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Molecular cloning, nucleotide sequencing and genome replication of bovine viral diarrhea virus

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The Ohio State University, 1992
MOLeULAR CLONING, NUCLEOTIDE SEQUENCING AND GENOME REPLICATION OF BOVINE VIRAL DIARRHEA VIRUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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* * * * *

The Ohio State University 1992

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To my wife and parents
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INTRODUCTION

Bovine viral diarrhea virus (BVDV), a small enveloped virus, is one of the most important viral pathogens of cattle. The virus is recognized as having worldwide distribution, with serum antibody prevalence in cattle ranging from 50% to 90% (Baker, 1987). Significant financial loss resulting from BVDV infections in cattle have been described (Duffell et al., 1986; Harkness, 1987). It was reported that the total annual cost of BVDV infection to the cattle industry in Great Britain was of the order of 32 million pounds (Harkness, 1987). BVDV infection is responsible for different clinical syndromes in cattle, such as abortion, persistent infection, mucosal disease and bovine viral diarrhea (Duffell and Harkness, 1985; Baker, 1987; Radostits and Littlejohns, 1988).

The most common infection of BVDV is the postnatal transient infection characterized by high morbidity, low mortality, development of serum neutralizing antibodies and elimination of the viruses from animals (Duffell and Harkness, 1985; Baker, 1987; Radostits and Littlejohns, 1988). About 25% of calves become infected with the virus during their first year of life (Harkness et al., 1978). Although such infections are subclinical or mild, there is potential for severe disease
because of the immunosuppressive activities of this virus in the host. By these effects, the virus may potentiate or enhance the pathogenicity of coinfecting microorganisms, such as parainfluenza virus type 3, infectious bovine rhinotracheitis virus, coronavirus, rotavirus, Pasteurella spp, Salmonella spp and coccidia (Baker, 1987).

The outcome of prenatal infections includes early embryonic mortality, abortion, stillbirth, congenital abnormalities and birth of persistently infected calves (Harkness, 1987; Baker, 1987; Radostits and Littlejohns, 1988). It has been estimated that more than three quarters of the financial losses with BVDV occur following prenatal infections (Harkness, 1987).

The persistently infected animal occurs as a result of infection with BVDV before the development of immunological competence of the fetus. These viremic carriers appeared either clinical normal or unthrifty, excreted large amounts of virus and produced no antibodies to the persisting virus (Radostits and Littlejohns, 1988; Brownlie, 1990; Moennig, 1990). Approximately 1% of cattle going to slaughter in Europe were persistently infected with BVDV (Meyling, 1984) and 1.7% of cattle tested in a survey in United states were identified to be persistently infected with BVDV (Bolin et al., 1985b). These viremic animals were the primary source of virus within herds and were responsible for spreading the virus to other susceptible animals.
The most severe outcome of BVDV infection is mucosal disease (MD). MD was usually sporadic in occurrence with low morbidity, but was almost invariably fatal although the course was chronic (Moennig, 1990; Brownlie, 1990; Moennig and Plagemann, 1992).

Because of the ubiquitous distribution of BVDV in the world and the multiple routes by which the virus may enter a herd, it is impossible to maintain BVDV-free herds (Baker, 1987). Control and prevention of BVDV may best be obtained by identification and removal of persistently infected animals, and prevention of transplacental infection (Duffell and Harkness, 1985; Baker, 1987; Radostits and Littlejohns, 1988). The identification of viremic carriers is entirely dependant on the availability of a rapid, accurate and cheap laboratory test. Although polymerase chain reaction (PCR) test is a good candidate, its false negative reactions due to the genetic heterogeneity between different BVDV strains would undermine the strategy entirely. Vaccination of the breeding female was recommended to prevent the transplacental infection (Harkness, 1987; Radostits and Littlejohns, 1988; Moennig, 1990). However, the efficacy of the currently available vaccines is a major question. One of the problems associated with modified live vaccines is the intrauterine transmission of the vaccine virus which has the same effects on the fetus as does natural BVDV prenatal infection (Radostits and Littlejohns, 1988). Because of their safety and absence of observed adverse
reactions, killed vaccines were widely used in cattle. However, it has been reported that an inactivated vaccine failed to protect approximately one-third of the fetuses against transplacental infection (Harkness et al., 1985). In addition, immunity may be adequate in response to vaccination to protect from homologous strain challenge, but may not protect from heterologous strain challenge. A thorough investigation of the molecular biology of the virus is an essential precondition for development of a new generation of BVDV vaccines.

BVDV has been divided into two biotypes based on cytopathogenicity in vitro (Baker et al., 1954; Underdahl et al., 1957; Gillespie et al., 1960). Cytopathic (CP) biotypes of BVDV induced cytopathic effects (CPE) in cell cultures, whereas noncytopathic (NCP) biotypes of BVDV replicated in cell culture without visual CPE. Inoculation of both CP and NCP BVDV into healthy cattle produced similar mild, postnatal infections (Pritchard, 1963; Thomson and Savan, 1963). On the other hand, the differences between CP and NCP BVDV were obvious in terms of the pathogenesis of persistent infections and mucosal disease. Only NCP BVDV is capable of establishing persistence following in utero infection and CP BVDV has never been isolated from persistently infected animals (Moennig and Plagemann, 1992). Mucosal disease occurred only when animals persistently infected with NCP BVDV were superinfected with an antigenically homologous CP BVDV. The pathogenesis of mucosal
disease is unique in that the disease is not a direct consequence of a simple "virus + host = disease" relationship. It was generated by the interaction between three entities: NCP BVDV, CP BVDV and the host. Based on the fact that in all the field investigations, CP BVDV was only recovered from cases of MD in association with NCP BVDV, and that certain epidemiological studies of field outbreaks of MD failed to show any introduction of BVDV diseased cattle into the herds (Brownlie et al., 1987; Howard et al., 1987; Corapi et al., 1988), it was proposed that CP BVDV developed from persistent NCP BVDV by viral genomic mutation (Brownlie et al., 1987; Howard et al., 1987; Corapi et al., 1988). Understanding the pathogenesis of the mucosal disease syndrome will depend on the elucidation of the molecular differences between CP and NCP biotypes of BVDV.

Because of the poor growth of BVDV in cell culture, difficulties in purifying large amounts of viruses and its sensitivity to manipulation, little progress in understanding molecular biology of BVDV was made until the middle 1980s. An understanding of the molecular biology of BVDV began crystallizing with the publication of the nucleotide sequence of a CP BVDV strain NADL (Collett et al., 1988a). To date, two strains of BVDV (NADL and Osloss) have been cloned and sequenced (Collett et al., 1988a; Renard et al., 1987a). Both strains, NADL and Osloss, were CP biotypes of BVDV. Only a partial genomic sequence (about 3.2 kb) in the viral
nonstructural protein p125 region has been determined for a NCP BVDV strain CP1 (Meyers et al., 1991). There are no sequence data available for the remainder of the genome for a NCP BVDV. As discussed previously, NCP BVDV played an important role in the persistent infection. Establishing a complete nucleotide sequence for NCP BVDV is highly desirable. Therefore, efforts were invested into cloning and sequencing the complete RNA genome of an NCP BVDV strain SD-1 in this study. In contrast to CP BVDV NADL and Osloss whose nucleotide sequence were determined from the virus stocks that had been adapted to and propagated in cell culture, the genomic RNA sequence of NCP BVDV SD-1 was established from the virus purified directly from a persistently infected animal without any passage in cell culture. The cDNA sequence of NCP BVDV SD-1, therefore, represents the genetic information of the virus in vivo, which may be different from that obtained in vitro due to the adaptive selection and spontaneous mutation of the virus during the cell culture passages. By comparing the nucleotide sequence of NCP BVDV SD-1 with CP BVDV NADL and Osloss, the molecular genetic difference between CP and NCP BVDV can be identified. This is important for understanding the difference in cytopathogenicity of both biotypes of BVDV. The establishment of the genomic sequence for a NCP BVDV is undoubtedly essential for studying and understanding the pathogenic mechanism for persistent infection, and the relationship and interaction between CP and NCP biotype of
BVDV in generating the mucosal disease. In addition, the sequence data provides important information about the genetic variation among different BVDV strains. It may contribute to the design and development of a new generation of BVDV vaccines, such as subunit vaccines. Practically, the sequence data may also provide important information on improving the PCR tests for diagnosis of persistently infected carriers by choosing the most conserved region for amplification. Finally, the determination of the entire genomic sequence of SD-1 is the prerequisite for construction of an infectious genomic cDNA construct for NCP BVDV.

It is known that the nucleotide sequence at the 5′-untranslated region (UTR) and 3′-UTR of viral RNA molecules harbors specific signals for viral RNA replication, transcription and translation (Strauss and Strauss, 1983). Limited data have been reported to analyze the termini of BVDV genomic RNA (Collett et al., 1989). Although the genomic nucleotide sequence of CP BVDV NADL has been almost completely determined, the extreme 5′ and 3′ terminal sequence remains to be established (Collett et al., 1988a). Although a cap structure at the 5′ terminus of the BVDV RNA genome was suspected (Collett et al., 1989), no direct experimental evidences have been presented to support this assumption. Therefore, another objective of this study was to characterize the 5′ and 3′ termini of BVDV genomic RNA and to establish the complete 5′ and 3′ terminal nucleotide sequence of CP BVDV.
NADL genome. Following the determination of the entire 5' and 3' terminal sequence for both CP BVDV NADL and NCP BVDV SD-1, the primary and secondary structure of the 5' and 3'-UTR of the genome was able to be analyzed and compared. The structural elements that may participate in viral RNA replication and translation, and their regulations were identified. This information may be important for further studies defining the functions of those structural elements for virus replication and viral RNA translation.

Asymmetric synthesis of positive and negative strand RNA during the viral replication has been described in a number of positive-stranded RNA viruses, including picornaviruses (Strauss and Strauss, 1983), flaviviruses (Cleaves et al., 1981; Chu and Westaway, 1985; Westaway, 1987), alphaviruses (Bruton and Kennedy, 1975; Sawicki and Sawicki, 1986b; Sawicki and Sawicki, 1987) and coronaviruses (Sawicki and Sawicki, 1986a; Perlman et al., 1986/87). The negative strand RNA identified in these viruses was exclusively in either replicative form (RF) or replicative intermediate (RI) form. However, little has been known about the replication of BVDV, neither RF nor RI RNA species in BVDV infected cells have been described (Collett et al., 1989) and no data on the synthesis of negative strand RNA of BVDV have been reported. In this study, the synthesis of both positive and negative strand RNA of BVDV during its replication cycles were investigated. The asymmetric synthesis of positive and negative strand RNA was
observed. In addition, the correlation between the viral RNA synthesis and the release of infectious virus from the infected cells was demonstrated.
Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens of cattle (Duffell and Harkness, 1985; Baker, 1987). It was first described by Olafson in 1946 as a transmissible disease with a high morbidity and low mortality in cattle (Olafson, 1946). Seven years later, Ramsey and Chivers (1953) reported a highly fatal disease of cattle with low morbidity rate. Because of the inability to experimentally reproduce this disease, it was described as a new disease, and was called "mucosal disease" (MD). Years later it was discovered that both BVD and MD were caused by the same virus, BVDV (Gillespie et al., 1961).

Together with the other two serologically and structurally related viruses; hog cholera virus (HoCV) of swine and border disease virus (BDV) of sheep, BVDV belongs to the pestiviruses in the family Flaviviridae (Collett et al., 1988c; Horzinek, 1991; Francki et al., 1991). The name of the group is derived from the infectious agent of *Pestis suum* meaning hog cholera virus, which was recognized much earlier than BVDV (1883, cited in Harkness and Roeder, 1988). Border
disease of sheep was first described by Hughes in 1959 (Hughes, 1959).

BVDV can cause a variety of clinical illness, including bovine viral diarrhea, mucosal disease, fetal disease, persistent infection and respiratory infection (Duffell and Harkness, 1985; Baker, 1987). The most common outcome of primary postnatal infection of BVDV was subclinical disease (Duffell and Harkness, 1985; Baker, 1987).

PERSISTENT INFECTION

Persistently infected cattle were first recognized by Von Borgen and Dinter (1961), then by Malmquist (1968) and Kendrick (1971). The virus was consistently isolated from cattle which were clinically normal, and had no detectable antibodies against BVDV. The relation between intrauterine infection and virus persistence was first described by Kendrick (1971) and Liess (1973). Following the infection of a nonimmune pregnant animal, the virus was capable of crossing the placental barrier and infecting the fetus. Intrauterine infections resulted in fetal death to persistent viremia throughout gestation and postnatal life (Kendrick, 1971; Brown et al., 1973; Brown et al., 1975; Roeder et al., 1986). The outcome of fetal infection was dependent on the stage of fetal development at which infection occurs and the infecting virus strain (Kendrick, 1971; Roeder et al., 1986). If the fetus was infected with BVDV before approximately 125 days of gestation,
prior to the development of immune competence, the virus was recognized as "self". Consequently, the fetus did not develop serum neutralizing antibodies and was carried normally to term and was born persistently infected with BVDV (Done et al., 1980; McClurkin et al., 1984; Roeder, 1984). These animals were immunotolerant to the virus and persistently viremic and appeared either clinically normal or unthrifty. The persistently infected calves continuously shed virus in secretions, even while carrying maternal antibodies. It was reported that the bovine fetus gains immune competence to BVDV around day 180 of gestation (Brown et al., 1979). If BVDV infection occurred after development of immune competence, the result was comparable to the acute postnatal infection characterized by subclinical disease, development of serum neutralizing antibodies and elimination of the virus from animal (Orban et al., 1983). At this stage, the bovine fetal immune system was sufficiently developed to recognize BVDV antigens and to mount an effective immune response. This was evidenced by the detection of neutralizing antibody to BVDV at birth prior to intake of colostrum. Recently, it was discovered that only NCP BVDV was capable of establishing persistent infection (Brownlie et al., 1984; McClurkin et al., 1985).

The immunotolerance of the persistently infected cattle has been shown to be highly specific. Persistently infected animals were immunoreactive to other antigens, such as
infectious bovine rhinotracheitis (IBR), parainfluenza virus type 3 (PI-3) and *pasteurella haemolytica* (McClurkin et al., 1984). In addition, they were capable of producing virus neutralizing antibodies following inoculation with antigenically heterogeneous BVDV strains (Liess et al., 1983; Bolin et al., 1985a; Bolin et al., 1987; Brownlie et al., 1987a; Brownlie et al., 1987b; Bolin, 1988). This phenomenon would explain the presence of antibodies to BVDV in persistently infected animals (Steck et al., 1980; Duffell and Harkness, 1985; Edwards et al., 1991).

Although the virus was present in all tissues of persistently infected animals (Moennig and Plagemann, 1992), lymphoid and certain epithelial tissues, especially the mucosae of the digestive tract, were the preferential sites for virus persistence (Ohmann, 1982; Ohmann, 1983; Ohmann, 1987; Ohmann, 1988a; Ohmann, 1988b; Liebler et al., 1991). The central nervous system (CNS) was another important location for virus persistence (Trautwein et al., 1985; Fernandez et al., 1989; Hewicker et al., 1990). The virus always was isolated from the serum of persistently infected animals. The virus titer in the serum was about $10^3$-$10^4$ CCID$_{50}$/ml (Ohmann, 1988), which was 3 logs lower than that in infected cell culture supernatants.

The birth of calves persistently infected with BVDV following introduction of BVDV in susceptible herds has resulted in serious economic losses (Duffell et al., 1986;
Harkness, 1987). Therefore, the persistent infection is central to the epidemiology and control of BVDV (Harkness et al., 1984). A persistently infected carrier may result from the following situations: (I) acute infection with noncytopathic (NCP) BVDV during the first trimester of gestation of a nonimmune pregnant cow. (II) persistently infected heifers reaching breeding age and producing offspring persistently infected. (III) a sero-negative cow inseminated with semen from a bull persistently infected with BVDV (Meyling and Jensen, 1988).

The prevalence of persistently infected cattle in the general population is not known, and there likely is considerable variation among herds. It was reported that 1% of cattle going to slaughter were persistently infected with BVDV (Meyling, 1984). In one survey in the United States, 9% of the herds were found to have persistently infected cattle, with a 1.7% detection rate in all cattle tested (Bolin et al., 1985b). Persistent infections appear to be a major mechanism by which the virus maintains itself in the cattle population (Duffell and Harkness, 1985; Baker, 1987; Radostits and Littlejohns, 1988). Therefore, elimination of persistently viremic animals from herds and the prevention of intrauterine infections are two major factors to controlling BVDV (Duffell and Harkness, 1985; Baker, 1987; Radostits and Littlejohns, 1988; Moennig and Plagemann, 1992).
MUCOSAL DISEASE

Mucosal disease (MD) was first recognized by Ramsey and Chivers (1953). However, their description of the spectrum of clinical disease was similar to that reported earlier by Olafson (1946) as "a gastro-enteritis with severe diarrhea". In contrast to the postnatal transient infection characterized by high morbidity and low mortality, MD usually occurred sporadically with low morbidity, but was invariably fatal. Although a connection between BVDV and MD was established experimentally (Gillespie et al., 1961; Pritchard, 1963; Thomson and Savan, 1963), the early experimental work prior to 1980 failed to fully define the relationship (Radostits and Littlejohns, 1988). There have been several important steps that have led to the present understanding of the pathogenesis of MD: (a) the recognition that BVDV can be one of two forms, noncytopathic (NCP) (Baker et al., 1954) or cytopathic (CP) (Underdahl et al., 1957; Gillespie et al., 1960); (b) the isolation and demonstration that viruses from both BVD and MD were serologically similar and induced the same mild experimental disease (Gillespie et al., 1961; Kniazeff and Pritchard, 1960; Hansen et al., 1962; Pritchard, 1963; Thomson and Savan, 1963); (c) the demonstration of transplacental transfer of virus to the fetus (Casaro et al., 1971; Kahrs, 1973) and the clinical evidence that abortions were a consistent finding of field outbreaks (Olafson et al., 1946; Dow et al., 1956); (d) the understanding that early fetal
infection with BVDV may lead to persistent viremia and failure to develop antibodies (Von Borgen and Dinter, 1961; Dinter et al., 1962; Malmquist, 1968; Kendrick, 1971); (e) the recognition that MD occurs exclusively in these persistently infected animals (Liess et al., 1974); (f) the observation that persistently viremic animals are infected with only the NCP BVDV and CP BVDV can not be isolated from these carriers (McClurkin et al., 1985, Brownlie et al., 1984); (g) the understanding that MD occurs only when the NCP BVDV carrier is superinfected with a CP BVDV strain (Brownlie et al., 1984); (h) successful experimental reproduction of MD (Brownlie et al., 1984; Bolin et al., 1985).

It was demonstrated that the experimental superinfection of two persistently infected animals with CP BVDV, isolated from a case of naturally occurring MD in the same herd where the two persistently infected animals were identified, induced typical mucosal disease in these two animals (Brownlie et al., 1984). Similar experiments were done by Bolin et al. (1985) by establishing experimental persistent infections in healthy cattle with defined NCP BVDV strains. Following the inoculation of six persistently infected animals with a CP BVDV strain, a severe disease typical of clinical MD developed in all six animals. However, some researchers failed to reproduce the disease by inoculating persistently infected animals with CP BVDV (Harkness et al., 1984). By neutralization assay, a study of paired CP and NCP BVDV
isolates from five outbreaks of MD demonstrated that there was a close antigenic relationship between each CP and NCP pair of the biotypes (Howard et al., 1987). Therefore, it was suggested that antigenic homology between CP BVDV and NCP BVDV was a prerequisite for the production of acute MD (Howard et al., 1987). The demonstration by monoclonal antibody (MAb) analysis of a close antigenic relationship between pairs of CP BVDV and NCP BVDV from cases of clinical MD supported this hypothesis (Corapi et al., 1988). This hypothesis was also proven by experimental observations (Brownlie et al., 1987a; Moennig et al., 1990). MD occurred after inoculation of persistently viremic cattle with CP BVDV that had been selected by MAb analysis for antigenic homology with the respective "endogenous" NCP BVDV. However, when viremic animals were inoculated with an antigenically heterogeneous CP BVDV strain, no disease developed until day 23 postinoculation but a strong antibody response directed against the superinfected virus was induced (Moennig et al., 1990).

Regarding the question of where superinfected, antigenically homologous, CP BVDV originated; Brownlie et al (1987a) and Howard et al. (1987) suggested that a mutation of the persisting NCP BVDV was the most likely source of the CP BVDV. This hypothesis was based on the fact that in all the field investigations, CP BVDV has only been recovered from cases of MD and in association with NCP BVDV, and detailed epidemiological studies of field outbreaks have failed to show
any introduction of BVDV infected animals. However, the possibility of superinfection due to the introduction of a new CP BVDV into the herds can not be excluded. It has been reported that mucosal disease outbreaks have occurred in the past following immunization of cattle using modified live CP BVDV vaccines (Monennig and Plagemann, 1992). Definitive evidence for a mutational origin of the cytopathogenic biotype must await further molecular studies.

The pathogenic mechanism of fatal MD infection remains unclear. It was proposed that the cytopathic potential of the CP biotype may be responsible for the tissue destruction, and that MD is largely induced by the unlimited growth of CP BVDV in animals immunotolerant to an antigenically homogeneous or identical NCP BVDV (Moennig and Plagemann, 1992). This hypothesis was supported by the observation that widespread tissue destruction occurred in areas where CP BVDV replicates (Liebler et al., 1991). Although CP BVDV causes cytopathic effects in vitro whereas NCP BVDV does not, there is no direct evidence to confirm that cytopathic effects of BVDV in vitro reflect the pathogenic properties of BVDV in vivo. In addition, it should be noted that cytopathogenicity may vary dependant on both genetic changes of the virus and different culture conditions. Furthermore, the pathogenicity of BVDV in persistently infected cattle, i.e. the ability of the virus to induce MD, has not been directly or genetically linked to cytopathogenicity in cell cultures (Løken, 1991). One
important observation is that acute infections with CP BVDV in cattle resemble those with the respective NCP BVDV. Therefore, direct cytopathic effects of CP BVDV may not be the only pathogenic mechanism for fatal MD infection. Immune complex disease has also been proposed to elucidate the pathogenic mechanism of MD fatal infection (Prager et al., 1976, Cutlip et al., 1976; Littlejohns and Walker, 1985; Littlejohns, 1985). The association of lesions suggestive of glomerulonephritis (Trautwein, 1965; Winter and Majid, 1984) may indicate that MD is due to an inappropriate immune response rather than to the direct effect of the virus. A superinfecting virus which is serologically homogeneous to the persistent virus, and hence is also able to persist, may differ from the original virus in regard to other antigens, i.e. antigens that are not involved in neutralization. This could conceivably provide a basis for immune complex disease, as the host’s immunotolerance is strictly limited to the antigens of the original virus strain. This hypothesis implied that the antigenic similarity but not the cytopathogenicity of BVDV was the prerequisite for producing MD. No direct experimental evidence is available to support this hypothesis. Further experiments are required to make conclusive remarks concerning the pathogenic mechanism of MD.

