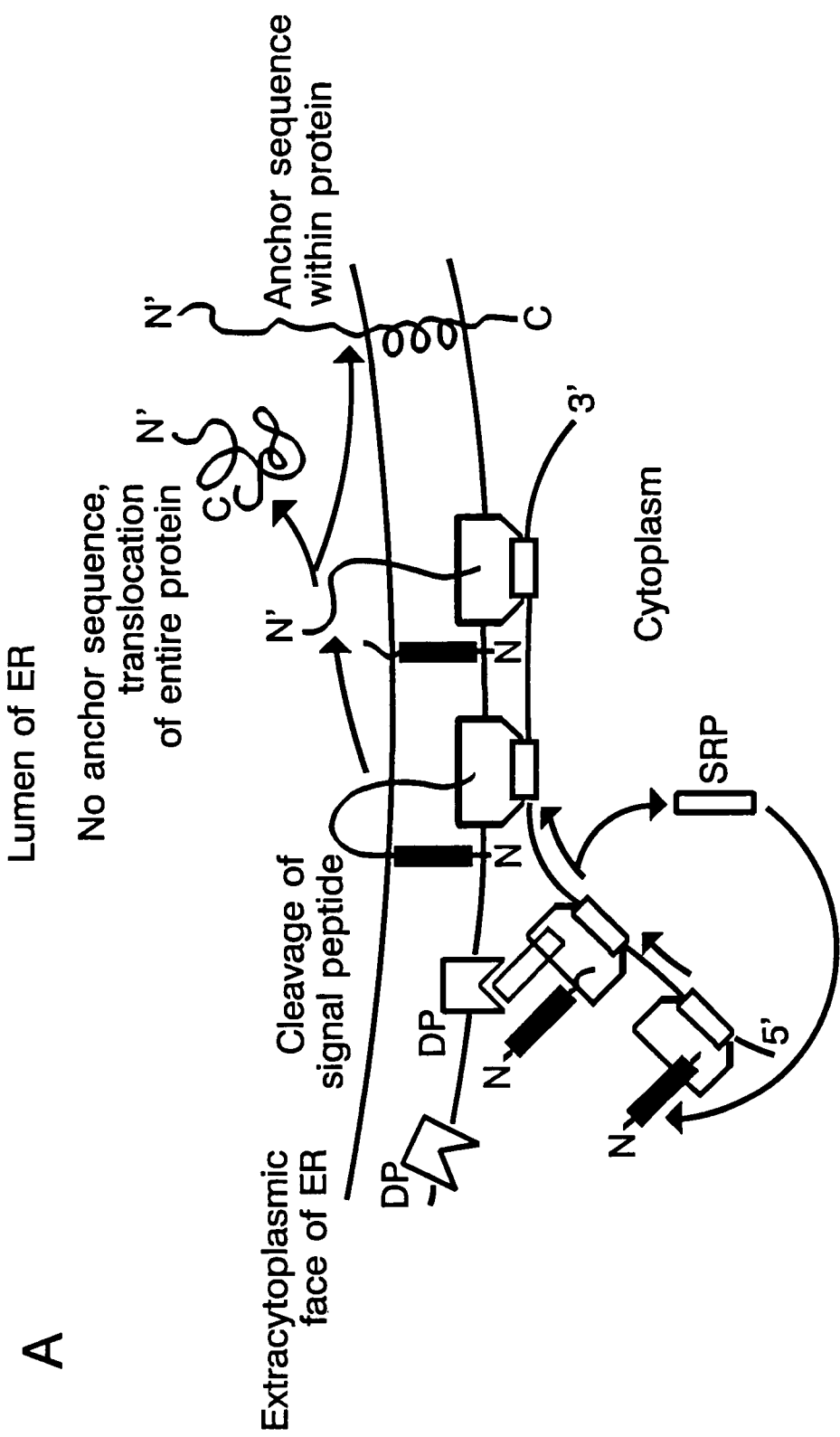
MEMBRANE PROTEIN INSERTION

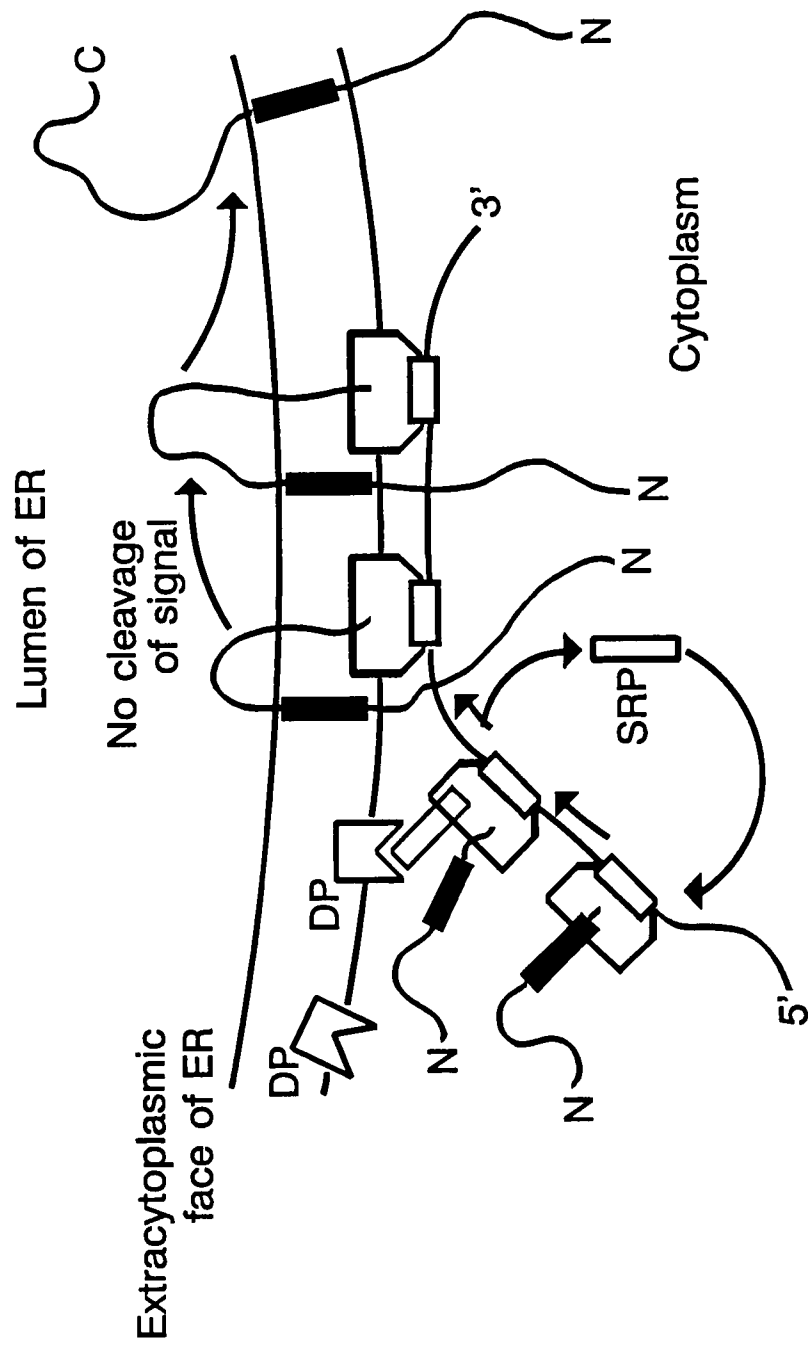
The mechanism of protein insertion, translocation, and anchorage in membranes of both prokaryotes and eukaryotes has been under intense investigation in recent years (Engelman et al., 1981; Gilmore et al., 1982; Perlman et al., 1983; Randall, 1983; Heinje, 1984; Adams et al., 1985; Davis et al., 1985; Wickner et al., 1985; Heinje, 1986; Markoff et al., 1986; Romisch et al., 1990). These investigations have resulted in several models to explain these phenomena.

Perhaps the most widely accepted theory is that of the signal hypothesis proposed by Blobel and Dobberstein in 1975 (Markoff et al., 1986). They postulated that proteins being synthesized that are destined for membrane insertion or secretion possess a "signal peptide" that is responsible for targeting the protein to specific membrane locations (Markoff et al., 1986). This signal sequence, positioned at the amino terminus of the protein, serves to recognize the target membrane. This recognition results in binding of the signal peptide to the membrane, initiation of pore formation within the membrane, and noncovalent binding of ribosomes to the membrane at the site of protein insertion. The elongating polypeptide is transported through the pore, using energy from chain elongation. Transport would continue until a "stop-transfer" amino acid sequence is encountered in the nascent polypeptide which ensures insertion of the protein into the membrane. If the latter amino acid sequence does not exist in the protein, the entire protein is secreted into the lumen of the endoplasmic reticulum (ER) (figure 1.6).

Experimental evidence to support this hypothesis includes the discovery, in recent years, of the cellular components of the machinery responsible for localization of the protein at the ER. The signal recognition particle (SRP) binds the nascent polypeptide, preventing its elongation, and also interacts with an integral membrane protein of the ER, the SRP receptor (Romisch et al., 1990). These two elements, the SRP and SRP receptor, have been isolated and characterized. The SRP consists of 7S RNA and six protein subunits with molecular weights of 9kD, 14kD, 19kD, 54kD, 68kD, and 72kD (Romisch et al., 1990). The 9kD and 14kD protein subunits bind to the 7S RNA component, and are responsible for retardation of nascent protein chain elongation (Romisch et al., 1990). The 68kD and 72kD subunits are required for interaction of the SRP with the SRP receptor (Romisch et al., 1990). The 19kD and 54kD subunits together are

Figure 1.6. Schematic illustration of the signal hypothesis for cotranslational insertion of membrane and secreted proteins in the endoplasmic reticulum (ER). (A) The signal recognition particle (SRP) binds to the nascent polypeptide at the signal sequence (solid box) and initiates interaction with the signal recognition particle receptor, or docking protein (DP) in the ER membrane. This results in insertion of the signal sequence into the membrane. The signal sequence is cleaved by a cellular protease, and the elongating polypeptide is extruded into the ER lumen. If a "stop-transfer" sequence is encountered, the protein becomes anchored with the amino terminus in the lumen of the ER; otherwise, the entire protein is secreted into the ER lumen. (B) If the signal sequence is not removed from the nascent polypeptide, it may serve as the membrane anchor, with the resultant membrane protein will have its amino terminus facing the cytoplasm (Wickner et al., 1985).





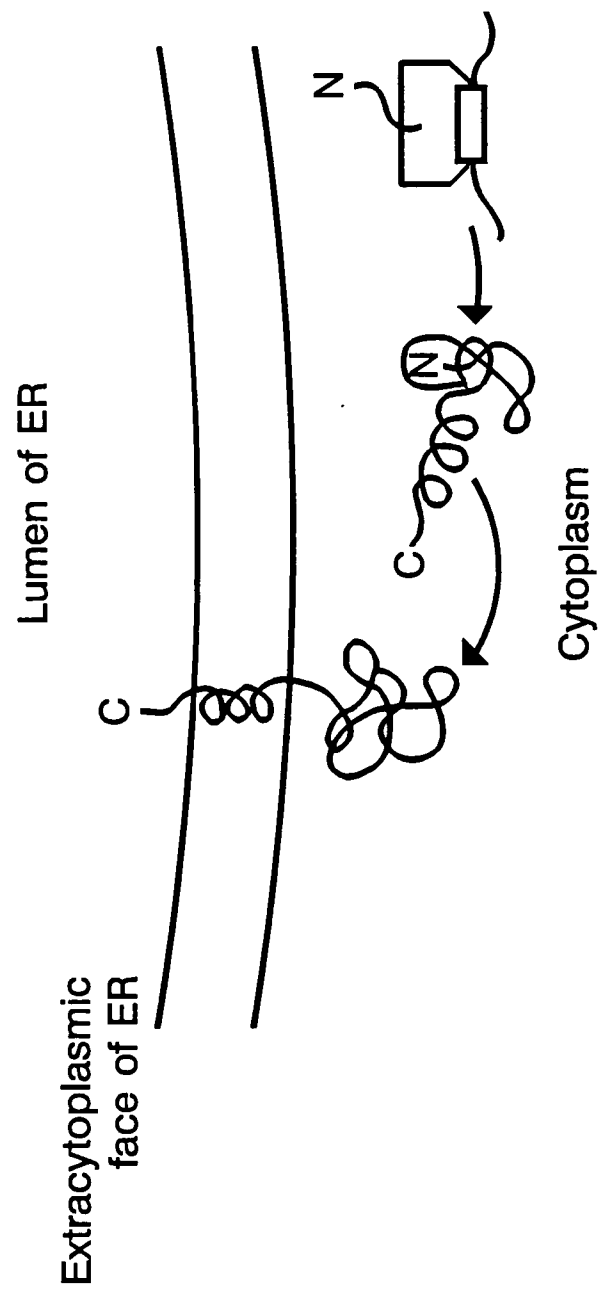
responsible for binding of the signal sequence of the nascent polypeptide to the SRP (Romisch et al., 1990). The SRP receptor is a 72kD integral membrane protein of the ER that interacts with the SRP and mediates translocation (Gilmore et al., 1982). Whether this interaction between the SRP and its receptor is sustained, or whether it is merely transient, serving only to initiate the process is unclear (Gilmore et al., 1982). The SRP and SRP receptor may recycle after translocation of the nascent protein (Markoff et al., 1986).

Variations on this hypothesis have been proposed (Engelman et al., 1981; Randall et al., 1983; Wickner et al., 1985). The direct transfer model postulates that the energy provided by polypeptide chain elongation is sufficient for translocation of the nascent polypeptide across the membrane, thus eliminating the need for a "pore" as such (Randall et al., 1983).

The helical hairpin theory proposes that the protein destined for membrane insertion forms two antiparallel helices, one of which is the signal sequence (Engelman et al., 1981). This helical hairpin inserts spontaneously into the membrane. If the second helix is polar, transfer of the protein across the membrane will continue until a stop-transfer amino acid sequence is encountered as it passes through (Engelman et al., 1981). Following this protein translocation, the leader peptide is removed by the signal peptidase within the lumen of the ER (Engelman et al., 1981). No additional proteins are required for this process (Engelman et al., 1981).

The final theory, proposed by W. Wickner, also suggests that translocation and insertion of membrane proteins occurs spontaneously based on the secondary and tertiary conformation of the nascent protein, and requires no protein machinery such as the SRP and SRP receptor (Randall et al., 1983). This model proposes that the translocation process occurs post- rather than cotranslationally (figure 1.7) (Randall et al., 1983). The nascent protein may be weakly associated with the ER membrane prior to translocation, and may even insert co-translationally if the folding of the protein into the secondary and tertiary conformation required for membrane insertion can occur before the protein is fully elongated (Randall et al., 1983). As with the previous theory, energy necessary for translocation are provided by protein folding (Randall et al., 1983).

Figure 1.7. Schematic illustration of post-translational spontaneous insertion of a completed membrane protein (Wickner et al., 1985).



A combination of the above models may be the reality of protein transport across membranes, but the exact mechanism remains unknown. However, agreement exists on the structural function of the amino-terminal amino acid sequence of proteins, the signal or leader peptides, which are secreted by or inserted into the membrane. These sequences have three basic domains, an amino-terminal domain that is basic, polar, and relatively short; a central hydrophobic region; and a post-hydrophobic domain that has a helix-breaking amino acid followed by amino acid residues with small side chains at positions -3 and -1 relative to the signal sequence cleavage site (Wickner et al., 1985). This leader is required for transport, but is unclear if it is sufficient for the process of translocation, or if other cellular machinery is required (Wickner et al., 1985).

Comparison of signal sequences from various secreted and anchored membrane proteins suggests that certain amino acids are conserved at certain sites within these domains (Heinje, 1984; Heinje, 1986; Perlman et al., 1983). The amino terminal domain usually has a positive charge, usually as a result of the presence of arginine and/or lysine residues (Perlman et al., 1983; Nothwehr et al., 1990). The most abundant amino acids within the central hydrophobic domain are leucine, alanine, valine, phenylalanine, and isoleucine (Perlman et al., 1983). A small number of threonine, cysteine, serine, and methionine residues may also occur (Perlman et al., 1983). Tryptophan, glycine, glutamine, tyrosine, proline, and asparagine are rarely present (Perlman et al., 1983). The post-hydrophobic domain contains the signal peptidase recognition site, and alanine, threonine, and serine are present frequently (Perlman et al., 1983). The enzyme which cleaves the signal sequence (signalase), removing it from the nascent polypeptide, recognizes a triplet of amino acids within the signal sequence, A-X-B, where A is alanine or one of the other aliphatic amino acids or a hydroxy amino acid, X is any amino acid, and B is a small aliphatic amino acid such as alanine, glycine, serine, cysteine, or threonine (Perlman et al., 1983). The entire length of the signal sequence is generally 9-14 residues (Perlman et al., 1983). The secondary structure may be either alpha helix or beta strand (Perlman et al., 1983).

The "-3, -1 rule" for the leader peptide amino acid sequence predicts which amino acids are present in the signal sequence at the third position upstream from (-3), and the position immediately preceding (-1) the signalase cleavage site (Heinje, 1984). This rule proposes that the amino acid in position -1 must be alanine, serine,

glycine, cysteine, threonine, or glutamine, and the amino acid at position -3 must not be aromatic, charged, or large polar residues. Proline must not be present in positions -3 to +1 (Heinje, 1984). This rule has been used with marked success in predicting cleavage sites of signalases (Heinje, 1986). The amino acid composition of the region preceding the signalase cleavage site is critical for successful signal peptide cleavage but not for translocation of the protein (Kuhn et al., 1985).

The "stop transfer" amino acid sequence may have to conform to certain structural requirements to function as a membrane anchor. The primary structural requisite is a stretch of amino acids with net hydrophobicity capable of spanning the membrane (Markoff et al., 1986). These regions differ in length and amino acid sequence, however, from the signal sequence (Perlman et al., 1983). They generally are longer, about 20 amino acids, and contain more glycine and serine residues, but less leucines than are present in the signal sequence (Perlman et al., 1983). This region can be as short as 14 amino acids and still function as an anchor (Adams et al., 1985).

The factors that may be involved in anchorage of membrane proteins are not fully known. It is known that an amino acid sequence of the transmembrane domain must stop translocation and create a stable membrane interaction (Davis et al., 1985). It is unclear if the mechanism of protein anchorage uses cellular components, such as the SRP and SRP receptor, that may be used in membrane insertion and translocation (Davis et al., 1985). The "stop-transfer" amino acid sequence may, in fact, initiate disruption of this machinery (Davis et al., 1985). If no cellular machinery participates in this process of protein transfer across the membrane, and no membrane tunnel or "pore" is formed, then the transmembrane domain must interact directly with the lipid bilayer (Davis et al., 1985). In the latter situation, the hydrophobic nature of the region serving as the anchor would be sufficient to stop the translocation because of the resulting low free energy of the membrane-protein interaction (Davis et al., 1985). The amino acid sequence of the anchor is random, and hydrophobicity may, in fact, be sufficient for function its (Davis et al., 1985). What requirements for membrane anchorage other than hydrophobicity exist must still be determined.

CHAPTER 2

MATERIALS AND METHODS

CELL CULTURES AND MEDIUM

Primary bovine turbinate cells, free of adventitious bovine viral diarrhea virus (BVDV), were used for viral propagation. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% horse serum.

VIRUS

Noncytopathic BVDV strain 2724 was used for this investigation (Potgeiter et al., 1984). Cells of confluent cell cultures were infected with BVDV at a multiplicity of infection of 0.1. On day 3 post-infection, the medium was replaced with fresh medium. Infected cells were harvested on day 10 post-infection. The presence of BVDV in cells was determined by indirect immunofluorescence.

TOTAL RNA ISOLATION

Ribonucleases were inactivated in liquids and on instruments and glassware used by a variety of procedures. Deionized water used in experiments was treated with diethylpyrocarbonate (DEPC) (final concentration of 0.1%), incubated overnight, sterilized by autoclaving at 121 C for 15 minutes. Glassware was soaked in the DEPC-treated water for 60 minutes, and then baked at 200 C for 4 hours according to the recommendations of Sambrook et al. with modifications (1989). Pipette tips and microfuge tubes were siliconized prior to sterilization by autoclaving according to the method described by Sambrook et al. (1989). Gloves were worn by laboratory personnel at all times.

Infected cells were collected with a cell scraper and pelleted by centrifugation at 10,000 x g for five minutes. The cell pellet was washed with ice-cold PBG (6mM Na₂HPO₄, 1mM KH₂PO₄, 300mM glucose). RNA extraction was done by methods described by Chomcynski and Sacchi (1987). Cell pellets were lysed with guanidinium isothiocyanate (GTIC) buffer (4M GTIC; 25mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1M 2-mercaptoethanol) and resuspended in 750-1000 ul of GTIC buffer. GTIC, a strong protein denaturant, ensured that protein was removed from RNA under conditions that inhibited endogenous ribonucleases (Macdonald et al., 1987).

RNA was separated from the other cellular components by selective precipitation with acidic sodium acetate. One hundred ul of 2M sodium acetate (pH 4.0), one ml of phenol, and 0.2 ml of chloroform/isoamyl alcohol (49:1) was added to the cell lysate. The mixture was shaken vigorously and placed on ice for 15 minutes. The sample then was centrifuged for 15 minutes at 10,000 x g. The aqueous phase was transferred to a clean tube, and precipitated with 0.6 volumes of isopropanol at -20 C for one hour. The RNA then was pelleted by centrifugation at 10,000 x g for 20 minutes. The pellet was resuspended in 0.3 ml of GTIC buffer to which one volume of isopropanol was added, and placed at -20 C for at least one hour. RNA not immediately used was stored at -20 C. Prior to use, the RNA again was pelleted by centrifugation at 10,000 x g for 20 minutes, washed with 70% ethanol, and dried in a Speed Vac Concentrator (Savant). RNA was quantified by spectrophotometry at 260nm. The ratio of absorbance at 260nm to absorbance at 280nm was determined in order to assess purity of the RNA. A ratio of 1.8-2.0 was considered acceptable for use in reverse transcription.

OLIGONUCLEOTIDE SYNTHESIS

The base sequence of oligonucleotides used as primers in cDNA synthesis and polymerase chain reaction procedures were designed according to sequence data on the NADL strain of BVDV (Collett et al., 1988a). Collett et al. postulated that the carboxy terminus of the major BVDV glycoprotein (gp53) is at amino acid residue 1063 of the polyprotein encoded by BVDV. The codon of this residue resides at nucleotides (nt) 3572 to 3574 of the BVDV genome. These investigators

determined that the nonglycosylated molecular weight of gp53 was 42kD. We estimated, by dividing this molecular weight by the average molecular weight of an amino acid (110D), that the number of amino acids in this protein was approximately 682. We predicted that the amino terminus of gp53 was near amino acid residue 681 (encoded by nt 2426 to 2428) by subtracting 682 amino acid residues from residue 1063 (the putative carboxy terminus of gp53 in the polyprotein).

The primer for first-strand cDNA synthesis (primer 1), which hybridized to the 3' end of the positive-stranded BVDV RNA template, contained the antisense BVDV NADL sequence from nt 3704 to 3724. The primer designed for second strand synthesis and polymerase chain reaction (primer 2), which hybridizes to the 3' end of the negative-stranded BVDV cDNA template, contained the sense BVDV NADL sequence from nt 2402 to 2423 (figure 2.1). The primers were designed to hybridize with locations immediately outside the 3' and 5' ends of the genomic region predicted to encode gp53. Primer 2 design included a codon for a methionine residue in frame so that the cDNA product contained the appropriate codon which could serve as an initiation codon for protein synthesis. Both primers were designed with restriction endonuclease sites at their respective 5' ends to allow cloning into plasmid vectors. Upstream (5') of the restriction enzyme sites were additional nucleotides to allow optimal restriction endonuclease digestion (figure 2.2). Two sets of two primers each were synthesized. Primer set 1 had different restriction endonuclease sites on the two primers to allow directional insertion of BVDV cDNA into the plasmid (primer 1 - Hind III, primer 2 - Sac I). Both primers of primer set 2 contained BamH I sites because the product of this primer set was to be used in a baculovirus expression system, and subcloning into a baculovirus transfer vector requires BamH I sites. Additionally, the 3' primer, designed to hybridize with the positive BVDV cDNA strand, of the latter primer set, contained stop codons in three reading frames to achieve protein synthesis termination in a baculovirus expression system. Both primer sets included the same NADL BVDV cDNA base sequence in their design.

Oligonucleotides used for DNA base sequencing of our plasmid cloned cDNA were designed by considering sequence data obtained from three clones of BVDV 2724 gp53. Initial base sequencing of cDNA ends used primers that hybridize to vector base sequences (T7 and Sp6; Promega). The internal primers

Figure 2.1. Genomic position represented by and base sequence of oligonucleotides used for cDNA synthesis and amplification of the putative gene encoding gp53 of BVDV strain 2724. Nucleotide (nt) number denotes nucleotide position in BVDV strain NADL nucleotide sequence (Collett et al., 1988a). Gp48 may be encoded by the 5' demarcated portion of the bracketed genomic area indicated, while gp25 may be encoded by the 3' portion, indicated by the arrow. ? denotes unknown identity of protein product from the genomic region indicated.

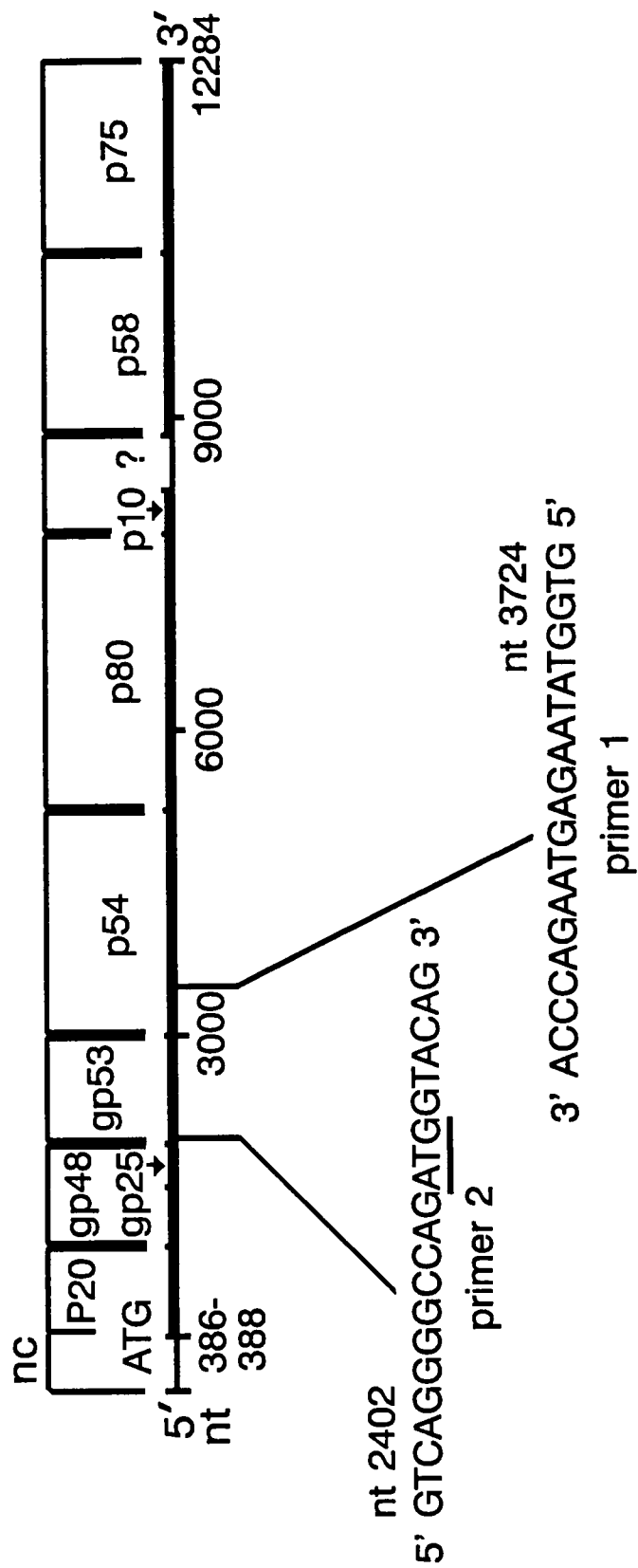


Figure 2.2. The complete sequence of oligonucleotides used for cDNA synthesis and amplification of the putative gene encoding gp53 of BVDV strain 2724 for cloning into the vector indicated. Underlines indicate restriction sites engineered into the primers. Asterisks indicate the 5' end of the BVDV-specific sequence of the primers (Collett et al., 1988a). Arrows indicate methionine codon which may be used for *in vitro* translation initiation. The box indicates stop codons in three reading frames engineered into primer 1 of primer set 2.

pGem3Zf(+)

primer 1 – 5' GGAAGCTT^{*}GTGTATAAGAGTAAGACCCA 3'

primer 2 – 5' GGGAGCTCGTCAGGGGCCAGATGGTACAG 3'
↑

pCR1000

primer 1 – 5' GGTGAGGATCCTCAATCAATCAAG^{*}GTGTATAAGAGTAAGACCCA 3'

primer 2 – 5' GGTGTTGGATCC^{*}GTCAGGGGCCAGATGGTACAG 3'
↑

used to base sequence further into the cDNA were analogous to appropriate BVDV strain 2724 sequences (figure 2.3).

Oligonucleotides were synthesized and purified by either the Molecular Biology Resources at the University of Tennessee or Oligos, Etc. Oligonucleotide purification was by HPLC and gel electrophoresis.

cDNA SYNTHESIS

Reverse transcription of BVDV strain 2724 for cDNA synthesis was done with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The optimal amount of RNA used in this procedure was empirically determined for each aliquot of RNA extracted. The amount of total RNA resulting in the most consistent reverse transcription results was 1-5 ug. The RNA template was denatured by heating at 65 C for 15 minutes and then rapidly cooled on ice in preparation for reverse transcription.

The reverse transcription reaction mixture contained RNase inhibitor (20 units per reaction volume; Bethesda Research Laboratories), dithiothreitol (DTT) (10mM), reaction buffer (50mM Tris-HCl, pH 8.3; 75mM KCl; 3mM MgCl₂), deoxyribonucleotide triphosphates (dNTP) (100uM each; Bethesda Research Laboratories), primer 1 (0.5-1.0uM), and reverse transcriptase (200 units/ug RNA). The RNA template was added and DEPC-treated water used to make a final volume of 50 ul. The reaction mixture was incubated at 37 C for one hour prior to its use in the polymerase chain reaction procedure.

POLYMERASE CHAIN REACTION

Second strand cDNA synthesis and DNA amplification were done by the polymerase chain reaction (PCR) procedure by adapting the methods of Mullis and Faloona (1987) and Saiki et al. (1988).

The 50 ul cDNA synthesis reaction mixture containing the cDNA template for polymerase chain reaction was divided into 25 ul aliquots. Deoxyribonucleotide triphosphates (dNTP) (100uM), PCR buffer (10mM Tris-HCl, pH 8.3; 50mM KCl;

Set 1: 5' primer	5'-TGCACAAGAGAACTAGATA - 3'
3' primer	5'-TGATAATCTCCTTTTAGCAT - 3'
Set 2: 5' primer	5'-AGGAACTGTGAGCTGTATGT - 3'
3' primer	5'-ACCAATCCTTGTGGTACTAT - 3'

Figure 2.3. Nucleotide sequence of internal primers used in DNA base sequencing. 5' and 3' designations denote oligonucleotide orientation relative to (+) strand of insert.

1.5mM MgCl₂; 0.01% gelatin), primer 2 (0.5uM), and Taq polymerase (2.5 units; Perkin Elmer Cetus) were added to each aliquot. DEPC-treated water was added to make a final volume of 100 ul. The reaction mixture was heated to 95 C for two minutes to denature any remaining proteases before the enzyme was added, and 50-75 ul mineral oil overlay was added prior to thermal cycling.

The mixture was brought to a variety of temperatures for specific periods (thermal cycling) in a GTC-1 Genetic Thermal Cycler (Precision Scientific) as described in table 2.1. Amplification products were analyzed by gel electrophoresis on a 1% agarose gel.

PURIFICATION OF THE AMPLIFIED cDNA

Purification of the amplification product for cloning was adapted from methods described in Sambrook et al. (1989). The PCR mixture was extracted with phenol/chloroform/isoamyl alcohol (24:24:1). The aqueous phase was recovered and extracted with chloroform/isoamyl alcohol (49:1). The aqueous phase recovered from the second extraction was transferred to a clean tube and 0.1 volume 3M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol were added and placed at -80 C for 30 minutes. The precipitated DNA was pelleted by centrifugation at 10,000 x g for 20 minutes, washed in 70% ethanol, and dried under vacuum in a Savant dryer. The dried DNA pellet was resuspended in 60 ul of water. Sixty ul of DNA was loaded in aliquots of 30 ul into wells and electrophoresed in a 1% agarose gel with ethidium bromide (1.0 ug/ml). Darkened conditions were used to avoid nicking of the DNA by the ethidium bromide. After the gel-loading buffer had migrated to the halfway point, the gel was examined briefly under long wave UV light (365nm) to assess the position of the DNA product and its separation from any nonspecific DNA products. A small slit then was cut in the gel in front of the target DNA. A piece of NA 45 paper (0.45 um; Schleicher and Schuell) cut to a length corresponding to two gel slots and width of 1.5 times the gel depth, and soaked in 1 x TBE (0.09M Tris; 0.09M boric acid; 0.0025M EDTA) was placed into this slit. Electrophoresis was continued for 10-15 minutes until all DNA had migrated onto the paper.

Table 2.1 Parameters of thermal cycling for polymerase chain reaction.

Step	Temperature	Time	# of cycles	Purpose
1	95°C	1.5 min	1	denaturation
2 ^a	50°C	1.0 min	30	primer annealing
3 ^a	72°C	1.5 min	30	DNA synthesis
4 ^a	94°C	1.5	30	denaturation
5	50°C	2.0 min	1	primer annealing
6	72°C	10.0 min	1	final strand synthesis
7	24°C	24.0 hr	1	holding

^aSteps 2, 3, and 4 were repeated in order 30 times before proceeding to step 5.

The paper was removed from the gel, placed in a 1.7 ml microfuge tube and covered with 400 ul of elution buffer (1M NaCl; 0.05M arginine free base, pH 9.0). The paper then was incubated at 65 C for one hour, removed, and placed under UV light to determine whether all DNA had been eluted. The DNA elution buffer mixture was extracted with phenol/ chloroform/isoamyl alcohol (24:24:1). The aqueous phase was transferred to a clean tube, 2.5 volumes of 100% ethanol were added, and kept at -20 C overnight to allow for DNA precipitation (sodium acetate was not necessary due to the high salt concentration of the elution buffer). DNA not immediately subjected to restriction enzyme digestion was stored at -20 C.

VECTOR pGEM3zf(+) PREPARATION

The primary vector used for cDNA cloning was pGem3zf(+) (Promega). Preparation of the vector for ligation has been described by Sambrook et al. (1989). Various cDNA preparations to be cloned had Sac I restriction endonuclease sites at their 5' ends and Hind III sites at their 3' ends (5' and 3' positions were relative to the positive cDNA strand). Therefore, sequential restriction endonuclease digestion of the vector with the appropriate enzymes was required.

Sac I restriction endonuclease digestion was done first, because it required a low-salt buffer. Five ug of pGem3zf(+) vector was digested with this enzyme. the total reaction mixture volume of 30 ul included three ul of assay buffer I (25mM Tris-HCl, pH 7.5; 10mM MgCl₂; 1mM DTT; 100 ug/ml bovine serum albumen; Stratagene), five ul of plasmid, and three ul of enzyme (21 units; Stratagene), and was incubated at 37 C for four hours. Two ul of this mixture was evaluated by electrophoresis on a 1% agarose gel to assess linearization of the plasmid. The remaining mixture was made up to a volume of 100 ul with water, extracted with phenol/chloroform, and precipitated with 100% ethanol. The DNA pellet was resuspended in 17 ul of water, to which was added two ul of assay buffer III (25mM Tris-HCl, pH 7.7; 50mM NaCl; 10mM MgCl₂; 10mM 2-mercaptethanol; 100 ug/ml bovine serum albumin (BSA); Stratagene), and one ul Hind III (40 units; Stratagene) for the second enzyme digestion. The mixture was incubated at 37 C for four hours. The reaction mixture volume then was made to a final volume of 100 ul with water, extracted with phenol/chloroform, and precipitated with ethanol.

Centrifugation, washing, and drying of the DNA followed, and it was resuspended in 60 ul of water.

After restriction endonuclease digestions of the vector, it was necessary to separate its multiple cloning site from the rest of the vector. This was done by agarose gel electrophoresis and electroelution. The vector DNA was loaded in 30 ul aliquots into two wells of a 1% agarose gel containing ethidium bromide, and electrophoresed in darkened conditions to the midpoint of the gel. The vector DNA was identified by long-wave UV light, excised from the gel and placed in dialysis tubing (MW exclusion 12,000-14,000D; BRL) containing approximately 400 ul of 1 x TBE. DNA within the tubing was electrophoresed for one to three hours to remove DNA from the gel slice (judged under UV light). DNA adsorbed onto the inner wall of the tubing was dislodged by electrophoresis in the opposite direction for 15 minutes and removed. Residual DNA in the tubing was removed by washing with approximately 100 ul 1 x TBE. The DNA preparation (about 500 ul) was extracted with phenol/chloroform/isoamyl alcohol and precipitated with 100% ethanol. Vector DNA then was pelleted by centrifugation, washed, and dried as previously described, resuspended in 50 ul of TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0), and quantified in a spectrophotometer.

RESTRICTION ENDONUCLEASE DIGESTION OF THE AMPLIFIED cDNA

Enzyme digestion of the PCR-amplified cDNA prior to ligation into pGem3zf(+) was done in the same manner as that for the vector. cDNA was pelleted by centrifugation, washed, and dried as previously described. The DNA pellet was resuspended in 16 ul water, to which was added two ul of assay buffer I (Stratagene), and two ul of Sac I (14 units; Stratagene). The reaction mixture then was incubated at 37 C for two hours, made up to a volume of 100 ul with water, extracted with phenol/chloroform/isoamyl alcohol, and precipitated with 100% ethanol. The DNA was pelleted by centrifugation, washed, dried, and resuspended in 17 ul of water. Two ul of assay buffer III (Stratagene) and one ul of Hind III enzyme (40 units; Stratagene) were added and the reaction mixture was incubated at 37 C for two hours. This was followed by addition of 80 ul water, extraction with phenol/chloroform/isoamyl alcohol and precipitation with 100% ethanol.

LIGATION OF pGEM3zf(+) AND AMPLIFIED cDNA

PCR-amplified cDNA was pelleted by centrifugation, washed, and dried as previously described. The entire DNA pellet was resuspended in four μ l of water and used for ligation into 200 ng of pGem3zf(+). The reaction mixture consisted of four μ l vector, four μ l cDNA, two μ l rATP (10mM, pH 7.5), two μ l ligation buffer (500mM Tris-HCl, pH 7.5; 70mM MgCl₂; 10mM DTT; Stratagene), and one μ l T4 ligase (4 units; Stratagene). The reaction mixture was made to a final volume of 20 μ l with water and kept at room temperature for three to four hours. The sample then was used for bacterial transformation.

BACTERIAL TRANSFORMATION

E. coli strains JM101 and JM109 (Promega) were prepared for transformation by a procedure adapted from the method described by Sambrook et al. (1989). One colony of E. coli was inoculated into 5 ml of Luria-Bertani (LB) broth (1% bacto-tryptone; 0.5% bacto-yeast extract; 1% NaCl) and grown overnight in a shaker incubator at 37 C. One ml of this start culture was inoculated into 100 ml of LB broth, and incubated at 37 C in a shaker until the UV light absorbance at 600 nm was 0.3-0.5. The bacterial cell suspension then was chilled on ice for 10 minutes.

The bacterial cell suspension was transferred to 50 ml Oak Ridge tubes and centrifuged at 10,000 rpm in a Sorvall GS-3 rotor at 4 C for five minutes. The cell pellet then was resuspended in 10 ml of ice-cold solution A (10mM NaCl; 50mM MnCl₂; 10mM NaC₂H₃O₂, pH 5.6) and placed on ice for 30 minutes. The cells were pelleted at 10,000 rpm for five minutes, and one ml of ice-cold solution B (75mM CaCl₂; 100mM MnCl₂; 10mM NaC₂H₃O₂, pH 5.6) was added. Cells either were used immediately for transformation or stored at -20 C in 15% glycerol.

For bacterial transformation, 100 μ l suspension of transformation competent cells were used. The mixture containing plasmid DNA into which cDNA had been ligated was diluted ten-fold with water, and 10 μ l were used to transform bacteria.

This DNA was added to the bacterial cells and chilled on ice for 20 minutes. The mixture then was heated rapidly to 37 C for five minutes, followed by addition of 0.5 ml of LB broth. The cells were incubated at 37 C with occasional shaking for 30 minutes and poured on agar containing ampicillin (1% bacto-tryptone; 0.5% bacto-yeast extract; 1% NaCl; 100ug/ml ampicillin; 40-60ug/ml 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal); 40-60ug/ml isopropylthio-B-D-galactoside (IPTG); 100 ul transformation reaction mixture/plate). Clone selection was based on growth (ampR) and white color {lacZ (-)}. The plasmid vector, by transfection, provided a gene for ampicillin resistance to the bacteria which did not have this gene. The bacterial strains used were β -galactosidase-deficient, and nonrecombinant vector supplied the missing lacZ gene resulting in a blue bacterial colonies from substrate catalysis. However, cDNA ligated into recombinant vector disrupted the lacZ gene, resulting in white bacterial colonies since bacteria were incapable of catalyzing the substrate. A control sample containing nonrecombinant plasmids was used in an identical manner with each transformation reaction to assess transformation efficiency.

TA CLONING

In order to express BVDV gp53 in a baculovirus system, cDNA with BamH I sites were cloned for incorporation in a suitable baculovirus transfer vector. Therefore, cDNA with these sites at either end was synthesized and amplified by PCR as previously described. The TA cloning system (Invitrogen) was used to clone this amplified cDNA. TA cloning (Invitrogen) capitalizes on deoxyadenosines (dA) at the 3' ends of the double-stranded products usually added by thermostable polymerases. The pCR1000 vector has deoxythymidine (dT) overhangs on the 3' ends of the site of cDNA insertion. These 3' dT-overhangs hybridize with the 3' dA-overhangs of the cDNA, thus avoiding any cDNA or vector preparation by restriction endonuclease digestion.

The cDNA was purified by agarose gel electrophoresis onto NA 45 paper as before. Following extraction and precipitation, the DNA was pelleted by centrifugation, washed, dried, and resuspended in seven ul water. Purified cDNA was mixed with two ul of pCR1000 vector (50 ng), one ul of ligation buffer (60mM

Tris, pH 7.5; 60mM MgCl₂; 50mM NaCl; 1mg/ml BSA; 70mM 2-mercaptoethanol; 1 mM ATP; 20mM DTT; 10mM spermidine), and one ul of T4 DNA ligase (4 units). The mixture was incubated at 12 C overnight. The ligation efficiency of the vector was assessed by self-ligation as recommended by the manufacturer.

Transformation of the competent cells (E. coli strain INV α F'; Invitrogen) by a method adapted from that recommended by the manufacturer. The ligation reaction mixture was centrifuged for 10 seconds at 10,000 x g and placed on ice. Two ul of 0.6M 2-mercaptoethanol was added to a vial containing competent bacterial cells (50 ul) and mixed gently. One ul of the ligation reaction mixture then was added to the competent cells. The transformation reaction was chilled for 30 minutes on ice, followed by rapid heating at 42 C for 60 seconds. The vials of transformed bacteria then were immediately placed on ice for two minutes. Four hundred and fifty ul of pre-warmed SOC medium (2% bacto-tryptone; 0.5% bacto-yeast extract; 10mM NaCl; 2.5mM KCl; 10mM MgCl₂; 10mM MgSO₄; 20mM glucose) was added to the transformation reaction, which then was placed in a shaking apparatus at 37 C for one hour and chilled on ice. The entire transformation reaction was plated on LB agar containing kanamycin (50 ug/ml) and X-gal (50 ug/ml). The INV α F' bacteria are deficient in both β -galactosidase and kanamycin resistance genes. The nonrecombinant plasmid vector provided both of these genes, but recombinant cDNA had disrupted β -galactosidase genes. Selection for transformed bacteria with recombinant vectors is based on growth (kanamycinR) and white color {lacZ(-)}. To assess transformation efficiency, a similar reaction using supercoiled nonrecombinant plasmid provided by the manufacturer was done as recommended by the manufacturer.

RECOMBINANT PLASMID DNA PURIFICATION

The procedure for isolation of plasmid DNA was adapted from methods described by Promega. An isolated colony of transformed bacteria was picked and inoculated into two ml of terrific broth (TB) (1.2% bacto-tryptone; 2.4% bacto-yeast extract; 0.04% glycerol; 0.17M KH₂PO₄; 0.072M K₂HPO₄; Tartof et al.,1987) containing the appropriate antibiotic (ampicillin at 100 ug/ml or kanamycin at 50 ug/ml). This medium supports excellent growth of the bacteria, and promotes

increased plasmid yields compared to that obtained with LB maintenance medium. The inoculated medium was placed in a shaking incubator at 37°C overnight. Two ml then was centrifuged at 10,000 x g for 10 minutes and the supernatant decanted.

The cell pellet was resuspended in 100 µl of lysis buffer (25mM Tris-HCl, pH 7.5; 10mM EDTA; 15% sucrose; 2 mg/ ml lysozyme), vortexed, and held at room temperature for five minutes. Two hundred µl of alkaline SDS (0.2M NaOH; 1% SDS) was added to the lysate, which then was mixed gently and chilled on ice for five minutes. One hundred and fifty µl of potassium acetate (5/3M, pH 5.0) was added to the mixture, which was mixed and chilled on ice for five minutes. The sample then was centrifuged at 10,000 x g for five minutes and the supernatant carefully collected. Fifty to 100 units of T1 RNase (Boehringer Mannheim Biochemicals) was added to the latter and incubated at 37 °C for 30 minutes. Plasmid DNA was extracted with phenol/chloroform followed by extraction with chloroform. The plasmid DNA in the aqueous phase was precipitated with 2.5 volumes of 100% ethanol for 20 minutes at -70 °C. DNA was pelleted by centrifugation at 10,000 x g for 15 minutes, washed with 70% ethanol, and dried. The DNA was dissolved in 16 µl of water to which four µl of 4 M NaCl, and 20 µl of 13% polyethylene glycol (MW 6000-8000) was added. The sample was chilled on ice for 30 minutes and then centrifuged at 10,000 x g for 15 minutes. The DNA pellet was washed twice in 70% ethanol, dried, and resuspended in 16-17 µl (for restriction endonuclease digestion), or 18 µl (for sequencing) of water. DNA not used immediately was stored at -20 °C.

Larger amounts of plasmid DNA were prepared in a similar manner with appropriate adjustments of the amounts of reagents used. Such preparations usually were done with 250 ml of bacterial culture obtained by inoculating one ml of an overnight culture from an isolated colony into 250 ml of TB.

EVALUATION OF TRANSFORMED BACTERIA

Initial screening for recombinant plasmids in bacteria was done by polymerase chain reaction. This method takes advantage of primers used in the PCR to produce the cDNA used in transformation. Plasmids from which an amplification product of appropriate size was produced were then evaluated further.

A reaction mixture containing 1.5 ul of PCR buffer (described previously), two ul of 1.25mM each dNTP, two ul of 10uM primers 1 and 2 was made up to a final volume of 15 ul. Transformed bacteria to be screened were picked from colonies and inoculated into the reaction mixture. The sample then was heated to 95 C for two minutes to lyse the bacteria. Taq polymerase (10 ul) (Perkin Elmer Cetus) diluted 1:80 in PCR buffer was then added, bringing the final volume to 25 ul. A mineral oil overlay was added and the reaction mixture was treated as before in a thermal cycler. Products were analyzed by agarose gel electrophoresis.

Initial evaluation of potential recombinants also was done by restriction endonuclease digestion. Approximately 3-5 ug of plasmid DNA was enzymatically digested with EcoR I and Hind III in assay buffer III (Stratagene) as previously described. EcoR I restriction endonuclease was used rather than Sac I for digestion of cDNA in pGem3zf(+) because the EcoR I restriction site was not removed from the vector during cDNA ligation into it. The cDNA from BVDV strain 2724, unlike BVDV strain NADL, did not have an internal EcoR I site in the region encoding gp53, and EcoR I was active in the presence of relatively high salt buffers. The latter property allowed simultaneous plasmid digestion with Hind III and EcoR I, thereby eliminating the need for separate sequential enzyme reactions. The reaction mixture was analyzed by agarose gel electrophoresis. Recombinants containing the properly sized product were analyzed further by dideoxy chain termination base sequencing to confirm the presence of BVDV cDNA.

STORAGE OF BACTERIA

Bacteria containing recombinant plasmids were frozen prior to further analysis as described in Sambrook et al. (1989). Five ml LB broth inoculated with transformed bacteria was incubated overnight in a shaker-incubator at 37 C. Eighty-five per cent concentrations of bacterial cultures were made in glycerol, and one ml aliquots were rapidly frozen in liquid nitrogen and stored at -70 C.

DNA BASE SEQUENCING

Plasmid DNA isolation for DNA sequencing was done as previously described. Denaturation of supercoiled plasmid DNA was adapted from methods described by Promega. Four to six μg of DNA was denatured by alkali. Two μl of 2M NaOH in 2mM EDTA was added to 18 μl of an aqueous solution of the DNA and incubated at 37 C for 30 minutes. The reaction mixture then was neutralized with two μl of 2M ammonium acetate, pH 4.6. The denatured DNA was precipitated with 100% ethanol at -70 C for 30 minutes, and pelleted by centrifugation at 10,000 x g for 20 minutes. The DNA pellet was washed with 70% ethanol, dried, and resuspended in seven μl of water.

Dideoxy chain termination DNA base sequencing was done with the Sequenase kit, version 2.0 (United States Biochemical) according to the manufacturer's recommendations. Two μl of annealing reaction buffer (200mM Tris-HCl, pH 7.5; 100mM MgCl_2 ; 250mM NaCl) and one μl of sequencing primer (0.5-1.0pmol) were added to seven μl of denatured plasmid DNA. The sample was heated to 65 C for two minutes, and then cooled slowly to below 35 C to achieve primer annealing. Radioisotope labelling of DNA then was done by addition of two μl of labelling reaction mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) diluted five-fold in water, one μl of DTT (0.1M), 0.5 μl of ^{35}S -ATP (10.0 mCi/ml), and two μl of Sequenase (USB) diluted eight-fold in enzyme dilution buffer (10mM Tris-HCl, pH 7.5; 5mM DTT; 0.5 mg/ml BSA) to the annealing reaction mix and incubation at room temperature for two to five minutes. Chain termination then was accomplished by dispensing 3.5 μl of the labelling reaction to tubes containing 2.5 μl of an appropriate dideoxynucleotide triphosphate mixture provided by the manufacturer and prewarmed to 37 C. Incubation was continued at 37 C for five minutes, and four μl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added. Samples which were not immediately evaluated by electrophoresis were stored at -20 C.