Another confusing aspect of BVDV infection is the chronic infection. A small proportion of infected cattle suffer from clinical illness for a prolonged period. The terms chronic BVD
and chronic MD have been used to describe these infections. To date, the disease has not been reproduced experimentally, although from natural cases there is evidence that affected animals were also persistently viremic. Chronic MD is a more descriptive term than chronic BVD and will be used throughout this discussion.

It was suggested that chronic MD might develop when viremic animals are superinfected with a CP BVDV sharing only partial antigenic homology with the "endogenous" NCP BVDV (Radostits and Littlejohns, 1988; Moennig and Plagemann, 1992). The partial homology could enable the superinfecting virus to take advantage of the host's immunotolerance and to survive for a prolonged period. Antibody against the heterogeneous antigenic epitopes of the superinfecting virus is usually produced (Radostits and Littlejohns, 1988). Both the direct cytopathic effect of the superinfecting CP BVDV and the lesions caused by immune complex may be responsible for the fatal consequence of chronic MD. The experiment done by Brownlie et al. (1987b) and Brownlie (1990) demonstrated that when persistently viremic animals were challenged with an antigenically different CP BVDV, an antibody response to the superinfecting virus was first induced, and after an incubation period of 98 to 138 days a disease characterized by mucosal lesions developed. This observation resembled naturally occurring chronic MD.
The genome of pestivirus was characterized as a single-stranded RNA (Horzinek, 1981). The viral RNA was shown to be infectious, establishing pestiviruses as positive-strand RNA viruses (Diderholm and Dinter, 1966). Genome size based on sedimentation data were somewhat discordant. Sedimentation values ranging from 24S to 45S have been described (Moennig, 1988. Collett et al., 1989). More precise data were obtained when viral RNA were analyzed electrophoretically under denaturing condition (Renard et al., 1985; Collett et al., 1989). Renard et al. (1985) reported the length of the RNA of the Osloss strain of BVDV to be 12.5 kilobases when compared with molecular weight standards. Collett et al. (1988a) reported a similar size for the RNA of the NADL strain of BVDV. The previously reported size of 8.2 kb for NADL RNA (Purchio et al., 1983) was an incorrect value based on an erroneous measurement (Collett et al., 1989). Comparison of the length of viral RNA from additional BVDV isolates, of both CP and NCP biotypes, with that of the NADL revealed their sizes to be indistinguishable (Collett et al., 1989). A size of about 12 kb for HoCV strains Alfort and Brescia has also been reported (Meyers et al., 1989; Moormann et al., 1990). Recently, a CP BVDV strain, CPI was described to have genomic size of 14 kb due to the cellular RNA insertion and viral gene duplication in its genome (Meyers et al., 1991).
In infected cells, only one species of virus specific RNA corresponding to the size of the viral genome has been demonstrated (Purchio et al., 1983, Renard et al., 1985, Collett et al., 1989, Rümenapf et al., 1989). No subgenomic RNA has been detected in infected cells (Purchio et al., 1983; Renard et al., 1985). To date, no reports have described the molecular features of pestiviral RNA replication. In addition, replicative form (RF) or replicative intermediate (RI) RNA species in pestivirus-infected cells have not been reported. Although the RNA found in BVDV NADL strain infected cells had features similar to RF RNA by virtue of its ability to bind to CF-11 cellulose in the presence of 15% ethanol (Purchio et al., 1984a) and to remain soluble in 2M LiCl (Collett et al., 1988a), it could be distinguished from RF by its sensitivity to RNase (Purchio et al., 1983). Synthesis of pestivirus RNA is not impaired by concentrations of actinomycin D, which completely inhibit host cell RNA synthesis (Horzinek, 1981; Purchio et al., 1983), implying that pestiviruses do not rely on host cell enzymes for their RNA synthesis. A viral nonstructural protein (p133) was speculated to function as RNA dependent RNA polymerase for BVDV RNA replication (Collett et al., 1989).

A high degree of secondary structure of pestivirus RNA was suggested based on the sedimentation data of viral RNA and the failure to translate undenatured BVDV RNA (Moennig, 1971; Purchio et al., 1984a). Observations that pestivirus
replication was sensitive to proflavine and acriflavine may be a consequence of the unusual secondary structure of pestiviral genomic RNA (Dinter and Diderholm, 1971; Diderholm et al., 1973; Collett et al., 1989).

Limited data have been reported to analyze the termini of pestivirus genomic RNA. The viral RNA lacks a poly A tail at the 3’ end as indicated by its inability to bind to oligo(dT) cellulose (Purchio et al., 1983, Renard et al., 1985). The 3’ end of the RNA was a suitable substrate for the addition of cytidine 3’, 5’-bisphosphate (pCp) by T4 RNA ligase (Renard et al., 1985; Collett et al., 1989). However, attempts to modify the BVDV RNA by addition of AMP residues to its 3’ end using E. coli poly(A) polymerase were unsuccessful (Renard et al., 1985; Collett et al., 1988c). Brock et al. (1988) and Moormann et al. (1990) succeeded in poly(A) tailing the 3’ ends of BVDV strain 72 and HoCV strain Brescia RNAs using E. coli poly(A) polymerase. The characteristics of the 5’ terminus of pestivirus RNA are unknown. Based on the observation that BVDV RNA failed to be radiolabelled with 32P-ATP using polynucleotide kinase with or without prior phosphatase treatment (Collett et al., 1989), a cap structure at the 5’ end of pestivirus RNA was suspected (Collett et al., 1989).

An important step in understanding the pestivirus genome was the molecular cloning and sequencing of their RNAs. Renard et al. (1985, 1987) first reported the genomic sequence of a CP BVDV strain, Osloss in a European patent application. In
1988, Collett et al. (1988a) published the genomic sequence of another CP BVDV strain, NADL. Meyers et al. (1989) and Moormann et al. (1990) reported the genomic sequence of HoCV strains Alfort and Brescia, respectively. The methods using synthetic random primer to obtain cDNA from viral RNA did not enabled them to clone the genomic ends, particularly the 3' termini. Several attempts have been made to sequence the termini of pestivirus RNAs. Enzymatical polyadenylation at the 3' end was not successful (Renard et al., 1985; Collett et al., 1989). Moorman et al. (1990) successfully polyadenylated the 3' end of HoCV RNA using poly(A) polymerase and obtained oligo (dT)-primed cDNA clones. Sequence analysis of the cDNA allowed the conclusion that the complete 3' region of HoCV strain Brescia had been cloned. However, additional efforts are necessary before this conclusion can be firmly accepted (Collett et al., 1992). For obtaining 5' end cDNA clones, a synthetic oligonucleotide that was complementary to the viral sequence of Brescia strain RNA was used to prime first-strand cDNA synthesis. However, because four cDNA clones covering the 5' end of the genome exhibited a few discrepancies in the terminal sequence, the authentic sequence of the 5' end was not unequivocally determined (Moormann et al., 1990). Therefore, a complete genomic sequence including the authentic 5' and 3' end sequence of a pestivirus genome remains to be established.
Both BVDV strains Osloss and NADL are CP BVDV. So far there is no sequence data available on NCP BVDV. As reviewed in the previous paragraphs, NCP BVDV plays an important role in the persistent infection and mucosal disease. In order to understand the cytopathogenicity of BVDV, the pathogenic mechanism for MD and the interaction between CP and NCP biotypes, the complete nucleotide sequence of an NCP BVDV strain is needed.

From the nucleotide sequence of cDNA clones, the size of the pestiviral genomic RNA ranges from 12,283 (HoCV Brescia) to 12,573 nucleotides (CP BVDV NADL). However, as mentioned previously, the exact size of the viral genome remains to be determined since the current sequence information may not be complete for both ends of their genomes. Comparison of the genomic nucleotide sequences of BVDV (strain NADL and Osloss) with that of HoCV (strain Alfort and Brescia) led to the finding of a cellular RNA insertion in the genomes of BVDV NADL and Osloss (Meyers et al., 1989; Collett et al., 1989). Analysis of the nucleotide sequences for potential protein-coding regions revealed significant open reading frames (ORF) in the positive polarity only (Renard et al., 1987; Collett et al., 1988a). Renard et al. (1987) reported two large ORFs for the Osloss strain. In contrast, Collett et al., (1988a) found only one large ORF for NADL strain. The possibility of a mistake in the Osloss sequence was suggested and an insertion of two nucleotides or a deletion of one nucleotide at or just
before Osloss nucleotides 4241 transformed the Osloss sequence to a single ORF (Collett et al., 1989). Recently, a sequence mistake in Osloss strain was confirmed (Meyers et al., 1989). Sequence analysis of HoCV strains Alfort and Brescia revealed that there is a single ORF in HoCV too. Therefore, all pestivirus genomes are characterized by possessing a single large open reading frame (ORF) capable of encoding almost 4000 amino acids. Although the ORF sizes of the two BVDV sequences are different from one another and both are larger than that of HoCV, if the amino acids contributed by the cellular insertion sequences are subtracted, the ORF of both CP BVDV Osloss and NADL become 3898 amino acids in size, which is identical to that of HoCV. Thus, despite the recombinational insertions in the genomes of CP BVDV, the fundamental size of the ORF for pestiviruses is constant. Preceding the large ORF is a 360 (HoCV Brescia) to 385 (BVDV NADL) nucleotide 5′-untranslated region (UTR). After the stop codon of the large ORF, the genomic sequence continues for another 186 (Osloss) to 229 (Brescia) nucleotides. With one single continuous ORF, pestiviral protein biosynthesis resembles flaviviruses and picornaviruses (Collett et al., 1989). A large polyprotein precursor is translated and processed by either cotranslational or posttranslational proteolytic cleavage (Collett et al., 1988b). This translation strategy is in good agreement with the absence of subgenomic viral RNAs during infection (Purchio et al., 1983; Renard et al., 1985) and the

VIRAL PROTEINS AND GENOME ORGANIZATION

The characterization of pestiviral proteins has been a subject of controversy. One constant difficulty has been the inability to unequivocally distinguish viral proteins, particularly less abundant ones, from host cell contaminants. This problem was a direct consequence of the low level of pestivirus replication in cell culture. Another complicating factor is that pestivirus seems to be fragile and membrane associated, which are properties that hamper efficient purification of viral particles. The classical methods including radiolabelling, gradient purification, and electrophoretic separation are not efficient enough to identify viral proteins. More recent investigations using immunological methods for identification of protein from lysates of infected cells or in vitro translated peptides have also failed to yield clear protein characterization. Much of the confusion may also be attributable to the inability to distinguish among "virus-encoded", "virus-induced," and "virus-associated" polypeptides (Collett et al., 1989).

Pritchett and Zee (1975) first reported the structural proteins of BVDV. After extensive purification of viral particles by differential and isopycnic zonal centrifugation, four electrophoretic components of viral origin were
identified. Two viral proteins (VC1 and VC3) migrated heterogeneously and had molecular weight (MW) of 93 to 110 KDa and 50 to 59 KDa, respectively. The other two components had MW of 70 KDa with VC2 and 25 KDa with VC4. In 1978, Enzmann and Weiland reported three structural proteins of hog cholera virus, two of them, gp55 and gp46 were glycoproteins. The third structural polypeptide, p36, was not glycosylated (Enzmann and Weiland, 1978). Similar results were obtained by Matthaeus (1979) for BVDV structural protein. The two large polypeptides, VP1 (gp57) and VP2 (gp44), were found to be glycosylated. The another structural protein, VP3, had MW of 34 KDa. The study done by Coria (1983) revealed four major structural proteins of purified BVDV. The MWs of these polypeptides were 75, 66, 54 and 26 KDa, respectively. The proteins of 75 and 54 KDa were glycoproteins as determined by staining with dansyl hydrazine. Purchio et al. (1984b) first used immunoprecipitation methods to identify BVDV proteins in infected cell lysates and virus preparations. Three major virus-specific polypeptides with MWs of 115, 80 and 55 KDa were observed in both infected cell lysates and virus preparations. Two minor proteins of 45 KDa and 38 KDa were also noted in infected cell lysates. Tryptic peptide mapping indicated that the 115 KDa and the 80 KDa polypeptides were structurally related (Purchio et al., 1984b). This is the first description for the structural relatedness among the putative BVDV-specific polypeptides.
In all the studies on viral proteins described above, only one biotype of BVDV, i.e. either CP BVDV or NCP BVDV, was used to analyze viral proteins. In 1987, Pocock et al. (1987) first characterized and compared both CP and NCP BVDV viral proteins. The most significant finding of their study was the observation that VP2, a 87 KDa protein, dominant in CP BVDV strain was absent in the corresponding NCP BVDV strain. In addition, eight BVDV-specific polypeptides (VP1 to VP8) with MW ranging from 23 to 120 KDa were identified, four of them were glycoproteins. Furthermore, variation of MW of some viral proteins in 3 CP BVDV Strains was noted. Similar results were obtained by Donis and Dubovi (1987a; 1987b) and Magar et al. (1988). When virus-induced polypeptides were compared between CP BVDV and NCP BVDV, one of the most surprising observations was that the 80 KDa protein was present exclusively in CP BVDV isolates and that NCP BVDV infected cells lack the 80 KDa polypeptide.

By combining radiolabelling of virus-infected cells in the presence of hypertonic translation initiation blockage and immunoprecipitation, Donis and Dubovi (1987a; 1987b; 1987c) succeeded in identifying 12 BVDV-specific polypeptides with MW ranging from 19 to 165 KDa. In addition to the 5 polypeptides described by Purchio et al. (1984b), seven unreported polypeptides with MWs of 165, 135, 75, 62, 32, 25 and 19 KDa were identified in infected cells. The sum of the molecular masses of these polypeptides was greater than the coding
capacity of the genome, suggesting the presence of precursor-product relationship between these polypeptides. Six proteins were reported to be glycosylated (Donis and Dubovi, 1987c). A summary of BVDV specific proteins reported by different investigators is presented in Table 1. The viral proteins of BDV were also reported by Akkina and Raisch (1990). Eleven viral polypeptides with MWs of 220, 165, 118, 84, 66, 58, 55, 53, 45, 37, 31 KDa, respectively, were detected in BDV infected cells. Nine glycosylated proteins were identified with MWs of 165, 118, 84, 66, 58, 55, 53, 45, 31 KDa.

As reviewed above, although certain polypeptides appear to be common in all these studies, no two groups have reported the same constellation of so-called "BVDV proteins". It was not until sequence-specific antibody reagents were generated that the virus-encoded nature of the observed proteins could be conclusively established. With the nucleotide sequence at hand, Collett et al. (1988b) invested considerable effort into producing these reagents. Both engineered proteins expressed in \textit{E. coli} and synthetic peptides were used to generate antisera in laboratory animals. These sera were then used in immunoprecipitation analysis to identify authentic BVDV-encoded proteins. Results from this excellent work allowed the identification of authentic BVDV proteins, positioning of BVDV gene products along the genome and demonstration of the precursor-product relationship of viral proteins (Collett et al., 1988b). The first and most comprehensive model for genome
organization and protein biogenesis was thus established by Collett et al. (1988b, 1989, 1991). To introduce a consistent nomenclature for pestivirus proteins, Collett et al. (1988b) proposed to use the Mr value to name the viral proteins. For a better understanding, this nomenclature and the designation will be used in this dissertation. A present model for BVDV genomic organization is summarized in Fig. 1.

The genes for viral structural proteins are located at the 5'—terminus of the genome. The first protein at the N-terminal portion is the p20. Based on the comparison of the genomic organization between pestiviruses and flaviviruses, p20 was proposed to be one of the structural proteins, perhaps the nucleocapsid protein (Collett et al., 1988b). However, it is controversial whether this protein is a capsid protein. Thiel et al. (1991) presented evidence that p20 is not a component of the virion as suggested by Collett et al. (1988b) and Wiskerchen et al. (1991). Instead, p14, a highly charged and conserved protein located between p20 and the first viral glycoprotein, was identified as the viral core protein. Furthermore, this newly identified protein was also confirmed in HoCV virions (Thiel et al., 1991). Downstream of the p14 the large glycoprotein precursor, gp116, was located which ultimately was processed into three viral glycoproteins, gp48, gp25 and gp53 (Collett et al., 1989; Stark et al., 1990). The region downstream of the structural protein genes encodes the highly conserved nonstructural protein, p125. As discussed
previously, NCP BVDV expresses the intact p125 molecule, whereas in CP BVDV, p125 was processed into two products, p80 and p54 (Collett et al., 1988b). The p80 was a dominant viral protein and was easily identified with polyclonal antisera (Purchio et al., 1984b; Pocock et al., 1987; Donis and Dubovi, 1987a; Magar et al., 1988), whereas p54 could only be detected by sequence-specific antisera (Collett et al., 1988b). Recently, a new protein, p175, was identified by two groups (Akkina, 1991; Deregt et al. 1991). Their results revealed that p175 is related to p125 and p80, suggesting that the p175 is the precursor protein for p125 and is processed into p125 and an unidentified protein. The carboxyl-terminus of the ORF encodes a precursor protein, p175, which is rapidly processed to yield the putative p42 and p133. The p42 is further processed into two products, p10 and an unidentified protein. The p133 is rapidly processed into two products: p58 and p75 (Collett et al., 1988b). The p58 is a rather stable product, whereas p75 is turned over rapidly (Collett et al., 1991). It should be stressed that an alternative cleavage scheme for this large precursor protein has been described (Akkina, 1991). It was alternatively processed into another two proteins: p96 and p72.

The preceding description of the pestiviral genomic organization almost covers the entire ORF of BVDV. However, there remain two small regions of the genome for which protein products have not been identified. In addition, the protein
coding boundaries depicted in Fig. 2 represent only estimated positions. There are no reports of terminal amino acid sequence data for any of the pestivirus proteins. This information is required for any conclusive determination of the boundaries and the relatedness of viral proteins.

Regarding the functions of the various viral proteins, most are unclear. p20, the first protein product of the ORF was reported to possess proteolytic activity and is responsible for autocatalytic cleavage at its carboxyl terminus (Wiskerchen and Collett, 1991a). All the glycoproteins, gp48, gp25 and gp53 are involved in the formation of disulfate-linked dimmers, demonstrable in infected cells and virions, respectively (Weiland et al., 1990; Thiel et al., 1991). The hydrophobic gp25 may function as a transmembrane protein serving as an anchor for gp53 (Collett, 1992). The gp53 was repeatedly reported to mediate neutralization of pestivirus (Magar et al., 1988; Donis et al., 1988; Bolin et al., 1988; Xue et al., 1990; Weiland et al., 1990; Rümenapf et al., 1991). The equivalent protein of HoCV gp48 was also described to induce neutralizing antibody (Weiland et al., 1992). These two glycoproteins, gp48 and gp53 were therefore predicted to be accessible on the surface of virion. In addition, gp48 seems to be a second viral polypeptide involved in BVDV-induced cytopathogenicity. Monoclonal antibodies (MAbs) against gp48 reacted to primarily biotype-specifically with CP BVDV (Peters et al., 1986;
Greiser-Wilker et al., 1991). Computer-assisted sequence comparisons revealed homologies between p80 and a class of serine proteinases and a helicase motif (Bazan and Fletterick, 1989; Gorbalenya et al., 1989). Recently, it was shown experimentally that p80 possesses proteinase activity and is responsible for all the nonstructural protein processing except for p20 (Wiskerchen and Collett, 1991b; Perric et al., 1992). Because of the lack of p80 in NCP BVDV, its precursor protein, p125, was proposed to have the p80 proteinase activity, which may be responsible for all the nonstructural protein processing in NCP BVDV (Wiskerchen and Collett, 1991b; Perric et al., 1992). Because p80 was characteristic for CP BVDV, it was suggested that this protein also plays an important role in the cytopathogenicity of BVDV (Megers et al., 1991). A "Zinc-finger" like domain has been described in p54, the N-portion of p125, implicating the possible nucleic acid binding function (Moerlooze et al., 1990). Based on hydrophobicity analysis, the N-terminal portion of p54 is extremely hydrophobic. Therefore, a membrane-associated function was predicted for this protein (Collett et al., 1991). The unidentified protein, p32, was proposed to be a cofactor of the p80 proteinase, involved in the cleavage of p133 into p58 and p75 (Wiskerchen and Collett, 1991). The short half-life of p75 may suggest a regulatory function in virus replication (Collett et al., 1991). In addition, p75 was proposed to be a candidate for viral RNA-dependent RNA
polymerase on the basis of identification of the highly conserved tripeptide in all the RNA replicases (Collett et al., 1991). The p58 was also suspected to be involved in virus RNA replication (Collett, 1992). The function of p10 is unknown.

The majority of the viral protein functions described above are based on sequence comparison and analysis. No direct experimental evidence has been established. Therefore, further work is required to elucidate these functions.

ANTIGENIC RELATIONSHIPS AND EPITOPE MAPPING

Darbyshire et al. (1960, 1962a, 1962b) first demonstrated the antigenic relationship between BVDV and HoCV. By using the Ouchterlony technique of double diffusion in agar gel, they found a continuous precipitation line between the homologous HoCV and BVDV systems in the same plate, suggesting a close serological relationship between the two viral species. The BVDV antigen involved in the formation of a single line of identity was described to be a soluble antigen because when concentrated BVDV pellets were used as antigens, no precipitation line was formed (Gutekunst and Malmquist, 1963; Pirtle and Gutekunst, 1964). The close antigenic relationship between BVDV and HoCV was also demonstrated by cross-immunofluorescent staining (Mengeling et al., 1963), complement-fixation tests (Gutekunst and Malmquist, 1964) and cross-protection tests (Sheffy et al., 1962).
By means of the gel precipitin test, Acland et al. (1972) first showed the antigenic relationship between BVDV and BDV. On the basis of the neutralization of BVDV by sera against BDV, Hamilton and Timoney (1972) implicated BVDV in the aetiology of BD.

In 1973, the relationship of the three virus species: BVDV of cattle, HoCV of swine and BDV of sheep was established (Plant et al., 1973; Osburn et al., 1973). In the gel precipitation test, a continuous line formed between BVDV, HoCV and BDV (Plant et al., 1973). Sera obtained from naturally occurring or experimentally produced BD lambs were able to neutralize BVDV and HoCV (Osburn et al., 1973). Based on their antigenic relationship, BVDV, HoCV and BDV were grouped in the same genus Pestivirus in the family Togaviridae (Horzinek, 1973).