Certain modifications were adapted to circumvent selected problems. To determine DNA base sequence near the primer, manganese buffer (0.15M sodium isocitrate; 0.1M MnCl_2) was added to the labelling reaction. Problems with secondary structure in the DNA template were reduced by using ITP reaction

mixtures, an analog for guanosine, in the labelling (15uM dITP, 7.5uM dCTP, 7.5uM dTTP) and termination reactions.

Electrophoresis of sequencing reactions was done in a 0.2mm thick 6% polyacrylamide gel with 8M urea on a 60 cm glass plate (vertical base runner nucleic acid sequencer; IBI). Two samples of each preparation were loaded and electrophoresed for long (six hours) and short (three hours) periods. Following electrophoresis, the gel was transferred to Whatman 3MM paper and soaked for 20 minutes in a solution of 5% acetic acid and 5% methanol to remove urea. Gels were dried at 90 C for 90 minutes (Biorad gel dryer model 583) and placed on radiograph film (Kodak X-omatic AR film) for at least 16 hours.

SEQUENCE ANALYSIS

Computer analyses of sequence data was done by sequence analysis programs of the Genetics Computer Group, version 6.0 of the University of Wisconsin (Devereux, 1989). Analyses on a variety of characteristics of nucleotide and predicted amino acid sequences of the putative gp53 gene of BVDV strain 2724 were done, and comparisons were made with comparable characteristics of flaviviruses and other pestiviruses.

IN VITRO TRANSCRIPTION

In vitro transcription was done as recommended by Promega. The recombinant plasmid construct pMK14, which contained the cDNA of the putative gene for gp53 in pGem3zf(+) plasmid, was used as the template for all transcription and expression assays. Analysis of pMK14 sequence data indicated that T7 polymerase would be required for transcription. Linearization of pMK14 recombinant plasmid was done with Hind III restriction endonuclease before producing appropriate run-off transcripts.

Recombinant plasmid construct pMK14 was isolated as previously described. Restriction enzyme digestion with Hind III was done as before. Fifteen ug of plasmid DNA was digested with one ul of Hind III (40 units; Stratagene). As Hind III restriction endonuclease digestion of double-stranded DNA does not produce 3' overhangs, further modification of the template was not necessary to eliminate any 3' overhangs which is required prior to transcription to avoid extraneous transcripts. Linearized plasmid DNA was extracted with phenol/chloroform and precipitated with 100% ethanol. The DNA then was pelleted by centrifugation, washed, dried, and resuspended in 30 ul of DEPC-treated water. DNA was quantified by spectrophotometry and transcription was done on five ug of linearized template.

The transcription reaction mixture consisted of 20 ul of transcription buffer (200mM Tris-HCl, pH 7.5; 30mM MgCl₂; 10mM spermidine; 50mM NaCl; Promega), one ul of 1M DTT, 100 units of RNasin (Promega), 2.5 ul each of 100mM nucleotide triphosphate, five ug template DNA, 30-50 units T7 polymerase (Promega), and DEPC-treated water to make a final volume of 100 ul. The mixture was incubated at 37 C for 60 minutes, 30-50 units of additional T7 polymerase were added, and incubation continued for 60 minutes.

The DNA template was removed from the reaction mixture by DNase digestion of the DNA after transcription had taken place. RNase-free DNase (RQ1; Promega) was added to the reaction mixture to obtain a concentration of one unit/ug DNA and incubated at 37 C for 15 minutes. Messenger RNA was extracted from the transcription reaction with phenol/chloroform and precipitated with ethanol. The RNA was pelleted by centrifugation, washed, and dried. The pellet was resuspended in 50 ul of DEPC-treated water, and a two ul aliquot was analyzed by agarose gel electrophoresis to determine whether all DNA had been eliminated from the preparation. The remaining RNA was quantified by spectrophotometry and stored at -20 C.

IN VITRO TRANSLATION

Preliminary data indicated that capping of the RNA transcript was not required for optimal translation as determined by comparison of products of translation by SDS-PAGE using capped and uncapped mRNA, and, thus, this step was omitted from mRNA translation reactions (data not given). Translations were done in a rabbit reticulocyte system as recommended by the manufacturer (Promega).

The optimal amount of RNA for translation was determined empirically for each lot of transcriptional product. The most efficient translation as determined by SDS-PAGE of the products was achieved with one to five ug of RNA template. The RNA was heated to 65 C for 10 minutes prior to translation to eliminate secondary structures. The reaction mixture consisted of 35 ul of rabbit reticulocyte lysate (Promega), 40 units of RNasin (Promega), one ul of 1mM amino acid mix (minus methionine) (Promega), four ul of ³⁵S-methionine (10.25 mCi/ml), one to five ug RNA template, and DEPC-treated water to make a final volume of 50 ul. The preparation was incubated at 30 C for 60 minutes. Some preparations not immediately evaluated or used in additional experiments were stored at -20 C. Positive controls for translation consisted of bromo mosaic virus (BMV) RNA provided by the manufacturer using their recommendations (Promega). Negative controls were identical procedures without template mRNA.

In order to assess cotranslational processing, translation reactions were done also in the presence of canine pancreatic microsomal membranes (Promega). The reaction mixture consisted of 17.5 ul of rabbit reticulocyte lysate, 0.5 ul of 1mM amino acid mixture (minus methionine), 20 units of RNasin, two ul of ³⁵S-methionine, 1.8 ul of microsomal membranes, the RNA template, and DEPC-treated water to make a final volume of 25 ul. As before, the sample was incubated for 60 minutes at 30 C. Positive controls for glycosylation was the α -factor gene product mRNA from *S. cerevisiae*, and positive controls for processing events such as signal peptide cleavage, membrane insertion, and translocation was the pre- β -lactamase mRNA from *E. coli* used as recommended by the manufacturer. Negative controls were done in an identical manner in the absence of mRNA.

Translation reaction products were analyzed by SDS gel electrophoresis using tris-glycine electrophoresis buffer in a 16 cm vertical gel apparatus. Gel and running buffer preparation were done according to Sambrook et al. (1989). The stacking gel was 5% polyacrylamide (pH 6.8), and the resolving gel was 12% polyacrylamide (pH 8.8). The gels were 0.75mm thick. SDS sample buffer (50mM Tris-HCl, pH 6.8; 100mM DTT; 2% SDS; 0.1% bromophenol blue; 10% glycerol; 5% 2-mercaptoethanol; Sambrook et al.) was added to each sample, and all samples were denatured at 95 C for two minutes prior to loading into the gel apparatus. 14C-labelled protein molecular weight markers were included with each assay. Samples were electrophoresed at low amperage (3-10 milliamps) overnight to achieve optimal protein migration and separation. Following electrophoresis, gels were treated with Coomassie brilliant blue (10% methanol, 7% glacial acetic acid, 2 g Coomassie brilliant blue R250/l) for four hours on a slowly rotating platform. Gels then were washed twice with the above solution without Coomassie brilliant blue for two to four hours. Gels were soaked in 1M sodium salicylate for 30 minutes followed by rehydration in a solution of 30% methanol and 3% glycerol for 30-60 minutes. Gels were transferred to 3MM Whatman paper, covered with Saran wrap, and dried at 65 C under vacuum for two to four hours. Problems of gel cracking were reduced by lengthening drying time to more than 16 hours. The dehydrated gel then was placed on radiograph film (Kodak X-omatic AR film) for 16-24 hours in a radiograph film cassette.

PROTEIN ANALYSIS

A variety of additional experiments were carried out to characterize the protein product of *in vitro* transcription and translation of pMK14. These experiments assessed the formation of intermolecular disulfide linkages, the membrane anchorage of the protein, and the immunologic identity of the protein.

In order to determine the formation of intermolecular disulfide linkages between the products of both the cotranslationally processed and non-processed translation reactions, samples were electrophoresed in an SDS-polyacrylamide gel with and without 2-mercaptoethanol in the loading buffer (Garfin, 1990). In this

way. The effects of this denaturant could be directly observed and aided in determination of the existence of oligomers of the protein *in vitro*.

Membrane anchorage was assessed by the procedure of Fujiki et al. (1982). Sodium carbonate was used to convert the closed membrane vesicles to open sheets. In this way, non-anchored, or secreted proteins, were released into solution, but anchored proteins remained attached to the membrane. Translation reactions with canine pancreatic microsomal membranes were done in a 50 μ l volume, and the volume of reagents adjusted accordingly. Preparations of processed translation products then were divided into three 15 μ l aliquots: one to remain untreated to assess translation and processing (described above), one to undergo sodium carbonate treatment, and a negative control to be treated with a neutral pH buffer which would not open the microsomal vesicles. Either 200 μ l of sodium carbonate (100mM, pH 11.5), or neutral buffer (10mM Tris-HCl, pH 7.4; 10mM KCl; 1.5mM MgCl₂) was added to the latter two preparation aliquots respectively, which then were placed for 30 minutes on ice. The samples were centrifuged at 10,000 x g for 30 minutes at 4 C to pellet membranes. The supernatant was transferred to a clean tube. The pellet was washed with 1 ml of immunoprecipitation wash buffer (50mM Tris-HCl, pH 7.5; 5mM EDTA; 150mM NaCl) and centrifuged at 10,000 x g for 15 minutes at 4 C. Twenty μ l of SDS loading buffer was added, and the entire sample was denatured at 95 C for two minutes and loaded onto the SDS-polyacrylamide gel.

One ml of acetone was added to the supernatant samples, followed by centrifugation at 10,000 x g for 15 minutes at 4 C. The resultant pellet then was rinsed with acetone and centrifuged at 10,000 x g for 10 minutes at 4 C. The sample was prepared for gel loading by the addition of 20 μ l of SDS loading buffer and denaturation at 95 C for two minutes. Gel electrophoresis was done as previously described for evaluation of translation reaction products. The appearance of the protein in either the pellet or supernatant portion of the sodium carbonate-treated sample revealed the presence or absence of membrane anchorage. A positive control for the sodium carbonate treatment consisted of the α -factor mRNA product (Promega). As the latter is secreted into the microsomal vesicle lumen, the protein should always appear in the supernatant fluid of the sodium carbonate reaction mixture, and in the membrane pellet aliquot of the neutral buffer reaction mix.

This experiment was taken further to analyze the location of the transmembrane region encoded by the cloned gene product. The locus of this region was predicted to lie between amino acid residues 360-387 encoded by the BVDV 2724 cDNA based on hydrophobicity analysis of the predicted amino acid sequence of the BVDV cDNA nucleotide sequence in pMK14 recombinant. A unique restriction endonuclease site upstream of the predicted membrane anchor was also identified based on nucleotide sequence data of the BVDV cDNA in pMK14 recombinant. This enzyme, Ssp I (Stratagene), was used to linearize the cDNA template in the recombinant using a procedure recommended by the manufacturer. The vector was also digested at three locations; at nucleotide residues 2142, 2580, and 2604 respectively. Disruption of the T7 promoter region of the vector, which lies between nucleotides 3183 and 3199, did not occur. The Ssp I-truncated cDNA template then was transcribed, the RNA translated, and the protein processed as before. Controls were done as before.

The final analysis of the *in vitro* BVDV strain 2724 cDNA expression product was immunoprecipitation of the protein with BVDV polyclonal antisera. This procedure was done by the methods described by Anderson et al. (1983). Antiserum used was obtained from M. Collett (Medimmune). This antiserum was generated against the putative carboxy-terminal half of a fusion protein of BVDV strain NADL gp53 and β -galactosidase by injection into rabbits. Nonimmune sera from gnotobiotic calves was acquired from K. Brock (Univ. of Ohio). mRNA derived from BVDV strain 2724 cDNA was translated with and without microsomal membranes in a 60 ul volume, and the reagent amounts adjusted accordingly. Immunoprecipitations were done on 50 ul of the sample, and 10 ul was saved for electrophoresis to assess translation efficiency of the RNA.

Twenty-five percent Triton X-100 to obtain a final concentration of 4% was added to the translation reaction (>1% final concentration is required when immunoprecipitating translation products from rabbit reticulocyte lysate systems to solubilize the proteins which are present in a high concentration in these preparations; Anderson et al., 1983). Four volumes dilution buffer (1.25% Triton X-100; 190mM NaCl; 60mM Tris-HCl; 6mM EDTA) and five ul antiserum were added to the translation reaction mixture, and held at 4 C overnight. Negative controls were identical procedures with nonimmune serum instead of immune serum. The sample then was centrifuged at 12,000 x g for two minutes and the supernatant

transferred to a new tube. Thirty μ l of a 1:1 suspension of protein G-sepharose beads (Pharmacia) in wash buffer without detergent (50mM Tris-HCl, pH 7.5; 5mM EDTA; 150mM NaCl) was added to the mixture. The sample was rocked at room temperature for at least two hours. The beads then were pelleted by centrifugation for 10 seconds at 10,000 x g and the supernatant was carefully aspirated. The beads were washed four times with wash buffer with detergent (0.1% Triton X-100, 0.02% SDS) and once with wash buffer without detergent. After the final wash, beads were pelleted, wash solution removed, and protein eluted from the beads with SDS loading buffer with urea (300 mg/ml) and 2-mercaptoethanol (5%). The sample was heated for four minutes to 100 C, and beads were pelleted by centrifugation. The supernatant then was loaded onto the SDS gel and electrophoresed as previously described for evaluation of translation products by SDS-polyacrylamide gel electrophoresis.

CHAPTER 3

AMPLIFICATION, CLONING, SEQUENCING, AND SEQUENCE ANALYSIS OF THE PUTATIVE GENE ENCODING THE MAJOR ENVELOPE PROTEIN OF A NONCYTOPATHIC STRAIN OF BOVINE VIRAL DIARRHEA VIRUS

INTRODUCTION

Bovine viral diarrhea virus (BVDV) causes some of the most important diseases affecting the cattle industry. The virus is ubiquitous; serum antibody prevalence in cattle ranges from 50% to 90% (Radostits et al., 1988). The course and consequences of BVDV infection are diverse, varying from inapparent infection to severe disease ending in death. The manifestations observed depend upon a number of factors, both host and virus related, and only now are beginning to be understood.

Three clinical syndromes that may occur following infection with BVDV are bovine viral diarrhea, fetal disease, and mucosal disease (Duffell et al., 1985; Ames, 1986). Bovine viral diarrhea is an acute infection of nonimmune immunocompetent bovine. Symptoms usually are mild, and may include fever, depression, inappetence, oculonasal discharge, oral erosions, and diarrhea (Ames, 1986; Radostits et al., 1988). Research suggests that a more serious consequence of primary postnatal infection in cattle is the transient immunosuppression induced by the virus (Baker, 1987; Potgieter, 1988; Radostits et al., 1988; Castrucci et al., 1990). This pathogen targets not only epithelial cells of the bovine digestive tract, but immunocompetent cells as well (Truitt et al., 1973; Baker, 1987; Ohmann et al., 1987; Potgieter, 1988; Bolin, 1990b). Development of opportunistic infections are often the result of this immune system dysfunction (Tyler et al., 1965; Potgieter, 1977; Bohac et al., 1980; Ames, 1986; Baker, 1987).

Infection of the pregnant cow may result in fetal infection and disease (Kendrick, 1971; Roeder et al., 1986a; Baker, 1987; Potgieter, 1988). The manifestations of fetal infection range from fetal death or congenital defects to an apparently normal calf depending upon the stage of gestation at the time of infection (Duffell et al., 1985; Baker, 1987; Potgieter, 1988). Calves infected with a noncytopathic biotype of the virus prior to development of immunocompetence may

become persistently infected, and, after their birth, constitute a constant source of the virus within the herd (Bolin et al., 1985d; Duffell et al., 1985; Baker, 1987; Radostits et al., 1988). These animals are at high risk for the development of mucosal disease (Radostits et al., 1988). Mucosal disease results from superinfection of persistently infected cattle with a cytopathic strain of BVDV (Brownlie et al., 1984; Bolin et al., 1985a; McClurkin et al., 1985; Baker, 1987; Radostits et al., 1988). The mortality rate from this disease syndrome is high (McClurkin et al., 1985; Potgieter, 1988; Radostits et al., 1988).

BVDV is a small, spherical virus containing single-stranded RNA of positive polarity (Diderholm et al., 1966). The genome is approximately 12.5 kb in length and may have one long open reading frame capable of encoding a polyprotein with molecular weight of 449kD (Collett et al., 1988a). The precise number and identity of BVDV-encoded proteins remains unclear. Classification of BVDV has been uncertain as well. It is classified within the genus *Pestivirus* along with the agents of hog cholera and border disease (Westaway et al., 1985; Moennig, 1990). Viruses in this genus are currently included in the family *Togaviridae*, but may soon be reclassified as *Flaviviridae* due to their similarities to members of this viral family (Horzinek et al., 1987; Collett et al., 1988c; Collett, personal communication).

Management practices aimed at preventing the spread of BVDV are, at best, difficult, and, at worst, unfeasible. Both killed and modified live vaccines are used for prevention of BVDV infection. The former are of dubious efficacy, while the latter may produce many of the sequelae associated with natural infection. Much work remains to both understand the biology of and to control this disease agent. Recombinant DNA technology may hold the key to achieving these goals. Therefore, we have placed investigative emphasis on the major envelope protein of BVDV. This glycoprotein is the only virally-encoded polypeptide consistently precipitated with BVDV antisera, and is the target for neutralizing antibodies (Bolin et al., 1990a). Characterization of this protein may well prove pivotal to control and prevention of BVDV infection in cattle. In this report, the amplification, cloning, and nucleotide sequencing of the putative gene encoding the major envelope protein of a noncytopathic BVDV strain is described.

RESULTS

Primer design

Oligonucleotide primers for cDNA production and polymerase chain reaction amplification were designed according to genomic sequence data of BVDV strain NADL (Collett et al., 1988a). The presumptive boundaries of the gene encoding gp53 was determined after analyses of immunoprecipitation data on BVDV-specific proteins using antisera generated to proteins expressed from cloned viral genomic sequences (Collett et al., 1988b). According to their investigation, the predicted carboxy terminus of gp53 was at amino acid residue 1063 of BVDV polyprotein.

We deduced that the amino terminus of gp53 should lie at or near the 681st amino acid. Our calculations included an estimate that the nonglycosylated molecular weight of this protein was 42kD and the assumption that the average molecular weight of an amino acid was 110 Daltons. Furthermore, we used data from the nucleotide and corresponding presumptive amino acid sequence of gp53 protein to aid in estimating its gene boundaries. A computer-generated hydrophobicity plot (Devereaux, 1989) of the NADL putative amino acid sequence of this region revealed significant areas of hydrophobicity at a stretch of approximately 16 residues near the predicted amino terminus of gp53 (residues 677-692), and a longer expanse of approximately 27 residues near the carboxy terminus (residues 1033-1059). We postulated that the former may serve as the signal sequence for this glycoprotein because this domain fulfills many of the requirements for leader peptide function. Additionally, precedence for this hypothesis exists from investigation of the analogous protein of flaviviruses as proposed by Collett et al., the NS1 glycoprotein (1988c). Falgout et al. determined that this flavivirus protein possesses a stretch of hydrophobic amino acid residues at its amino terminus capable of targeting this protein to the endoplasmic reticulum (1989). Also, we assumed that the larger hydrophobic amino acid region may serve as the transmembrane domain. The primers delineating gene boundaries of gp53 were designed to lie outside these hydrophobic regions to incorporate the latter into the construct.

Amplification of the putative gene encoding BVDV gp53

A noncytopathic strain of bovine viral diarrhea virus was chosen for analysis of the putative envelope protein because this biotype is often incriminated in diseases caused by this virus and characterization of this biotype has not been as extensive as that of cytopathic strains. Virus was propagated in bovine turbinate cells grown in medium supplemented with 10% horse serum. Fetal calf serum was not used because it often is contaminated with BVDV (Nuttall et al., 1977; Erickson et al., 1988). Maximal viral RNA production and yield from infected cells was obtained after at least 10 days post-infection.

Total RNA was isolated from infected cells using the procedure described by Chomczynski and Sacchi (1987). This technique resulted in consistent and sufficient yields of RNA suitable for use as a template for cDNA synthesis. Reverse transcription for cDNA synthesis was done with Moloney murine leukemia virus reverse transcriptase using the manufacturer's recommendations. Primer 1 of each primer set was used to prime first strand cDNA synthesis.

Second strand cDNA synthesis and amplification of the double-stranded cDNA product was done by polymerase chain reaction (PCR) procedure. The efficient amplification of the gp53 gene product required optimization of several parameters. Of primary importance was the amount of total RNA used in the initial reverse transcription reaction. Quantities of RNA greater than five ug resulted in reduced yield and specificity of the PCR product, while 0.5-5.0 ug of RNA was optimal. Analysis of the primers to determine their potential for hybridization to each other (formation of "primer-dimer") and for internal secondary structure was required (Williams, 1989). The optimal amount of each primer used (0.5-1.0uM) was determined empirically. Magnesium concentrations in the PCR buffer were important for efficient amplification, and amounts of 1.0-2.0mM were optimal. Concentrations of deoxyribonucleotide triphosphates (dNTP) greater than 200uM decrease the fidelity of Taq polymerase, and, therefore, the concentration used in reactions was 100uM (Saiki, 1989). Finally, optimum temperature and times of the various thermal cycles of the reaction resulting in the highest specificity and yield of DNA product were determined. Primer annealing at 55 C resulted in acceptable specificity. Primer extension time of one minute was the shortest time that allowed for efficient amplification, and 1.5 minutes was used for denaturation of double-

stranded DNA. The number of temperature cycles which produced maximal cDNA in the PCR reaction with primers designed to encompass gp53 gene of BVDV were 25-30.

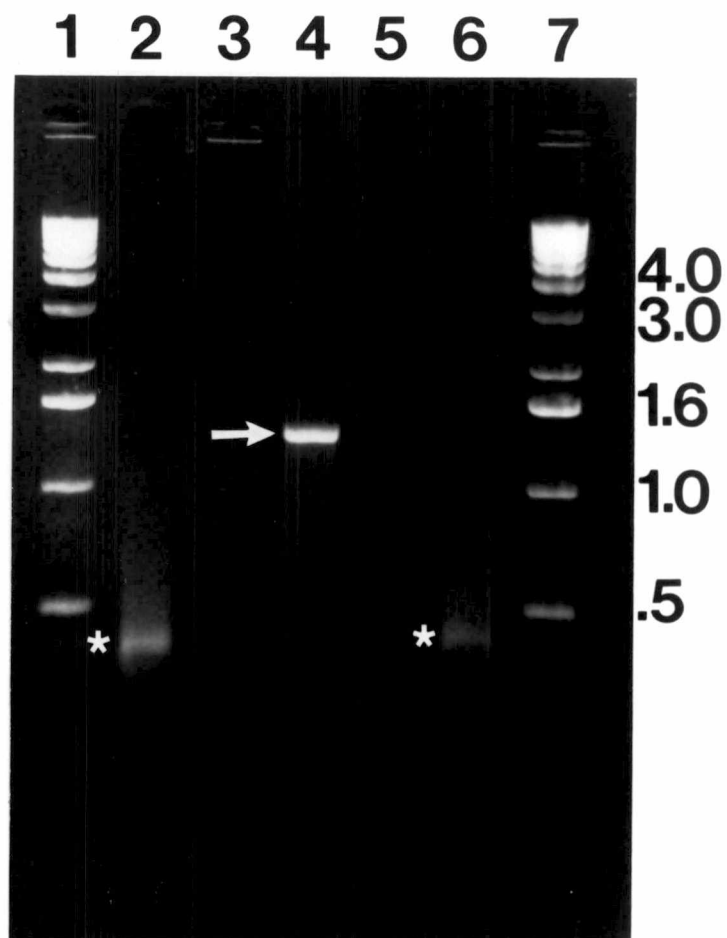
PCR products were analyzed by electrophoresis in an agarose gel (figure 3.1). The reaction mixtures containing the appropriately-sized cDNA were used in recombination experiments.

Recombination experiments

PCR-amplified cDNA was isolated and purified. Appropriate cDNA was electrophoresed onto nitrocellulose and eluted with an alkaline arginine solution. Following purification, the cDNA was digested with the appropriate restriction endonucleases and ligated into the plasmid vector pGem3zf(+) or pCR1000. Restriction enzyme sites which differed from one another were engineered into the two primers of primer set 1 to allow for directional cloning of cDNA into pGem3zf(+), to eliminate the need for the addition of restriction enzyme site linkers onto the cDNA termini, and to reduce the background of blue plaques because the vector, having been digested with two different restriction endonucleases, is far less likely to self-ligate (Scharf et al., 1986). As the individual BVDV genes do not contain stop codons, primer 1 of primer set 2 was synthesized with stop codons in all three reading frames so that, following amplification, they were incorporated at the 3' end of the cDNA positive strand. This was done to allow the construct to be used in an appropriate expression system.

The vector pGem3zf(+) permitted directional recombination of cDNA by taking advantage of the many different restriction enzyme sites within the multiple cloning site, and efficient transcription of the cDNA using its T7 promoter. Also, recombinants of this vector were stable. The pCR1000 vector circumvented the need for restriction endonuclease digestion of cDNA and vector prior to recombination thereby avoiding modifications of the ends of the cDNA insert and vector from over-digestion or "nibbling" by the restriction endonuclease. Although ligation of cDNA into pCR1000 vector was less efficient than into pGem vector, this cloning method proved to be very useful. This vector contained fewer available restriction enzyme sites for later use, but this problem was overcome by engineering

Figure 3.1. Product of polymerase chain reaction with primers encompassing the putative gene encoding gp53 of BVDV strain 2724. Numbers on right are size of the DNA standards (lanes 1 and 7) in kilobases. Lanes 2 and 6, positive controls for polymerase chain reaction using primers specific for amplification at the 3' end of the BVDV genome of a 300 nucleotide base region (asterisk). Amplification was done as described in the text on total RNA from BVDV-infected cells. Lanes 3 and 5, negative controls using total RNA from uninfected cells. Lane 4, amplification using primers encompassing the putative gp53-encoding region produced a 1300 base pair product (arrow).



the desired site into the primers used for amplification. This vector also provided a stable recombinant.