Meanwhile, using cross-neutralization tests, distinct antigenic differences between BVDV and HoCV were reported by Sheffy et al. (1962), Coggins and Seo (1963), Dinter (1963), Kumagai et al. (1962), Stewart et al. (1971) and Fernelius (1973). In their studies, antisera against BVDV or HoCV neutralized the homologous virus, but no cross-neutralizing activity was observed. However, cross-neutralization testing was not able to distinguish between BVDV and BDV, suggesting that the ruminant pestiviruses were more closely related to each other than to HoCV (Laude and Gelfi, 1979).
The antigenic relationship between different BVDV strains was first studied by Gillespie et al. (1961). Using virus neutralization tests, five strains of BVDV and one MD virus were shown to be antigenically related, establishing the same aetiology for BVD and MD. More extensive studies done by Kniazeff (1961) revealed that antisera against a number of BVDV strains from various parts of the world were capable of neutralizing the BVDV strain Oregon C24V. These results indicated that an antigenic relationship existed between the Oregon strain and other BVDV strains. The extent of the relationship was not revealed by this study.

The antigenic variation among BVDV strains was first reported by Castrucci et al. (1968). Serum neutralization tests using homologous and heterologous antisera were done to analyze the antigenic relationships among different BVDV strains. The results revealed five serological groups for all the BVDV strains tested. Fernelius et al. (1971) described three serotypes of BVDV on the basis of the degree of neutralization for each strain tested. Antigenic heterogeneity of BVDV strains isolated from different countries including the U.S.A., Germany, Denmark, France, Sweden and Hungary was also reported by Güneri (1968). However, these in vitro serological differences did not correlate with the in vivo immunity following challenge with both homologous and heterologous strains of viruses (Castrucci et al., 1975).
Previous studies of antigenic variation among pestivirus have been mainly based on the virus neutralization tests. The preparation of anti-BVDV monoclonal antibodies (MAbs) has enabled a new approach to characterization. Peters et al. (1986) first developed MAbs against BVDV to analyze the antigenicity of different strains of BVDV and HoCV by indirect immunofluorescence assays. One MAb reacted with all 12 BVDV strains and 4 HoCV strains tested. Other MAbs showed different degrees of cross reactivity with BVDV and HoCV strains. MAbs against HoCV were first developed by Wensvoort et al., (1986). Thirteen MAbs reacted by varying degree with all but one of the HoCV strains tested, and none of the MAbs recognized BVDV strains. Similar results were obtained by Greiser-Wilke et al. (1990) in which 7 MAbs reacted with 14 different isolates of HoCV, but not with any BVDV strains tested. This suggests that the viral epitopes recognized by MAbs were unique and highly conserved among HoCV. Wensvoort et al. (1989a) used 13 MAbs against HoCV to identify the antigenic variations of pestiviruses and none of the BVDV or BDV strains were detected by the 13 MAbs. Seven MAbs reacted with all 94 HoCV strains, and six other MAbs detected heterogeneity among and within HoCV strains.

Based on the MAb reactivity with different strains of pestivirus, antigenic groups were proposed by a number of authors (Edwards et al., 1988; Magar et al., 1988; Bolin et al., 1988; Wensvoort et al., 1989a and 1989b). Edwards et al.
(1988) used 38 MAbs against BVDV and HoCV to characterize 101 field isolates of pestivirus. The results revealed that the MAbs can be divided into three panels: (1) pestivirus group specific, (2) ruminant pestivirus specific, (3) HoCV specific. Based on the reaction patterns with panel 2 MAbs, field isolates could be divided into two main groups: Group A predominate in cattle and Group B in sheep. Magar et al. (1988) described three antigenic groups within BVDV on the basis of neutralization and immunofluorescence reactions of a single MAb with 10 field isolates: group 1, CP BVDV NADL-like; group 2, CP BVDV Oregon C24V-like; and group 3, NCP New York like. This supported the proposal by Fernelius et al. (1971) for three serotypes, including NADL, Oregon C24V and NCP BVDV group. Bolin et al. (1988) identified four groups of BVDV by neutralization using nine MAbs with ten field isolates: group 1 included three CP BVDV; group 2 consisted of one CP BVDV and two NCP BVDV; group 3 had 3 NCP BVDV and group 4 only NADL. Based on cross-neutralization tests, Wensvoort et al. (1989a) described four distinct serologic groups. One group was composed of established BVDV, BDV and porcine BVDV strains. One group was consisted of established HoCV strains. The other two groups included HoCV strains and unidentified pig origin strains.

The extensive antigenic heterogeneity among BVDV strains and isolates has been described by Howard et al. (1987), Xue et al. (1990), Drèze et al. (1991) and Donis et al. (1991). In
contrast to the suggestion that BVDV strains can be divided into distinct serotypes or groups (Castrucci et al., 1968; Fernelius et al., 1971; Magar et al., 1988; Bolin et al., 1988), Howard et al. (1987) reported that an antigenic spectrum, within a single related group, existed for BVDV strains; rather than distinct serotypes.

In order to compare the reactivity of MAbs with strains and isolates of pestivirus available in different laboratories, an international workshop was held at the Hannover Veterinary School (Cay et al., 1989). A total of 74 MAbs from 13 laboratories, including 19 MAbs against HoCV, 42 MAbs to BVDV and 13 MAbs against BDV were used to characterize 43 pestivirus strains. Seven MAbs reacted with all pestivirus strains tested, eight MAbs detected only the seven HoCV strains and three recognized only the 16 BVDV strains. No MAb was found specific for BDV. BVDV and BDV strains were broadly cross-reactive with the MAbs. A close relationship between these two species was readily demonstrated, whereas HoCV strains were characterized as distinct from BVDV and BDV. One of the important findings from this workshop was that the same strain of pestiviruses (NADL and Oregon C24V) from different laboratories and having varying passage histories, displayed numerous minor antigenic differences as evidenced by their different reactivity with some of the MAbs. A high frequency of genomic mutations was proposed to be responsible for this intra-strain antigenic variation (Cay et al., 1989).
The MAbs, available at present, are directed to only a few apparently immunodominant viral proteins, i.e. the major glycoprotein gp53, the minor glycoprotein gp48, and the nonstructural protein p125/p80.

Most MAbs directed against gp53 of BVDV and HoCV were shown to neutralize virus (Donis et al., 1988; Donis et al., 1991; Paton et al., 1991; Wensvoort, 1989c; Bolin et al., 1988; Onisk et al., 1991), suggesting that gp53 is one of the envelope glycoproteins of BVDV or HoCV. Consequently, most antigenic variations on the interspecies and to a lesser extent on the intraspecies level were observed on the gp53. Some of MAbs against gp53 of BVDV and HoCV lack neutralizing activity (Donis et al., 1988; Greiser-Wilke et al., 1990; Paton et al., 1991; Moening et al., 1989; Magar et al., 1988).

Most MAbs against gp48 were directed against rather conserved domains (Peters et al., 1986; Greiser-Wilke et al., 1991). This result was consistent with the genomic sequence data that demonstrated the gp48 sequence was more conserved than gp53 sequence (Moormann et al., 1990). The antigenic variation of the gp48 of BVDV and HoCV was also demonstrated by Paton et al. (1991) and Weiland et al. (1992). Some MAbs against gp48 possessed some neutralizing activity (Weiland et al., 1992). The presence of gp48 on the virion surface was demonstrated by immunogold electron microscopy (Weiland et al., 1992). In addition, some of the MAbs seem to identify an
antigenic marker on gp48 associated with cytopathogenicity of BVDV (Peters et al., 1986; Greiser-Wilke et al., 1991).

MAbs against the nonstructural p125 of pestiviruses were generally directed against very conserved antigenic structures and their reaction spectrum was characterized as panpestivirus specific (Edwards et al., 1989; Paton et al., 1991; Moennig and Plagemann, 1992). These results are in accord with genomic sequence data indicating strong homologies in the respective regions of pestivirus genomes (Collett et al., 1989; Moormann et al., 1990).

The epitope mapping was mainly done with gp53 of BVDV and HoCV. Extending the work of Bolin et al. (1988), an epitope map of the BVDV gp53 was proposed by Moennig et al. (1989) using 47 pestiviruses in competitive binding assay with MAbs specific for 10 different epitopes. A total of 8 epitopes were relevant for neutralization and 7 of them were clustered in one antigenic domain, whereas a single epitope was located outside this domain. In these studies, binding of a single MAb species was sufficient for virus neutralization.

Wensvoort (1989c) proposed a topographical and functional map of the HoCV gp53 using 13 MAbs recognizing different epitopes of the Brescia strain. Four distinct domains (A, B, C, D) were identified by competitive binding studies, antigen capture assays, neutralization test and isolation of neutralization escape mutants. Domain A was subdivided into
A1, A2, A3. Domain A1 and A2 were shown to be highly conserved for HoCV, whereas A3, B, C and D displayed some variability.

The relationship between antigenicity and biotype of BVDV was subject to confusion. Fernelius (1964) compared the antigenicity of CP and NCP BVDV by serum neutralization test and immunofluorescent assay. Differences of intensity of fluorescence and neutralization titers between CP and NCP existed. The cross-neutralization of CP and NCP strains of BVDV with homologous and heterologous antisera revealed that the serotypes were also biotypically related to the cytopathic effect, and the biotype and serotype of BVDV were postulated to be genetically linked (Fernelius et al., 1971). However, these results were contrary to the data obtained by a number of investigators. Using complement-fixation tests, Gutekunst and Malmquist first (1964) reported that CP and NCP BVDV were antigenically similar. Howard et al. (1987) used cross-neutralization tests to compare the antigenicity of pairs of CP and NCP BVDV isolated from animals that died of MD. The results revealed that each pair of CP and NCP BVDV strains from the same outbreak was antigenically indistinguishable. In contrast, when comparisons were made between isolates from different outbreaks, significant antigenic differences were observed. This excellent work led to the present understanding of the relationship between antigenicity and biotype of BVDV. Mainly, antigenic variations exist among BVDV strains regardless of cytopathogenicity, and a pair of CP and NCP BVDV
strains was antigenically indistinguishable. This observation was consistent with the hypothesis that CP BVDV strains from natural outbreaks of MD arise by mutation from the homologous NCP BVDV strains. Thereafter, similar observations were obtained by other investigators. Donis et al. (1988) described that MAbs against a set of NCP BVDV isolates gave radioimmunoprecipitation and serum neutralization results essentially identical to those obtained with CP BVDV strains, suggesting that, for BVDV, biotypic and antigenic variation are two independent variables. Bolin et al. (1988) identified MAbs reactive to both CP and NCP BVDV, indicating that there is little correlation between viral biotype and the pattern of reactivity with MAbs. The indistinguishable antigenicity between CP and NCP BVDV was also reported by Magar et al. (1988). Four CP BVDV strains and five NCP BVDV strains reacted with the same MAb as showed by immunofluorescent staining.

Meanwhile, biotype specific MAbs were reported by some investigators. Peters et al. (1986) identified two MAbs specific for CP BVDV strains tested. Bolin et al. (1988) described the MAbs specific for either CP BVDV or NCP BVDV. Extending the work of Peters et al. (1986), Greiser-Wilke et al. (1991) characterized the antigenic differences between CP and NCP BVDV strains by an enzyme immunoassay on fixed infected cells. In the majority of cases, the MAb could discriminate between cells infected with each of the two viral biotypes by different reactivity patterns. It is important to
note that only limited strains were used to test the biotypic specificity of the MAbs. Therefore, if the number of BVDV strains tested was increased, the "biotype specific" MAbs may not remain biotype specific. This occurred with one of the MAbs described by Peters et al. (1986); although originally reported to be CP BVDV specific (Peters et al., 1986), it was later found to react with a number of NCP BVDV isolates (Edwards et al., 1988).

In summary, all three species of pestiviruses: BVDV, BDV and HoCV are antigenically related. BVDV and BDV are more closely related to each other, whereas HoCV is a distinct viral entity, easily discriminated by a number of MAbs or cross-neutralization tests. Both antigenic homology and extensive heterogeneity exist among different strains of BVDV. Although serotypes or groups were proposed to classify BVDV strains, there have been no consistent results defining these groups or serotypes. As proposed by Howard et al. (1987), it is generally accepted that a relatively broad spectrum of overlapping antigenic variation exists for BVDV, rather than distinct serotypes or groups. Viral biotype and antigenicity are two independent variables and there is little correlation between these two properties of BVDV. The major glycoprotein gp53 is the immune dominant protein. A number of epitopes that elicit neutralizing antibodies are present on this protein. In addition, extensive antigenic variation of this protein is present. The minor glycoprotein gp48 also has neutralizing
epitopes, although the majority of the MAbs against the gp48 have no neutralizing activity. The p80 is an immunodominant protein and MAbs against this protein have panreactivity with different strains but no neutralizing activity.

LABORATORY DIAGNOSIS AND VACCINATION

Virus Detection

A number of laboratory techniques are applicable for the diagnosis of all pestiviruses. However, current routine procedures are tedious, expensive and often inaccurate, due to the cross-antigenic relationship between pestiviruses and difficulties in their handling (Carbrey, 1988; Liess, 1988.)

During the initial phase of BVDV research, inoculation of susceptible cell lines was the only and most reliable method to isolate and diagnose BVDV. Viruses can be isolated from buffy coat cells or organ suspensions of viremic animals. Because some BVDV strains are the NCP biotype, conjugated antibodies with fluorescent dyes or enzymes are a prerequisite for the identification of viral isolates (Bolin et al., 1991; Moennig and Plagemann, 1992). A major improvement in the diagnosis of BVDV was the development of MAbs with defined properties, allowing a reliable and easy identification and discrimination of BVDV from other pestiviruses (Wensroort et al, 1986; Hess et al., 1988).

Persistently infected animals are an efficient reservoir for spreading the BVDV in herds (Bolin et., 1985; Howard et
al., 1986; Peters et al., 1987). In general, viremic animals are seronegative, therefore virus detection is the only method for diagnosis of BVDV in these animals. The conventional isolation of BVDV from buffy coat cells is a reliable and sensitive, though time-consuming technique. Enzyme immunoassays were developed for the direct demonstration of viral antigen in lysed peripheral blood lymphocytes of cattle (Fenton et al., 1990; Fenton et al., 1991; Mignon et al., 1991; Moenning and Plagemann, 1992). In these studies, the broadly reactive MAbs against the conserved p125/p80 were used as capture antibodies to coat microtiter plates. Because of the excellent correlation with standard cell culture isolation, these tests are promising new tools for a rapid and reliable diagnosis for BVDV carrying animals.

With cDNA clones and sequence data of BVDV available, detection of viral nucleic acids has become an additional method for laboratory diagnosis (Brock et al., 1988; Potgieter and Brock, 1989; Brock and Potgieter, 1990; Brock, 1991; Kwang et al., 1991; Lewis et al., 1991; Cruciere et al., 1991). The application of the polymerase chain reaction (PCR) also proved to be useful for the diagnosis of BVDV (Schroeder and Balassuchan, 1990; Brock, 1991; Ward and Misra, 1991; Boye et al., 1991; Hertig et al., 1991; Belak and Ballagi-Pordany, 1991; Roehe and Woodward, 1991; Van Iddekinge et al., 1992).
Antibody Detection

An accurate serodiagnostic procedure is the prerequisite of any measure for the control and/or eradication of BVDV. Standard techniques for the serodiagnosis of BVDV are based on the demonstration of neutralization antibodies (Holm-Jensen, 1981; Liess, 1988; Bolin et al., 1991; Meonnig and Plagemann, 1992). The development of an enzyme-linked immunosorbent assay (ELISA) was hampered by the difficulty in producing a sufficiently purified test antigen. Recently, ELISAs for the diagnosis of BVDV antibodies have been described (Chu et al., 1985; Straver et al., 1985; Howard et al., 1985; Katz and Hanson, 1987). The expression product of a 2200-nucleotide fragment of BVDV, including part of the p125 gene, has been successfully employed in an ELISA (Lecomte et al., 1990).

Vaccination

Because of the world wide presence of BVDV, vaccination plays an important role in the prevention of BVDV. Both inactivated and live virus vaccines are available (Baker, 1987). Most live vaccine strains have been attenuated through cell culture passage (Coggins et al., 1961). Although vaccination with modified live vaccines provides protection from BVDV infections, there are a few disadvantages associated with the use of modified live vaccines (Peter et al., 1967; Lambert, 1973). The response to vaccine virus resembles a mild natural infection and the animals's immune functions might be
suppressed temporarily (Roth and Kaeberle, 1983). It was recommended that pregnant animals must not be vaccinated with modified live vaccines because the vaccine virus might cross the placenta, infecting the fetus. A temperature sensitive mutant has been developed to reduce the risk of intrauterine spread of the vaccine virus (Lobman et al., 1984; 1986). Because most vaccine viruses are CP BVDV, vaccination of persistently infected animals with live vaccine might induce MD if there is antigenic homology between the endogenous NCP BVDV and the vaccinated CP BVDV (Bolin et al., 1985a). Neither subunit vaccines nor engineered vaccines have been described for BVDV. There is substantial need for the development of improved vaccines against BVDV.

COMPARISON OF CP BVDV WITH NCP BVDV

Since the recognition of both CP and NCP BVDV in the middle of 1950s (Baker et al., 1954; Underdahl et al., 1957; Gillespie et al., 1960), great progress has been made in understanding the properties of both biotypes. Inoculation of both CP and NCP BVDV into healthy cattle produced a similar mild postnatal infection (Pritchard, 1963; Thomson and Savan, 1963). A pair of CP and NCP BVDV isolated from naturally occurring MD was antigenically indistinguishable (Howard et al., 1987; Donis et al., 1988; Bolin et al., 1988; Magar et al., 1988). These observations indicated that both CP and NCP BVDV share similar properties. On the other hand, the
differences between CP and NCP BVDV are also obvious. First, CP BVDV causes cytopathic effects on cell cultures and destroys the cells, whereas NCP BVDV grows on cell cultures without visibly causing CPE. Second, only NCP BVDV is capable of establishing persistence following in utero infection (Von Borgen and Dinter, 1961; Dinter et al., 1962; Malmquist, 1968; Kendrick, 1971). CP BVDV has never been recovered from persistently infected animals (Moennig and Plagemann, 1992). Third, when an animal persistently infected with NCP BVDV is superinfected with a antigenically homologous CP BVDV, a fatal MD occurs (Brownlie et al., 1984; Bolin et al., 1985; Brownlie et al., 1987). However, there has been no evidence that superinfection of persistently viremic animals with NCP NVDV could produce MD. Fourth, viral protein analysis indicated that the nonstructural protein p80 is only present in CP BVDV and has never been detected in NCP BVDV (Pocock et al., 1987; Donis and Dubovi, 1987; Magar et al., 1988; Collett et al., 1988b; Meyers et al., 1991). Fifth, nucleotide sequence comparisons between CP BVDV and HoCV led to the identification of a cellular RNA insertion in both CP BVDV strains, Osloss and NADL, at the N-terminal portion of p125 (Meyers et al., 1989; Collett et al., 1989).

With respect to the main issue about the origin of CP BVDV and NCP BVDV, at present one hypothesis is that CP BVDV originates from NCP BVDV by genomic mutation (Brownlie et al., 1987; Howard et al., 1987; Corapi et al., 1988). Based on the
identification of a cellular RNA insertion in the CP BVDV strains Osloss and NADL, Meyers et al. (1989) proposed that cellular RNA insertion into NCP BVDV is responsible for the development of CP BVDV. In the case of CP BVDV strain Osloss, the insertion was found to be a 228-nucleotide sequence encoding a ubiquitin monomer (Meyers et al., 1989). The genome of CP BVDV strain NADL, on the other hand, carries a 270-nucleotide insert, which was reported to represent a cellular RNA unrelated to ubiquitin (Collett et al., 1989, Meyers et al., 1989b). The presence of insertions in some CP BVDV strains is in accord with the observations that the pl25 protein of these strains migrates slower in SDS gel electrophoresis than does the pl25 of NCP BVDV and other CP BVDV (Akkina, 1991; Greiser-Wilke et al., 1992). Recently, Meyers et al. (1991) cloned and sequenced partial genomic sequence of a pair of BVDV, CP BVDV strain CP (5.7 kb) and NCP BVDV strain NCP1 (3.2 kb) in the pl25 region. A ubiquitin insert and p80 nucleotide sequence duplication were found in the CP1, but not in the NCP1 within this region. However, whether there are other differences in the remaining region requires the establishment of a complete nucleotide sequence of the NCP BVDV strain.

Probably as a consequence of cellular RNA insertion, pl25 acquires properties that may lead to its cleavage, resulting in the formation of p80 and p54 in CP BVDV (Collett et al., 1988b) and p80 and p41 in CP BVDV Osloss (Meyers et al.,
In case of CP1 strain, only p80 was detected in infected cells, whereas the p54 equivalent protein was not demonstrable. This suggested that p125 was not processed in CP1 and p80 was likely a translation product of the duplicated p80 gene rather than a cleavage product of p125. Therefore, the only characteristic common to these three CP BVDV strains described above is the production of p80 in infected cells, either as a cleavage product of p125 or by translation of a newly generated p80 gene. The cytopathogenicity of BVDV seems to be not dependent on processing of p125 or the presence of a protein like p54 of NADL or p41 of Osloss. The p80 represents the only obvious marker for CP BVDV (Meyers et al., 1991). The finding that these CP BVDV strains carry an insertion in the p125 gene, leading to the production of p80, has led to the notion that such an insertion may be essential for the generation of CP BVDV. However, this hypothesis has been challenged by the observation that some CP BVDV strains lack such insertions (Moerlooze et al., 1990; Desport and Brownlie, 1991; Qi et al., 1992). Using PCR to amplify the region corresponding to the carboxyl-terminal portion of the p125, Moerlooze et al. (1990) confirmed the cellular RNA insertions in CP strains NADL and Osloss but did not find comparable insertions in this region of 5 other CP BVDV strains. Using the same strategy, Desport and Brownlie (1991) amplified and sequenced the p125 coding regions of a "homologous" pair of BVDV, no insertion of host sequence was
observed in that region. However, one of the disadvantages of PCR is that duplications as described by Meyers et al. (1991) may not have been detected, particularly if they spanned large areas. Qi et al. (1992) used the PCR strategy that can detect either insertion or duplication to analyze the possible cellular insertion or viral gene duplication for 4 pairs of CP and NCP BVDV. A large duplication of the p80 gene and a ubiquitin gene insertion was identified in three of the four CP BVDV isolates. However, neither insertion nor duplications were detected in one of the CP BVDV isolates.