Various recombinants served different purposes (figure 3.2). The construct utilizing the pGem3zf(+) plasmid was used for *in vitro* transcription and expression, while the construct of plasmid pCR1000 was designed primarily for use in a baculovirus expression system. Both constructs were used for DNA base sequencing.

The recombinant plasmids were transfected into *E. coli* strains JM101 and JM109 {pGem3zf(+) recombinants}, and INV α F' (pCR1000 recombinants). Screening for potential recombinants was done by two separate methods. Polymerase chain reaction of lysed transformants was done with the original primers used for cDNA synthesis. Recombinants of pGem3zf(+) were amplified also with primers which hybridize to T7 and Sp6 promoters of the vector. Amplification products were analyzed by agarose gel electrophoresis (figure 3.3). Recombinant plasmids that resulted in an appropriately-sized amplification product were digested with the appropriate restriction enzymes to release cloned cDNA. This was followed by agarose gel electrophoresis to determine whether a DNA fragment of the correct size had been released from the recombinant (figure 3.4). Recombinant plasmids identified by both screening methods were used for DNA base sequencing for final confirmation of the presence of cloned BVDV gp53 cDNA in the vectors.

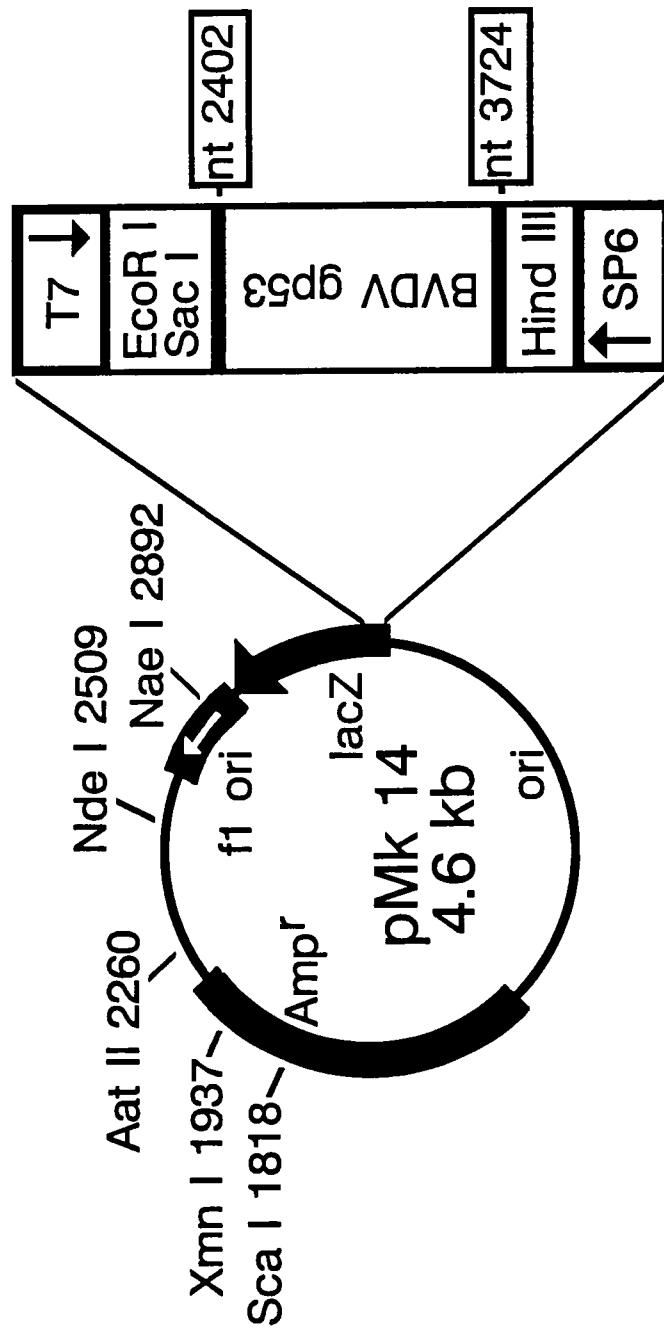
DNA base sequence analysis

The nucleotide sequence of the putative gene encoding gp53 of BVDV 2724 was determined from three independent clones acquired from three separate PCR reactions to minimize mistakes due to Taq polymerase infidelity during cDNA amplification. A schematic diagram of the sequencing protocol is given in figure 3.5. Few differences existed among the clones. Discrepancies were resolved by assuming that the base present in two out of the three clones was the correct base. Differences in all three clones at one site were not encountered.

Figure 3.6 gives the nucleotide and the predicted amino acid sequence of BVDV 2724 gp53-encoding region. The sequence was 1323 nucleotides long encoding 441 amino acids. A small portion of the BVDV genome encoding the downstream nonstructural protein, p125, may have been included in the plasmid constructs, because the region encompassed by the primers used for cDNA synthesis

Figure 3.2. Construction of recombinant BVDV strain 2724 cDNA in plasmid vectors. (A) cDNA of the putative gene encoding gp53 engineered into the Sac I and Hind III restriction endonuclease sites of vector pGem3zf(+). (B) cDNA of the putative gene encoding gp53 engineered into the cloning site of vector pCR1000. Nucleotide (nt) number denotes nucleotide position of cDNA relative to BVDV strain NADL nucleotide sequence (Collett et al, 1988a).

A



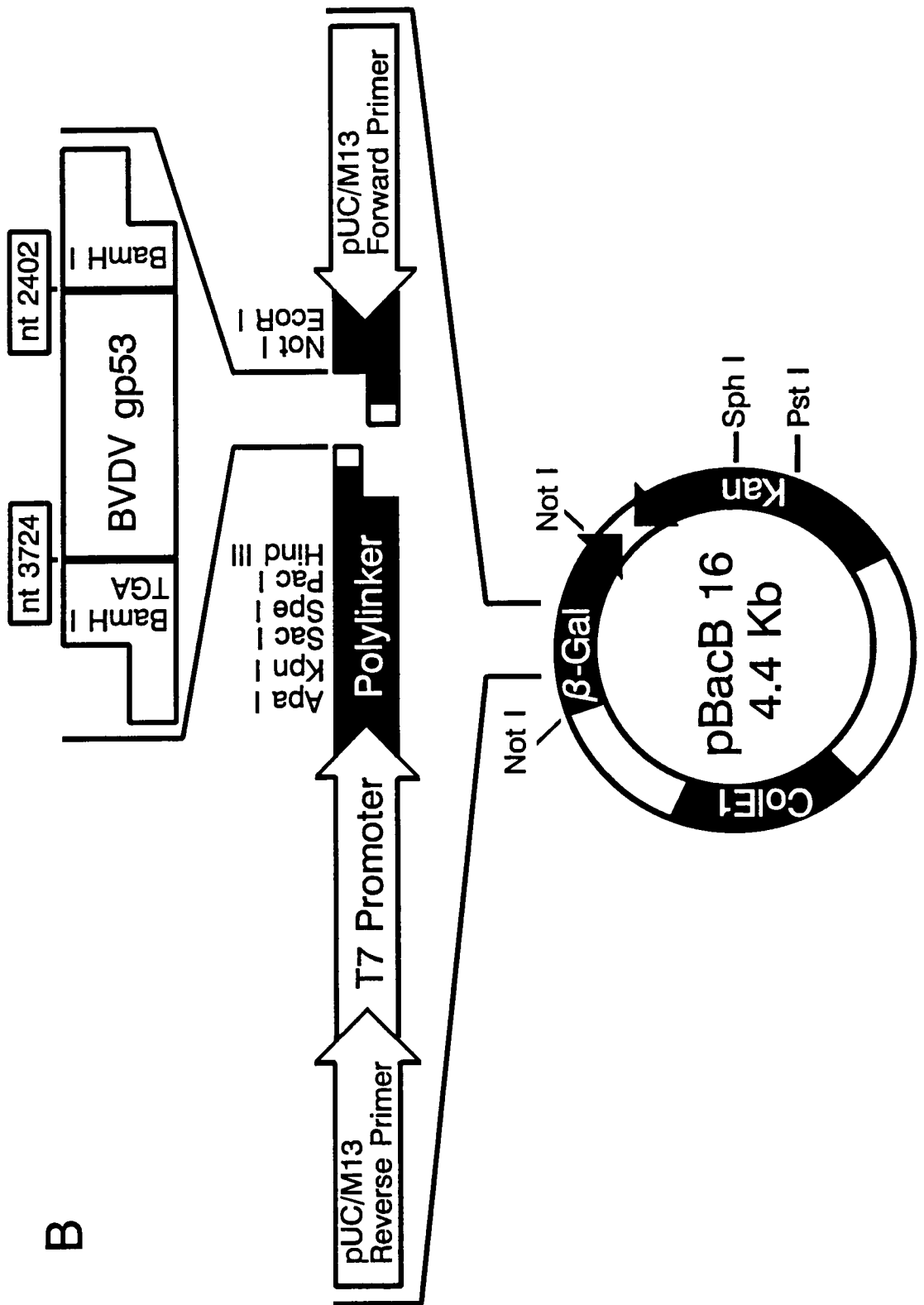


Figure 3.3. Evaluation of transformed bacteria using polymerase chain reaction. Numbers on right are size in kilobases of DNA standards. Lanes 1-12 are the products of amplification of lysed E. coli transformants. All lanes positive for amplification have the appropriate size product of approximately 1300 base pairs.

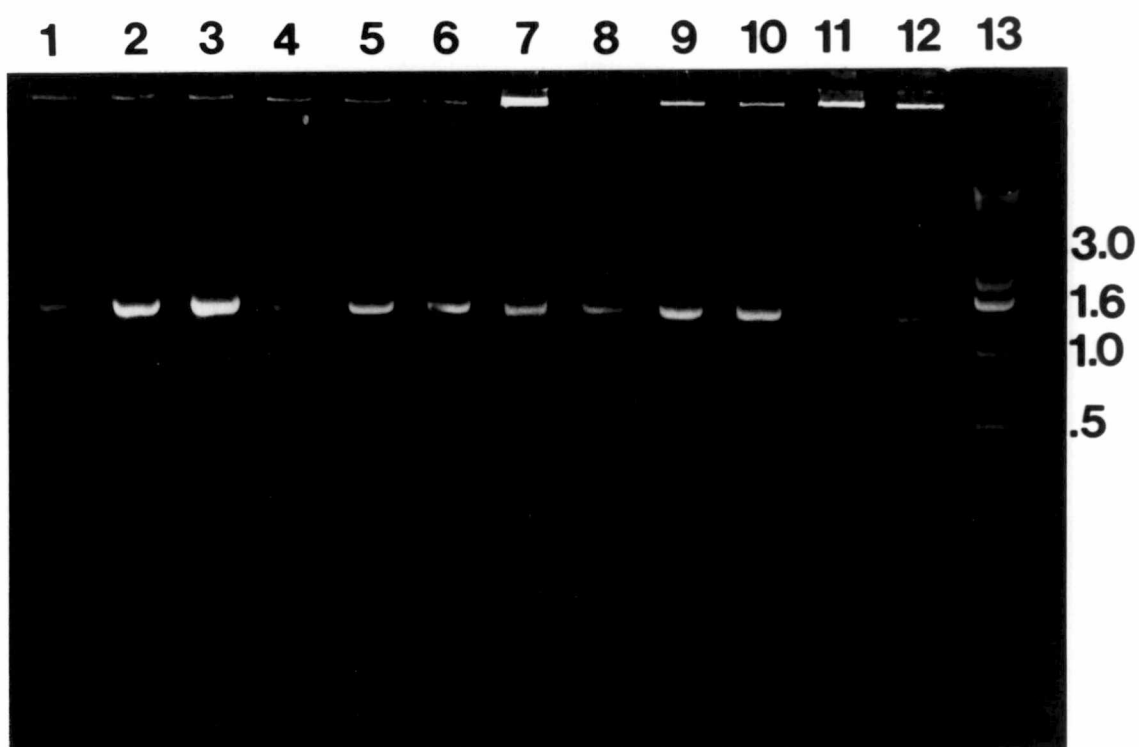


Figure 3.4. Evaluation of transformants by restriction endonuclease digestion of recombinant plasmids. Numbers on right are size in kilobases of DNA standards (lane 7). Lane 1, pMK14 digested with Hind III and EcoR I restriction endonucleases. The 1300 base pair cDNA insert is indicated by the arrow. Lane 2, recombinant pMK14 digested with Hind III. Lane 3, recombinant pMK14 digested with EcoR I. Lane 4, undigested recombinant pMK14. Lane 5, nonrecombinant pGem3zf(+) digested with Hind III and EcoR I. Lane 6, undigested nonrecombinant pGem3zf(+).

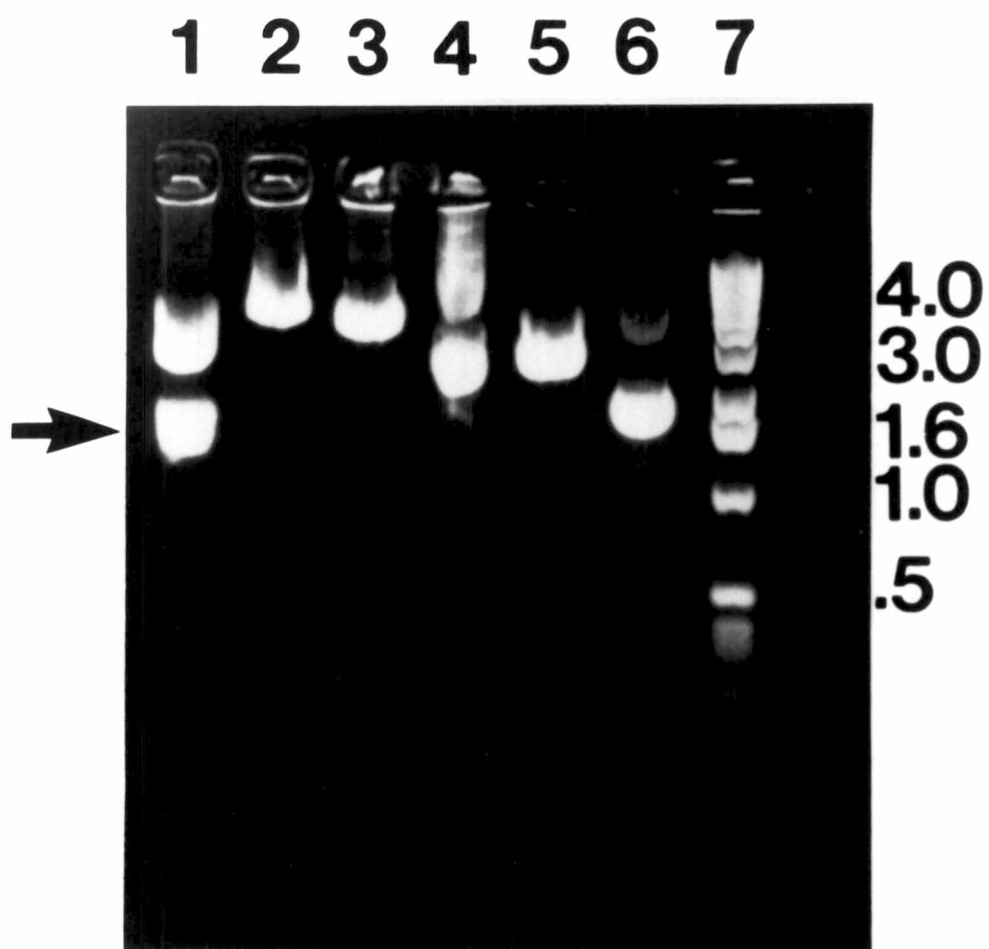


Figure 3.5. Schematic illustration of cDNA sequencing procedure. Nucleotide (nt) numbers denote nucleotide position of cDNA relative to BVDV strain NADL (Collett et al, 1988). T7 and Sp6 denote primers which hybridize to vector nucleotide sequences immediately outside the cDNA (T7 and Sp6 promoter regions). Open boxes denote primers which were synthesized for hybridization to internal nucleotide sequences of the cDNA. Squiggly arrows denote direction of DNA synthesis during sequencing.

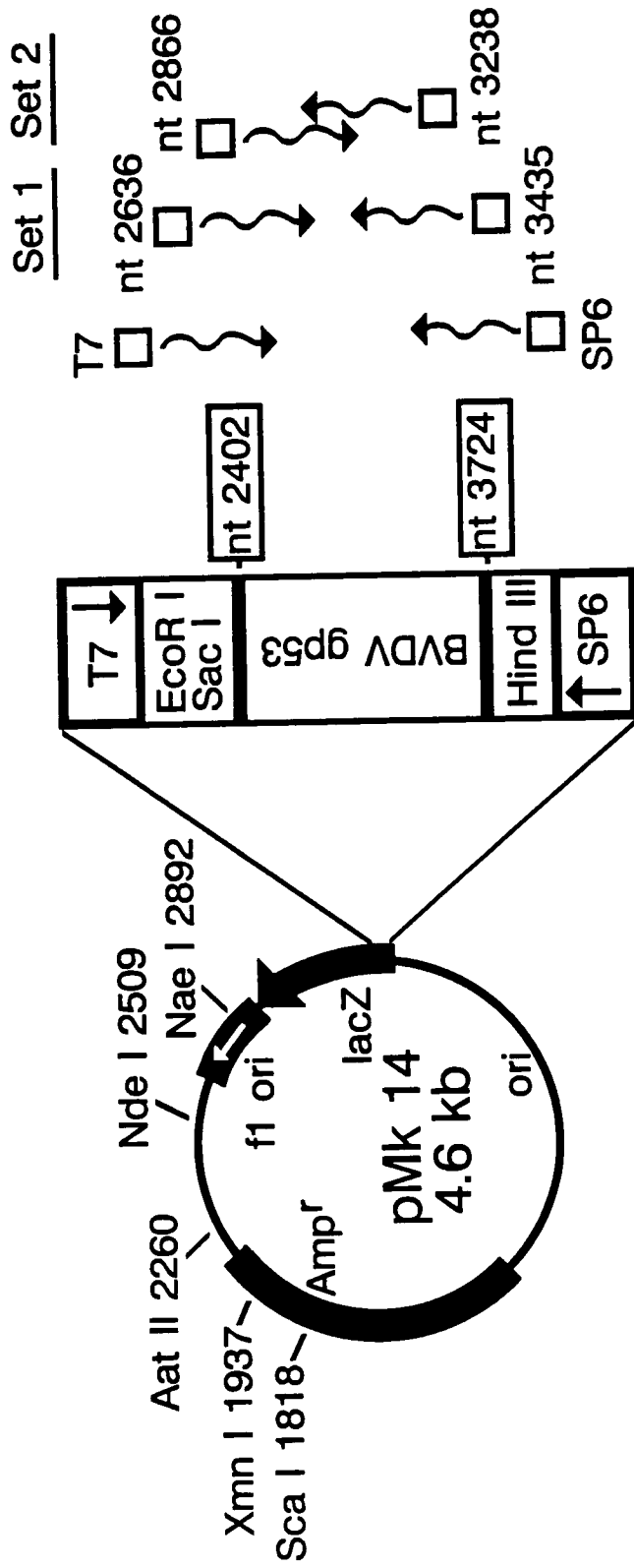


Figure 3.6. Nucleotide sequence of BVDV strain 2724 cDNA. Amino acids encoded by the cDNA are given also. Nucleotides and amino acids are numbered from the 5'- and amino-termini respectively. The sequence begins near the predicted 5' end and extends over 100 nucleotide residues beyond the predicted 3' terminus of the putative gene encoding gp53 (Collett et al., 1988b). Boxes denote amino acid sequences which may function as signal sequence (amino acid residues 5-20) and transmembrane domain (amino acid residues 361-387). Solid arrows denotes predicted amino- and carboxy-termini of gp53. Open arrow denotes possible C-terminal cleavage site following dibasic amino acid residues. Asterisks denote potential N-linked glycosylation sites (see text for full description).

1	GTC	AGG	GGC	CAG	ATG	GTA	CAG	GCG	ATC	CTA	30
1	Val	Arg	Gly	Gln	MET	Val	Gln	Gly	Ile	Leu	10
31	TOG	CTA	CTA	CTG	ATA	ACA	GCG	GTA	CAA	GCG	60
11	Trp	Leu	Leu	Leu	Ile	Thr	Gly	Val	Gln	Gly	20
61	GAC	ATT	GAC	TOC	AAA	CCT	GAA	CAC	TCA	TAC	90
21	Asp	Ile	Asp	Cys	Lys	Pro	Glu	His	Ser	Tyr	30
91	GCC	ATA	GCC	AGG	AAT	GAT	AGA	ATT	GCG	CCA	120
31	Ala	Ile	Ala	Arg	Asn	Asp	Arg	Ile	Gly	Pro	40
121	TTA	GGA	GCT	GAA	GCG	CTC	ACC	ACT	GTT	TOG	150
41	Leu	Gly	Ala	Glu	Gly	Leu	Thr	Thr	Val	Trp	50
151	AAO	GAT	TAC	TCA	CAT	GAA	ATG	AAG	CTG	GAA	180
51	Lys	Asp	Tyr	Ser	His	Glu	MET	Lys	Leu	Glu	60
181	GAC	ACA	ATG	GTC	ATA	GCT	TOG	TOC	AAA	GAC	210
61	Asp	Thr	MET	Val	Ile	Ala	Trp	Cys	Lys	Asp	70
211	GCT	AAG	TTT	ACA	TAC	CTC	TCA	AOG	TOC	ACA	240
71	Gly	Lys	Phe	Thr	Tyr	Leu	Ser	Arg	Cys	Thr	80
241	AGA	GAA	ACT	AGA	TAT	CTT	GCA	ATT	CTG	CAT	270
81	Arg	Glu	Thr	Arg	Tyr	Leu	Ala	Ile	Leu	His	90
271	TCA	AGA	GCC	TTG	CAG	ACC	AGT	GTC	GTA	TTC	300
91	Ser	Arg	Ala	Leu	Gln	Thr	Ser	Val	Val	Phe	100
301	AAA	AAA	CTT	TTC	GAG	GCG	CAA	AOG	CRA	GCG	330
101	Lys	Lys	Leu	Phe	Glu	Gly	Gln	Arg	Gln	Gly	110
331	GAA	ACA	TTT	GAA	ATG	GCT	GAC	GAC	TTT	GAA	360
111	Glu	Thr	Phe	Glu	MET	Ala	Asp	Asp	Phe	Glu	120
361	TTT	GGA	CTC	TOC	CCA	TOC	GAT	GCC	AAT	CCC	390
121	Phe	Gly	Leu	Cys	Pro	Cys	Asp	Ala	Asn	Pro	130
391	GTA	GTA	AGA	GCG	AAG	TTC	AAT	ACA	ACA	CTG	420
131	Val	Val	Arg	Gly	Lys	Phe	*Asn	Thr	Thr	Leu	140
421	CTA	AAC	GGA	CCG	GCC	TTC	CAG	ATG	GTA	TOC	450
141	Leu	Asn	Gly	Pro	Ala	Phe	Gln	MET	Val	Cys	150
451	CCT	ATA	GGA	TOG	ACA	GGA	ACT	GTC	AOC	TGT	480
151	Pro	Ile	Gly	Trp	Thr	Gly	Thr	Val	Ser	Cys	160
481	ATG	TTA	GCT	AAT	AOG	GAC	ACC	CTA	GAC	ACA	510
161	MET	Leu	Ala	Asn	Arg	Asp	Thr	Leu	Asp	Thr	170
511	GCA	GTA	GTC	GCT	GTC	TAT	AAG	AOG	TCC	AAA	540
171	Ala	Val	Val	Arg	Val	Tyr	Lys	Arg	Ser	Lys	180
541	CCA	TTC	CCT	TAT	AGA	CAA	GCT	TGT	ATC	ACC	570
181	Pro	Phe	Pro	Tyr	Arg	Gln	Gly	Cys	Ile	Thr	190
571	CAA	AGA	ACT	CTG	GCG	GAG	GAT	CTC	TAT	AAC	600
191	Gln	Arg	Thr	Leu	Gly	Glu	Asp	Leu	Tyr	Asn	200
601	TGT	GAT	CTT	GGA	GCG	AAT	TOG	ACT	TGT	GTC	630
201	Cys	Asp	Leu	Gly	Gly	*Asn	Trp	Thr	Cys	Val	210
631	ACT	GCG	GAC	CAG	CTA	CAA	TAC	ACA	GGA	GCG	660
211	Thr	Gly	Asp	Gln	Leu	Gln	Tyr	Thr	Gly	Gly	220
661	CCT	GTC	GAA	TCT	TOC	AAG	TOG	TGT	GCT	TAT	690
221	Pro	Val	Glu	Ser	Cys	Lys	Trp	Cys	Gly	Tyr	230