In summary, although rapid progress in understanding the cytopathogenicity of BVDV biotypes has been made, much work is still required to conclusively elucidate the mechanism. One of the critical steps is to establish the complete nucleotide sequence of a NCP BVDV.
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Fig. 1. The genome organization of bovine viral diarrhea virus. Open boxes denote the viral nonstructural proteins. Black boxes indicate the viral capsid protein. Shaded boxes represent the viral glycoproteins. Lined box indicates the unidentified viral protein. The genome organization presented was proposed by Collett et al. (1988b, 1991) and modified by Theil et al. (1991).
CHAPTER II

Nucleotide Sequencing of 5' and 3' Termini of Bovine Viral Diarrhea Virus by RNA Ligation and PCR

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most important disease pathogens of cattle that is distributed worldwide. An understanding of the molecular biology of BVDV and other Pestiviruses began crystallizing with the publishing of the nucleotide sequence of a cytopathic strain (CP) of BVDV (NADL strain) (Collett et al., 1988a). Elucidating the pathogenic mechanisms of BVDV infections remains a challenge to BVDV researchers. To date, there are at least two important findings related to the molecular biology of BVDV; (1) biotypic differences between CP and noncytopathic (NCP) strains of BVDV correlate with a dysfunctional insertional mechanism involving the p80 viral protease (Meyers et al., 1991) and (2) there is considerable antigenic and genomic nucleotide sequence diversity among BVDV strains (Bolin et al., 1991a; Ridpath et al., 1991; Kwang et al., 1991).

Due to the complex biology of BVDV, further investigation of the mechanisms of viral replication and viral pathogenesis may depend on the development of an infectious RNA clone for
in vivo experiments. To further develop this potential, the complete nucleotide sequence of the NADL isolate of BVDV must be established. Therefore, confirmation and completion of the 5' and 3' terminal nucleotide sequence was done using RNA ligase and specific primer-directed amplification (PCR) using Taq polymerase.

MATERIALS AND METHODS

Virus stocks and cell culture

Cytopathic BVDV, strain NADL, was kindly provided by Dr. Marc Collett, (Gaithersburg, MD). This strain was stock virus from which the published BVDV NADL nucleotide sequence was determined (Collett et al., 1988a). Another CP BVDV isolate, strain 72, also was used (Smith, 1980). Serum collected from a persistently infected heifer was used for purification of NCP BVDV strain SD-1 genomic RNA. Secondary bovine turbinate (BTU) cells were grown in DEME medium supplemented with 10% horse serum. The horse serum was treated to eliminate the possible contaminated virus and antibody against BVDV. Typically, after thawing at 37°C, the horse serum was heat-inactivated at 56°C for 30 minutes. After cooled to room temperature, 4 ml of 40% (w/v) polyethylene glycol (6000-8000 MW) and 25 μl of β-propiolactone were added into 100 ml of the horse serum and mixed. After 12 hours at room temperature, the horse serum was heated to 37°C for 1 hour and then stored at -20°C for further use.
Extraction of viral RNA

Genomic RNA was extracted from infected supernatant fluids and serum as previously described (Brock, 1991). Confluent BTU cell cultures were inoculated with BVDV at a moi of 0.5 and incubated at 37°C for 36 hours. Infected culture supernatant fluids or serum from a heifer persistently infected with SD-1 were clarified at 10,000 g for 30 minutes at 4°C and pelleted by centrifugation at 110,000 g for 4 hours at 4°C. The viral pellet was resuspended in 1.0 ml of TE buffer (50 mM Tris-HCl and 1 mM EDTA, pH 7.5). To the suspension, 5.0 ml of solution D (4 M guanidinium isothiocyanate; 25 mM sodium citrate, pH 7.0; 0.5 % Sarcosyl; and 0.1 M 2-mercaptoethanol), 0.5 ml of 2 M sodium acetate (pH 4.0), 5.0 ml of phenol, and 1.0 ml of chloroform-isoamyl alcohol (24:1) was added and the mixture was shaken vigorously for 15 seconds and placed on ice for 30 minutes. The mixture was centrifuged at 10,000 g for 30 minutes at 4°C. The aqueous phase was removed and viral RNA was precipitated with the addition of an equal volume of isopropanol and placed at -20°C for 12 hours. The viral RNA was pelleted by centrifugation at 10,000 g for 30 minutes at 4°C, resuspended in 200 µl of diethyl pyrocarbonate (DEPC) treated water, and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was
ethanol precipitated and placed at -70 °C. The extracted BVDV RNA finally was dissolved in 50 μl of DEPC-treated water.

Decapping of RNA

The viral RNA was decapped using tobacco acid pyrophosphatase (TAP) according to manufacturers instructions (Promega Corp., Madison, WI). RNA decapping was done in the following 50 μl reaction mixture: 50 mM sodium acetate (pH 5.3), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 2 mM ATP, and 8 units of TAP. The reaction mixture was incubated at 37 °C for 2 hours and heat inactivated at 65 °C for 7 minutes. Following decapping, the RNA was phenol:chloroform and chloroform extracted, ethanol precipitated, pelleted, and resuspended in 20 μl of DEPC-treated water.

Ligation of viral RNA

Decapped and nondecapped BVDV RNA were ligated using T4 RNA ligase in a reaction mixture of 50 mM HEPES (pH 8.3); 3 mM DTT, 10 mM MgCl₂, 10 % (v/v) DMSO, 10 μg/ml BSA, 2 mM ATP, and 180 μg/ml T4 RNA ligase according to manufacturers instructions (GIBCO/BRL, Gaithersburg, MD). The mixture was incubated for 10 hours at 15 °C and the ligated RNA was phenol:chloroform and chloroform extracted, ethanol precipitated, pelleted, and resuspended in DEPC-treated water.
Primer-directed amplification

Oligonucleotide primers were custom synthesized according to nucleotide sequence information from BVDV strain NADL (Collett et al., 1988a). The upstream oligonucleotide primer (primer 12434) began at nucleotide 12434 and continued for 18 nucleotides to 12451 and was homologous to the genomic RNA. The downstream primer (primer 231) was selected from nucleotide 231 and extended to nucleotide 249 and was complementary to BVDV RNA. The first strand reaction contained 1X reverse transcriptase (RT) buffer (5X RT buffer contains 50 mM Tris-HCl, pH 8.3; 40 mM KCl; 6 mM MgCl$_2$; and 0.1 mg/ml BSA), 1 mM of each deoxynucleotide (dATP, dGTP, dTTP, dCTP), 12.5 units of RNasin (Promega Corp.), 300 ng random hexanucleotides, 200 units M-MLV reverse transcriptase, and extracted BVDV RNA in a reaction volume of 20 µl as previously described (Brock, 1991). The reaction mixture was incubated at 37 ℃ for 45 minutes, heat inactivated at 95 ℃ for 5 minutes, and 80 µl of the following reaction mixture was added: 1X PCR buffer (10X PCR buffer contains 500 mM KCl; 100 mM Tris-HCl, pH 8.3; 15 mM MgCl$_2$), 1.25 µM of primer 12434, 1.25 µM of primer 231, and 2.5 units of AmpliTaq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT). The reaction mixture was initially denatured at 94 ℃ for 2 minutes followed by 30 cycles of the following reaction parameters: template denaturation at 94 ℃ for 1 minute, primer annealing at 55 ℃ for 1.5 minutes, and extension at 72 ℃ for 3 minutes. A final extension step was
done at 72 °C for 7 minutes to complete the amplification reaction. A diagram showing the 5' and 3' ligation and PCR amplification is given in Fig. 2.

5' Terminal tailing and amplification of cDNA

First-strand cDNA was synthesized from genomic RNA using reverse transcriptase and random hexanucleotides as previously described. The first-strand cDNA was homopolymer tailed at the 3' end using terminal deoxytransfersase (TdT) (Roychoudhury and Wu, 1978) and dATP. Conditions used were those that yielded the addition of 15-20 dAMP's at the 3' end. For tailing, the first strand cDNA was incubated for 15 minutes at 37 °C in a reaction volume of 50 μl that consisted of 1X tailing buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 25 mg/ml BSA, 5 mM CoCl$_2$ ), 10 mM dATP, and 30 units of TdT. The reaction was stopped by adding 5 μl of 0.5 M EDTA and heating at 68°C for 5 minutes, and the product was precipitated with ethanol. PCR amplification was done using an oligo-d$_{18}$T primer and primer 231 as previously described. A diagram showing the poly(A)-tailing and PCR amplification is given in Fig. 3.

Preparation of BVDV-specific DNA probe

DNA probe was made by nick translation using pBV-18 NADL cDNA clone which has the insertion sequence of BVDV from nucleotides 24 to 1308, kindly provided by Dr. Marc Collett (Collett, 1988a). The cDNA clone was first digested with
restriction enzyme Pst I, and the insert was separated by electrophoresis in a 1% agarose gel in 1X Tris-acetate (TAE) buffer (40 mM Tris-acetate and 10 mM EDTA). The BVDV cDNA specific bands were excised for electroelution. Using a Model 422 Electro-Eluter (Bio-Rad, Richmond, CA), the elution was done at 15 mA/glass tube with constant current for 1 hour in 1X TAE buffer. After reversing the polarity for approximately 1 minute to remove the DNA from the membrane, the tube was removed and the buffer remaining the tube was discarded. The liquid remaining in the membrane cap was transferred to a microfuge tube, extracted once with an equal volume of phenol/chloroform (1:1) and once with chloroform. After centrifugation for five minutes, the aqueous phase was transferred to a fresh microfuge tube and precipitated by addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold (-20°C) ethanol. The tube was placed at -70°C for 1 hour and the DNA was pelleted by centrifugation at 16,000 g for 15 minutes at room temperature. After washing with 70% of cold (-20°C) ethanol once, the DNA pellet was dried under vacuum and resuspended in TE buffer (50 mM Tris-HCl and 1 mM EDTA, pH 7.5). The DNA was quantitated by spectrophotometric measurement at 260 nm. Using purified DNA and a nick translation kit (Promega, Madison, WI), BVDV specific probe was made in the following reaction conditions; in a total volume of 50 μl containing 1 μg of DNA, 50 mM Tris-Cl (pH 7.2), 10 mM MgSO₄, 0.1 mM DTT, 50 μM dATP, 50 μM dGTP,
50 μM dTTP, 10 mCi/ml of [α-32P]dCTP (Amersham, Arlington Heights, IL), 5 units of DNA polymerase and 1 ng of DNase I. After incubation of the mixed solution at 15°C for 1 hour, the reaction was stopped by addition of 5 μl of stop solution (0.25 M EDTA, pH 8.0). The unincorporated dNTPs were removed by self-prepared spun column of Sephadex G-50. Briefly, a hole was made at the bottom of the microfuge tube with a 18 gauge needle and the bottom of the tube was plugged with a small amount of sterile glass wool. After filling with Sephadex G-50 equilibrated in the TE buffer (50 mM Tris-HCl, 1 mM EDTA), the microfuge tube was inserted into a 15-ml glass tube and the resin was packed down by centrifugation at 1,600 g for 5 minutes at room temperature in a swinging-bucket rotor. After more resin was added to the microfuge tube, the resin was packed down again by centrifugation at 1,600 g and washed once with 100 μl of TEN (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 100 mM NaCl). After the spun column was placed in a fresh microfuge tube, a total volume of 100 μl of the DNA probe sample (using 1X TEN buffer to make up the volume) was applied to the column, followed by centrifugation at 1600 g for five minutes. Finally, the eluted DNA was collected and stored at -20°C until needed.

Analysis of PCR products

Following amplification, the products were characterized by electrophoresis in 1% agarose gel in 1X TAE buffer. The
virus-specificity of the amplified products were determined by Southern blots hybridization with BVDV specific DNA probe. After the PCR products were separated on 1% agarose gel, the DNA bands were transferred to nitrocellulose membranes using the alkaline vacuum transfer method with a VacuGene XL Vacuum Blotting System (Pharmacia LKB Biotechnology, Piscataway, NJ). Briefly, the gel was gradually placed onto the nitrocellulose membrane which was previously wet with distilled water and placed under the plastic mask so that each side of the membrane overlapped the plastic mask by approximately 5 mm. After a stable vacuum was obtained between 50 and 55 mbar, approximately 50 ml of 0.2 N HCl solution were pipetted onto the gel. After approximately 30 minutes when the bromophenol blue turned yellow, the excess liquid on the gel was removed, followed by the addition of enough 1 M NaOH to the blotting chamber to cover the gel to twice its depth. After 1 hour, the vacuum was released and the membrane was removed from the blotting system, washed with 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M trisodium citrate, PH 7.0) for 10 minutes to eliminate agarose, stored at room temperature until it became dry and baked at 80°C for 45 minutes. The membrane was prehybridized for 8 h at 42°C in a solution of 5X SSC, 50% formamide, 1X Denharts (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 1% SDS and 25 μg/ml of denatured salmon sperm DNA. The hybridization was performed at 42°C for 14 hours in the prehybridization solution containing BVDV-specific DNA probe.
which was denatured by boiling for 3 minutes before it was added to the prehybridization buffer. Following the hybridization, the membrane was washed twice for 5 minutes with 2X SSC and 0.1% SDS at 42°C, and twice for 15 minutes with 0.1X SSC and 0.1% SDS at 68°C. Then the washed membrane was laid on a piece of Whatman paper, taped on the paper and covered with a piece of wrap. Autoradiography was done by exposing the membrane to X-ray film (Kodak) for 6 hours at -70°C and developed.

**Molecular cloning**

Following PCR amplification and identification of the virus-specific DNA band, the size-specific PCR product was purified by electroelution as described previously. To determine the optimal method for cloning the PCR products, three methods were applied; blunt ligation, linker addition and C-tailing.

a. blunt ligation: The pGEM-3z(+) (Promega, Madison, WI) vector was linearized with restriction enzyme Sma I in the following reaction conditions: 1X reaction buffer, 5 µg of plasmid DNA, 100 µg/ml of acetylated BSA, 25 units Sma I enzyme, in a total volume of 50 µl, at room temperature for 4 hours. The reaction was stopped by heating to 75°C for 10 minutes. To remove the 5′-phosphate of the plasmid, 10 µl of 10X calf intestinal alkaline phosphatase (CIAP) buffer, 1 µl of 1 unit/µl CIAP (Promega, Madison, WI) and 39 µl of
distilled sterile water were added directly to the digestion solution. After incubation at 37°C for 1 hour, the reaction was stopped by adding 2 μl of 0.5 M EDTA (pH 8.0). The sample was extracted with equal volume of phenol/chloroform once and chloroform once. The plasmid DNA was ethanol-precipitated, pelleted, washed with 70% of cold (-20°C) ethanol, dried, resuspended in 30 μl of TE buffer and stored at -20°C until needed. A blunt ligation reaction was set up as following: 1X ligase buffer, 100 ng of Sma I linearized pGEM plasmid DNA, 40 ng of purified PCR product (about 450 bp), 1 unit of DNA ligase (GIBCO BRL, Gaithersburg, MD), in a total volume of 10 μl, at room temperature for 4 hours. After ligation reaction, the sample was placed on ice ready for transformation.

b. linker addition: The purified PCR product was first treated with Klenow fragment (Promega, Madison, WI) in the following conditions: 5 μg of PCR product, 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 0.5 mM DTT, 10 μg/ml BSA and 6 units Klenow enzyme in a total volume of 30 μl, at room temperature for 30 minutes. The reaction was stopped by extraction of the sample with phenol/chloroform and chloroform. The DNA was recovered as described above and resuspended in 10 μl of TE buffer. To add the Pst I linker to the PCR product, 2 μg of Klenow treated DNA was incubated in 1X ligation buffer, 100 μg/ml BSA, 0.02 A₂₆₀ units of Pst I linker and 3 units DNA ligase, at 15°C for 15 hours. The reaction was stopped by heating at 75°C for 10 minutes. The DNA was recovered, resuspended and
digested with restriction enzyme Pst I as described before. Meanwhile, the pGEM vector was linearized with Pst I enzyme and treated with CIAP as described previously. The ligation reaction was performed in a total volume of 10 µl of the mixture containing 1X ligase buffer, 100 ng plasmid DNA, 20 ng PCR product and 1 unit DNA ligase. The mixture was incubated at room temperature for 1 hour and then placed on ice until needed for transformation.

c. C-tailing: The purified PCR product was C-tailed at the 3' end in the solution containing 1 µg purified PCR product, 200 mM potassium cacodylate, 25 mM Tris-HCl (pH 6.6), 250 µg/ml BSA, 0.75 mM cobalt chloride, 5 µM dCTP and 50 units terminal transferase (Boehringer Mannheim, Indianapolis, IN), at 37°C for 20 minutes. After the reaction was stopped by heating at 75°C for 10 minutes, the sample was extracted with phenol/chloroform and chloroform, and the DNA was ethanol-precipitated, recovered and resuspended in 10 µl of TE buffer. The C-tailed PCR product was then annealed to Pst I-digested, G-tailed pUC 9 vector (Pharmacia, Piscataway, NJ) in a reaction volume of 10 µl consisting of 10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 100 ng of pUC 9 plasmid DNA, 20 ng of PCR product. The mixture was incubated at 65°C for 5 minutes, at 58°C for 3 hours, then gradually cooled to 4°C over 30 minutes and finally maintained at 4°C for at least 1 hour before use for transformation. A diagram showing the G/C-tailing method for cloning PCR products is given in Fig. 4.
After the PCR product was ligated with or annealed to plasmid vectors, transformations were done using *E. coli* strain JM109 (Promega, Madison, WI) based on the method of Hanahan (Hanahan, 1983). Typically, the stock cells were spread on the YT plate\(^a\) and incubated at 37°C overnight. A colony was picked with sterile loop, dispersed in 1 ml of SOB medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl\(_2\), 10 mM MgSO\(_4\)) and used to inoculate 30 ml SOB medium in a 300 ml flask. The cells were incubated at 37°C with shaking at 225 revolutions/minute until a density of 5 \(\times\) 10\(^7\) colony forming units/ml was reached. After the bacterial cultures were transferred to a 50 ml polypropylene tube and placed on ice for 15 minutes, the cells were collected by centrifugation at 1,200 \(g\) for 12 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 1/3 volume of transformation buffer (TFB) (10 mM potassium-2-[N-morpholino] ethanesulfonic acid pH 6.2, 0.1 M RbCl, 45 mM MnCl\(_2\)-4H\(_2\)O, 10 mM CaCl\(_2\)-2H\(_2\)O, 3 mM HACOCl\(_3\)) and placed on ice for 10 minutes. The suspension was centrifuged at 1,200 \(g\) for 10 minutes at 4°C and the pellet was resuspended in 1/12.5 of original cell volume of TFB and aliquoted into 200 \(\mu\)l in the microfuge tubes. DMSO was added to obtain a final concentration of 3.5% and the mixture was placed on ice for 5 minutes. DTT was added to obtain a concentration of 75 mM and the mixture was placed on ice for 10 minutes. Then the same amount of DMSO was added and the
cell suspension was placed on ice for another 5 minutes and transferred to a chilled 17 mm x 100 mm polypropylene tube. Next, 10 µl of the ligated or annealed DNA was added as previous and the mixtures were incubated on ice for 30 minutes, followed by heat pulse at 42°C for 90 seconds. After the samples were cooled on ice for 2 minutes, 800 µl of SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) at 20°C was added. The mixtures were incubated at 37°C for 1 hour with shaking at 225 rpm. Finally, 80 µl of the transformed cell suspension was spread on each X-gal plate with a bent pasteur pipette, followed by the incubation of the plates at 37°C overnight to establish colonies. To determine the transformation efficiencies of the different cloning methods, both white and blue colonies were counted. White colonies were picked up, inoculated on the fresh X-gal plates, incubated at 37°C for 12 hours and stored at 4°C ready for screening.

**Colony screening**

A nitrocellulose filter was placed on the surface of the plate and removed when wet. The bacterial cells sticking to the filter were lysed by placing the filter on 10% SDS for 3 minutes and then on Southern solution 1 (0.5 M NaOH and 1.5 M NaCl) for 5 minutes. After exposing to Southern solution 2 (3 M NaCl and 0.5 M Tris-HCl, pH 7.0) for 5 minutes, the filter
was washed twice with 2X SSC, each for 5 minutes, air-dried at room temperature and then baked at 80°C for 1 hour. The prehybridization and hybridization were performed as described before. Positive colonies were identified based on the hybridization signals. To store the positive clones, an equal volume of 40% glycerol YT solution was added to the overnight bacterial culture and the mixture was placed at -70°C until needed.

Preparation and identification of plasmid DNA

Bacteria from positive colonies were inoculated into 5 ml of YT broth (8 g Bacto tryptone, 5 g Bacto yeast extract and 5 g NaCl/liter) supplemented with ampicillin (0.1 mg/ml). Mini-plasmid preparations were carried out according to the procedure described by Sambrook et al. (1989). After overnight incubation at 37°C, 1.5 ml of bacterial culture was transferred to a microfuge tube and the cells were pelleted by centrifugation at 16,000 g for 30 seconds. The cell pellet was resuspended in 100 μl of 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. After incubation at room temperature for 5 minutes, 50 μl of 2 mg/ml of RNAse was added, followed by addition of 200 μl of 0.2 N NaOH and 1.0% SDS and the mixture was placed on ice for 5 minutes. Then 150 μl of ice-cold 3 M KOAc was added and mixed. After another 5 minutes incubation on ice, the microfuge tubes were spun at 16,000 g for 5 minutes at room temperature and the aqueous phase was
transferred to a fresh microfuge tube. The plasmid DNA was ethanol-precipitated, pelleted, resuspended in 50 μl of distilled sterile water and stored at -20°C. The plasmid DNA was digested with restriction enzyme Pst I as described previously and analyzed by 1% agarose gel electrophoresis.

**Nucleotide sequencing**

The double stranded plasmid DNA was sequenced using a sequencing kit (U. S. Biochemical, Cleveland, OH) according to the procedure recommended by the manufacture.

a. Denaturing plasmid DNA: 5 to 10 μg of plasmid DNA was denatured in 0.2 M NaOH at room temperature for 10 minutes. The mixture was neutralized by adding 0.4 volume of 5 M NH₄OAc and the DNA was precipitated by adding 4 volumes of cold (-20°C) ethanol. After washing the pelleted DNA with 70% of ethanol, it was redissolved in 7 μl of distilled water, 2 μl of Sequenase reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂ and 250 mM NaCl) and 1 μl of 0.5 pmol/μl of primer.

b. Annealing template and primer: The capped sample tube was warmed to 65°C for 5 minutes and was cooled slowly to room temperature over a period of about 30 minutes. Once the temperature was below 30°C, annealing was complete and the tube was placed on ice ready for next step.

c. Labeling reaction: To the annealing DNA mixture, 1 μl of DTT (0.1 M), 2 μl of 1:5 diluted labeling Mix (5X concentrated mix was 7.5 μM dGTP, 7.5 μM dCTP and 7.5 μM
dTTP), 0.5 μl of 10 uCi/ml of [³⁵S]dATP (Amersham, Arlington Heights, IL) and 2 μl of 1:8 diluted sequenase (1.6 units/μl) were added. The mixture was incubated at room temperature for 2-5 minutes.

d. Termination reaction: Four tubes were labeled A, C, G and T, respectively. 2.5 μl of the ddATP termination mix (80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, 8 μM ddATP and 50 mM NaCl) was added to the tube labeled A. Similarly, 2.5 μl of the ddCTP (80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, 8 μM ddCTP and 50 mM NaCl), ddGTP (80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, 8 μM ddGTP and 50 mM NaCl) and ddTTP termination mixes (80 μM dATP, 80 μM dCTP, 80 μM dGTP, 80 μM dTTP, 8 μM ddTTP and 50 mM NaCl) were added to the C, G and T labeled tubes, respectively. After capping the tubes, the samples were pre-warmed at 37°C for at least 1 minute. Then 3.5 μl of the labeling reaction solution was transferred to each termination tube. The mixture was incubated at 37°C for 5 minutes. To stop the reaction, 4 μl of the stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) was added to each tube. The samples were stored at -20°C until the sequencing gel was run.

e. Preparation of sequencing gel: To make an 8% denaturing polyacrylamide gel, 48 ml of 20% acrylamide stock and 72 ml of urea stock were mixed. When ready to pour the gel, 1.2 ml of a freshly prepared 10% NH₄ persulfate solution and 50 μl of TEMED solution were added. After mixing the
solution for a few seconds, the acrylamide was poured slowly to fill the plates. A comb was inserted and several clamps were placed at the top and both sides of the plates to hold the comb. The gel was polymerized within 1 hour.

f. Running sequencing gel: When the polymerization of the gel was complete, the clamps were taken off, the gel mold was attached to the electrophoresis apparatus and the comb was removed carefully. After both the top and bottom reservoirs were filled with 1X TBE buffer (90 mM Tris-borate and 4 mM EDTA pH 8.0), the gel was pre-warmed at 40 mA for about 1 hour. After heating the sequencing reactions at 80°C for 3 minutes, 3 μl of each sample was loaded into adjacent wells. The gel was run at 35 mA. When the xylene dye had migrated out of the gel (about 5-6 hours), the second loading of the sequencing samples was applied, followed by another 4 hour running of the gel at 35 mA.

g. Autoradiography of the sequencing gel: After the electrophoresis was complete, the gel mold was removed from the apparatus, the plates were separated, the gel was transferred to a shallow bath containing the gel fixation solution (10% methanol and 10% acetic acid in water) and fixed for 20 minutes. Then the gel was lifted out of the fixation fluid, a piece of Whatman paper was placed on the top of the gel and the gel was removed from the plate, covered with a piece of wrap and dried at 80°C under vacuum overnight. After
exposure of the gel to X-ray film for 18-36 hours, the film was developed in Kodak Developer.

h. Reading and analyzing the sequences: The sequences were read from the bottom to top. Each sequence was read twice to eliminate the possible reading error. The sequence close to the primer (the first 20 nucleotides) was not able to read. From each reaction, 300 to 350 nucleotide sequence could be obtained. To analyze the sequences, the sequence data were entered into the HIBIO DNASIS program (Hitachi Software Engineering America, Ltd., Brisbane, CA). The homologies of the obtained sequences with the published BVDV NADL genomic sequence were determined using DNASIS computer program (Lipman and Pearson, 1985).

RESULTS

Following ligation of the 5' and 3' termini the amplification produced a band of approximately 450 bp in length. Similar migration patterns were obtained for the CP NADL, CP 72, and NCP SD-1 strains. The amplified products from all 3 isolates hybridized with the pBV-18 NADL cDNA clone. Amplification was successfully obtained with decapped genomic RNA and also genomic RNA that was not previously decapped. The nucleotide sequence of the ligation product from the NADL strain was 100% homologous with the previously published nucleotide sequence (Collett, 1988a). However, an additional nucleotide sequence "CCCCC" was found at the point of 5' and
3' ligation (Fig. 5). Following amplification and sequencing of the CP strain 72 and NCP SD-1 BVDV genomic regions, the additional nucleotide sequence ("CCCCC") also was identified at the ligation point. This indicated that an additional nucleotide sequence "CCCCC" existed at either the 5' and/or 3' terminus of the BVDV genome. The identical nucleotide sequence was obtained from the 3 isolates when genomic RNA was not decapped using TAP prior to ligation compared with RNA that was decapped with TAP.

To determine the distribution of the "CCCCC" nucleotide sequence, the 5' terminus of the first strand cDNA was tailed with dATP using TdT and amplified using the 231 primer and oligo-d18T. Using this strategy, the 5' sequence was found to not begin with any dCTP nucleotides, but that the 5' terminus was identical to that previously reported by Collett et al., 1988b. This result indicated that the additional "CCCCC" sequence identified at the point of 5' and 3' ligation was the authentic sequence at the 3' end of the BVDV genome.

The cloning efficiencies of the PCR products using blunt ligation, linker addition and C-tailing methods were given in the Table 2. No positive colony was found using the blunt ligation method and only 2.3% of the colonies were identified to be positive using the Pst I linker addition method. The highest cloning efficiency was obtained when the C-tailing strategy was used and 31.1% of the colonies were found to be positive clones.
Table 2. Cloning efficiencies of PCR products with different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Total colonies</th>
<th>Blue colonies</th>
<th>White colonies</th>
<th>Positive colonies</th>
<th>% positive colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ligation</td>
<td>146</td>
<td>132</td>
<td>15</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>Blunt ligation</td>
<td>135</td>
<td>118</td>
<td>17</td>
<td>0</td>
<td>0 %</td>
</tr>
<tr>
<td>Linker addition</td>
<td>848</td>
<td>707</td>
<td>141</td>
<td>16</td>
<td>2.3 %</td>
</tr>
<tr>
<td>C-tailing</td>
<td>571</td>
<td>405</td>
<td>166</td>
<td>132</td>
<td>31.1 %</td>
</tr>
</tbody>
</table>

DISCUSSION

No information has been published on the termini of pestivirus RNA to date with the exception of unpublished observations (Collett et al., 1988a) (Collett et al., 1989). Although a cap structure has been identified with the flaviviruses (Chambers and Rice, 1987), no information has been published concerning whether BVDV possess a cap structure. The terminal nucleotide sequence of several tick-borne flavivirus genomes has been determined using TAP, RNA ligase, and primer-directed amplification as described in this study (Mandl et al., 1991).

The nucleotide sequence obtained from the NADL strain was 100% homologous with the nucleotide sequence reported by Collett et al., 1988b. From the data obtained in this report, the 3' terminus of the NADL BVDV genome must include an additional 5 cytosine nucleotides ("CCCCC"). This would increase the number of nucleotides in the complete genome of CP NADL to 12,578. The 5' termini nucleotide sequence as reported by Collett et al., 1988b was confirmed. It is
important to note that the additional sequences also were identified in other NCP (SD-1) and CP (72) BVDV isolates in addition to the CP NADL initially reported by Collett et al., 1988b.

The conclusion that the nucleotide sequence "CCCCC" was from the 3' terminus was made following the results of the dATP tailing and amplification. Although the cDNA was ethanol precipitated prior to dATP tailing, low levels of contaminating free nucleotides (dGTP, dCTP, and dTTP) were incorporated at random into the tailing reaction. The addition of these free nucleotides was found to be random and no additional consensus nucleotide sequence was identified on the 5' termini from the previously reported 5' terminal nucleotide sequence (Collett et al., 1988a). To verify that random incorporation had occurred, 5 independent clones from 2 different reactions were sequenced.

Recently, the re-classification of Pestiviruses has been considered as a member of the Flaviviridae due to similar genomic organization and replication strategies (Collett et al., 1989). Although the Flaviviridae are known to possess a terminal cap structure, to date, no cap structure has been identified for BVDV. Different reactivity of modifying enzymes have been reported (Collett et al., 1988a). The 5' terminus failed to be radiolabelled with $[^{32}P]$ATP using polynucleotide kinase with or without previous phosphatase treatment (Collett et al., 1988c) (Collett, et al., 1989).
Making the assumption that the BVDV genomic RNA was capped, a decapping step was done prior to RNA ligation using TAP. However, identical amplification products as well as nucleotide sequence data was obtained when the RNA ligation was done without a previous decapping reaction for all three strains of BVDV (CP NADL, CP 72, and NCP SD-1). It may be argued that incomplete replicative RNA intermediates lacking a cap structure may have been present and were ligated and amplified. This was not probable due to the fact that virus from CP NADL and CP 72 were purified from supernatant fluids prior to the development of cytopathic effect. Additionally, NCP SD-1 virus was purified from serum collected from a persistently-infected animal which should not have contained incomplete RNA replicative intermediates. Although this is indirect evidence these results would suggest that BVDV genomic RNA may not possess a cap structure at the 5' end of the genome.

In conclusion, the RNA ligation and amplification strategy used in this report is useful for determining the 5' and 3' terminal nucleotide sequences of viral genomes. The complete nucleotide sequence of CP NADL BVDV includes an additional "CCCCC" sequence at the 3' terminus in addition to the 12,573 nucleotides reported by Collett et al., 1988a. Indirect evidence would suggest that BVDV may not posses a cap structure although further studies must be done for direct determination.
REFERENCES


FOOTNOTES

a. Preparation of YT plates: 8 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl and 15 g agar were added to a 2 liter flask and q.s. to 1 liter with Milli-Q-filtered water. The mixture was autoclaved 15 lb/in² (121°C, 256°F) for 30 minutes. When the medium was removed from the autoclave, it was gently swirled to distribute the melted agar evenly throughout the solution. About 30 ml of the solution was poured into each plates. When the medium became hardened completely, the plates were inverted and stored at 4°C until needed.

b. Preparation of X-gal plates: 8 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl and 15 g agar were added to a 2 liter flask and q.s. to 1 liter with Milli-Q-filtered water. The mixture was autoclaved 15 lb/in² (121°C, 256°F) for 30 minutes. When the medium was removed from the autoclave, it was gently swirled to distribute the melted agar evenly throughout the solution. After incubation of the flask in the water bath (56°C) to cool the medium to about 56°C, 4 ml of ampicillin stock (25 mg/ml), 10 ml of 0.1 M of isopropylthio-β-D-galactoside (IPTG) and 2 ml X-gal solution (40 mg of X-gal in 2 ml dimethyl formamide) were added. About 30 ml of the mixture was poured into each plate. When the medium became hardened completely, the plates were inverted and stored at 4°C until needed.

c. Preparation of 20% acrylamide stock: 96.5 g acrylamide, 3.35 g bisacrylamide, 233.5 g urea and 50 ml of 10X TBE (0.9 M Tris-borate and 20 mM EDTA) were mixed. To the mixture Milli-Q-filtered water was added to the final volume of 500 ml. The solution was sterilized by filtration and stored at 4°C in the dark.
d. Preparation of urea stock: 233.5 g urea and 50 ml of 10X TBE were mixed. To the mixture Milli-Q-filtered water was added to the final volume of 500 ml. The solution was sterilized by filtration and stored at 4°C in the dark.
Fig. 2. 5' and 3' ligation of BVDV genomic RNA and PCR amplification. The genomic RNA was ligated by T4 RNA ligase to generate either tandem or circular molecules. After synthesis of the first strand cDNA, two virus-specific primers were used to amplify the 5' and 3' terminal sequences of the BVDV genome.
Fig. 3. Cloning of the 5' terminal sequence of BVDV genome by oligo(A)-tailing and PCR amplification. After the synthesis of the first strand cDNA by random primer (open box), poly(A) tail (lined box) was added at the 3' end of the cDNA by terminal transferase. Then a poly(T) primer (shaded box) and a virus specific primer (dot box) was used to amplify the 5' terminal sequence of the BVDV genome.
Fig. 4. Cloning of PCR products by G/C-tailing method. The PCR amplified DNA was C-tailed by terminal transferase. The pUC 9 vector was digested with Pst I followed by G-tailing. After annealing between C-tailed PCR amplified DNA and G-tailed pUC 9 vector, the plasmid was transformed into E. coli strain JM109.
Fig. 5. Homology of the nucleotide sequence of the 5' and 3' ligation clone of CP BVDV NADL with the published NADL sequence (Collett et al., 1988a). Note the additional 5 cytosine nucleotides present at the point of ligation. The rest of the sequence is 100% homology with the published sequence.
CHAPTER III

Molecular Cloning and Nucleotide Sequence of
A Pestivirus Genome, Noncytopathic Bovine
Viral Diarrhea Virus Strain SD-1

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a small enveloped virus, is one of the most important viral pathogens of cattle (Duffell and Harkness, 1985). BVDV infection can result in a variety of clinical diseases in cattle, such as abortion, persistent infection and mucosal disease (MD). Its genome is a single-stranded RNA with positive polarity and consists of about 12,500 nucleotides (Renard et al., 1987a, Collett et al., 1988a). Together with the other two serologically and structurally related viruses: hog cholera virus (HoCV) of swine and border diseases virus (BDV) of sheep, BVDV belongs to the Pestivirus group. Based on their similarities of genome organization and strategy of gene expression with that of the Flaviviruses, pestiviruses were recently reclassified into the Flaviviridae family (Collett et al., 1988c; Horzinek, 1991; Francki et al., 1991). However, differences in virion composition with the Flaviviridae (Thiel et al., 1991) and the absence of a 5' cap structure of its RNA genome (Brock et al.,
1992a), reflecting different mechanisms of viral RNA translation, are some objections to this reclassification.

Based on the cytopathogenicity in cell culture, BVDV has been divided into two biotypes: cytopathic (CP) BVDV and noncytopathic (NCP) BVDV (Bolin et al., 1985c). Only NCP BVDV is capable of establishing persistent infections in cattle following in utero infection (Brownlie et al., 1984). Furthermore, mucosal disease, a severe clinical syndrome, occurs only in persistently-infected animals when they are superinfected with a second, antigenically indistinguishable CP BVDV (Brownlie et al., 1984; Bolin et al., 1985c; Corapi et al., 1988). These observations led to the hypothesis that CP BVDV may originate from NCP BVDV by genomic mutation (Corapi et al., 1988).

To date, 2 CP strains of BVDV: NADL (Collett et al., 1988a) and Osloss (Renard et al., 1987a), and 2 strains of HoCV: Alfort (Meyers et al., 1989a) and Brescia (Moormann et al., 1990) have been cloned and sequenced. Recently, a partial genomic sequence located in the p125 region of a pair of BVDV, CP BVDV strain CP1 (about 5.7 kb) and NCP BVDV strain NCP1 (about 3.2 kb) were published (Meyers et al., 1991). Comparison of the genomic sequence between BVDV and HoCV led to the finding of cellular sequence inserts in a region coding for the N-part of p125 in the CP BVDV (Meyers et al., 1989b; Collett et al., 1989). Therefore, a hypothesis was proposed by Meyers et al. (1991) that the insertion of a cellular RNA
sequence by RNA recombination into the NCP BVDV genome was responsible for the development of CP BVDV from NCP BVDV. However, this hypothesis was challenged by the observations that some CP BVDV strains lack the insertion in their genomes (Moerlooze et al., 1990; Desport et al., 1991; Akkina, 1991; Greiser-Wilke et al., 1992). This suggests that the knowledge of BVDV cytopathogenicity is far from complete.

In this report, the complete nucleotide sequence of NCP BVDV, strain SD-1, is presented for the first time. In addition, analyses and comparisons of the nucleotide and amino acid sequences are made with that of other pestiviruses.

MATERIALS AND METHODS

Persistently infected animal and virus

A persistently-infected heifer was maintained in an isolation facility to prevent exposure and infection with other BVDV strains. The virus isolated from this heifer was a NCP BVDV and was designated as strain SD-1. Blood was collected from the persistently-infected heifer and serum recovered for virus purification and extraction of viral RNA.

Virus purification and RNA extraction

Partial purification of virus by differential centrifugation was done as described in Chapter II. Viral RNA extraction by the guanidine thiocyanate method were performed as described in Chapter II.
cDNA synthesis, PCR amplification and cloning

cDNA synthesis using random primer and genomic viral RNA extracted from partially purified viral particles was done as previously reported (Brock et al., 1992a). Following the first strand cDNA synthesis as described in Chapter II, PCR was carried out to amplify the defined viral segments. The two primers used to amplify the first SD-1 fragment were designed based on the sequence of the NADL strain in the nonstructural protein region. After determination of the nucleotide sequence of the first SD-1 cDNA clone, some of the primers were designed based on SD-1 sequence. The size of amplified segments was chosen to be 1.5 kb-2.0 kb in order to obtain optimal amplification. Primer length was chosen to be 18 to 24 bases in order to maintain the Tm Value above 55°C. PCR amplification was performed with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) for 30 to 35 cycles. The working profiles were as follows: 94°C for 1 minute to denature the DNA, 42°C to 60°C for 1.5 minutes to allow primer annealing and 72°C for 2 to 5 minutes for DNA extension. All the primers utilized were purchased from either Biochemical Instrument Center of The Ohio State University (Columbus, OH) or Genosys (Woodlands, TX).

Following PCR amplification, C-tailing strategy was used to clone all the PCR products. After the PCR products were purified by electroelution, C-tailing of the PCR products was done as described previously. C-tailed PCR products were
cloned into G-tailed pUC 9 plasmid and used to transform competent *E. coli* strain JM109 (Hanahan, 1983). Colony blots were done using nitrocellulose membranes and positive clones were screened by the corresponding $^{32}$P-dCTP labelled NADL cDNA fragments. The cloning of 5' and 3' end sequences of viral RNA was done by 5'-3' ligation and PCR as previously described in Chapter II.

**Subcloning**

Prior to nucleotide sequencing, restriction enzyme mapping was performed to determine the appropriate restriction enzyme sites in the cDNA clones for subcloning. The plasmid DNA prepared by mini-preparation method described previously was digested with commonly used restriction enzymes including Pst I, Sma I, EcoR I, Hind III, Acc I, Kpn I, Sal I, Nhe I, Ava I, BamH I, Sac I and Xba I. After the map of these restriction enzyme sites was obtained, the appropriate restriction enzymes were used to digest the original cDNA clones and the inserts (about 700 to 300 bp) were subcloned into the pGEM-3Z(+) vector (Promega, Madison WI). A general method for subcloning is shown in Fig. 6. To subclone the S2817-4285 cDNA clone which did not have commonly used restriction enzyme sites for subcloning, the plasmid DNA was transformed the *E. coli* strain JM110 (American Type Culture Collection-ATCC, Rockville, Maryland) which has the genotype of *dam* and *dcm*. The plasmid DNA prepared from JM110 was not
methyalted at the N⁸ position of the adenine residue in the DNA sequence 5'...GATC...3' which is the site for the methylation-sensitive restriction enzyme Mbo I. After the plasmid DNA of S2817-4285 clone was prepared from JM110, it was digested with restriction enzyme Mbo I, the fragments were separated on a 1.5% agarose gel, purified by electroelution and subcloned into BamH I linearized pGEM-3z(+) vector.

**Preparation of plasmid DNA**

a. **Large-scale preparation**: the alkaline lysis and polyethylene glycol (PEG) precipitation method was used to extract and purify plasmid DNA (Birnboim, 1983; Lis and Schleif, 1975). A single colony of *E. coli* containing the desired plasmid was used to inoculate 5 ml of YT broth complemented with 0.1 mg/ml of ampicillin. After growing at 37°C with vigorous shaking overnight, it was used to inoculate 500 ml of YT broth containing 0.1 mg/ml of ampicillin in a 2 liter flask. The cultures were incubated at 37°C with shaking until saturated. The cells were collected by centrifugation at 6000 g for 10 minutes at 4°C and the pellet was resuspended in 4 ml of glucose/Tris/EDTA solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. To the suspension 100 mg of hen egg white lysozyme was added. After 10 minute incubation of the mixture at room temperature, 10 ml of freshly prepared NaOH/SDS solution (0.2 N NaOH and 1% SDS) was added. The solution was mixed by stirring gently until it
became homogeneous and clear. After incubation on ice for 10 minutes, 7.5 ml of potassium acetate solution (3 M KOAc and 1.18 M formic acid, pH 5.5) was added to the tube and the solution was mixed by stirring gently until viscosity was reduced and a large precipitate formed. After it was placed on ice for another 10 minutes, the sample was spun at 10,000 g for 10 minutes at 4°C, the supernatant was transferred into a clean centrifuge tube and the plasmid DNA was precipitated by adding 0.6 volume of isopropanol. After incubating the tube for 30 minutes at -20°C, the DNA was recovered by centrifugation at 10,000 g for 30 minutes at 4°C, and the pellet was washed twice with 70% ethanol, dried under vacuum and resuspended in 1 ml of glucose/Tris/EDTA solution. To the mixture RNase was added to a final concentration of 20 µg/ml, followed by incubation at 37°C for 20 minutes. Then 2 ml of freshly prepared NaOH/SDS solution was added. After the sample was incubated at room temperature for 10 minutes, 1.5 ml of potassium acetate solution was added and the mixture was incubated at room temperature for 10 minutes. The mixture was spun at 10,000 g for 20 minutes at 4°C and the supernatant was transferred to a clear tube and extracted with phenol/chloroform and chloroform. The plasmid DNA was precipitated by adding 1/4 volumes of 10 M ammonium acetate and 2 volumes of cold ethanol. After the tube was placed at -70°C for 30 minutes, the plasmid DNA was recovered by centrifugation at 10,000 g for 30 minutes at 4°C, the pellet
was washed with 70% cold ethanol, dried under vacuum and redissolved in 2 ml of TE buffer. The DNA was precipitated again by adding 0.8 ml PEG solution (30% PEG 8000 and 1.6 M NaCl). After incubation at 4°C overnight, the plasmid DNA was recovered by centrifugation at 10,000 g for 30 minutes at 4°C, the pellet was resuspended in 1 ml TE buffer and the DNA was ethanol-precipitated, recovered, redissolved in 1 ml TE buffer and stored at -20°C until needed. The yield of the plasmid DNA prepared by this method was approximately 1 mg/500 ml culture.

b. Medium-scale preparation: As described previously, at least 3 days were required to prepare plasmid DNA using the large-scale preparation method. Since more than one hundred clones and subclones needed to prepare plasmid DNA in this study, a more rapid, medium-scale preparation was developed to prepare plasmid DNA. This method was a combination of minipreparation and large-scale preparation methods. A single recombinant *E. coli* colony was suspended in 1 ml of YT broth and used to inoculate 50 ml YT medium. After incubation with vigorous shaking at 37°C for about 6-8 hours in a 300 ml flask, the cells were collected by centrifugation at 10,000 g for 10 minutes at 4°C and the pellet was resuspended in 2 ml of glucose/Tris/EDTA solution. To the suspension 1 ml of 2 mg/ml RNAse was added, followed by the addition of 4 ml of freshly prepared NaOH/SDS solution. After the sample was mixed gently and placed on ice for 5 minutes, 3 ml of potassium acetate solution was added and the mixture was placed on ice.
for another 5 minutes. Then the sample was spun at 10,000 g for 20 minutes at 4°C, the supernatant was transferred to a clear tube and extracted with phenol/chloroform and chloroform. The aqueous phase was transferred to a fresh tube and ethanol-precipitated. The plasmid DNA was recovered by centrifugation, dried under vacuum, redissolved in 2 ml TE buffer and precipitated by adding 0.8 ml of PEG solution (30% PEG 8000 and 1.6 M NaCl). After incubation of the sample on ice for 1-4 hours or at 4°C overnight, the DNA was recovered by centrifugation and resuspended in 500 μl TE buffer. Finally, the plasmid DNA was ethanol-precipitated again, recovered and stored at -20°C until needed. The yield of plasmid DNA by this method was about 150-200 μg/50 ml culture.