691	AAA	TTC	CAA	AAA	AGT	GAG	GGG	TTG	CCA	CAC	720
231	Lys	Phe	Gln	Lys	Ser	Glu	Gly	Leu	Pro	His	240
721	TAC	CCC	ATC	GGC	AAG	TGT	AGG	TTG	AAG	AAT	750
241	Tyr	Pro	Ile	Gly	Lys	Cys	Arg	Leu	Lys	*Asn	250
751	GAG	ACT	GGC	TAC	AGA	TTT	GTA	GAC	GGC	ACC	780
251	Glu	Thr	Gly	Tyr	Arg	Phe	Val	Asp	Gly	Thr	260
781	ACT	TGC	AAC	AGA	GAG	GGT	GTA	GCC	ATA	GTA	810
261	Thr	Cys	Asn	Arg	Glu	Gly	Val	Ala	Ile	Val	270
811	CCA	CAA	GGA	TTG	GTA	AAG	TGT	AAG	ATA	GGA	840
271	Pro	Gln	Gly	Leu	Val	Lys	Cys	Lys	Ile	Gly	280
841	GAC	ACA	ATC	GTA	CAG	GTC	ATA	GCT	CTT	GAC	870
281	Asp	Thr	Ile	Val	Gln	Val	Ile	Ala	Leu	Asp	290
871	ACC	AAA	CTT	GGG	CCT	ATG	CCT	TGC	AAG	CCA	900
291	Thr	Lys	Leu	Gly	Pro	MET	Pro	Cys	Lys	Pro	300
901	TAT	GAG	ATC	ATA	CCA	AGT	GAG	GGG	CCT	GTA	930
301	Tyr	Glu	Ile	Ile	Pro	Ser	Glu	Gly	Pro	Val	310
931	GAA	AAG	ACG	GCA	TGC	ACC	TTC	AAC	TAC	ACG	960
311	Glu	Lys	Thr	Ala	Cys	Thr	Phe	*Asn	Tyr	Thr	320
961	AGG	ACA	TTA	AAA	AAT	AAA	TAT	TTT	GAG	CCC	990
321	Arg	Thr	Leu	Lys	Asn	Lys	Tyr	Phe	Glu	Pro	330
991	AGA	GAC	AGT	TAC	TTC	CAG	CAA	TAC	ATG	CTA	1020
331	Arg	Asp	Ser	Tyr	Phe	Gln	Gln	Tyr	MET	Leu	340
1021	AAA	GGA	GAT	TAT	CAA	TAC	TGG	TTC	GAC	CTG	1050
341	Lys	Gly	Asp	Tyr	Gln	Tyr	Trp	Phe	Asp	Leu	350
1051	GAG	GTC	ACT	GAC	CAT	CAT	GGG	GAT	TAC	TTC	1080
351	Glu	Val	Thr	Asp	His	His	Arg	Asp	Tyr	Phe	360
1081	GCC	GAG	TCC	ATA	TTG	GTC	GTC	GTC	GTA	GCT	1110
361	Ala	Glu	Ser	Ile	Leu	Val	Val	Val	Val	Ala	370
1111	TTA	CTG	GGT	GGA	AGA	TAC	GTC	CTC	TGG	TTA	1140
371	Leu	Leu	Gly	Gly	Arg	Tyr	Val	Leu	Trp	Leu	380
1141	CTG	GTA	ACA	TAC	ATG	GTC	CTA	TCA	GAA	CAA	1170
381	Leu	Val	Thr	Tyr	MET	Val	Leu	Ser	Glu	Gln	390
1171	AAG	GCC	TTG	GGG	ACC	CAA	TAT	GGG	GCA	GGG	1200
391	Lys	Ala	Leu	Gly	Thr	Gln	Tyr	Gly	Ala	Gly	400
1201	GAA	GTC	GTC	ATG	ATG	GGT	AAC	TTG	CTA	ACA	1230
401	Glu	Val	Val	MET	MET	Gly	Asn	Leu	Leu	Thr	410
1231	CAT	GAC	AGT	ATT	GAA	GTC	GTC	ACA	TAT	TTC	1260
411	His	Asp	Ser	Ile	Glu	Val	Val	Thr	Tyr	Phe	420
1261	TTG	TTG	TTA	TAC	CTA	CTG	CTA	AGA	GAG	GAG	1290
421	Leu	Leu	Leu	Tyr	Leu	Leu	Leu	Arg	Glu	Glu	430
1291	GCT	GTA	AAG	AAG	TGG	GTC	TTA	CTC	TTA	TAC	1320
431	Ala	Val	Lys	Lys	Trp	Val	Leu	Leu	Leu	Tyr	440
1321	CAC										1323
441	His										441

extended beyond the predicted 3' terminus of the gp53 gene. No stop codons were encountered in the cDNA open reading frame analogous to the appropriate portion of the long open reading frame of the BVDV NADL genomic sequence (Collett et al., 1988a).

Computer analyses revealed several properties of the protein predicted from the cDNA nucleotide sequence (table 3.1). Amino acids with apolar side chains made up 40% of the amino acid residues, those with uncharged polar side chains 36%, and charged amino acids 24% of the polypeptide encoded by the BVDV strain 2724 cDNA. Stretches of uncharged amino acid residues occurred at both the amino-terminal and carboxy-terminal regions. Distribution of acidic and basic amino acid residues in the protein were fairly uniform. Cysteine residues constituted 3.85% of all amino acids in the protein. Four potential N-linked glycosylation sites were present (figure 3.7).

The hydrophobicity plot was calculated using GCG software (figure 3.7). The protein is hydrophilic, but several expanses of hydrophobic regions were evident. A stretch of approximately 15 hydrophobic amino acid residues occurred at the amino terminus. A major span occurred from amino acid residue 361 to 385 of the protein. This region was followed by two shorter areas of hydrophobicity, one near amino acid residue 402 of approximately 10 residues, and one near amino acid residue 415 of approximately 12-15 residues.

Secondary structure predictions and antigenic index were also determined using GCG software. The major areas of hydrophobicity were predicted to form beta-sheets (figure 3.7). The antigenic index was calculated according to GCG software (figure 3.7). Dominant antigenic areas occurred from amino acid residues 175 to 275, and amino acid residues 290 to 360.

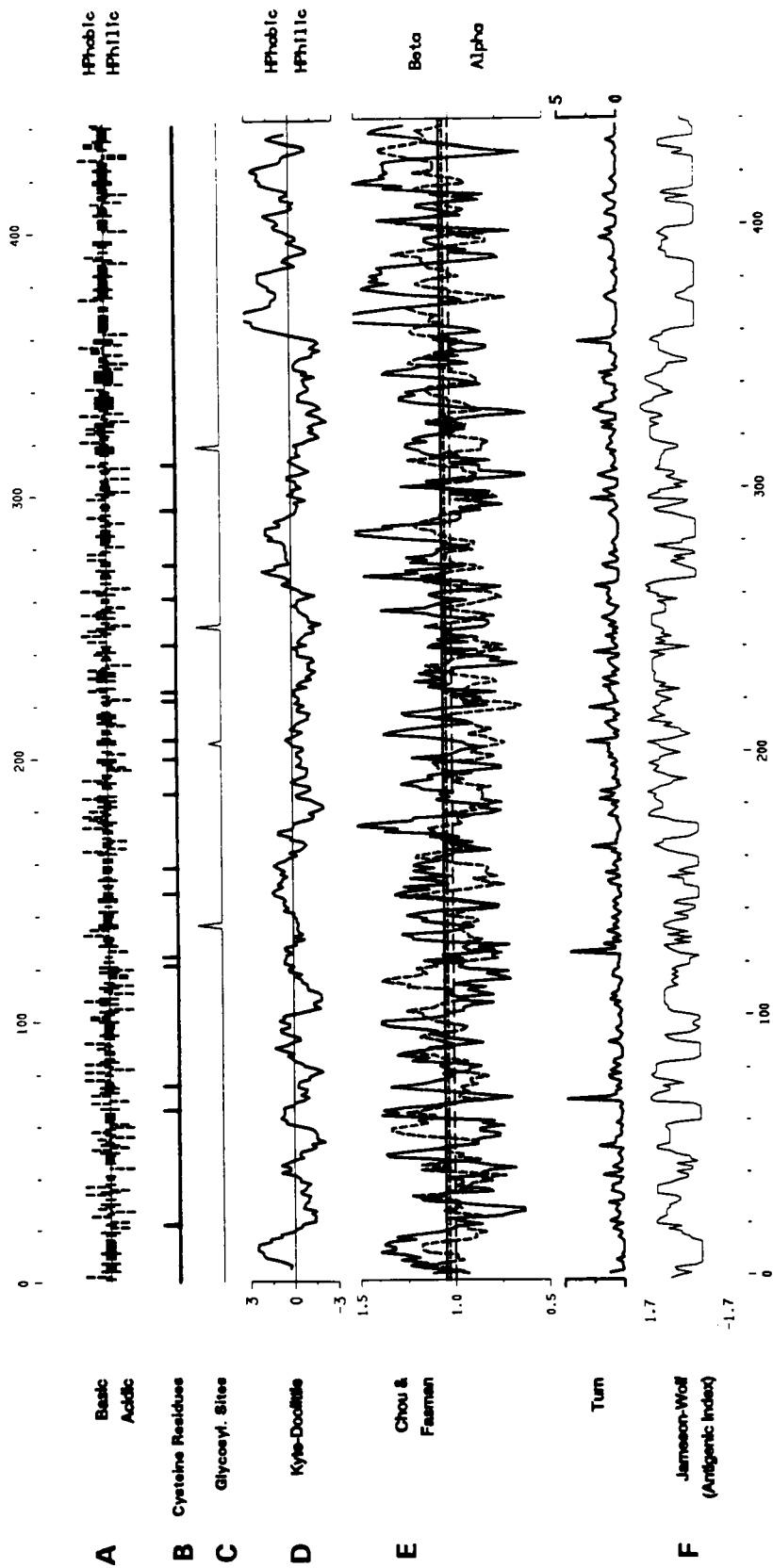
Comparison to Pestiviruses and Flaviviruses

Comparisons were made between the nucleotide base sequence of the putative BVDV strain 2724 gene encoding gp53 and the predicted amino acid sequence encoded by this gene to those of BVDV NADL strain (Collett et al., 1988a) and BVDV Osloss strain (Renard et al., 1987a), to those of gp55 of hog cholera virus Brescia strain (Moorman et al., 1990a) and Alfort strain (Meyers et al., 1989), and to those of the NS1 flavivirus protein of dengue fever (Hahn et al., 1988)

Table 3.1 Properties of putative gp53 of BVDV strain 2724.

Property	Values
Uncharged apolar amino acids	40%
Uncharged polar amino acids	36%
Charged amino acids	24%
Cysteine residues	4%
N-linked glycosylation sites	4
Isoelectric point	7.21

Figure 3.7. Features of the protein encoded by BVDV strain 2724 cDNA. The left-hand side of the figure represents the amino-terminal end of the open reading frame. Numbered hash marks along the top and bottom margins indicate amino acid position within the predicted amino acid sequence of the cDNA. (A) Distribution of acidic and basic residues within the amino acid sequence encoded by BVDV strain 2724 cDNA. Hashed marks extending only above (basic) or below (acidic) the line represent hydrophilic charged residues. Short marks extending above and below the line represent hydrophilic uncharged amides or hydrophobic aliphatic residues. Long marks extending above and below the line represent hydrophilic uncharged alcohols or hydrophilic aromatic residues. Unmarked positions represent alanine, glycine, or cysteine. (B) Distribution of cysteine residues within the amino acid sequence encoded by BVDV strain 2724 cDNA. (C) Distribution of potential N-linked glycosylation sites (asparagine-any amino acid-threonine or serine) within the amino acid sequence encoded by BVDV strain 2724 cDNA. (D) Hydropathy plot of the protein encoded by BVDV strain 2724 cDNA calculated based on methods of Kyte and Doolittle. The curve is the average of a residue specific hydrophobicity index over a window of nine residues. Curves in the upper half indicate a hydrophobic region, curves in the lower half indicate a hydrophilic region. (E) Predicted secondary structural characteristics of the polypeptide encoded by BVDV strain 2724 cDNA. Upper panel represents the Chou and Fasman propensity measures for alpha-helix (hashed line) and beta-sheet (solid line). Curves are the average of a residue-specific attribute over a window of four amino acids. Curves rising above the threshold satisfy one criterion for propagation of an alpha-helix or beta-sheet structure. Curves falling below the threshold indicate termination of the structure. The lower panel represents regions of the amino acid sequence encoded by BVDV strain 2724 cDNA found in turns. The curve is the result of a residue-specific, position-dependent attribute multiplied across a window of four amino acid residues. (F) Distribution of antigenic regions within the amino acid sequence encoded by BVDV strain 2724 cDNA. The antigenic index is calculated by summing several weighted measures of secondary structures and protein characteristics including hydrophilicity, surface probability, flexibility, and turns. All calculations were done using GCG software (Devereux, 1989).



and yellow fever (Rice et al., 1985). These comparisons also were made to the NS1 protein and the gene encoding it of hepatitis C virus, which, although it is not classified in the family Flaviviridae, is similar to pestiviruses in many respects (Miller et al., 1990; Weiner et al., 1991). The flavivirus gene encoding NS1 protein of the latter three viruses was selected for comparison because it may be analogous to gp53 of BVDV (Collett et al., 1989). The results are given in table 3.2.

The gene base sequence and amino acid sequence comparisons of these viruses were done with the GAP program of GCG software (table 3.2). This program makes the optimal alignment between two complete sequences using the algorithm of Needleman and Wunsch. The greatest degree of nucleotide and amino acid sequence similarity of BVDV strain 2724 gp53 region was to the NADL strain of BVDV. A nucleotide homology of 85%, and amino acid homology of 84% were identified. When similar amino acids (those having comparable charge, size, structure, etc.) were included in the homologous percentile, homology rose to 90%. Homology of BVDV strain 2724 gp53 to the Osloss BVDV strain was slightly lower, with a nucleotide identity of 74% and amino acid identity of 75%. Again, amino acid similarity between the two proteins increased to 87% when like residues were included in the analysis. Nucleotide comparisons of BVDV strain 2724 putative gp53-encoding gene to the genomic sequences encoding the major envelope proteins of hog cholera virus strains Alfort and Brescia revealed an identity of 60%. Amino acid sequences of the latter proteins had an identity of 61% and similarity of 75% respectively to BVDV 2724 gp53. No gaps, in which nucleotide or amino acid residues of the two sequences being compared were unpaired, were required to optimize alignment of nucleotide and amino acid sequences of BVDV strain 2724 to BVDV strain NADL, or to either of the hog cholera strains. Three gaps between nucleotide positions 476 and 477 were required to align BVDV strain 2724 with the Osloss strain in the nucleotide sequence comparison, and one gap following amino acid residue 155 was needed to align BVDV strain 2724 for amino acid sequence.

Nucleotide and amino acid sequence comparisons of BVDV strain 2724 gp53 to the NS1 protein of the flaviviruses of yellow fever and dengue fever, and to the virus of hepatitis C did not indicate the same high degree of homology (table 3.2). Nucleotide identity of the proposed BVDV strain 2724 gp53 gene was 39% for the NS1 gene of all three viruses, while amino acid identity for the respective proteins was 20%, 17%, and 19% for yellow fever virus, dengue fever virus, and hepatitis C

Table 3.2 Homology of the genomic region encoding gp53 of BVDV 2724 with Pestiviruses and Flaviviruses^a

	BVDV NADL ^b	BVDV Oslon ^c	HC Alfort ^d	HC Brescia ^e	Hep C ^f	Dengue 2 ^g	Yellow Fever ^h
nucleotide	85%	74%	60.5%	60%	39%	39%	39%
amino acid	84% (90%) ⁱ	75% (87%)	61% (75%)	61% (75%)	19% (43%)	17% (43%)	20% (48%)
glycosylation sites ^j	4/4	3/4	4/4	3/4	0/4	0/4	1/4
cysteine res ^j	17/17	16/17	15/17	15/17	3/17	3/17	1/17

^aHepatitis C, dengue, and yellow fever virus comparison to the NS1 protein-encoding region

^bCollett, et al. (1988a).

^cRenard, et al. (1987a).

^dMeyers, et al. (1989).

^eMoormann, et al. (1990a).

^fTakamizawa, et al. (1991).

^gHahn, et al. (1988).

^hRice, et al. (1985).

ⁱnumbers in parenthesis denote % similarity.

^jvalues indicate number of sites from the various viruses conserved in BVDV 2724.

virus respectively. Amino acid similarity (which considers residues of like structure, size, charge, etc.) of these latter proteins to the protein of BVDV strain 2724 was notably higher at 48%, 43%, and 43%. All nucleotide and amino acid sequence alignments of the cDNA and predicted protein of BVDV strain 2724 cDNA to the flavivirus and hepatitis C virus NS1 genes and proteins contained significant gaps in order to optimize the matches.

Cysteine residues were highly conserved among the pestiviruses, but not so between BVDV strain 2724 gp53 and the flaviviruses or hepatitis C virus (table 3.2). Of 17 cysteine residues encoded by the putative gp53 gene of BVDV 2724, 17 of 17 residues in BVDV NADL strain, 16 of 17 in BVDV Osloss strain, 15 of 17 in hog cholera virus Alfort strain, and 15 of 17 in hog cholera virus Brescia strain were present. The gp53 gene of the Osloss BVDV strain encodes one fewer cysteine, and both hog cholera virus strains encode two cysteines fewer in this region than do BVDV strains 2724 and NADL. Only one cysteine residue encoded by NS1 gene of the flaviviruses and hepatitis C virus, was present at the same site in the aligned sequences of BVDV 2724 and yellow fever viruses, and only three were encoded in this region of dengue fever and hepatitis C viruses. The latter three viruses contain a total of 12, 11, and 15 cysteines in their respective NS1 proteins.

Glycosylation sites of the predicted gp53 were well conserved between BVDV strain 2724 and the other pestiviruses, but were not comparable to those of the flaviviruses or hepatitis C virus (table 3.2). Four N-linked sites were identified in gp53 of strain 2724 and were similar to those of BVDV NADL strain, and to three of four sites in the BVDV Osloss strain. The latter virus lacks one site present in BVDV strain 2724. All four glycosylation sites of strain 2724 gp53 were present in hog cholera virus strain Alfort gp55. However, the latter contained two additional glycosylation sites. Three of the four glycosylation sites of BVDV strain 2724 gp53 were present in hog cholera virus strain Brescia gp55, but the latter possesses two additional sites not present in the former. Yellow fever virus NS1 protein contains one glycosylation site present in gp53 of BVDV 2724, while neither dengue fever virus nor hepatitis C virus NS1 proteins contain any glycosylation site in common with BVDV strain 2724 gp53. The NS1 proteins of the yellow fever, dengue fever, and hepatitis C viruses contain a total of two, four, and nine N-linked glycosylation sites respectively.

The hydrophobicity plots of gp53 and gp55 of the pestiviruses and NS1 protein of the flaviviruses are in figure 3.8. These plots were derived with GCG software and have both the measure of hydropathy according to Kyte and Doolittle analysis, and nonpolar transbilayer helices according to Goldman, Engelman, and Steiz analysis. The basic pattern of these plots was very similar among the pestiviruses. While the flaviviruses' pattern was distinct from that of the pestiviruses, it had some similarity to that of the pestiviruses in that the respective proteins are predominantly hydrophilic, with areas of hydrophobicity at the amino and carboxy termini.

The distribution of basic and acidic residues for the viruses are in figure 3.9. The basic pattern of distribution of charged amino acid residues appears similar among all the pestiviruses, while the flavivirus distributions of these residues are distinct from that of pestiviruses.

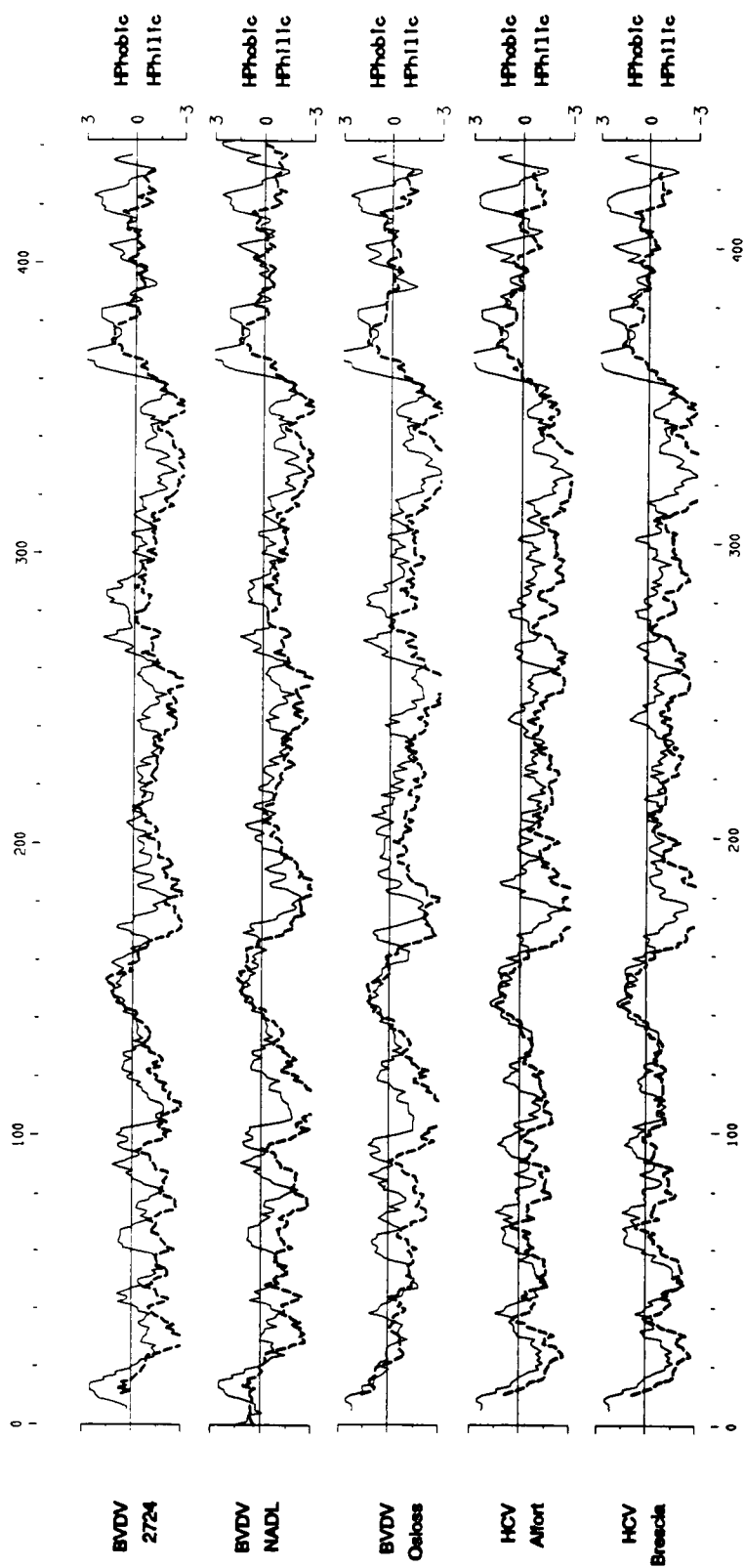
DISCUSSION

Polymerase chain reaction (PCR), which involves repeated cycles of DNA denaturation, primer annealing, and primer extension, proved very useful. As BVDV is difficult to grow adequately and manipulate in the laboratory, viral RNA yields are small making molecular cloning by conventional methods laborious (Renard et al., 1985). Cloning of PCR-amplified genes eliminates the cloning and screening of complete genomic libraries (Scharf et al., 1986). Additionally, the high temperature at which the thermostable polymerase functions in PCR provides greater specificity and fidelity in amplification than DNA polymerase I Klenow fragment, and facilitates research with nucleic acids with considerable secondary structure (Oste, 1988). Finally, because PCR primers can be modified at their respective 5' ends without affecting hybridization to the template, alterations in the termini of the PCR product can be made easily.

Primer design was based primarily on sequence data of BVDV NADL strain (Collett et al., 1988a). Analysis of the nucleotide sequence, and the amino acid sequence of the protein predicted by the nucleotide sequence, of the putative gp53 gene BVDV NADL strain revealed features important in assessment of the function and structure of this protein, and served to identify the likely gene boundaries encoding this protein in strain 2724. Analysis of the sequence of BVDV strain 2724

Figure 3.8. Hydrophobicity comparisons of the protein encoded by BVDV strain 2724 cDNA to the major envelope protein of pestiviruses and NS1 protein of flaviviruses. Numbered hash marks along the top and bottom margins indicate amino acid position within the predicted amino acid sequence. Solid lines represent the hydropathy measure calculated according to the methods of Kyte and Doolittle (see description in figure 3.7). Bold hashed lines represent the curve for nonpolar transbilayer helices calculated according to the methods of Goldman, Engelman, and Steiz (GES). Curves in the upper half denote a hydrophobic region, while curves in the lower half denote a hydrophilic region. The curve is the hydrophobicity average of a residue based on the GES scale over a window of 20 residues. (A) Hydrophobicity plots of the putative major envelope protein of BVDV strain 2724, BVDV strain NADL (Collett et al., 1988a), BVDV strain Osloss (Renard et al., 1987), HCV strain Alfort (Meyers et al., 1989), and HCV strain Brescia (Moorman et al., 1990a). (B) Hydrophobicity plots of NS1 protein of dengue-2 virus (Hahn et al., 1988), yellow fever virus (Rice et al., 1985), and hepatitis C virus (Takamizawa et al., 1991).