**Sequencing**

The nucleotide sequence of the inserts was determined by the dideoxy chain termination method (Sanger et al., 1977) using sequencing kits (United State Biochemicals, Cleveland, OH). The nucleotide sequence of the clones that lacked the appropriate restriction enzyme sites for subcloning were determined by a progressive oligonucleotide primer method (Sambrook et al., 1989). The procedure for the sequencing reactions was described in Chapter II. Considering the potential of sequence errors created by Taq polymerase the entire genomic sequence of NCP BVDV SD-1 was determined by completely sequencing a minimum of 2 clones from independent
PCR reactions for each region. If a different nucleotide sequence was obtained from the 2 clones, the consensus nucleotide sequence was verified by sequencing a third or even fourth clone from other independent PCR reactions. Most of the sequences were determined by sequencing both strands of the plasmid DNA. Some of the sequences were obtained by multiple determinations of the sequence on one strand of the plasmid DNA. The 5' and 3' end sequences were confirmed by sequencing 9 independent 5'-3' ligation clones as described in Chapter II.

Computer analysis

Nucleotide sequence comparison and analysis were made with HIBIO DNASIS (Hitachi Software Engineering Co., Ltd., Brisbane, CA) (Lipman and Pearson, 1985; Needleman and Wunsch, 1970). The predicted amino acid sequence was analyzed and compared by using HIBIO PROSIS (Hitachi Software Engineering Co., Ltd., Brisbane, CA) (Kyte and Doolittle, 1982; Lipman and Pearson, 1985).

RESULTS

Molecular cloning of BVDV SD-1 RNA

To determine the nucleotide sequence, viral RNA was directly extracted from serum obtained from a persistently-infected heifer. The virus titer in the serum was $10^3$-$10^4$ CCID$_{50}$/ml. Viral RNA extracted from 1 to 5 ml of serum was
enough to carry out the PCR amplification and cDNA cloning. To optimize PCR amplification and minimize the nonspecific priming, PCR profiles varied for each set of primers. The annealing temperatures ranged from 42°C to 60°C depending on the Tm value of the primer and the homology between the primer and the sequence to be amplified. Several cloning methods were tried to clone PCR products. Compared with either blunt end ligation or AT annealing and ligation cloning methods for PCR products, the GC-tailing method had a higher cloning efficiency. In order to eliminate the potential sequence errors created by Taq polymerase, repeat cDNA cloning of three independent PCR reactions for each viral RNA region was done. The corresponding restriction fragments of NADL cDNA clones were used as probes for identification of positive cDNA clones of SD-1 genomic RNA. To determine the extreme 5' and 3' end sequences of the genome, genomic RNA ligation was performed before PCR amplification and cloning. Based on previous results suggesting there is no cap structure at the 5' end of BVDV genome (Brock et al., 1992a), genomic RNA of SD-1 was directly ligated without the treatment of pyrophosphatase to remove a 5’ cap structure. After restriction enzyme mapping, about 70% of inserts in the original pUC 9 vector were subcloned into pGEM vectors for sequencing. A total of 29 cDNA clones that almost overlapped the whole genome three times were used to determine the nucleotide sequence of SD-1 genome
Nucleotide sequence of NCP BVDV strain SD-1

The complete SD-1 sequence was determined by sequencing at least two clones from independent PCR reactions. Seventy percent of the sequence was determined from both strands of cDNA clones. The remainder was obtained from multiple determinations on a single strand. A total of 36 nucleotides were different by comparison of the two independent nucleotide sequences in the cDNA sequence of about 30,000 nucleotides. It is probable that most of the different nucleotide sequences were errors incorporated by Taq polymerase. In those cases, the consensus nucleotide sequence was obtained by sequencing a third or even fourth independent clone. Interestingly, one of the errors found in one of the cDNA clones has been previously described for BVDV NADL (Collett et al., unpublished data) and HoCV Brescia cDNA clones (Moormann et al., 1990), involving a stretch of 5 sequential adenosines, where 6 adenosines were the final correct nucleotide sequence.

The 5' and 3' end sequences of the SD-1 genome were determined from the 5'-3' ligation clones. Out of 9 clones sequenced, 4 clones have the 3' end sequence of 5'...CAGCCCCC 3', 4 clones have 5'...CAGCCCC 3' and 1 clone has 5'...CAGCCC 3'. Therefore, the dominant and longest sequence, 5'...CAGCCCCC 3', is the authentic 3' end sequence of SD-1 genome, which is identical to the 3' end sequence of NADL. The
complete nucleotide sequence consists of 12,308 nucleotides (Fig. 8), which is less than CP BVDV strain NADL (12,578 nucleotides) and Osloss genomes (12,430 nucleotides), but closer to HoCV strain Alfort and Brescia genomes (12,284 and 12,283 nucleotides, respectively). Base composition of the entire genome of SD-1 is 32.2% A, 22.0% U, 25.6% G and 20.2% C and is similar to NADL RNA (Collett et al., 1988a).

Analysis of the SD-1 sequence for translation revealed one large open reading frame (ORF) in the second phase of one strand. The other two reading phases of this strand and the three reading phases of the complementary strand contain multiple stop codons throughout the sequence (Fig. 9). No other significant ORF can be predicted in these reading phases. The large ORF starts with the AUG at position 386 to 388 and ends with a stop codon UGA at position 12080 to 12082. This ORF is capable of encoding a polyprotein of 3898 amino acids with the calculated molecular weight of 438 kDa. The predicted amino acid sequence of the large ORF is given in Fig. 8. The 5' untranslated region (5'-UTR) preceding the large ORF consists of 385 nucleotides, including 6 AUG start codons and several small ORFs. One of the small ORFs, which was maintained in NADL but not in Osloss and HoCV, starting with a AUG codon at nucleotides 131 and ending with a stop codon UAA at nucleotides 266, is in the same reading phase as the large ORF and could potentially encodes a polypeptide of 45 amino acids. Whether this small ORF is functional is
unknown. Following the stop codon of the large ORF, the 3' untranslated region (3'-UTR) continues for another 229 nucleotides.

**Amino acid sequence of NCP BVDV strain SD-1**

The large ORF encodes a polyprotein with 3898 amino acid residues (Fig. 8). The hydrophobicity analysis of this sequence revealed that along the entire amino acid sequence, two regions are characteristic. The first region, including 250 residues and located at the N-terminal of the polyprotein, is highly hydrophilic, particularly in the region from residues 165 to 250 which was reported to encode the viral capsid protein p14 (Thiel et al., 1991). Out of 85 amino acid residues, 25 are positively charged residues (22 lysines, 3 arginines) and 12 are negatively charged residues (6 aspartic acids, 6 glutamic acids). The second region located at position 1036 to 1301 consists of 266 amino acid residues and is highly hydrophobic. This region contains 51 leucines, 35 valines, 29 isoleucines and 20 threonines.

Twenty eight potential glycosylation sequences (Asn-X-Ser or Asn-X-Thr) were predicted along the polyprotein. Fourteen sites are located in the glycoprotein region between residues 271 and 992 (Fig. 8). Out of the 14 sites, 8 were conserved among all the pestiviruses and 3 other sites were conserved among the BVDV strains. A cysteine-rich stretch, reported to conform to a "zinc finger" binding domain (Moerlooze et al.,
was also found in the SD-1 polyprotein at amino acid residues 1484 to 1512 (Fig. 8). The tripeptide sequence, Gly-Asp-Asp, highly conserved in viral RNA dependent RNA polymerases (Kamer and Argos, 1984), was uniquely identified in the nonstructural protein p75 region at position 3626 to 3628 (Fig. 8). This finding supports the proposal that this protein is a candidate BVDV replicase (Collett et al., 1991). Sequence comparisons and structural pattern analysis predicted the BVDV p80 protein to be a trypsin-like serine proteinase (Bazan and Fletlerick, 1989; Gorbalenga et al., 1989a; Moormann et al., 1990). Recently, Wiskerchen and Collett (1991b) experimentally demonstrated that this protein is a viral proteinase and is responsible for all the nonstructural protein processing. The catalytic triad of His, Asp and Ser residues was also observed in the SD-1 sequence and located at positions 1658, 1659 and 1752, respectively (Fig. 8).

Comparison of nucleotide and amino acid sequences

The complete genome of SD-1 is 12308 nucleotides, which is 270 nucleotides shorter than NADL genome and 124 nucleotides shorter than Osloss. The inserts identified in CP BVDV Osloss (Meyers et al., 1989b) and NADL (Collett et al., 1989) and a deletion of 41 nucleotides in the 3'-UTR of Osloss (Deng and Brock, in preparation) are responsible for this variation of genome sizes (Fig. 10). There is a 270 nucleotide cellular RNA insert in NADL between nucleotides 4992 and 4993.
of SD-1 and a 228 base ubiquitin RNA insert in Osloss between nucleotides 5152 and 5153 of SD-1. Our results reveal that, in contrast to CP BVDV, NADL and Osloss, NCP BVDV SD-1 has no RNA insertions along its genome.

The overall nucleotide sequence homologies of SD-1 are 88.6% with NADL, 78.3% with Osloss, 67.1% with HoCV Alfort and 67.2% with HoCV Brescia. The most conserved region is located in the 5'-UTR in which the degree of homologies of SD-1 sequence are 93% with NADL, 86% with Osloss, 74% with HoCV Alfort and Brescia (Fig. 10). A moderately high homology is found along most of the viral nonstructural protein region. The viral structural proteins (p14, gp48, gp25, and gp53) and nonstructural proteins p54 and p58 have the lowest homology between SD-1 and other pestivirus sequences (Fig. 10).

The predicted amino acid sequence is more conserved than nucleotide sequence among all the pestiviruses. The overall amino acid sequence homologies of SD-1 are 92.7% with NADL, 86.2% with Osloss, 72.5% with HoCV Alfort and 71.2% with HoCV Brescia. The p80 is the most conserved viral protein with sequence homologies of SD-1 with NADL of 98%, with Osloss of 95%, and with HoCV Alfort and Brescia of 86% (Fig. 10). Similar to the nucleotide sequence, the amino acid sequence of the viral structural proteins and nonstructural proteins p54 and p58 is more variable than other regions. Extensive analysis of amino acid sequence led to the identification of 4 conserved domains (designated C1, C2 C3, C4) and 3 highly
variable domains (designated V1, V2, V3) in the viral structural proteins and nonstructural protein p54 region. The regions that had average amino acid sequence homologies of above 90% between SD-1 and other pestiviruses were defined as conserved domains. The average percentages of homology between SD-1 and other pestiviruses in C1, C2, C3 and C4 domains are 95%, 92%, 94% and 90%, respectively. A comparison of amino acid sequence in those four conserved domains is shown in Fig. 11. The regions that had average amino acid homologies of below 61% between SD-1 and other pestiviruses were defined as variable domains. The average percentages of homology between SD-1 and other pestiviruses in V1, V2 and V3 domains are 54%, 61% and 48%, respectively. A comparison of amino acid sequence in those three variable domains is shown in Fig. 12. The C1, C2 and C3 domains are located in the viral capsid protein p14, glycoproteins gp48 and gp25, respectively. The C4 domain is located in the junction between gp53 and p54. Out of 3 highly variable domains, 2 (V1 and V2) are located in the same viral glycoprotein gp53 (Fig. 13), which has been reported to induce neutralizing antibodies in the host (Magar et al., 1988a; Donis et al., 1988; Bolin et al., 1988b; Xue et al., 1990; Weiland et al., 1990). The hydrophobicity analysis of the amino acid sequence in this area show that the V1 and V2 domains identified in gp53 are hydrophilic (Fig. 13), indicating that these domains may be located on the outside of the viral envelope. The third variable domain, the largest
one, is located in the N-terminal of nonstructural protein p54. Although the primary deduced amino acid sequence is hypervariable in the V3 domain, the hydrophobic feature of this domain is still maintained in all the pestiviruses.

**DISCUSSION**

Strain SD-1 is a NCP BVDV obtained from a persistently-infected heifer maintained in isolation. In order to eliminate the possibility of the adaptive selection of virus during cell culture passage, which may change the dominance of virus in the population, and to eventually evaluate the naturally occurring mutation rate of the viral genome in the persistently infected animal in vivo, the persistently infected animal was the source of virus for cDNA cloning. However, the virus titer in serum obtained from the persistently infected heifer was approximately $10^3$ logs lower than that in infected cell culture supernatants. Initially, direct cloning of cDNA from SD-1 RNA extracted from serum was attempted. Because of low concentrations of viral RNA in the preparations, no positive clones of SD-1 were obtained after screening of the cDNA library. To overcome this problem, PCR was used to amplify the lower viral RNA levels in the serum before cloning. Based on dot-blot hybridization results (Brock et al., 1992b), there was a high homology between SD-1 and NADL nucleotide sequences. It was also reported that the highest sequence homology was located in the nonstructural
protein region, particularly in the p80 region (Meyers et al., 1989a; Moormann et al., 1990). Therefore, the first two primers used to amplify SD-1 RNA were designed based on NADL sequence in the p80 region, which is located in the middle of the genome. Because of the lower homology between NADL and SD-1 nucleotide sequence in the viral glycoprotein and the nonstructural protein p76 regions, NADL sequence primers were not able to amplify SD-1 sequence in these regions. These two gaps, therefore, were filled in following cloning and determination of the 5' and 3' UTR nucleotide sequences of SD-1 and the use of specific SD-1 sequence primers. The cDNA sequence of SD-1 reported represents the genomic sequence of the virus in vivo, which may be different from that of the virus obtained in vitro due to the adaptative selection of virus during cell culture passage.

It was reported that the error rate of Taq polymerase during the DNA polymerization is about 2 x 10^{-5} errors/nucleotide/cycle under standard conditions (Eckert et al., 1990; Lundberg et al., 1991)). A total of 36 nucleotide sequence "errors" were identified in the total cDNA sequence of about 30,000 nucleotides. The actual error rate was therefore 4 x 10^{-5} errors/nucleotide/cycle, which is two times higher than the theoretical error rate of Taq polymerase. Therefore, out of 36 "errors", some may have been created by either reverse transcriptase or DNA polymerase. In addition,
some nucleotide differences may have been due to the sequence heterogeneity in the viral RNA population.

The cDNA sequence of NCP BVDV strain SD-1 RNA was determined to be 12308 nucleotides in length using a 5'-3' ligation strategy to determine the extreme 5' and 3' end sequence. Therefore, it is reasonable to state that the sequence of our cDNA clones represents the complete nucleotide sequence of NCP BVDV SD-1 genome. Out of nine 5'-3' ligation clones sequenced, 4 had 1 nucleotide, and 1 had 2 nucleotides shorter than the authentic 3'end sequence. Whether this variation represents the heterogeneity in the viral RNA population or indicates the presence of a low level of RNA exonuclease activity in the viral RNA preparations, resulting in cleavage of 1 or 2 bases at the 3' end of viral RNA prior to the 5'-3' ligation, is unknown.

Comparison of the nucleotide sequence between CP BVDV and HoCV led to the identification of a cellular RNA and a ubiquitin sequence insert within the region coding for the p54 in CP BVDV NADL and Osloss, respectively (Meyers et al., 1989b; Collett et al., 1989). Because the NADL and Osloss strains are cytopathic, it has been proposed that cellular RNA insertion into NCP BVDV genome is responsible for the development of cytopathogenicity (Meyers et al., 1989b). Recently, Meyers et al. (1991) cloned and determined the partial genomic nucleotide sequence in the p125 region of a pair of CP BVDV strain CP1 and NCP BVDV strain NCP1. A
ubiquitin insert and p80 nucleotide sequence duplicate were found in the CP1, but not in the NCP1 within this region, which supports the previous hypothesis. However, whether other differences are present in the remainder of the genome is unknown. Comparison of the complete nucleotide sequence of NCP BVDV SD-1 with that of CP BVDV NADL and Osloss revealed that the most remarkable difference is the absence of an inserted cellular RNA sequence in the p125 region. These results, therefore, support the working hypothesis for the cytopathogenicity of BVDV proposed by Meyers et al. (1991). This hypothesis, however, has been challenged by the observations that some CP BVDV strains lack such cellular insertions in their genome (Moerlooze et al., 1991; Desport et al., 1991; Akkina, 1991; Greiser-Wilke et al., 1992). It has been reported that the p80 protein, generated by either cleavage of p125 or expression from a p80 duplicated region, is the only marker of CP BVDV (Meyers et al., 1991). Therefore, the release of the p80 protein from the viral polyprotein is the key event associated with the development of cytopathogenicity. Although the p125 proteins of the different CP BVDV strains were heterogeneous in size, the processed p80 had the identical size (Akkina, 1991; Greiser-Wilke et al., 1992). These results indicate that the size variation of p125 is determined in the p54 region and the cleavage site at the N-terminal of p80 is present in the authentic BVDV sequence rather than in the cellular inserts.
(Greiser-Wilke et al., 1992). Some of the CP BVDV strains had almost identical sized p125 with that of NCP BVDV (Akkina, 1991; Greiser-Wilke et al., 1992), suggesting that minor base changes in these CP BVDV, such as base substitutions, small insertions or deletions, are responsible for the release of p80 from the polyprotein. Comparison of NCP BVDV SD-1 nucleotide sequence with CP BVDV NADL and Osloss at the N-terminal part of p125 revealed that, in addition to the obvious difference of cellular RNA insertion, there are many of base substitutions in NADL and Osloss, which result in the change of amino acid sequence, particularly in the V3 domain. Secondary structure prediction from amino acid sequence in this region reveals that some of the amino acid changes alter the secondary structure of the N-terminal portion of p125 (data not shown). Therefore, in addition to the insertion, the base substitutions resulting in conformational change of the N-terminal part of p125 may also contribute to the release of p80 from the polyprotein. It is tempting to propose that the mutations that occurred in NCP BVDV, such as insertion, deletion, duplication and substitution, result in conformational changes in the N-terminal portion of p125. Following the conformational change, the cleavage site at the N-terminus of p80 becomes functional and the p80 is released from the viral polyprotein, resulting in the development of CP BVDV from NCP BVDV. The construction of an infectious cDNA clone and the following experimental conversion of both
biotypes will help to elucidate the mechanism for cytopathogenicity of BVDV.

The most conserved nucleotide and amino acid sequences are located in the 5′ UTR and nonstructural protein p80 respectively, which reflect the functional importance of these two regions for either virus replication or viral RNA translation or viral polyprotein processing. The p80 was reported to possess protease activity which is responsible for the processing of all the viral nonstructural proteins (Wiskerchen and Collett, 1991b). In addition, a helicase motif was also predicted within p80 region (Gorbalenka et al., 1989b; Moormann et al., 1990). The possible regulatory elements and the secondary structure of 5′-UTR of BVDV RNA will be analyzed and reported elsewhere (Deng and Brock, in preparation).

The comparison of both nucleotide and amino acid sequence of SD-1 with that of other pestiviruses revealed the high heterogeneity in the region of viral structural proteins and nonstructural proteins p54 and p58. Four conserved domains and three variable domains were identified in these regions. The four conserved domains are located in the viral capsid protein p14, glycoprotein gp48, gp25 and the junction between glycoprotein gp53 and nonstructural protein p54, respectively. It is reasonable to assume that these domains are under highly functional selection and are critical for either viral RNA packaging or virion assembly or important for viral
interaction with receptors on infected cells. However, the functions of these domains remain to be determined. It has been reported repeatedly that neutralizing monoclonal antibodies against BVDV bound to and immunoprecipitated glycoprotein gp53 (Magar et al., 1988a; Donis et al., 1988; Bolin et al., 1988b; Xue et al., 1990; Weiland et al., 1990). Antigenic variation of this protein among BVDV isolates was demonstrated by the fact that none of the monoclonal antibodies neutralized all the BVDV isolates tested (Magar et al., 1988a, Xue et al., 1990). The amino acid sequence analysis revealed that two hypervariable domains (V1, V2) are located in the viral glycoprotein gp53. The hydrophobicity analysis indicated that these two domains are hydrophilic, suggesting that V1 and V2 domains are present on the outer surface of virions. Therefore, it is proposed that V1 and V2 domains in gp53 may represent the protective epitopes of this protein, which are the important targets for the immune response of the host, hence under the high immunological pressure. The high diversity of amino acid sequence within the protective epitopes of gp53 among BVDV and HoCV implies the difficulty in developing effective vaccine for preventing pestivirus infections by expression of this immunodominant glycoprotein. Recently, Weiland et al. (1992) reported that antibodies to a second envelope glycoprotein gp44/48 was able to mediate neutralization of HoCV. Whether the corresponding glycoprotein gp48 in BVDV has the same ability to induce
neutralizing antibodies remains to be investigated. Amino acid sequence analyses indicated that glycoprotein gp48 was more conserved than gp53 among pestiviruses. Furthermore, a highly conserved domain (C2) was observed within this protein. Therefore, gp48 may be a candidate for a subunit vaccine as well. Similar to pestiviruses, the variable and hypervariable domains were also identified in the regions of Hepatitis C virus (HCV) corresponding to the gp53 of pestivirus (Weiner et al. 1991). However, no similar hypervariable regions have been reported in the corresponding envelope E protein and NS1 protein of flaviviruses (Chu et al., 1989; Deubel et al., 1986; Lobigs et al., 1988; Hahn et al., 1988; Irie et al., 1989).

The largest variable domain was identified within the first nonstructural protein p54, the N-part of precursor protein p125. The characteristic feature of this protein is that a highly hydrophobic stretch including 266 amino acids is present in this protein and it was usually not detected by immunoprecipitation (Moennig and Plagemann, 1992). The V3 domain is located in the highly hydrophobic stretch of this protein. Although the primary amino acid sequence is hypervariable, the hydrophobic character of this domain is maintained in all the pestiviruses, indicating that the hydrophobicity rather than the primary sequence of this protein is under the functional selection. Although a membrane associated function of this protein was proposed (Wiskerchen
and collett, 1991b), further work is needed to elucidate its function.

REFERENCES


Fig. 6. A sample of subcloning for the original SD-1 cDNA clones. The original BVDV SD-1 cDNA clone was digested with restriction enzyme EcoRI and NheI. The pGEM vector was digested with EcoRI and XbaI. Because the stick ends produced by NheI and XbaI were compatible. The small insert fragment was cloned into pGEM vector.
Fig. 7. Distribution of cDNA clones of NCP BVDV strain SD-1 along the genome of 12308 nucleotides. Only the clones used to determine the nucleotide sequence are shown here. The 5'-3' ligation clones are indicated by stars. The restriction enzyme sites of cDNA clones utilized to perform subcloning are shown: A: Acc I, B: BamH I, H: Hind III, K: Kpn I, M: Mbo I, N: Nhe I, P: Pst I.
Fig. 8. The complete genomic nucleotide sequence of the genomic RNA of NCP BVDV strain SD-1. The deduced amino acid sequence of the large ORF is shown in three-letter code below the nucleotide sequence. Boxed sequence at the N-terminal of viral glycoprotein indicates the viral signal sequence. The putative glycosylation sites in the viral glycoprotein region are underlined. The double underlined sequence is the cysteine-rich stretch. ■■■■ indicates the catalytic triad of His, Asp and Ser residues of the viral p80 proteinase. ◊◊◊ denotes the tripeptide sequence highly conserved in viral RNA dependent RNA polymerases.
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12266  CCG ACG TCT ATA GGG CTA GGA ACC TCT AAC AGG CCC C
Fig. 9. Distribution of open reading frame in 6 phases of 2 orientations of NCP BVDV cDNA sequence. The vertical bars above the line indicate the AUG start codon. The vertical bars below the line indicate the stop codon. The large ORF is identified in the second phase of the 5'-3' orientation.
Fig. 10. Comparison of nucleotide and amino acid sequences of NCP BVDV SD-1 with that of other pestiviruses: CP BVDV NADL and Osloss, HoCV Alfort and Brescia. Sequences were compared on basis of the genome organizations of BVDV (Collett et al., 1988b) and HoCV (Thiel et al., 1991). The percentages of homology of nucleotide sequences are given in the upper line and the percentages of homology between amino acid sequences are given in the next line parenthetically. The black bars below the sequences of NADL and Osloss indicate the cellular sequence insertion within the p54 region.
Fig. 11. Comparison of the predicted amino acid sequence of SD-1 in the four conserved regions within the viral structural protein and p54 region with that of other pestiviruses: BVDV strains NADL and Osloss, HCV strains Alfort and Brescia. The sequence shown is for SD-1. Hyphens indicate the identity with SD-1 sequence. Stars indicate the deletions compared with each other. The positions of the amino acid residues are given above the amino acid sequence.
Fig. 12. Comparison of the predicted amino acid sequence of SD-1 in the three variable regions within the viral structural protein and p54 region with that of other pestiviruses: BVDV strains NADL and Osloss, HCV strains Alfort and Brescia. The sequence shown is for SD-1. Hyphens indicate the identity with SD-1 sequence. Stars indicate the deletions compared with each other. The positions of the amino acid residues are given above the amino acid sequence.
Fig. 13. Location and hydrophobicity of three variable domains and four conserved domains identified among the pestiviruses in the region of viral structural proteins and nonstructural protein p54. The gene organization given was proposed by Collett et al. (1988b) and Thiel et al. (1991). The black box and symbol V indicate the variable domain. The shaded box and symbol C indicate the conserved domain. The hydrophobicity plot in this region is proportionally given above the viral proteins. Solid and dot line present the hydrophobicity of SD-1 and NADL sequence, respectively. The star indicates the insertion region in NADL. The amino acid number is given at the bottom of the figure. S indicates the signal sequence.
CHAPTER IV

5' and 3' Untranslated Regions of Pestivirus Genome:
Primary and Secondary structure analyses

INTRODUCTION

Bovine viral diarrhea virus (BVDV) of cattle, hog cholera virus (HoCV) of swine and border disease virus (BDV) of sheep make up the pestivirus group. The genome of pestivirus is a single stranded, positive sense and nonpolyadenylated RNA molecule with a size of about 12.5 kb (Renard et al., 1987a; Collett et al., 1988a). It encodes a larger polyprotein which is proteolytically processed during virus replication to produce a series of structural and nonstructural proteins (Collett et al., 1988b). The genomic RNA sequences of 5 strains of pestivirus: cytopathic (CP) BVDV strains NADL (Collett, et al., 1988a) and Osloss (Renard et al., 1987b), noncytopathic (NCP) BVDV strain SD-1 (Deng and Brock, 1992a), HoCV strains Alfort (Meyers et al., 1989a) and Brescia (Moormann et al., 1990), have been reported. In addition, the complete 5' and 3' end sequence of the genome of strains NADL and SD-1 have been determined (Brock et al., 1992. Deng and Brock, 1992a).

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It is known that the nucleotide sequence at the termini of the viral RNA molecules harbors specific signals for viral RNA replication, transcription and translation (Strauss and Strauss, 1983). The signals include both primary and secondary structure and are specifically recognized by a virus-specific replicase (RNA dependent RNA polymerase) and cellular translational complexes. Recently, a specific internal sequence within the 5’ untranslated region (UTR) of picornavirus, called internal ribosome entry site (IRES), was reported to mediate the direct binding of ribosome to viral RNA template and initiate viral RNA translation (Jang et al., 1988; Pelletier and Sonenberg, 1988; Jackson, 1988; Jang et al., 1989; Iizuka et al., 1991; Borman and Jackson, 1992). A similar IRES sequence within the 5’-UTR of a closely related pestivirus, hepatitis C virus (HCV), and some cellular mRNAs have also been identified (Tsukiyama-Kohara et al., 1992; Macejak and Sarnow, 1991). In addition, it has been reported that the consensus primary and secondary structures in the 5’- and 3’-UTR is functionally important for replication, phenotype and virulence of the viruses (Strauss and Strauss, 1983; Racaniello and Meriam, 1986; Pilipenko et al., 1989; Lipton et al., 1991; Macadam et al., 1991).

In this report, the primary and secondary structures of the 5’- and 3’-UTR of five pestiviruses were compared and analyzed. A consensus model of secondary structure of the 5’- and 3’-UTR of pestivirus RNA is proposed. In addition, the
structural similarity of the 5′-UTR of pestivirus RNA to picornavirus and hepatitis C virus RNA is demonstrated.

MATERIALS AND METHODS

Computer Analysis

Primary structural analysis was made with HIBIO DNASIS (Hitachi Software Engineering Co., Ltd., Brisbane, CA) (Lipman and Pearson, 1985) to determine nucleotide sequence homology, nucleotide sequence repetition and open reading frame prediction. One limitation of this program imposed when performing nucleotide sequence homology searches is that a deletion or insertion of more than 10 nucleotides can not be aligned by the program. In this case, the sequence upstream and downstream the deletion or insertion must be aligned separately.

Secondary structure prediction was done using the FOLD program (Zuker and Stiegler, 1981) and HIBIO DNASIS program (Zuker and Stiegler, 1981). The first 500 nucleotides of the genome, which included the entire 5′-UTR and part of the coding sequence, was folded to predict the secondary structure of 5′-UTR. The nucleotide sequence downstream of the stop codon of the viral polyprotein was folded to predict the secondary structure of 3′-UTR. To derive a consensus model of the secondary structure, the nucleotide sequences of the 5′-UTR and 3′-UTR from five pestiviruses were folded separately and elements common to or dominant in these five pestiviruses
were retained. Next, the intervening segments were refolded until a consensus structure maximizing total base pairing was achieved. Taking into account the conservation of primary structure, the substitution of one base pair for another and the thermodynamic stability of the structure, some adjustments were made to maintain the structural details among all the five pestiviruses.

Source of the sequence information

Primary structures of the pestivirus genome were taken from the references indicated in the parentheses: CP BVDV strain Osloss (Renard et al., 1987a), CP BVDV strain NADL (Collett et al., 1988a; Brock et al., 1992a), NCP BVDV strain SD-1 (Deng and Brock, 1992a), HoCV strain Alfort (Meyers et al., 1989a) and HoCV strain Brescia (Moormann et al., 1990).

RESULTS

Primary structural feature of 5'-UTR of pestivirus genome

The genome of the pestiviruses has a relatively long 5'-UTR, ranging from 360-363 nucleotides for HoCV to 383-385 nucleotides for BVDV. Compared with other regions of the genome, the nucleotide sequence of the 5'-UTR is the most conserved among pestiviruses with an average nucleotide sequence homology of 82%. Three highly variable regions (designated as region I, II and III) were identified within the 5'-UTR when the nucleotide sequence of 5'-UTR from five
pestiviruses was compared (Fig. 14). Region I, containing 72 nucleotides with an average homology of 71.5%, extends from nucleotides 1 to 72. Region II, containing 15 nucleotides with an average homology of 35%, extends from nucleotides 206 to 221. Region III, containing 40 nucleotides with an average homology of 61.9%, extends from nucleotides 280 to 320. One of the characteristic features of pestiviral 5'-UTR is the presence of multiple cryptic AUG start codons preceding the authentic AUG. The number of cryptic AUGs within the 5'-UTR is 5 in BVDV Osloss, 6 in BVDV NADL and SD-1, 7 in HoCV Alfort and 8 in HoCV Brescia. Two of them, one located at nucleotides 108 to 110 and the other at nucleotides 379 to 381, are conserved among all the five pestiviruses. Most of these AUGs can potentially open a reading frame containing 7 to 57 amino acid residues. Four of these small ORFs are present in the 5'-UTR of SD-1 and NADL, 5 in Osloss and Alfort, and 6 in Brescia (Fig. 14).

Inspection of the context of AUGs revealed that the polyprotein initiation AUG has a weak context, where the nucleotide at position -3 is a C rather than an A or G. The most favorable context was found in two of the cryptic AUGs. One has the context sequence of AXXAUGA from nucleotides 128 to 134 and can potentially open a reading frame encoding a polypeptide with 45 amino acid residues in both BVDV NADL and SD-1. The other has the context sequence of GXXAUGG from nucleotides 309 to 315 and can potentially open a reading
frame encoding a polypeptide with 17 amino acid residues in both HoCV Alfort and Brescia. In addition, another two cryptic AUGs, located at nucleotides 165 to 168 and 369 to 371 respectively, have the similar context to the polyprotein initiation AUG. One AUG can potentially open a reading frame encoding a polypeptide with 8 amino acid residues and is conserved in three BVDV strains. The other, 7 bases upstream of the polyprotein initiator AUG, can potentially open a reading frame encoding a polypeptide with 8 amino acid residues and is conserved in all the five pestiviruses. In addition, the nucleotide sequence around the authentic AUG from -25 to +10 is identical among all the five pestiviruses (Fig. 14).

Two oligopyrimidine tracts (designated as track a and b) were identified at the 5′-UTR of pestivirus (Fig. 14). Tract a, 34 bases upstream of the cryptic AUG at position 108 to 110, has the nucleotide sequence of AUCCCUCUAG and 83.3% of the sequence is complementary to the consensus sequence at 3′ terminus of eucaryotic 18S rRNA (Hagenbüchle et al., 1978) speculating by G:U base pairing (Fig. 15). Track b, 13 nucleotides upstream of the cryptic AUG at position 379-381 and 20 nucleotides upstream of the authentic AUG, has the nucleotide sequence of AUCUCUGCUAG and 83.3% the nucleotide sequence is complementary to the consensus sequence of eucaryotic 18S rRNA (Hagenbüchle et al., 1978) (Fig. 15). A comparison of the primary structural features of the 5′-UTR of
pestivirus with that of other related viruses is given in Fig. 15 and Table 3. The results indicate that the primary structural features of the 5'-UTR of pestivirus resemble that of picornavirus and hepatitis C virus rather than a number of Flaviviridae.

**Secondary structure of 5'-UTR of pestivirus genome**

A consensus RNA secondary structure model for the 5'-UTR of pestivirus was created through the FOLD program of Zuker and Stiegler (1981). The model shows a linear series of stem-loop like structures which can be divided into 5 domains: domain A, B, C, D and E (Fig. 16). About 70% of the nucleotides in the 5'-UTR participate in the base pairing to conform to the secondary structure. The optimal $\Delta G$ values of the structure calculated by the FOLD program are -103.8 Kcal/mol, -109.6 Kcal/mol, -117.3 Kcal/mol, -110.8 Kcal/mol and -102 Kcal/mol for BVDV SD-1, BVDV NADL, BVDV Osloss, HoCV Alfort and HoCV Brescia, respectively. Domain A is a stable stem-loop structure in BVDV strains and located in the variable region I of the primary sequence. The nucleotide sequence maintaining the stem structure starts from the first base of the genome and is conserved in three BVDV strains. The variable sequences including base substitutions and deletions are exclusively located in the loop part of this domain. Because of the size variation of the sequence at the extreme 5' end of the genome between BVDV and HoCV where the reported
5' end sequence of HoCV genome starts about 15 nucleotides downstream of the 5' end of BVDV genome (Fig. 14), domain A is not conserved between these two groups. In HoCV Brescia, domain A is a less stable stem-loop structure and in HoCV Alfort, domain A is a little larger and imperfect stem-loop structure. Domain B is also located in the variable region I of primary sequence and shows little conservation among the pestiviruses, particularly for HoCV Alfort where no equivalent structure was formed. Domain C is a conserved, relatively large and imperfect stem-loop structure. It can be folded independently in four strains of pestivirus. However, in BVDV SD-1 the structure of this domain can be interfered with by the upstream nucleotide sequence and a more favorable structure (smaller ΔG) could form when the upstream sequence was involved in the prediction. Domain D is a stable stem-loop like structure with ΔG values of -74.2 Kcal/mol, -76.6 Kcal/mol, -82.8 Kcal/mol, -78.1 Kcal/mol and -72.7 Kcal/mol for SD-1, NADL, Osloss, Alfort and Brescia respectively. It contains two third of 5'-UTR nucleotide sequence from 139 to 361 and can be divided into 5 subdomains, designated as D1, D2, D3, D4, D5. Although two of the variable regions (II and III) of primary sequence are located in this domain, the secondary structure is almost identical among the pestiviruses. The conserved part of primary nucleotide sequence in this region is a factor contributing to the maintenance of the common secondary structure. The other,
perhaps more important, contributing factor is a large number of compensatory mutations, that is the simultaneous alterations of two noncontiguous nucleotides that result in the substitution of one base pair for another. When comparing the structure between BVDV and HoCV, about 36% of the base pairs maintaining the common secondary structure are substituted in this domain. The longest distance between the two compensatory mutated nucleotides is 214 nucleotides, where a G:C base pair in BVDV is substituted by a C:G base pair in HoCV at the bottom of the stem structure. In addition to the compensatory mutations in the stem structure, the other heterogenous nucleotide sequence consisting of a number of base substitutions and small stretches of nucleotide deletions is exclusively located in the loop structure, mainly in the loops of D2 and D4 domain. Among the 5 subdomains, domain D5 exhibits almost the identical primary structure, domain D1 and D2 has a relatively conserved primary structure, and domain D3 and D4 are located in the variable region III of the primary structure and show less conservation of the primary structure. Domain E is a conserved, imperfect and unstable stem-loop structure with ΔG value of approximately -2.3 Kcal/mol.

Primary structure feature of 3′-UTR of pestivirus genome

The pestiviruses also have a relatively long 3′-UTR, 188 nucleotides for BVDV Osloss and approximately 228 nucleotides for the other four strains. It has been reported that the 3′-
UTR sequence of BVDV NADL and SD-1 represented the complete 3' end nucleotide sequence of their genomes (Brock et al., 1992; Deng and Brock, 1992). It is not known whether the 3'-UTRs of BVDV Osloss, HoCV Alfort and Brescia cover the complete nucleotide sequence of the authentic 3' end of their genomes. However, when these sequences are aligned with BVDV NADL and SD-1, BVDV Osloss genome ends at the same position as BVDV NADL and SD-1, HoCV Alfort and Brescia genome end 4 bases and 2 bases upstream the 3' end of BVDV NADL and SD-1 genome respectively. Therefore, it is reasonable to assume that the reported 3'-UTR sequence of those viruses cover almost, if not completely, the authentic nucleotide sequence of 3' end of their genomes (Fig. 17). Comparison of the nucleotide sequence among those 5 strains of pestivirus led to the identification of a variable region (designated as 3'V region) and a conserved region (designated as 3'C region) within the 3'-UTR (Fig. 17). The 3'V region, starting at the AUG stop codon of the polyprotein at position 12080 and ending at nucleotides 12206, contains 127 bases and shows an average nucleotide sequence homology of 59.3% between SD-1 and other pestiviruses. The nucleotide sequence variation in this region is particularly remarkable between BVDV group and HoCV group where the average nucleotide sequence homology is only 44.3% between HoCV and BVDV. The most obvious difference in this region is the presence of a 41 base deletion in BVDV Osloss (Fig. 17), which is responsible for the size variation of 3'-
UTR between Osloss and other pestiviruses. The 3′C region was composed of 102 nucleotides at the 3′ terminal portion of 3′-UTR and shows an average nucleotide sequence homology of 83.7% between SD-1 and other pestiviruses. This region was particularly conserved among BVDV. Compared with SD-1 nucleotide sequence, only 3 and 10 nucleotide substitutions were found in NADL and Osloss respectively, with homology of 96% between SD-1 and NADL and 90% between SD-1 and Osloss (Fig. 17). When the nucleotide sequence of 3′-UTR was compared with complementary sequence of 5′-UTR, no significant similarity of the primary sequence was found.

An AU rich stretch containing 58 nucleotides in BVDV and 47 nucleotides in HoCV were found respectively. Both are located within the 3′V region but in the different positions (Fig. 17). Repeat sequence searching revealed that there is no perfect, relatively large repeat sequence within the 3′-UTR of pestivirus. However, one stretch of imperfect repeat sequence containing about 56 nucleotides with a 35 bases identity was identified in the 3 BVDV strains, and two stretches of imperfect repeat sequence containing 18 nucleotides with a 14 bases identity and 32 nucleotides with a 25 bases identity respectively, were found in 2 HoCV strains (Fig. 18). The nucleotide sequences present in the repeats are not necessarily conserved between different viruses, and the repeat stretches can also be located in the different positions for different viruses.
**Predicted secondary structure of 3′-UTR of pestivirus genome**

The secondary structure of the 3′-UTR from the 5 pestiviruses was predicted separately. No consensus structure was found when DNASIS was used to predict the secondary structure of 3′-UTR. However, when FOLD was used, the predicted secondary structure for five pestiviruses was very similar to one another. The consensus model contains a series of 4 stem-loop structures, designated as stem-loop I, II, III and VI (Fig. 19). The optimal ΔG values of the structure are -48.7, -44.1, -56.3, -46.6 and -42 for SD-1, NADL, Osloss, Alfort and Brescia, respectively. Stem-loop I, which contains 60 nucleotides and is located at the 3′ portion of the 3′C region, is conserved among all the 5 pestiviruses. This stem-loop can be folded independently in NADL, Osloss, Alfort and Brescia. However, in BVDV SD-1, this stem-loop could be interfered with by the surrounding sequence. It also should be noted that the intervening sequence ACAGCACUUUA between stem-loop I and II was also conserved in the five pestiviruses (Fig. 17). Stem-loop II was composed of part of the 3′V region and part of the 3′C region of the primary structure and its structural detail was relatively conserved among. Stem-loop III and VI, located at the 5′ terminal portion of the 3′-UTR and within the 3′V region, were less conserved in terms of the length of the stem and structural configuration, particularly between BVDV group and HoCV group.
DISCUSSION

Interactions between viral template RNA and the proteins of the replication and translation complexes are presumed to be mediated by a signal sequence present in the viral RNA template. The pestivirus genome is a single-stranded, positive sense RNA molecule, and no subgenomic RNA has been detected (Purchio et al., 1983; Renard et al., 1987b). The genomic RNA of pestivirus must serve as the template for both replication of viral genome and translation of viral polyproteins. The signals for RNA replication and polyprotein translation is presumed to be located in either 5'-UTR or 3'-UTR of pestivirus genome.

Pestivirus has a relatively long 5'-UTR. The characteristic feature of the primary structure of 5'-UTR is the presence of multiple cryptic AUGs and several small ORFs. Because most of the small ORFs are poorly conserved among pestiviruses and no virus specific polypeptide have been detected in vivo or in vitro from this region, it was believed that these small ORFs had no coding function. Studies on the cryptic AUG codons of poliovirus RNA indicate that the upstream ORFs are not essential for virus replication (Pelletier et al., 1988). However, the utilization of the cryptic AUGs upstream from the authentic AUG in the 5'-UTR of human rhinovirus has been demonstrated by the fact that a number of unexpected polypeptides appeare early in time-course experiment in addition to the expected authentic products.
(Borman and Jackson, 1992). It has been also reported that two
AUG start codons, separated by 84 bases, were used to initiate
viral protein synthesis of foot-and-mouth disease virus (FMDV)
both in vivo and in vitro (Belsham, 1992). Limited data of in
vitro translation of BVDV RNA revealed that in addition to
cellular proteins, a large number of other proteins
evidenced by high levels of background were synthesized in a
in vitro translation system (Purchio et al., 1984a), possibly
suggesting abnormal initiation of viral RNA translation.
Therefore, whether the cryptic AUGs in the 5'-UTR of
pestivirus are functional remains to be determined by
carefully designed experiments.