A



B

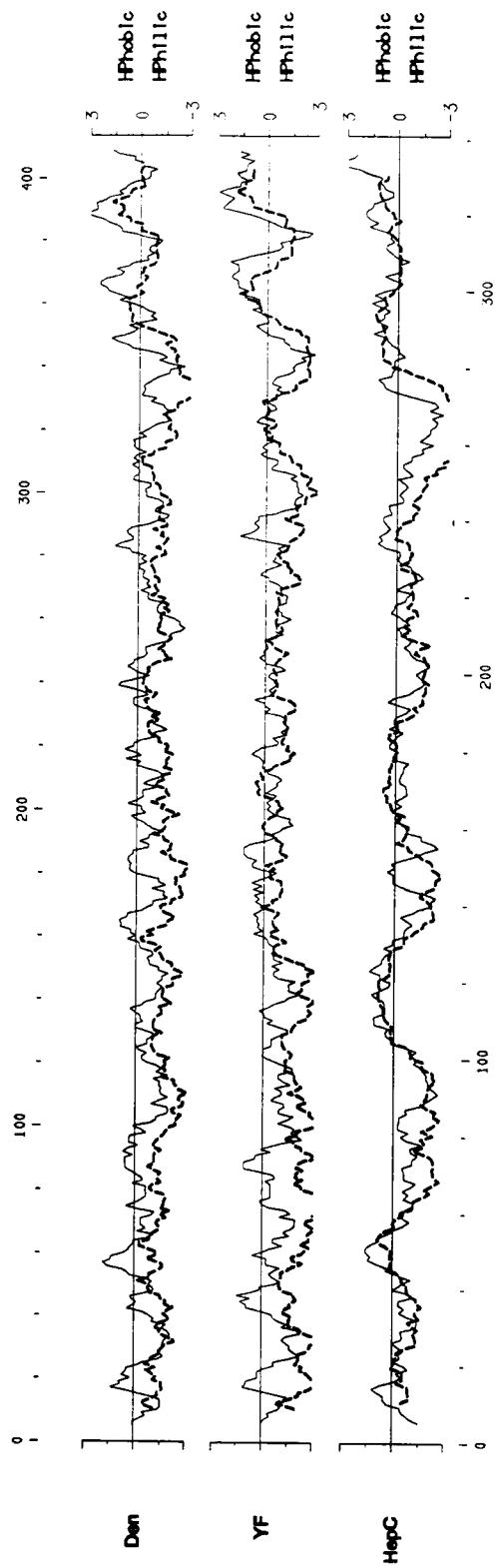
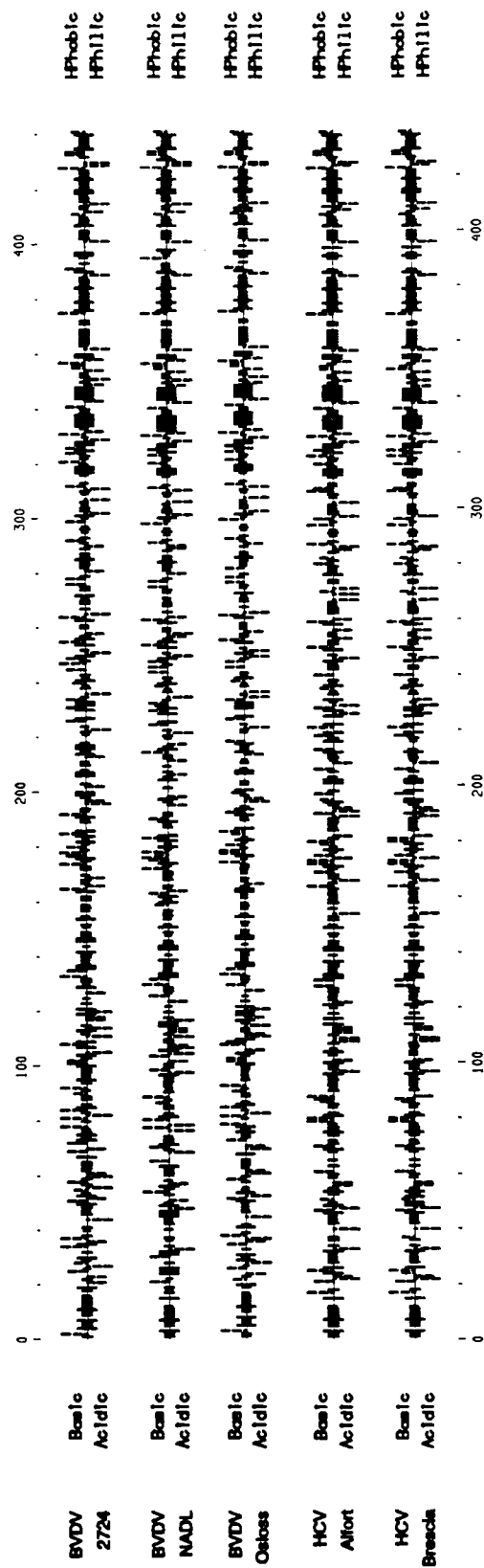
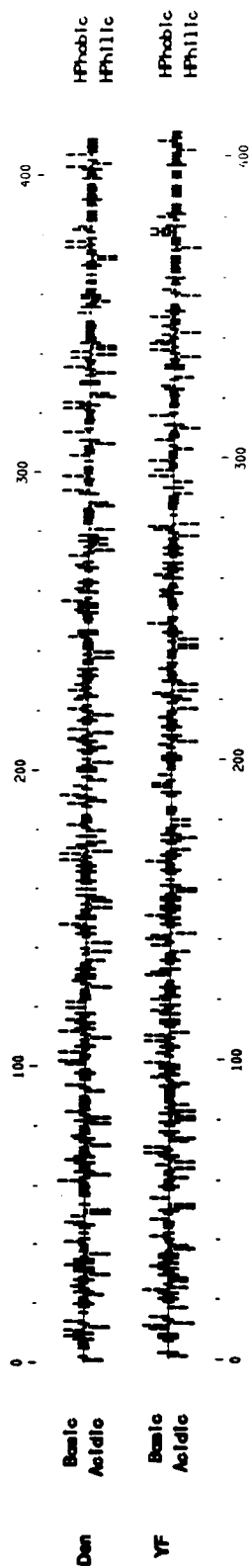


Figure 3.9. Comparison of acidic and basic amino acid residue distribution within the protein encoded by BVDV strain 2724 cDNA to the putative major envelope protein of pestiviruses and NS1 of flaviviruses (see description of calculations in figure 3.7). (A) Basic/acidic residues of putative major envelope protein of BVDV strain 2724, BVDV strain NADL (Collett et al., 1988a), BVDV strain Osloss (Renard et al., 1987), HCV strain Alfort (Meyers et al., 1989), and HCV strain Brescia (Moorman et al., 1990a). (B) Basic/acidic residues of putative NS1 protein of dengue-2 virus (Hahn et al., 1988), and yellow fever virus (Rice et al., 1985).

A



B



gp53 cDNA from this region indicated properties similar to those of the NADL strain. At the amino terminus of the predicted region encoding gp53 of the NADL and 2724 BVDV strains, a stretch of hydrophobic amino acid residues occurred which may serve as the signal sequence. The amino acid residues immediately preceding the hydrophobic core of the putative signal sequence in BVDV NADL gp53 were polar, and a basic residue, arginine, was present at amino acid position 674 of the polyprotein encoded by the NADL strain viral genome. This conforms to the predictions of Perlman et al. (1983) for amino acid positions within signal peptides. These authors proposed that the pre-core region of signal sequences, which is 1-3 residues long, is positively charged due to the presence of one or more basic residues. The positive charge in this region aids in binding and orientating the signal sequence to the negatively-charged endoplasmic reticulum (ER) membrane surface thereby facilitating insertion of the signal peptide into the membrane (Perlman et al., 1983; Coleman et al., 1985). Additionally, the arginine residue is followed by a glycine residue which may serve to disrupt secondary structure in the signal peptide and thereby allow the preceding charged residues to interact with the ER membrane surface (Perlman et al., 1983). We could not determine whether this positively charged pre-core region occurs in BVDV 2724, as this area was included within primer 2 used for cDNA amplification. The hydrophobic core of both BVDV strain NADL and strain 2724 gp53 started at its amino terminus with methionine (corresponding to amino acid position 677 of BVDV strain NADL polyprotein, and encoded by the fifth codon of BVDV strain 2724 cDNA). Eight of the 16 residues in this region consisted of leucine, isoleucine, and valine, which are the amino acids found most frequently in the hydrophobic core region of leader peptides (Perlman et al., 1983). This core region may actually terminate at glycine 689 of NADL and glycine at position 17 of our cDNA-encoded protein, as this residue is known to disrupt protein secondary structure (Perlman et al., 1983). The residues following these glycines then would function as the post-core region described by Perlman et al. (1983).

Structural proteins of flaviviruses may be processed by cellular signalases (Moorman et al., 1990b). Moorman et al. predicted that the amino-terminal cleavage site of the major envelope protein of hog cholera virus strain Brescia occurred at amino acid residue 689 of the polyprotein based on analysis by the method of von Heinje for predicting signalase cleavage sites (1986). This

corresponds to glycine at amino acid residue 692 of BVDV NADL virus strain polyprotein and glycine at amino acid residue 20 encoded by the BVDV strain 2724 cDNA. The residues preceding these glycine residues in all three of these pestiviruses fulfill the "(-3,-1)-rule" of von Heinje which states that position -1 (relative to the cleavage site) must be either alanine, serine, glycine, cysteine, threonine, or glutamine; position -3 must not be aromatic, charged, or large and polar; and proline can not occur in positions -3 to +1. The residues valine at position -3 and glycine at position -1 in BVDV NADL and 2724 gp53 at this site form an acceptable recognition sequence for signalase cleavage (Perlman et al., 1983). Additionally, the residues at positions +1, +3, and +5 relative to the putative cleavage site of both BVDV NADL and 2724 gp53 are charged, since histidine, aspartic acid, and lysine occur in NADL virus strain gp53 and aspartic acid, aspartic acid, and lysine in 2724 virus strain cDNA respectively. This is in agreement with von Heinje's prediction that the region from +1 to +5 is rich in charged and large polar residues (1984). Further evidence that this region of hydrophobicity near the amino-terminus of the putative gp53 may serve as a signal sequence is offered by the predicted beta secondary structure of this region for both BVDV NADL and 2724. Beta structure may be a characteristic of signal sequences according to Perlman et al. (1983). These authors argued this conformation of the leader peptide would allow for interactions between the leader peptide and signal recognition particle receptor proteins in the ER membrane.

Various pestivirus investigators postulated that the signal sequence for all the glycoproteins occurs at the amino-terminus of the first glycoprotein encoded by the genome, gp48 of BVDV and gp44/48 of hog cholera (Wiskerchen et al., 1991; Rumenapf et al., 1991). However, each flavivirus glycoprotein may possess its own leader peptide, and upstream sequences also may be involved in targeting the protein to the ER (Falgout et al., 1989). The region of hydrophobicity which occurs near the amino-terminus of the protein encoded by the 2724 virus strain cDNA (as was predicted from analysis of the nucleotide and amino acid sequence of the NADL strain of BVDV) was included in our construct to determine if this domain would function as a signal sequence for in vitro protein expression and processing.

One reason for defining the exact 5' gene boundary of gp53 cDNA used in this study was to include by appropriate design of primer 2 of both primer sets, for cDNA synthesis and amplification, an in-frame methionine-encoding codon within

this region. The purpose for ensuring the presence of methionine codon in frame at the 5' end of the positive strand of the cDNA was to provide an initiation codon for *in vitro* expression.

The primer used for first strand cDNA synthesis defined the 3' gene boundary for gp53 cDNA used in this study, and its design was based on several factors present in the genome of BVDV strain NADL. An expanse of hydrophobic amino acid residues found in the carboxy terminal region of BVDV NADL gp53 (amino acid residues 1033-1059) was identified. This stretch contained 27 amino acid residues, and at least 20 amino acid residues may be needed for optimal membrane spanning function (Perlman et al., 1983). Thus, this region may be the transmembrane domain of gp53 and the 3' primer was designed to include it in our construct. Downstream of this region, two smaller stretches of hydrophobic residues were present. Whether these regions serve as multiple membrane-spanning domains remains to be determined. All of these hydrophobic regions were found to be conserved in BVDV strain 2724.

Additional data used in primer design was based on the phenomenon that some flavivirus proteins are produced from the polyprotein, encoded by the genome, by cleavage after double basic amino acid residues (Westaway, 1987). Therefore, lysine residues at amino acid positions 1105-6 of BVDV strain NADL polyprotein were included in our construct and our data confirmed that these residues were conserved in BVDV strain 2724 at amino acid positions 433-4 encoded by the cDNA. Cleavage after single basic amino acid residues in flavivirus proteins also has been documented (Biedrzycka et al., 1987). Thus, Collett's hypothesis that the cleavage site of gp53 at the carboxy terminus of the protein follows the lysine at amino acid position 1063 of the polyprotein is feasible (1988b). However, by including nucleotides encoding the dibasic residues at amino acid positions 433-4 in our construct, we hoped to ensure cloning of at least the entire gp53 protein gene even though its exact boundary limit at the 3' end of the gene is unknown.

Nucleotide base sequence data of the BVDV strain 2724 cDNA revealed also other features of the protein encoded by this genomic region. The relatively high cysteine content encoded by our cDNA implies significant tertiary and quaternary structure of the protein product formed by intra- and intermolecular disulfide bonds. Previous work on the major envelope protein gp55 of hog cholera virus identified oligomers formed with the minor glycoprotein gp33, as well as some

formed with itself (Weiland et al., 1990). Disulfide-linked dimers between gp53 and gp25 of BVDV formed *in vitro* were described also in the latter study. A study by Rumenapf et al. supported these findings (1991). These researchers determined that the major envelope protein of both BVDV and hog cholera virus form homodimers, as well as heterodimers *in vivo* with the minor glycoprotein gp25 or gp33 respectively. Cysteine residues in the gp53-encoding region are highly conserved among the pestiviruses. The latter is also true for the noncytopathic BVDV strain 2724. This phenomenon suggests that these residues may be important in protein structure and/or function.

The sites of potential N-linked glycosylation may be conserved among the pestiviruses. These sites occur within the central portion of the putative gp53 coinciding with areas predicted by computer analysis to have significant antigenicity. Differences exist between BVDV and hog cholera virus in number and location of glycosylation sites. The extent of glycosylation and the role of these oligosaccharide chains in immunogenicity of the putative envelope protein, and the function of these carbohydrates in the viral replication cycle remain to be elucidated.

The high degree of amino acid homology among the pestiviruses is reflected in the hydrophobicity profiles of and acid/base distributions in the major envelope proteins of these viruses. The analysis patterns of these properties bear a striking similarity to one another, demonstrating the relatedness of these viruses. The comparisons of the gp53 gene and its product of BVDV strain 2724 to the NS1 gene and its product of flaviviruses and hepatitis C virus do not reveal the same degree of relatedness. The NS1 protein, while present in infected cells in significant concentrations, likely does not occur in mature virions whereas the gp 53/55 of pestiviruses is present in virions. However, comparisons based on hydrophobicity analyses indicate that BVDV gp53 may be analogous to flavivirus NS1 protein (Collett et al., 1988c). The nucleotide sequence and amino acid sequence of the gp53 gene and product and NS1 gene and product respectively have minimal homology to one another. Hydrophobicity profiles of gp53 of pestiviruses and NS1 of flaviviruses have certain similarities since small areas of relative hydrophobicity occur at the amino-termini, and larger regions lie at the carboxy-termini of these proteins. Additionally, both gp53 of BVDV strain 2724 and flavivirus NS1 protein are relatively hydrophilic. However, these similarities cannot necessarily be interpreted as viral relatedness, only, perhaps, as a similarity of structure and

function in that both are membrane proteins.

Bovine viral diarrhea is an important disease of cattle resulting in significant economic losses to the cattle industry (Moennig, 1990). An effective safe vaccine is critical to controlling this disease. A recombinant vaccine or synthetic peptide vaccine may prove to be the most viable option, offering safety, effectiveness, and affordability (Harkness, 1987). The putative envelope protein gp53 of BVDV is very immunogenic (Donis et al., 1988). Additionally, it is well documented that neutralizing antibodies are produced against this glycoprotein (Donis et al., 1988; Magar, 1988; Bolin, 1988b; Bolin et al., 1990a). In one study, gp53 was the only viral-induced polypeptide consistently precipitated with antisera from vaccinated cattle (Bolin et al., 1990a). This viral protein is the likely candidate for exploitation in a genetically engineered vaccine. The use of a recombinant virus such as Infectious Bovine Rhinotracheitis Virus (IBRV) expressing the envelope protein of BVDV may offer safe, effective protection to both viruses. Additionally, cloning and expression of this protein from a noncytopathic strain of BVDV rather than a cytopathic strain for use in immunization may be preferred. The etiology of biotypic variation between viral strains of BVDV is unknown. However, in the event that the major envelope protein plays a role in this phenotypic difference, expression of the protein from a cytopathic virus strain in a recombinant vaccine may result in cell damage and cytolysis in the recipient animal. Expression of this protein from a noncytopathic virus template would ensure that this deleterious effect would not occur. Additionally, as the noncytopathic strains of BVDV are more commonly associated with disease in cattle, it would be advantageous to use a strain of this biotype in immunoprophylaxis.

The approach to immunoprophylaxis of using the major envelope protein in a genetically engineered vaccine has been explored with flaviviruses, and with hog cholera virus. Rabbits immunized with purified envelope protein of yellow fever virus produced neutralizing antibodies, and mice immunized with the same protein were protected against intracerebral challenge inoculation with the virus (Brandriss et al., 1990). Swine vaccinated with a recombinant vaccine expressing the complete major envelope protein of hog cholera virus were fully protected against disease (Zijl et al., 1991). These authors concluded that this glycoprotein is sufficient to induce protective immunity against hog cholera virus. Another study utilized the entire structural protein-encoding region of the HCV genome in a recombinant to

generate an immune response (Rumenapf et al., 1991). Again, protective immunity against a lethal dose of the virus was induced. A recombinant lacking the major envelope protein provided only partial protection. Neutralizing antibodies were not elicited in vaccinated swine with the latter. The same approach with BVDV may be useful. Several properties of this protein must first be investigated before utilization of it in a genetically engineered vaccine will be feasible. The extent of variability of this protein among different BVDV strains, and the degree of conservation of epitopes important for the induction of protective immunity must be determined. Additionally, the effect of immunization of persistently BVDV-infected animals with a recombinant vaccine must be assessed. The cloning and subsequent analysis of the envelope protein-encoding gene reported here are important tools for achieving this goal. Expression of the cloned gene and investigation into its structure are the logical next steps.

CHAPTER 4

CELL-FREE TRANSLATION, TRANSLOCATION, PROCESSING, AND ANCHORAGE OF THE PUTATIVE MAJOR ENVELOPE PROTEIN OF A NONCYTOPATHIC STRAIN OF BOVINE VIRAL DIARRHEA VIRUS

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a significant pathogen of cattle worldwide (Radostits et al., 1988). This agent is the source of tremendous economic losses to the cattle industry primarily because of its capacity to induce reproductive and immune system dysfunction (Baker, 1987; Potgieter, 1988; Radostits et al., 1988). Consequences of BVDV infection in cattle are varied and include fetal death, congenital defects, neonatal death, respiratory disease, opportunistic infections, and mucosal disease (Harkness, 1987).

This virus has remained an enigma primarily because of the difficulty in growing and manipulating the virus in the laboratory (Renard et al. 1985). Recent research advances have provided information about the molecular virology of this pathogen. The organization of the viral genome, and the identity and function of the proteins it encodes, are under investigation. We have focused on characterization of the major envelope protein because this viral protein is immunogenic, and induces neutralizing antibodies (Donis et al., 1988; Magar et al., 1988; Bolin et al., 1988a; Bolin et al., 1988b; Bolin et al., 1990a). Furthermore, it is the only viral-specific protein consistently precipitated with antisera from vaccinated animals (Bolin et al., 1990a). This protein is a prime target for use in a recombinant vaccine.

In a previous report, we described the amplification, cloning, and nucleotide base sequencing of the putative gene encoding this protein in a noncytopathic BVDV strain (Kennedy, unpublished results). In this report, we have analyzed the *in vitro* expression of this gene. The gene encoding the major envelope protein was transcribed and translated in a cell-free translation system. Analyses of the gene product have revealed several properties of the putative major envelope protein, including the identification of a putative membrane-spanning region. Previous studies suggested that the major envelope protein of the pestivirus of hog cholera has the capacity to induce protective immunity against this disease in swine (Zijl et

al., 1991; Rumenapf et al., 1991). Ultimately, we hope to define the immunogenicity of the major envelope protein of BVDV strain 2724 and utilize it for immunization of cattle against this serious virus infection of cattle. This strain of BVDV is noncytopathic in biotype. Vaccines currently used are of dubious efficacy, and can result in many of the sequelae that occur following infection with the virus (Rosner et al., 1968; Bittle et al., 1973; Lambert, 1973; Orban et al., 1983; Stober, 1984; Neaton, 1986; Baker, 1987; Radostits et al., 1988; Ames et al., 1991). Therefore, it is desirable that the vaccine be not only effective, but that it produce no deleterious effects. Noncytopathic strains of BVDV are the most common BVD viruses in nature, and are more commonly associated with disease than are cytopathic strains, and thus are a logical choice for immunoprophylaxis (Potgieter et al., 1987). Also, since the viral property responsible for cytolysis following infection by cytopathic strains is not known, in order to ensure that cytopathic effects do not occur following vaccination, a major envelope protein from a noncytopathic strain may be the ideal template for expression of this protein in a genetically engineered vaccine.

RESULTS

In vitro transcription and translation of gp53

The putative gene encoding the major envelope protein of BVDV strain 2724 was cloned into pGem3zf(+) as previously described (Kennedy, unpublished results). This recombinant plasmid was used as the template for *in vitro* transcription and translation in a nuclease-treated rabbit reticulocyte lysate. Glycosylation was done by including canine pancreatic microsomal membranes to the translation reaction.

Nucleotide base sequencing of the cDNA revealed that the positive strand of the putative gp53 gene was downstream from the T7 promoter of the pGem vector and, thus, establishing that the T7 polymerase was required for transcription in order to produce the appropriate mRNA. The recombinant plasmid was linearized with Hind III restriction endonuclease and run-off transcripts were produced. The resultant RNA was used for translation because preliminary experiments indicated

that, unlike eukaryotic mRNA, a 5' cap was not necessary for efficient translation. Transcription products were assessed by agarose gel electrophoresis (figure 4.1).

The potential for translation initiation at the first ATG codon of the viral cDNA sequence was explored (Kozak, 1989). The context in which this methionine codon exists in BVDV RNA does not conform fully with the consensus sequence of the scanning model for translation initiation suggested by Kozak, in that nucleotide position -3 relative to the ATG is not a purine, but a pyrimidine, cytosine. However, nucleotide at position +4 is a guanosine which is essential for efficient translation in the absence of a purine at -3 (Kozak, 1989). Additional methionine codons occur downstream of this codon which fulfill Kozak's rules better than the first methionine codon of the cDNA. The first three of these methionine codons occur 53, 59, and 111 codons downstream of the first methionine codon. The methionine codons at positions 59 and 111 possessed the preferred nucleotides at both position -3 and +4. It was not known which of these initiation codons would be functional in our translation system.

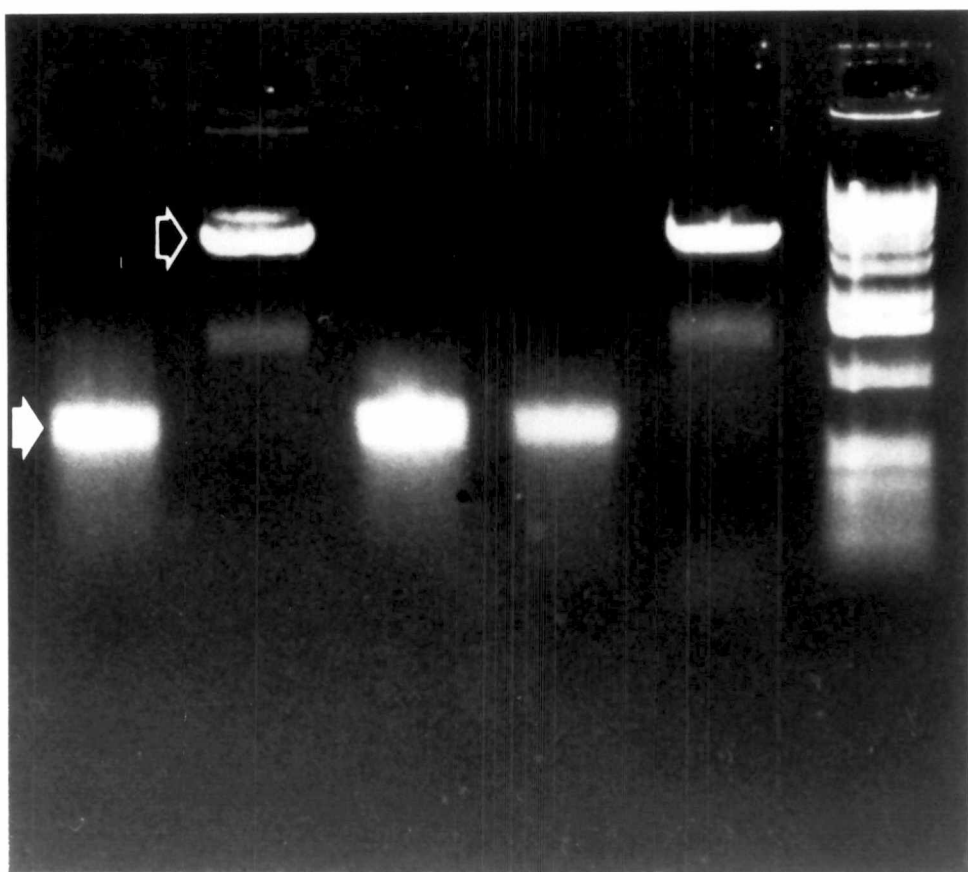
Translation reactions were carried out in a rabbit reticulocyte lysate according to the manufacturer's recommendations. The proteins produced were radiolabelled with ³⁵S-methionine to allow analysis of products by autoradiography following gel electrophoresis. Initial analyses were done on the nonglycosylated and glycosylated proteins generated from the complete transcript.

Figure 4.2 gives the translation products of the full-length transcript. Lanes 1 and 2 contain the translation reaction, while lanes 3 and 4 contain the glycosylation reaction products.

The primary product observed in the translation reaction is a protein of molecular weight 42-44kD. Two minor protein species with a molecular weight of 36-38kD and 32-34kD, indicated by the asterisks in figure 4.2, were also evident. Following glycosylation, a protein doublet of molecular weight 50-54kD was synthesized in addition to the products of simple translation. This doublet may have been the only glycosylated product made, because additional products were not noted. Also, only the major protein product may have been glycosylated because the concentration of the major nonglycosylated product decreased following cotranslational processing, while that of the other products remain unchanged. Several faint bands of products of higher molecular weight than the primary product were apparent in both translation and glycosylation reactions. The identity of these

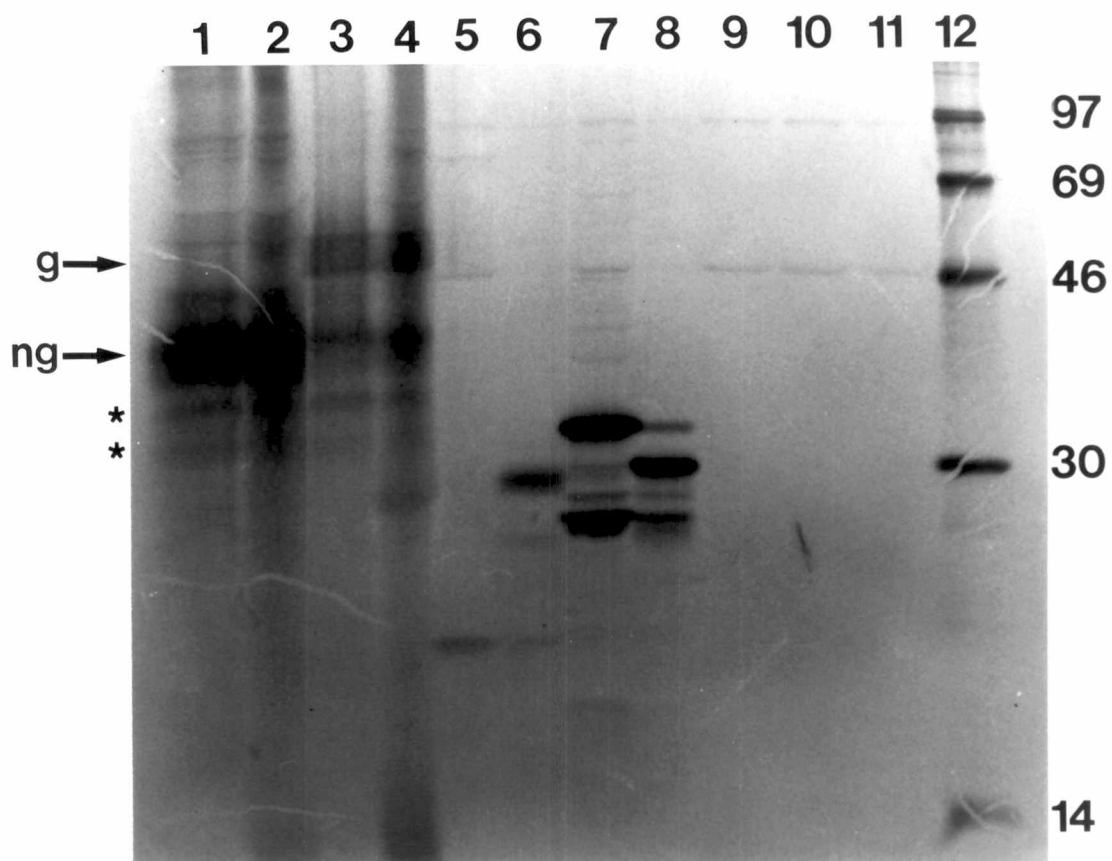
Figure 4.1. RNA product from transcription of linearized recombinant pMK14. Numbers on right indicate size of DNA standards in kilobases (lane 6). Lanes 1, 3, and 4, RNA transcript (solid arrow) following DNase treatment to remove the DNA template. Lanes 2 and 5, Hind III restriction endonuclease-linearized recombinant pMK14 (open arrow) which served as the template for transcription.

1 2 3 4 5 6



4.0
3.0
1.6
1.0
.5

Figure 4.2. SDS-PAGE analysis of products of recombinant pMK14 mRNA translation and glycosylation. Numbers on right denote molecular weight of protein size standards in kilodaltons (lane 12). Lanes 1 and 2, nonglycosylated products from recombinant pMK14 mRNA translation. Lanes 3 and 4, glycosylated products from recombinant pMK14 mRNA translation. Lanes 5 and 6, positive control for glycosylation using yeast α -factor mRNA, nonglycosylated and glycosylated products, respectively. Lanes 7 and 8, positive control for post-translational processing using pre- β -lactamase mRNA, unprocessed and processed products, respectively. Lanes 9 and 10, translation products of linearized and nonlinearized pGem3zf(+) DNA. Lane 11, products of translation without RNA. G denotes glycosylated recombinant pMK14 mRNA translation product; ng denotes nonglycosylated recombinant pMK14 mRNA translation product; asterisks denote minor protein translation products of pMK14 (see text for full description).



products was not known, and their presence was inconsistent. Additionally, two faint bands of products were evident in the positive and negative control reactions with molecular weight of 46kD and 100kD. Their identity, too, was not known. All of these high molecular weight products may have been the result of translation of residual reticulocyte mRNA or the labeling of preexisting protein present in the reticulocyte mixture (Jackson et al., 1983; Promega, personal communication).

The molecular weight of the nonglycosylated protein product of the full-length gp53 gene transcript was 42-44kD, but the predicted molecular weight of this protein was 49kD. The predicted length of the protein was 437 amino acids if initiation occurred at the first methionine codon. The predicted molecular weights of proteins initiated at the internal methionine codons and terminated at the last amino acid residue codon, histidine 437, are given in table 4.1. Translation initiation at amino acid residue 53 or 59 could result in a protein of 43-44kD which could correspond to the major protein product obtained in our study.

The molecular weights of proteins initiated at each of the first four methionine residue codons of the cDNA and terminated at the lysine residue predicted to be the carboxy terminus of gp53 by Collett et al. (1988b), codon 387 of the cDNA, are given also in table 4.1. Note that the predicted size of the nonglycosylated protein initiated at the first methionine of the construct and terminated at this lysine residue is 43kD. This would suggest that autocatalytic cleavage and removal by the viral cDNA-encoded protein of the carboxy-terminal 50 amino acid residues occurs, because cellular enzymes equivalent to signalases are not present in the rabbit reticulocyte translation system. Proteins initiated at any of the internal methionine codons and terminated at lysine residue at amino acid 387 encoded by the cDNA would result in polypeptides of the appropriate molecular weight for the smaller-sized product (approximately 38kD molecular weight), but not the major translation product of 42-44kD. Also given in table 4.1 are the molecular weights of the proteins whose synthesis may have initiated at the various methionine codons and terminated at the dibasic residues lysine-lysine codons at codon positions 429-430 of the cDNA. It is known that some flavivirus proteins are cleaved at dibasic residues (Rice et al., 1985; Rice et al., 1986a; Biedrzycka et al., 1987; Westaway, 1987; Speight et al., 1988; Chambers et al., 1989; Nowak et al., 1989; Wengler et al., 1991). However, the size of the nonglycosylated translation product, if it is cleaved at this residue and if initiation occurs at the first

Table 4.1 Predicted molecular weights in kilodaltons of *in vitro* translation products differing in initiation and termination sites.

Terminus ^a		Met 1 ^b	Met 53 ^b	Met 59 ^b	Met 111 ^b
His	437	49.2	44.5	43.9	36.3
Lys	430	48.2	43.5	42.9	35.3
Lys	387	43.4	38.7	38.1	30.5
Lys	322	35.4	30.7	30.1	22.5

^aProposed carboxy termini of *in vitro* translation products.

^bProposed initiation codons of *in vitro* translation products.

methionine, would be greater than that observed in our studies. However, if translation initiation occurred at methionine codon 53 or 59, and terminated at these dibasic residues, the size would be closer to that which we observed for the major translation product. This, too, may require an autocatalytic reaction by the cDNA-encoded protein for cleavage at these residues to occur, because cellular enzymes are not present in the translation system used. Finally, in table 4.1, the predicted sizes of proteins resulting from translation of a transcription template that was truncated by restriction endonuclease cleavage at the unique restriction site Ssp I, and whose synthesis was initiated at the various methionine codons are given.

The molecular weight of the major nonglycosylated protein product was approximately 8-12kD less than that of the glycosylated product. Assuming 2-3kD of carbohydrate are added per attachment site, this suggested the existence of 3-4 glycosylation sites. The synthesis of a doublet of glycosylated product probably reflects a variation in the degree of glycosylation.

Nonglycosylated and glycosylated protein products of *in vitro* translation were electrophoresed with and without 2-mercaptoethanol in order to assess disulfide-linked homodimer formation (figure 4.3). Lanes 6 and 7 contain the translation products of the RNA transcribed from BVDV strain 2724 cDNA electrophoresed with and without 2-mercaptoethanol respectively. Lanes 8 and 9 contain the translation and glycosylation products of this RNA electrophoresed with and without 2-mercaptoethanol respectively. Oligomers of the BVDV major envelope protein were not detected, as electrophoretic migration of proteins with and without 2-mercaptoethanol were similar.

Immunoprecipitation of the *in vitro* expression product

Immunoprecipitation of the translation reaction products was done to confirm that the latter were BVDV-specific. The antiserum used was generated to the carboxy terminal half of BVDV strain NADL gp53 fused with β -galactosidase. Translation reactions were carried out as before. A portion of each was used to assess the efficiency of translation by electrophoresis (figure 4.4, lanes 1 and 2). The remaining portion (50 μ l) was used in the immunoprecipitation reaction. Immunoprecipitation reactions were done according to the methods of Anderson (1983). The nonionic detergent Triton X-100 was used to solubilize the lysate

Figure 4.3. SDS-PAGE analysis of products of recombinant pMK14 mRNA translation and glycosylation electrophoresed with and without 2-mercaptoethanol. Numbers on right denote molecular weight of size standards in kilodaltons (lane 10). Lane 1, positive control for translation using BMV mRNA. Lanes 2 and 3, positive control for glycosylation using yeast α -factor mRNA, nonglycosylated and glycosylated products, respectively. Lanes 4 and 5, positive control for post-translational processing using *E. coli* pre- β -lactamase mRNA, unprocessed and processed products, respectively. Lane 6, nonglycosylated products of recombinant pMK14 mRNA translation electrophoresed with 5% 2-mercaptoethanol. Lane 7, nonglycosylated products of recombinant pMK14 mRNA translation electrophoresed without 2-mercaptoethanol. Lane 8, glycosylated products of recombinant pMK14 mRNA translation electrophoresed with 5% 2-mercaptoethanol. Lane 9, glycosylated products of recombinant pMK14 mRNA translation electrophoresed without 2-mercaptoethanol. G denotes glycosylated product of recombinant pMK14 mRNA translation; ng denotes nonglycosylated product of recombinant pMK14 mRNA translation; asterisks denote minor protein translation products using recombinant pMK14 mRNA (see text for full description).

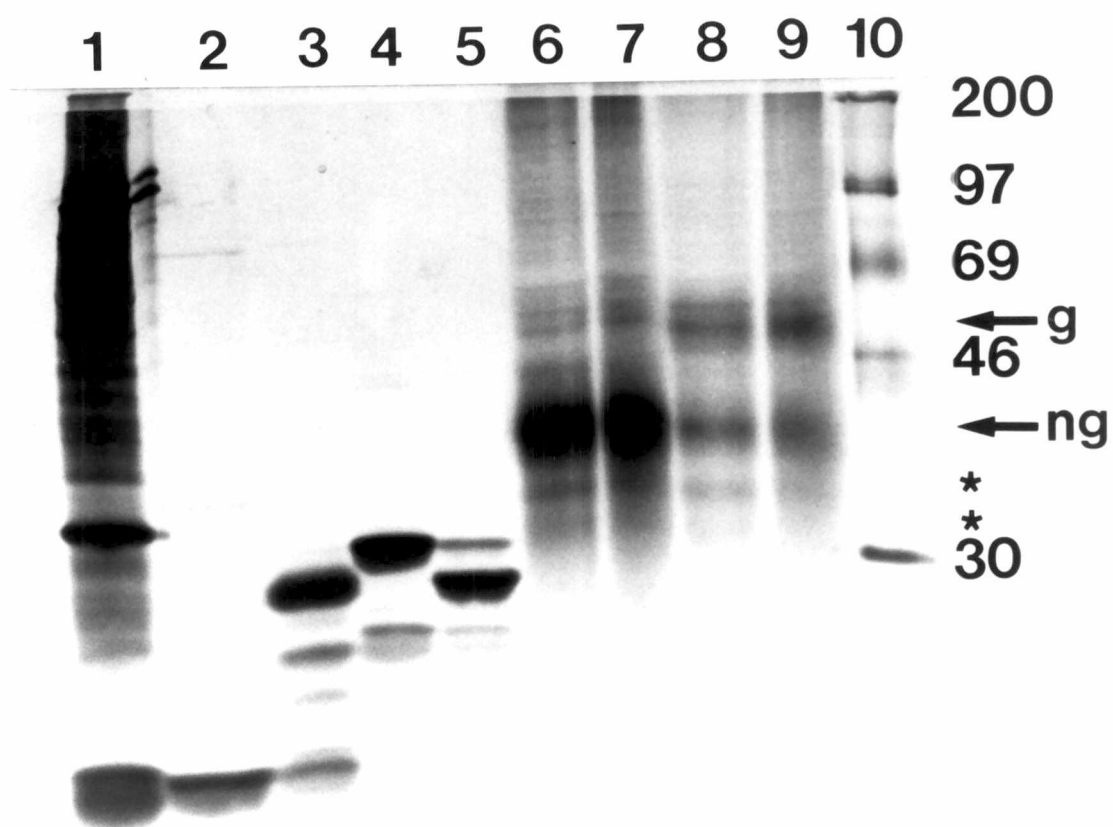
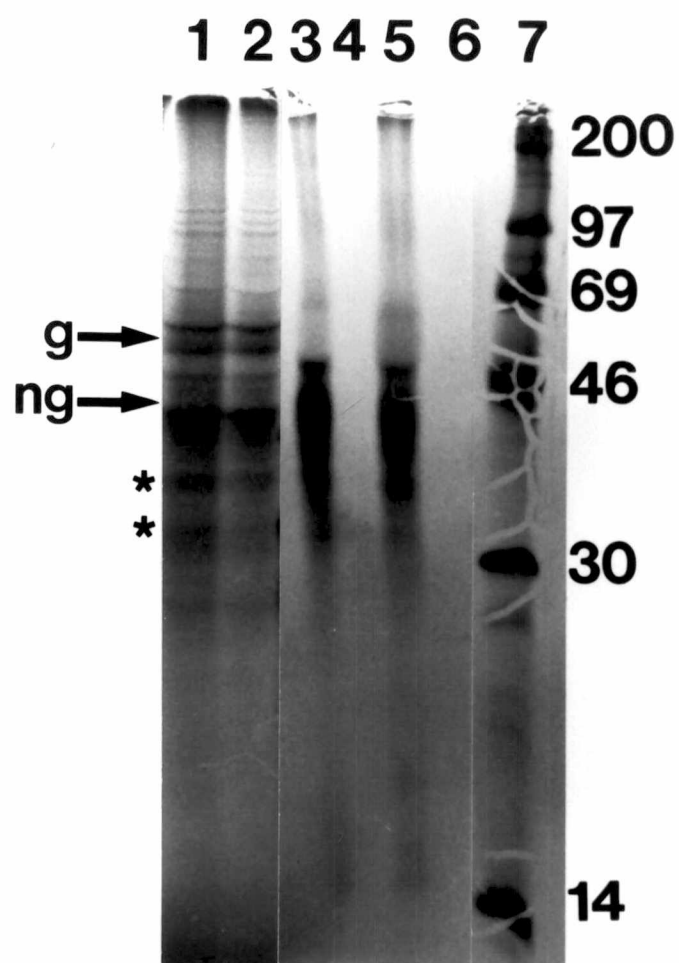


Figure 4.4. Immunoprecipitation assay of products of recombinant pMK14 mRNA translation and glycosylation. Numbers on right denote molecular weight of protein size standards in kilodaltons (lane 7). Lanes 1 and 2, nonglycosylated (ng) and glycosylated (g) products of recombinant pMK14 mRNA translation. Lane 3, immunoprecipitation of nonglycosylated recombinant pMK14 mRNA translation products only, using gp53-specific antiserum. Lane 4, immunoprecipitation of nonglycosylated recombinant pMK14 mRNA translation products only, using nonimmune antiserum. Lane 5, immunoprecipitation of glycosylated and nonglycosylated recombinant pMK14 mRNA translation products, using gp53-specific antiserum. Lane 6, immunoprecipitation of glycosylated and nonglycosylated recombinant pMK14 mRNA translation products, using nonimmune antiserum. Asterisks denote minor products of pMK14 mRNA translation which are immunoprecipitated with gp53-specific antiserum.



proteins at a final concentration of 4%. Following overnight incubation with the gp53 antiserum, the major translation product as well as the two minor proteins of lower molecular weight were precipitated (figure 4.4, lanes 3 and 5). The immunoprecipitation of the nonglycosylated protein was easily detected. However, the possible precipitation of the glycosylated protein was obscured by immunoglobulin heavy chain protein. This protein, even when present in small amounts, was sufficient to compress and conceal proteins of similar molecular weight. Nonimmune antisera did not precipitate any of the proteins (figure 4.4, lanes 4 and 6).

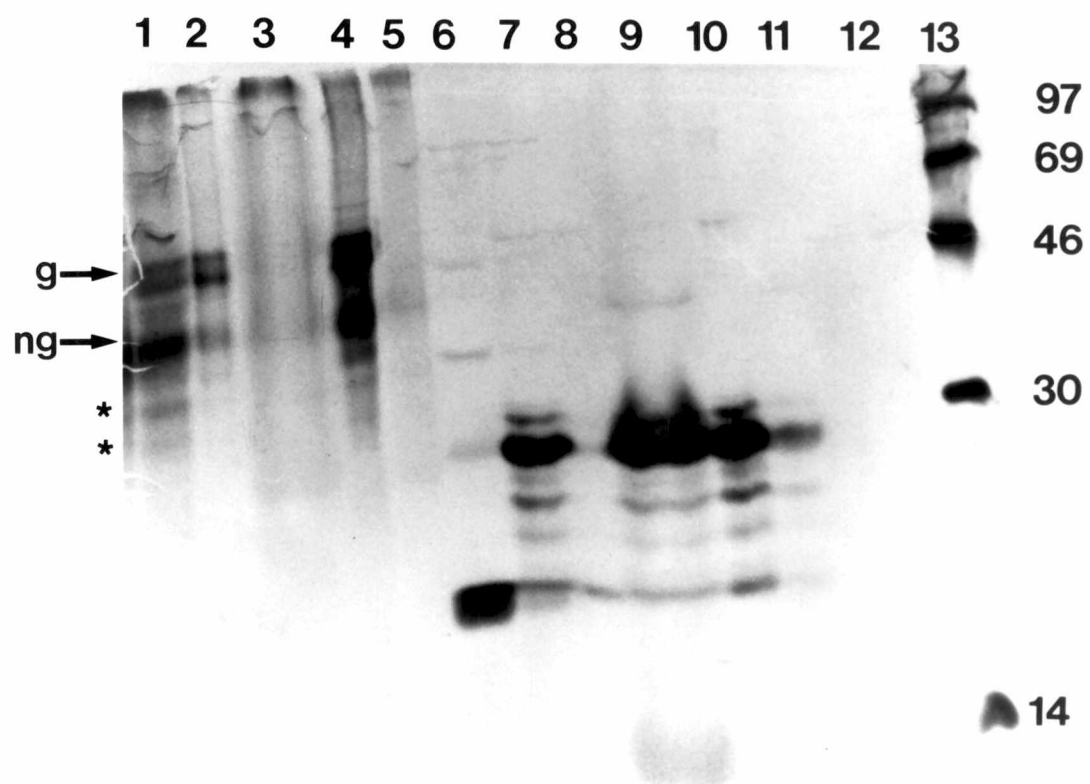
Analysis of gp53 membrane anchorage

The translation studies confirmed that the protein encoded by the cDNA was translocated and glycosylated. It would have been useful to know whether this protein is anchored in the microsomal membrane or secreted into the lumen, and, if anchored, what region serves as the transmembrane domain.

Characterization of gp53 membrane anchorage was done by the method of Fujiki et al. (1982). Closed microsomal membrane vesicles were converted to open sheets with an alkaline sodium carbonate solution. The membrane components of the reaction then were separated from soluble proteins and analyzed by gel electrophoresis. The fraction in which the labelled protein appears reveals whether the protein has been anchored or not.

The initial experiments were done on full-length cDNA of BVDV strain 2724 gp53 transcription template by linearizing the recombinant plasmid with Hind III restriction endonuclease. The results are given in figure 4.5. The untreated portion of the translation and glycosylation reaction products are in lane 1. After treatment with alkaline sodium carbonate, the glycosylated protein was present only in the membrane portion of the reaction mixture, indicating it had been anchored (figure 4.5, lane 2). No protein of equal size was present in the soluble portion of the reaction (figure 4.5, lane 3). The negative control reaction treated with a neutral buffer instead of the sodium carbonate solution, which does not open the microsomal vesicles, resulted in the translocated glycosylated protein product remaining with the membrane fraction (figure 4.45 lane 4) and not appearing in the

Figure 4.5. SDS-PAGE analysis following sodium carbonate treatment of products of Hind III restriction endonuclease-linearized recombinant pMK14 mRNA translation and glycosylation. Numbers on right denote molecular weight of protein size standards in kilodaltons (lane 13). Lane 1, glycosylated (g) and nonglycosylated (ng) translation products of Hind III-linearized recombinant pMK14 mRNA. Lane 2, membrane fraction of sodium carbonate-treated recombinant pMK14 glycosylated translation products. Lane 3, supernatant fraction of sodium carbonate-treated recombinant pMK14 glycosylated translation products. Lane 4, membrane fraction of neutral buffer-treated recombinant pMK14 glycosylated translation products. Lane 5, supernatant fraction of neutral buffer-treated recombinant pMK14 glycosylated translation products. Lanes 6 and 7, nonglycosylated and glycosylated translation products of α -factor mRNA respectively. Lane 8, membrane fraction of sodium carbonate treated glycosylated α -factor translation product. Lane 9, supernatant fraction of sodium carbonate-treated glycosylated α -factor translation product. Lane 10, membrane fraction of neutral buffer-treated glycosylated α -factor translation product. Lane 11, supernatant fraction of neutral buffer-treated glycosylated α -factor translation product. Lane 12, translation without RNA. Asterisks denote minor products of pMK14 mRNA translation.



soluble fraction (figure 4.5, lane 5). The experiment was done also on the α -factor glycosylated protein from the yeast *S. cerevisiae*. The products of translation and glycosylation of the α -factor mRNA are in lanes 6 and 7 of figure 4.4. As this protein is secreted, it was not observed in the membrane fraction (figure 4.5, lane 8) but was observed in the soluble portion of the sodium carbonate reaction mixture (figure 4.5, lane 9). In the negative control reaction with neutral buffer, it remained with the membrane portion (figure 4.5, lane 10), as the microsomal vesicles remained intact. However, small amount of glycosylated α -factor was evident in the soluble fraction of the reaction mixture, which was, perhaps, due to membrane disruption during the processing (described in Materials and Methods) of the reaction mixture. Lane 12 of figure 4.5 contains the translation mixture without RNA, and no product is evident.

The location of the transmembrane domain of the putative gp53 of BVDV strain 2724 was also investigated using the sodium carbonate procedure. The extended stretch of hydrophobic residues near the carboxy terminus of gp53 may serve as the membrane anchor. Our nucleotide base sequence analysis identified a unique restriction site, Ssp I, immediately upstream of this region. The recombinant plasmid was linearized with this enzyme, effectively truncating the template and resultant transcript. The protein synthesized from this transcript lacked this hydrophobic area, ending at amino acid residue 322 encoded by the cDNA (figure 4.6). The translation products produced from the truncated RNA transcript were first analyzed by gel electrophoresis (figure 4.7). The translation reaction mix contained three distinct products with molecular weights of 26kD, 32kD, and 36kD (figure 4.7, lanes 1 and 2). Following glycosylation, a wide band indicating a product with molecular weight of 44-46kD, which in some reactions also appeared as a doublet, was observed (figure 4.7, lanes 3 and 4). Only the concentration of the higher molecular weight product of the nonglycosylated fraction decreased following cotranslational processing, suggesting it was the only protein translocated and glycosylated.

Immunoprecipitation of the nonglycosylated translation products of the truncated template from BVDV strain 2724 cDNA indicated that all three proteins were BVDV-specific (figure 4.8, lane 2). Immunoprecipitation reactions with nonimmune serum of the nonglycosylated translation products from this template did not precipitate any proteins (figure 4.8, lane 3). The largest protein may be the full-length product of the Ssp I-digested template. The identity of the two lower

Figure 4.6. Hydrophobicity plot of the predicted protein encoded by the putative gp53 cDNA of BVDV strain 2724 (see figure 3.7 for full description). Arrow denotes position of Ssp I restriction endonuclease site in the transcription template relative to the encoded protein.

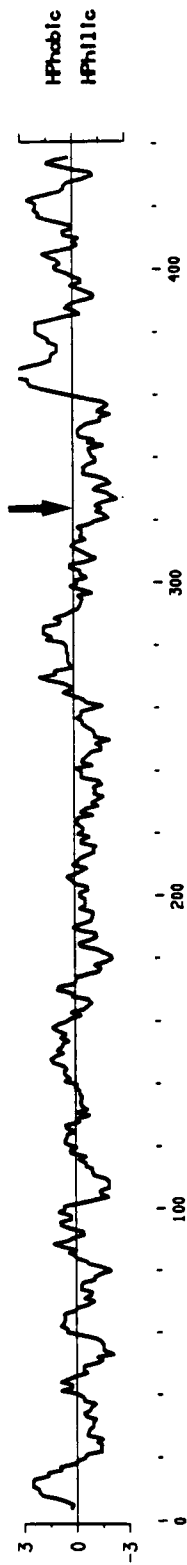


Figure 4.7. SDS-PAGE analysis of products of truncated recombinant pMK14 mRNA translation and glycosylation. Numbers on right denote molecular weight of protein size standards in kilodaltons (lane 8). Lanes 1 and 2, nonglycosylated translation products (ng) using mRNA from Ssp I-linearized transcription template. Lanes 3 and 4, glycosylated (g) and nonglycosylated translation products using mRNA from Ssp I-linearized transcription template. Lanes 5 and 6, nonglycosylated and glycosylated translation product of α -factor mRNA. Lane 7, translation without RNA. Asterisks denote additional nonglycosylated translation products of Ssp I-linearized pMK14 mRNA.

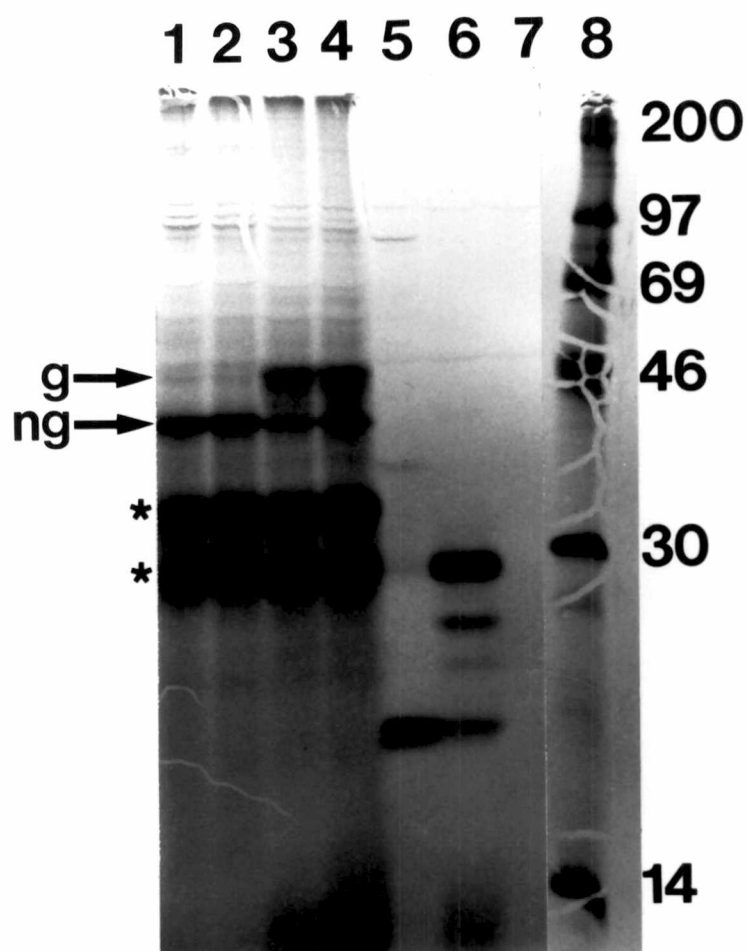
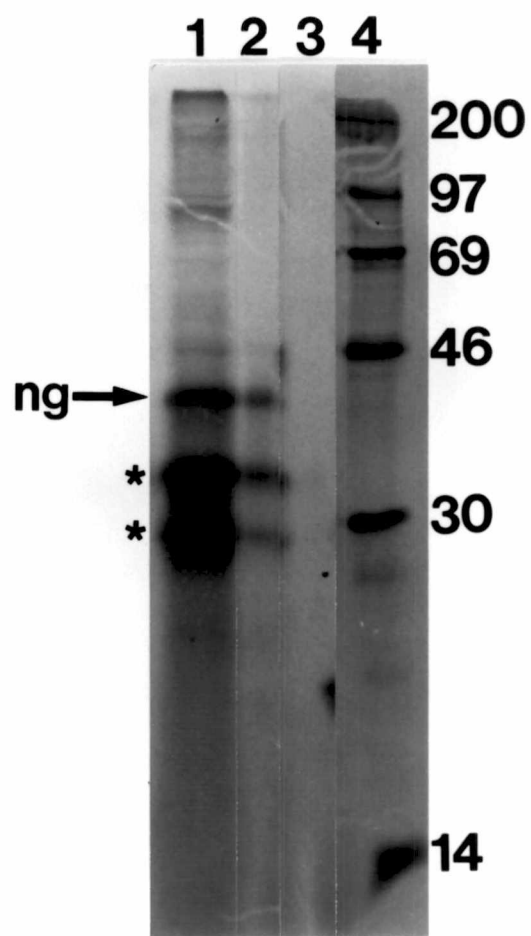


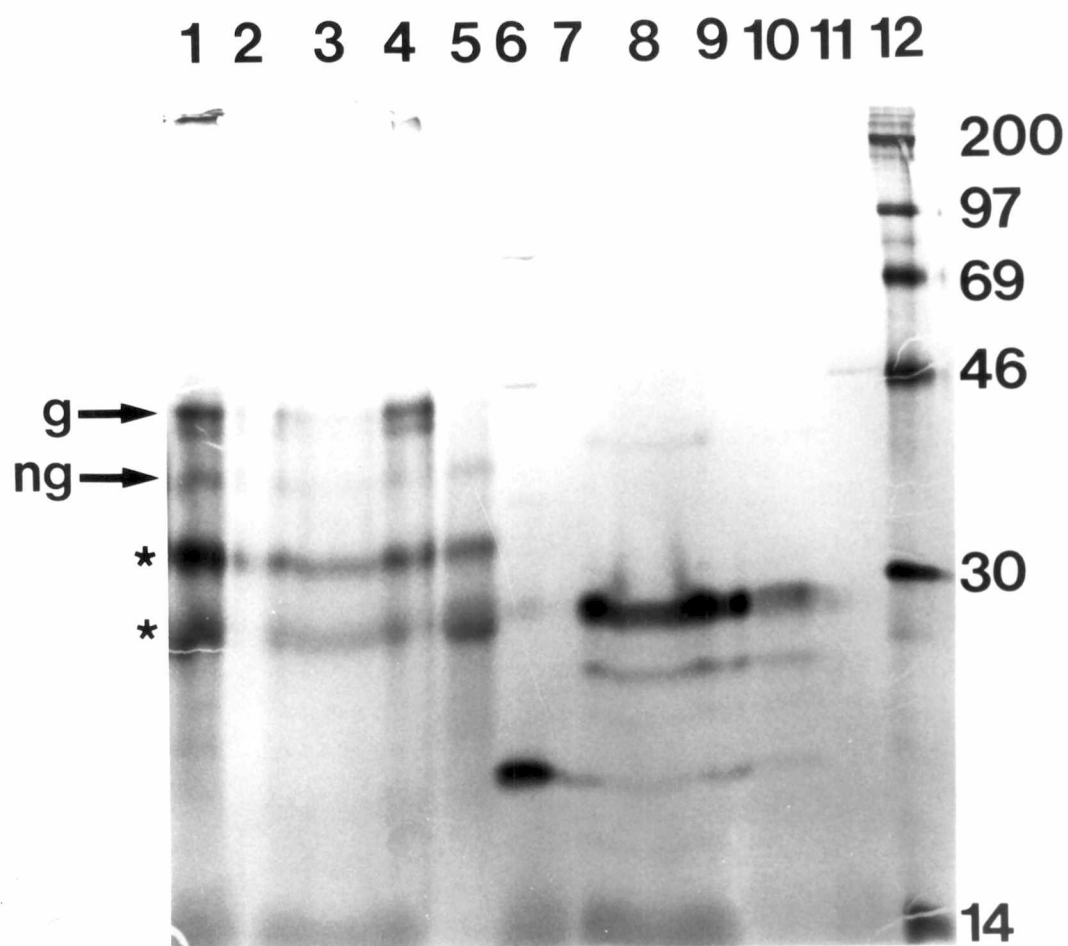
Figure 4.8. Immunoprecipitation assay of products of truncated recombinant pMK14 mRNA translation. Numbers on right denote molecular weight of protein size standards in kilodaltons (lane 4). Lane 1, nonglycosylated products of Ssp I-linearized recombinant pMK14 mRNA translation. Lane 2, Immunoprecipitation of nonglycosylated products of Ssp I-linearized recombinant pMK14 mRNA translation using gp53-specific antiserum. Lane 3, immunoprecipitation of nonglycosylated products of Ssp I-linearized recombinant pMK14 mRNA translation using nonimmune antiserum. Asterisks denote additional nonglycosylated products of Ssp I-linearized recombinant pMK14 mRNA translation which are also immunoprecipitated with gp53-specific antiserum.



molecular weight bands is unknown. Several possibilities exist, and include anomalous electrophoretic migration of the translation products, premature termination of transcription or protein synthesis, deletion of RNA product from transcription, or initiation of translation at internal methionines. The predicted molecular weights of proteins which may have initiated at the first four methionine codons and terminated at amino acid residue 322 are given in table 4.1. The predicted weight of the protein initiated at the first methionine is 35kD which corresponds to the size of the largest nonglycosylated protein product. The predicted weight of proteins initiated at the various internal methionine codons differs from that observed by electrophoretic migration of the lower molecular weight proteins. This may have been due to the reasons described above.

The glycosylated translation products were subjected to the alkaline sodium carbonate experiment, using the same controls as before. The results are given in figure 4.9. Lane 1 of figure 4.9 contains the translation and glycosylation reaction products from the truncated RNA. The labelled glycosylated protein was not retained in the membrane portion (figure 4.9, lane 2), but was present in the soluble portion (figure 4.9, lane 3). The results of treatment of the glycosylated translation products with neutral buffer are also given in figure 4.9. The glycosylated product remained with the membrane fraction, because the membrane vesicles were not opened by the neutral buffer to release the secreted protein (figure 4.9, lane 4). This product was not present in the soluble portion (figure 4.9, lane 5). Control reactions were done as before, and are in lanes 6-11 of figure 4.9. This experiment indicated the anchor of the putative gp53 had been lost due to truncation of the RNA by restriction enzyme digestion of the DNA template. It was concluded that the long stretch of hydrophobic residues from amino acid 361 to 387 serves as a transmembrane domain. Whether the shorter stretches of hydrophobicity downstream of this region also serve as membrane-spanning elements remains to be determined, though their relatively short length would seem to preclude their functioning in this capacity.

Figure 4.9. SDS-PAGE analysis following sodium carbonate treatment of products of truncated recombinant pMK14 mRNA translation and glycosylation. Numbers on right denote molecular weight of protein size standards in kilodaltons (lane 12). Lane 1, glycosylated (g) and nonglycosylated (ng) translation products of mRNA from truncated (Ssp I restriction endonuclease-linearized) transcription template. Lane 2, membrane fraction of sodium carbonate-treated glycosylated translation product of truncated recombinant pMK14 mRNA. Lane 3, supernatant fraction of sodium carbonate-treated glycosylated translation product of truncated recombinant pMK14 mRNA. Lane 4, membrane fraction of neutral buffer-treated glycosylated translation product of truncated recombinant pMK14 mRNA. Lane 5, supernatant fraction of neutral buffer-treated glycosylated translation product of truncated recombinant pMK14 mRNA. Lane 6, nonglycosylated translation product of α -factor mRNA. Lane 7, membrane fraction of sodium carbonate-treated glycosylated α -factor translation product. Lane 8, supernatant fraction of sodium carbonate-treated glycosylated α -factor translation product. Lane 9, membrane fraction of neutral buffer-treated glycosylated α -factor translation product. Lane 10, supernatant fraction of neutral buffer-treated glycosylated α -factor translation product. Lane 11, translation without RNA. Asterisks denote additional products of mRNA from truncated recombinant pMK14 mRNA translation.



DISCUSSION

In vitro translation of the gp53 gene cDNA of BVDV strain 2724 revealed several important properties of the protein encoded by this gene. Expression can be initiated *in vitro* by a codon other than that used by the virus *in vivo*. A methionine codon well within the structural protein-encoding region of the BVDV genome functioned in this translation system. Immunoprecipitation studies confirmed that the protein expressed by the cDNA recombinant is an authentic BVDV-specific polypeptide.

Our data suggested that BVDV strain 2724 gp53 cDNA encoded a signal peptide, as the protein product is translocated and glycosylated. The amino acid residues in position 5 to 20 of the cDNA-encoded protein conform to the requirements for signal sequences described by von Heinje, and Perlman et al. (1986; 1983; respectively). No genomic regions downstream of internal methionine codons of the cDNA encode hydrophobic peptide stretches that comply as well with these requirements. This supports our hypothesis that the primary translation products of the full-length transcript and the truncated transcript were initiated at the first methionine of the cDNA. Whether this leader peptide functions as a signal domain *in vivo* remains to be determined.

Our data suggested also that the translation product is membrane anchored. Experiments with truncated cDNA suggested that the amino acid residues 361 to 387 of the cDNA-encoded protein serve as the membrane-spanning domain of this protein. Other hydrophobic stretches, either the small regions downstream of the large hydrophobic span, or segments not encoded by our cDNA may also serve in this capacity. However, the long hydrophobic stretch of amino acids near the carboxy terminus of the protein encoded by our cDNA is undoubtedly a major component of the membrane anchor. As the location of this domain lies near the carboxy terminus, gp53 is likely to be oriented such that the amino terminus is extracytoplasmic.

The apparent anomalous migration in electrophoresis gels of the primary translation product of the full-length cDNA transcript is the source of some uncertainty, as this protein did not appear to migrate at the molecular weight, predicted from the cDNA-encoded amino acid sequence, of 49kD. Several plausible explanations for this discrepancy exist. Anomalous electrophoretic migration of the

protein, premature termination of RNA or protein synthesis, or deletion of regions of the RNA template during transcription may explain the phenomenon. Internal initiation at a downstream methionine may also have occurred, because the context in which the internal initiation codons occur more closely follows the consensus sequence for translation initiation codons predicted by M. Kozak than does the first methionine encoded by the cDNA (1989). The protein produced is translocated efficiently *in vitro* which implies the existence of a functional signal peptide. The 16 amino acid residues at the amino terminus of the cDNA product downstream of the first methionine adequately fulfill the requirements described by von Heinje and Halvorson and Perlman for the role of leader peptide (1986; 1983; respectively). No downstream region equals this domain in conformity to the amino acid consensus sequence for signal peptides. This, and the fact that the first methionine codon encountered by the translational machinery is favored normally for initiation, suggest that this codon may have been the functional initiation codon in the translations described in this study. The other methionine codons may be functional too, as smaller molecular weight proteins were produced. The frequency at which various methionine codons were used for initiation in the cDNA translation can be determined through mutational studies in which these residues are deleted or altered. Additionally, deletion or alteration of the codons encoding the amino acids of the putative signal sequence would clarify the role of the latter in translocation and processing.

The possibility of autocatalysis of the protein encoded by this cDNA cannot be dismissed. The p20 of BVDV, the putative capsid protein, has autocatalytic properties (Wiskerchen et al., 1991; T. Poole, personal communication). Also, protein domains encoded downstream of that encoding the NS1 protein of flaviviruses within the region encoding NS2a are cis-acting proteases responsible for cleaving the carboxy terminus of NS1 protein (Falgout et al., 1989). Furthermore, the amino terminal region of the NS3 protein of flaviviruses may possess serine protease activity that may process viral proteins by intramolecular reactions (Wengler et al., 1991). The amino terminal region of the p80 portion of BVDV protein p125 may function as a serine protease (Gorbalenya et al., 1989). This protein may be equivalent to the NS3 protein of flaviviruses (Collett et al., 1988c). Our cDNA incorporates an unknown amount of genetic material encoding amino acids downstream from gp53, and these downstream peptides may be instrumental

in processing the translation product produced. However, the entire NS2a flavivirus protein was required for NS1 protein processing, and the amino-terminal 178 amino acid residues of the NS3 protein was necessary for its enzymatic activity (Falgout et al., 1989; Wengler et al., 1991). Thus, the length of downstream amino acids required for effective processing is substantial making it unlikely that the protein encoded by our cDNA possessed this capability. This phenomenon will require further analysis.

Further investigation is necessary on gp53 gene boundaries (as well on those of the other structural proteins of BVDV), on the identification of the signal sequence that is actually functional *in vivo* for glycoprotein translocation and processing, and on the existence in the virus of gp53 homodimers and/or heterodimers with other structural proteins. These will increase our basic understanding of the molecular virology of BVDV. Toward this end, expression of this protein in a system such as the baculovirus expression system will allow for the production of large amounts of recombinant BVD viral protein. Antibodies generated against this protein could then be used to isolate the native viral protein using affinity chromatography. In this way, this important BVD viral protein could be analyzed and characterized. Of paramount importance is the characterization of the antigenicity of this protein, and the immune response it elicits. Ultimately, this knowledge may be used to develop a safe and effective recombinant vaccine, as well as improved diagnostics. The former could be accomplished by exploiting the major envelope protein gene and its product in a recombinant or subunit vaccine. The latter may be approached by using the recombinant gene product in an enzyme-linked immunosorbent assay (ELISA). The protein could be used to capture antibody from sera of exposed animals, thereby identifying them as antibody positive; alternatively, antibodies generated to this recombinant protein could be used to capture antigen from viremic cattle, aiding in the identification of persistently BVDV-infected cattle.

Our current investigations are focused on the use of baculoviruses as expression vectors, a system first developed by Summers et al. (1987). These viral vectors have several distinct advantages over other expression systems. Foreign genetic material can be incorporated readily into the polyhedrin gene of the virus. The modification of this gene through insertional deletion results in viruses that are phenotypically different from wild type, allowing relatively easy screening for

recombinants. This gene's promoter is strong and results in high levels of protein production from genes inserted into it. As the virus replicates in eukaryotic cells, the proteins produced undergo appropriate posttranslational modifications, such as glycosylation, phosphorylation, signal sequence processing, and protein transport (Kang, 1988). This system not only provides large amounts of properly processed proteins for analysis, but has been used to produce immunogens as well.

Various viral proteins have been expressed using baculoviruses. The hemagglutinin gene of influenza virus was expressed and transported to the plasma membrane in a baculoviruses expression system (Possee, 1986). This hemagglutinin protein was similar antigenically to the natural protein. The envelope glycoproteins of Rift Valley Fever virus, a bunyavirus, have been produced by recombinant baculoviruses (Schmaljohn et al., 1989). These proteins were indistinguishable from the authentic glycoproteins by gel electrophoresis analysis. Mice immunized with these recombinant proteins generated protective immune responses.

Envelope proteins of flaviviruses also have been expressed from recombinant baculoviruses. The envelope protein of Japanese encephalitis virus was produced from recombinant baculovirus (Matsuura et al., 1989). The amount produced was equivalent to that produced by JEV-infected cells. Mice immunized with this recombinant baculovirus-infected insect cells developed neutralizing antibodies, and, though not strongly immunogenic alone, the recombinant protein induced an adequate priming response for development of protective immunity. The structural protein genes of dengue-2 virus have also been engineered into a baculovirus (Deubel et al., 1991). The dengue-2 virus glycoproteins were processed and transported to the surface of cells infected with recombinants. One recombinant encoding a carboxy-truncated E protein that lacked an anchor produced a soluble protein in infected cells. This secreted form of the E protein was synthesized at a higher level than the full-length membrane-bound form, and was more soluble and more easily purified. Both the truncated and full-length E protein reacted with type-specific and neutralizing monoclonal antibodies. In another study, mice immunized with dengue structural proteins produced by a recombinant baculovirus were protected from fatal dengue encephalitis (Zhang et al., 1988).

It is our goal to engineer the envelope protein initially, and eventually, all the structural proteins of BVDV, into a baculovirus. This will allow production of sufficient amounts of recombinant proteins for analysis. Native structure analysis,

epitope mapping, immunodominant antigenic determinant location, and the study of immunogenicity and nature of the immune response to these proteins will be facilitated. Additionally, proteins expressed in this system may be useful in effective immunization against BVDV infection and improved diagnostics for identification of this virus infection in cattle.

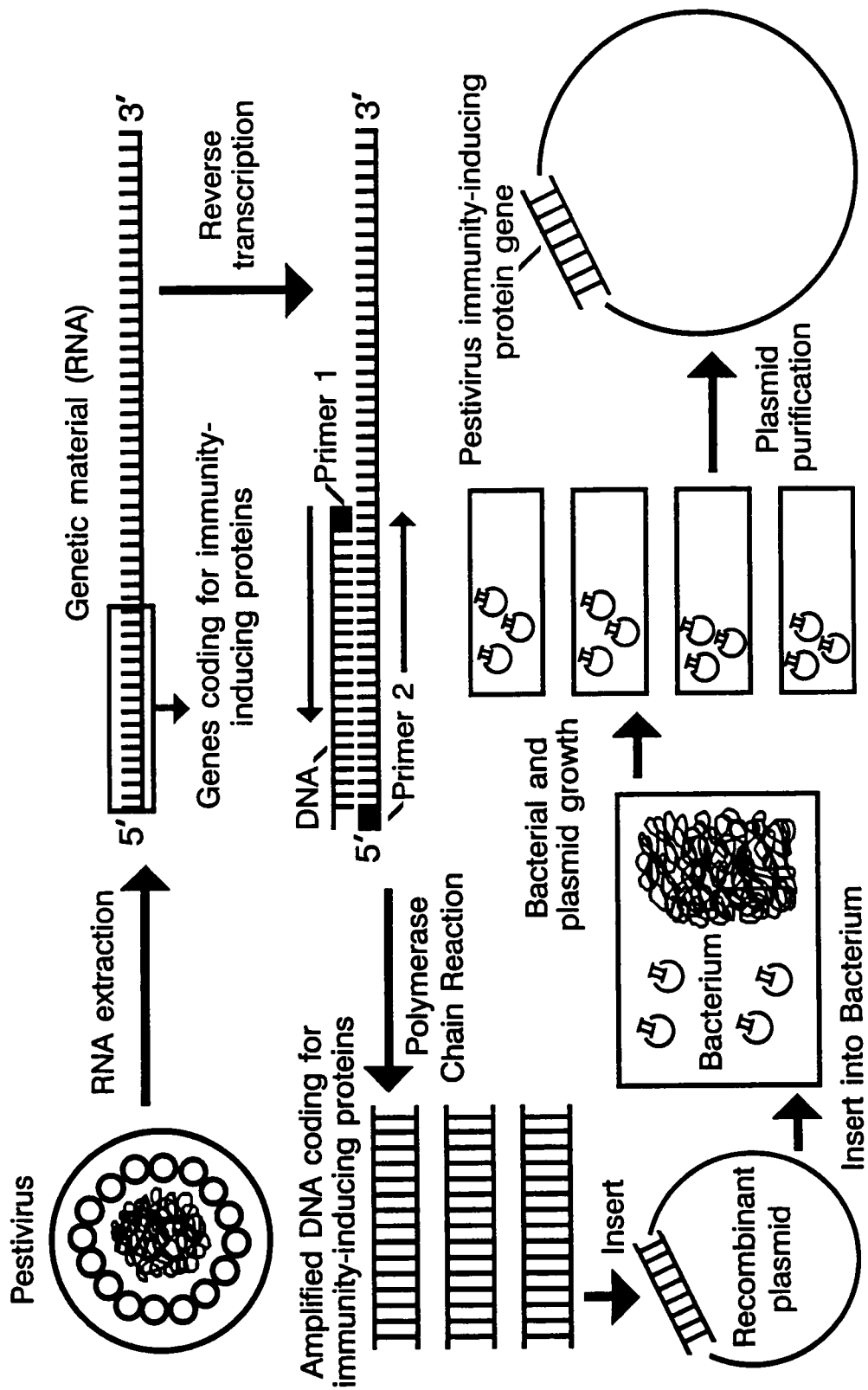
SUMMARY

The investigation presented in this dissertation involved analysis of the putative major envelope protein of BVDV strain 2724. A schematic diagram of the research is given in figure 4.10.

The data acquired from this investigation is summarized below.

- 1) Polymerase chain reaction using primers derived from published sequence data allowed specific amplification of BVDV genetic material.
- 2) Base sequence data of BVDV strain 2724 cDNA encoding the putative gp53 gene furthered characterization of the protein encoded by this genomic region of BVDV, and revealed significant homology of strain 2724 to the pestiviruses, but not to the flaviviruses.
- 3) Expression of this gene in a cell-free translation system was done. Initiation of protein synthesis occurred primarily at the first methionine codon of the cDNA, a codon not used by the virus *in vivo*.
- 4) Results from immunoprecipitation of the translation product suggested the cDNA translation product was an authentic BVDV-specific protein.
- 5) The translation product was translocated and glycosylated, implying the existence of a signal sequence in the cDNA translation product.
- 6) The translation product was membrane-anchored, and the putative transmembrane domain was identified.

Figure 4.10. Schematic diagram illustrating the investigation of the putative gene of BVDV strain 2724 encoding gp53 and its product. Procedures depicted include viral propagation, RNA extraction, cDNA synthesis and amplification, cloning, and recombinant cDNA isolation.



The research presented here represents preliminary characterization of the immunodominant protein of a significant veterinary pathogen. It is our intent to expand upon this data through further structural and immunogenic characterization of the protein. It is our hope that this protein may be exploited in a genetically engineered vaccine.

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VITA

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