Compared with the consensus context of AUG start codon
for efficient initiation (Kozak, 1986), the authentic AUG of
pestivirus has a weak context for initiation, where the
nucleotide at position -4 is C rather than A or G. The weak
context of the authentic AUG may be one of the reasons that
pestivirus can not efficiently replicate in vitro and possibly
in vivo. The most favorable context for efficient initiation
was found in two of the cryptic AUGs, one of which can
potentially open a reading frame encoding a polypeptide of 45
amino acid residues in both NADL and SD-1 and the other can
potentially open a reading frame encoding a polypeptide with
12 amino acid residues. Whether these small ORFs have a coding
function remains to be determined. It has been proposed that,
similar to the Shine-Dalgarno sequence in procaryotic mRNA, an
oligopyrimidine tract upstream the authentic AUG in a number of eucaryotic mRNA and viral RNA mediates the base-pairing with the consensus sequence of 3' terminus of eucaryotic 18S rRNA and directs the translation initiation (Hagenbüchle et al., 1978; Agol, 1991; Pestova et al., 1991; Pilipenko et al., 1992). Interestingly, two rather than one such oligopyrimidine tracts were identified in 5'-UTR of pestivirus and both of these are located closely upstream to the AUGs that are conserved among all the pestiviruses, including the authentic AUG (Fig. 14). Whether either of the oligopyrimidine tracts mediate ribosome binding to and direct the translation initiation at the cryptic AUG sites remains to be determined. However, the second oligopyrimidine tract, 20 bases upstream the authentic AUG, most probably mediates the recognition of RNA template by ribosomes and directs the translation initiation of the polyprotein at this site. It should be noted that this oligopyrimidine tract does not participate in the base pairing to conform to the secondary structure of 5'-UTR and, as linear sequence. This tract is located in the intervening region between domain D and E, indicating the ease accessibility of this stretch to the 18S rRNA to mediate base pairing.

It has been reported that the insertion of a tract with an AUG codon upstream to the authentic AUG reduced virus growth, conferring a small plaque phenotype (Kuge et al., 1988). In this study, all the large plaque revertants had lost
the inserted AUG codon as a result of point mutations. It has also been reported that an AUG, created by point mutation 8 bases upstream the authentic AUG, acted as a barrier to initiating at the downstream authentic AUG (Kozak, 1986). In the 5′-UTR of pestivirus, just 7 bases upstream the authentic AUG is a conserved cryptic AUG in a different reading phase. It is possible that this closely located cryptic AUG could functions as a negative regulator to the downstream authentic AUG and decrease the initiation efficiency of viral RNA translation, resulting in relatively poor growth of pestivirus in vitro and possibly in vivo.

With the completion of the genomic sequence of BVDV NADL and SD-1 (Brock et al., 1992a; Deng and Brock, 1992a), we have now been able to perform secondary structural analysis for the entire 5′-UTR of the pestivirus genome. Although the secondary structure model presented here has not been demonstrated by experiments, the high conservation of the structure through a number of compensatory mutations among all the pestiviruses indicates the possibility of the existence of such a structure in vivo. Particularly, domain D, which covers two third of the 5′-UTR, is almost identical among all the pestiviruses, although two highly variable stretches of primary structure are located in this region.

Recent evidence suggests that picornavirus behaves unlike most other eucaryotic mRNA in its translational strategy in that it appears to initiate translation internally through the
internal ribosome entry site (IRES) within 5′-UTR (Jang et al., 1988; Pelletier and Sonenberg, 1988; Jackson, 1988; Jang et al., 1989; Iizuka et al., 1991; Borman and Jackson, 1992). It also has been demonstrated that the translation initiation of HCV occurs by direct entry of ribosomes to the internal sequence (IRES) within 5′-UTR (Tsukiyama-Kohara et al., 1992). The features of the primary and secondary structure of 5′-UTR of pestivirus resemble that of picornaviruses and HCV. The translation initiation of pestivirus RNA is not satisfactorily accommodated by the scanning model proposed by Kozak (Kozak, 1989a). According to this model, ribosomes and associated factors initially bind to the capped 5′ end of mRNA and scan the RNA molecule to reach the authentic initiator AUGs which is usually the first AUG and is in favorable sequence context, and the secondary structure upstream the authentic AUG dramatically decrease the translation initiation efficacy (Pelletier and Sonenberg, 1985; Kozak, 1989b). Therefore, it is tempting to speculate that, similar to the translational strategy of picornavirus and HCV, an internal translational initiation mechanism could be predicted in pestivirus based on the following considerations. (I) It has been proposed that pestivirus RNA lacks a cap structure at the 5′ end of the genome (Brock et al., 1992), suggesting a possible cap-independent translation initiation mechanism. (II) Pestivirus has a relatively long 5′-UTR in which multiple cryptic AUGs and several small ORFs are present upstream the authentic AUG
and it is unlikely that before reaching the authentic initiation site, which is in a weak context, the scanning ribosome could bypass 6 to 8 upstream AUGs, some of which have an equal or more favorable context for efficient initiation.

(III) Since a conserved and stable secondary structure was predicted in the 5′-UTR of pestivirus, it seems unlikely that ribosome could traverse such a great length of secondary structure by binding at the 5′end of the genome and scanning to reach the authentic translational initiation site. It has also been reported that secondary structure rather than merely a linear nucleotide sequence functions as IRES in picornavirus (Agol, 1991; Haller and Semler, 1992) and HCV (Tsukiyama-Kohara et al., 1992). The conserved domain D of pestivirus consisting of the sequence corresponding to the IRES sequence in HCV and may be the candidate for IRES in pestivirus.

Nucleotide sequence comparison between 5′-UTR and 3′-UTR of pestivirus did not show any significant similarity of primary structure. However, secondary structure prediction indeed led to the identification of a conserved stem-loop structure (domain A) in the extreme 5′end of the genome among BVDV strains and a conserved stem-loop structure (stem-loop I) in the extreme 3′ end of the genome among all the pestiviruses. These stem loop structures may be responsible for specific template recognition by viral RNA-dependent RNA polymerase. A 100:1 asymmetry of the synthesis of positive and negative strand viral RNA in the BVDV infected cells was
observed (Deng and Brock, 1992c). The different structural
details between domain A and stem-loop I may reflect the
different efficiency of template activity for positive and
negative strands of viral RNA. No similar stem loop structure
was identified at the extreme 5′ end of HoCV strains, probably
because the authentic 5′end of HoCV genome has not been cloned
and sequenced. The other possible explanation is that the
recognition signals for positive strand RNA replication is
different between BVDV and HoCV due to the heterogeneity of
RNA replication complexes. It has been proposed that the
nonstructural protein p135 in pestivirus is the viral RNA
replicase (Gorbalenga et al., 1989a, Moormann et al., 1990).
Amino acid sequence comparison of p135 between BVDV and HoCV
did show a supervariable stretch consisting of 114 amino acid
residues with homology of only 42% between BVDV and HoCV.

Recently pestivirus was reclassified into family
Flaviviridae (Collett et al., 1989; Horzinek, 1991; Francki et
al., 1991). However, the features of the primary and secondary
structure of 5′-UTR of pestivirus resemble that of
picornavirus and HCV rather than flaviviruses. In addition,
the predicted cap-independent translation mechanism of
pestivirus is totally different from that of a number of the
family Flaviviridae. Therefore we agree with the suggestion
that establishment of a new family may be desirable for
classification of pestivirus (Meyers et al., 1989a; Moormann
et al., 1990; Thiel, et al., 1991). HCV, which has been
reported to be more closely related to pestiviruses than flaviviruses (Han et al., 1991; Weiner et al., 1991; Choo et al., 1991; Tsukiyama-Kohara et al., 1992) and has significantly different properties from pestivirus (Han et al., 1991), probably should be classified as an independent genus in the same family.

REFERENCES


**Fig. 14.** Primary structure for the 5'-UTRs of five pestiviruses. The sequence shown in the top line is for SD-1 and the coordinates of the nucleotides for SD-1 are given above the sequence. Hyphens indicate the identity with SD-1 sequence. Asterisks denote the deletions compared with one another. AUG codons within the 5'-UTR are bolded. The polyprotein initiation AUGs are underlined. Three variable regions (I, II and III) are boxed. Two oligopyrimidine tracts (a and b) are shaded.
Pestivirus (tract a)

18S rRNA 3’ AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU 5’

SD-1 5’ (74) AUCCCU CAG 20 GCC AUG (111) 3’
NADL 5’ (74) AUCCCU CAG 20 GCC AUG (111) 3’
OSLOSS 5’ (73) AUCCCU UAG 20 GCC AUG (110) 3’
ALFORT 5’ (58) ACCCUCC- CAG 19 GCC AUG (94) 3’
BRESCIA 5’ (56) CUGCCUC- CAG 19 GCC AUG (91) 3’

Pestivirus (tract b)

18S rRNA 3’ AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU 5’

SD-1 5’ (366) AUCUCUGCUGUAC AUGGCACAUGGA (390) 3’
NADL 5’ (366) AUCUCUGCUGUAC AUGGCACAUGGA (390) 3’
OSLOSS 5’ (364) AUCUCUGCUGUAC AUGGCACAUGGA (388) 3’
ALFORT 5’ (344) AUCUCUGCUGUAC AUGGCACAUGGA (368) 3’
BRESCIA 5’ (341) AUCUCUGCUGUAC AUGGCACAUGGA (365) 3’

Picornavirus

18S rRNA 3’ AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU 5’

PV1M 5’ (556) GUGUUUCC 18 GCUAUG (589) 3’
COXBl 5’ (559) GUGUUUGA 19 GCUAUG (593) 3’
RHIN01B 5’ (557) GUGUUUCA 18 GCUAUG (590) 3’
EMCV-R 5’ (809) UUUCGUUU 14 UAAUGG (837) 3’

Hepatitis C virus

18S rRNA 3’ AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU 5’

HCV-1 5’ (190) GGCUUUUCC 10 GCUAAUGG (219) 3’
HCV-J 5’ (177) GGCUUUUCC 10 GCUAAUGG (206) 3’
HCV-H 5’ (189) GGCUUUUCC 10 GCUAAUGG (218) 3’

Fig. 15. The complementarity of oligopyrimidine tracts in pestivirus, picornavirus and hepatitis C virus to eucaryotic 18S rRNA. The 18S rRNA sequence was taken from Hagenbüchle et al. (1978). The viral names are abbreviated as follows: PV1M, poliovirus type 1 strain Mahoney; COXBl, coxsackievirus strain B1; RHIN01B, rhinovirus type 1 strain B; EMCV-R, encephalomyocarditis virus strain Rueckert; HCV-1, HCV-J and HCV-H, hepatitis C virus strain 1, J and H, respectively. The viral nucleotide sequences are taken from the following sources: PV1M, COXBl and RHIN01B, Pilipenko et al. (1992); EMCV-R, Duke et al. (1992); HCV-1, Choo et al. (1991); HCV-J, Kato et al. (1990); HCV-H, Inchauspe et al. (1991). The coordinates of the first and last nucleotides of the oligopyrimidine tracts are given parenthetically. The conserved AUG codons are shadowed. The numbers of omitted nucleotides in the middle of the tracts are given. Asterisks denote the possibility of base pairing between 18S rRNA and the viral RNA sequences.
Table 3. Primary Structural Features of the 5'-UTR of Pestiviruses and Other Related Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>5'Cap</th>
<th>Size</th>
<th>Number of AUGs</th>
<th>Number of ORFs</th>
<th>Initiator AUG context</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pestiviruses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CxxAUGG</td>
<td></td>
</tr>
<tr>
<td>BVDV SD-1</td>
<td>No</td>
<td>385nt</td>
<td>6</td>
<td>4</td>
<td>CxxAUGG</td>
<td>Deng and Brock, 1992a</td>
</tr>
<tr>
<td>BVDV NADL</td>
<td>No</td>
<td>385nt</td>
<td>6</td>
<td>4</td>
<td>CxxAUGG</td>
<td>Collett et al., 1988a</td>
</tr>
<tr>
<td>BVDV Osloss</td>
<td>No</td>
<td>383nt</td>
<td>5</td>
<td>5</td>
<td>CxxAUGG</td>
<td>Renard et al., 1987a</td>
</tr>
<tr>
<td>HoCV Alfort</td>
<td>No</td>
<td>363nt</td>
<td>7</td>
<td>5</td>
<td>CxxAUGG</td>
<td>Meyers et al., 1989a</td>
</tr>
<tr>
<td>HoCV Brescia</td>
<td>No</td>
<td>360nt</td>
<td>8</td>
<td>6</td>
<td>CxxAUGG</td>
<td>Moormznn et al., 1990</td>
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<td><strong>Hepatitis C Virus</strong></td>
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<tr>
<td>HCV1</td>
<td></td>
<td>341nt</td>
<td>5</td>
<td>3</td>
<td>AxxAUGA</td>
<td>Han et al., 1991</td>
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<td><strong>Piconavirus</strong></td>
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<tr>
<td>EMCV-R</td>
<td>No</td>
<td>834nt</td>
<td>10</td>
<td>6</td>
<td>AxxAUGG</td>
<td>Duke et al., 1992</td>
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<tr>
<td>YFV</td>
<td>Yes</td>
<td>119nt</td>
<td>0</td>
<td>0</td>
<td>AxxAUGU</td>
<td>Brinton et al., 1988</td>
</tr>
</tbody>
</table>

1: The viral names are abbreviated as follows: HCV1, hepatitis C virus strain 1; EMCV-R, encephalomyocarditis virus strain Rueckert; YFV, yellow fever virus.
Fig. 16. The consensus model of secondary structure for 5'-UTRs of five pestiviruses. Five stem-loop like structures are designated as domain A to E. Domain D was divided into five subdomains as D1 to D5. Compared with SD-1 structure, the base pair substitutions contributing to the maintenance of the same structure of domain D in other four pestiviruses are shaded. The bolded AUGs denote the polyprotein initiation codon.
Fig. 16.
Fig. 16 (continued)

**Domain D**

```
180-195
G U A 195 A U C G
199-262
100-G C A U
```

**Domain C**

```
A A 180-A A G C U A C G C G 195 A U C GG
G U AGUCGGG GUGG UGCA GCUU A D2
A ACAG CCC ACCU UGGA A
```

**Domain B**

```
A A 160-G C A A G C G A U U A
G U
```

**Domain A**

```
A A A A U A A A A G C G C A A G A G C G C A A-G C G C G A A C G A A
```

**Domain E**

```
A A 160-G C A A G C G A U U A
G U
```

**Domain F**

```
A A A A U A A A A G C G C A A G A G C G C A A-G C G C G A A C G A A
```

---

**BVDV NADL**

```
5' 1-G C-----G C-----AAA-----U G-----U G-----AAA U CUCUGC------U A-421 3'
```
Fig. 16 (continued)
Fig. 16 (continued)

HoCV ALFORT
Fig. 16 (continued)
**3'V Region**

| 12080 | SD-1 | 5' UGA**GAAC**AUAAUGCUAUAAUUAAUAAAGGAUAUUACCUCUGUA*CAUAGUGUAUAAGCAUAGUGGGGU |
|       | NADL  | 5' UGA---------------G---------------*---G--------UA-----U-A---A- |
|       | OSLOSS | 5' UGA**AU---------G-C---------------*---CUGUAU----------U--A---A-U-A- |
|       | ALFORT | 5' UGA**GC-UG**UGGCCC-UG**UCGG-CCC--U-AG-A-A-C-C-------A-----*--UAACUUA- |
|       | BRESCIA | 5' UGA**GUGCGGG**G-C-CCCGG-UC**G-G-CCCGG-AG-A-G-C-C-------G------CAC--A-**UU- |

**3'C Region**

| 12207 | SD-1 | UCAUCUACCUC*AAGCUAUACU**A**CAUACACAGCUAAACAG**UAGG**UGAGA*UUAU |
|       | NADL  | C-G-C---------------G---------------A-G-C-CG-C---------------**---**CA----*--- |
|       | OSLOSS | -U-GU--------------------------------------------------------*--GA-**AU-U-G---- |
|       | ALFORT | -A--UAAUUA**G**-U**-CUU---U--U-U-UU-UU-UU-UU-UU-UUG---UG---CA---AC-GU**CA-A |
|       | BRESCIA | -U--U--U-UAG-U**-UAA----U--U-U-UU-UU-UU-UU-UU-UG---UG-**-AGAACUG-UACA-A |

Fig. 17. Primary structure of the 3'-UTRs of five pestiviruses. 3'V region and 3'C region denote the variable and conserved region, respectively. The nucleotide sequence shown in the top line is for SD-1 and the coordinates of the nucleotides for SD-1 are given above the sequence. Hyphens indicate the identity with SD-1 sequence. Asterisks indicate the deletions compared with one another. The polyprotein stop codon UGAs are bolded. A 41 base deletion in Osloss are underlined. The AU rich stretches are shaded.
Fig. 18. Impefect repeat sequences in the 3'-UTRs of five pestiviruses. The coordinates of the first and last nucleotides of the repeat sequences are given. The identity of nucleotide between the repeats are indicated by colon. Asterisks denote the deletions of nucleotides comparing with each other.
Fig. 19. Consensus model of secondary structure for 3'-UTRs of five pestiviruses. Four stem-loop structures are designated as stem-loop I to IV in 3' to 5' direction. The polyprotein stop codon UGAs are underlined. The conserved linear sequence between stem-loop I and II are double underlined.
Fig. 19.
Fig. 19 (continued)

BVDV OSLOSS

HoCV ALFORT
Fig. 19 (continued)

HoCV BRESCIA
CHAPTER V

Bovine Viral Diarrhea Virus Replication: Asymmetric Synthesis of Positive and Negative Strand Viral RNAs

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a small enveloped virus with a single stranded, positive sense, RNA genome approximately 12.5 kb in size (Horzinek, 1981; Renard et al., 1987a; Collett et al., 1988a; Deng and Brock, 1992a). A single large open reading frame (ORF) spanning almost the entire length of the genome encodes all the viral proteins (Collett et al., 1988b). It was proposed that a large polyprotein precursor is translated and processed by either cotranslational or post-translational proteolytic cleavage (Collett et al., 1988b). Genomes of two cytopathic (CP) strains, Osloss and NADL, and one noncytopathic (NCP) strain, SD-1, have been sequenced (Renard et al., 1987a; Collett et al., 1988a; Deng and Brock, 1992a). However, little information regarding the BVDV replication has been presented (Collett et al., 1989; Moennig and Plagemann, 1992). In infected cells only one species of BVDV RNA was demonstrable and no subgenomic RNAs were reported (Purchio et al., 1983;
Renard et al., 1985; Collett et al., 1989; Rümenapf et al., 1989). BVDV RNA synthesis was not inhibited by actinomycin D (Horzinek, 1981; Purchio et al., 1983). A viral nonstructural protein was speculated to be the RNA dependent RNA polymerase responsible for BVDV RNA replication (Collett et al., 1989).

Asymmetric synthesis of positive and negative strand RNA during viral replication cycles has been described for a number of positive-stranded RNA viruses, including picornaviruses (Strauss and Strauss, 1983), flaviviruses (Westaway, 1987), alphaviruses (Sawicki and Sawicki, 1987) and coronaviruses (Sawicki and Sawicki, 1986a; Perlman et al., 1986/87). Negative strand RNA detected during replication of these viruses were either a replicative form (RF) or replicative intermediate (RI) form. However, in BVDV infected cells neither RF nor RI RNA species have been detected (Collett et al., 1989). No data on the synthesis of BVDV negative strand RNA have been reported.

In this study, synthesis of both positive and negative strand RNA of BVDV during its replication were detected, and a correlation between viral RNA synthesis and the release of virus from infected cells was investigated.

MATERIALS AND METHODS

Virus and viral RNA preparation

A cytopathic BVDV, strain NADL, was obtained from Dr. M. S. Collett (Gaithersburg, MD) (Collett et al., 1988a). A
noncytopathic BVDV, strain SD-1, was isolated from a persistently infected heifer (Brock et al., 1992). Secondary bovine turbinate (BTU) cell cultures, which was tested to be BVDV free, were grown in DMEM supplemented with 10% horse serum. After washing with medium, the cell monolayers were inoculated with BVDV NADL at 0.5 moi. After 1 hour incubation at 37°C, cell monolayers were washed again to remove unattached viruses and replaced with fresh DMEM medium. Following incubation of cell cultures at 37°C for 2 h, 4 h, 8 h, 12 h, 16 h, 24 h, 30 h, 36 h and 50 h, respectively, total RNA was extracted from infected cells at these intervals by the method of Sambrook et al. (1989). To prepare RNA from virions, virus was partially purified from infected culture supernatant and serum obtained from the heifer persistently infected with SD-1 by differential centrifugation as previously described (Brock et al., 1992a), and viral RNA was extracted with guanidine thiocyanate method (Brock et al., 1992a).

**Preparation of strand specific RNA probes**

A pBV4-p80 NADL cDNA clone containing a cDNA insert encompassing nucleotides 5644 to 7949 of the BVDV genome (Collett et al., 1988a), which corresponded to the nonstructural protein p80 region, was kindly provided by Dr. M. S. Collett (Gaithersburg, MD). To prepare a positive sense RNA probe, plasmid DNA was linearized with restriction enzyme
Sma I at nucleotides 6956, and transcribed with SP6 RNA polymerase (Promega, Madison, WI). The RNA probe produced was 1312 bases in length. Briefly, 1 μg of linearized DNA template was incubated in 40 mM Tris-HCl (pH 8.0), 6 mM MgCl₂, 10 mM DTT, 2 mM spermidine, 10 mM NaCl, 1 unit/μl Rnasein, 1 mM each of ATP, GTP and CTP, 0.65 mM UTP, 0.35 mM digoxigenin-11-uridine-5'-triphosphate (DIG) (Boehringer Mannheim, Indianapolis, IN), and 40 units of SP6 RNA polymerase for 2 h at 37°C. The transcription reaction was stopped by heating to 65°C for 10 minutes followed by the addition of 2 μl of 4 M LiCl and precipitation with 60 μl of ethanol. The RNA pellet was suspended in 60 μl of DEPC treated water. Synthesis of a negative sense RNA probe was accomplished as described above with T7 RNA polymerase (Promega, Madison, WI) using EcoR I linearized plasmid DNA at nucleotides 6524 as template. The negative sense probe was a 1425 base RNA fragment.

**Slot-blot hybridization**

Total RNA (50 μg) extracted from a flask (75 cm²) was split into two equal samples for detection of positive and negative strand viral RNA, respectively. Each RNA sample was serially dilated 10 fold and 100 μl aliquots were applied to HYBRI-slot apparatus (GIBCO BRL, Grand Island, NY) containing a nitrocellulose membrane previously wetted with distilled water. After baking at 80°C for 45 minutes, filters were prehybridized for 8 h at 42°C in a solution of 5 x SSC, 50%
formamide, 1 X Denhart's solution, 1% SDS and 25 μg/ml of denatured salmon sperm DNA. Hybridization was performed at 42°C for 14 h in prehybridization buffer containing 340 ng/ml of digoxigenin-labelled RNA probes. Hybridized filters were washed twice with 2 X SSC and 0.1% SDS for 5 minutes, and twice with 0.1 X SSC and 0.1% SDS for 15 minutes. Slot-blot hybridizations were also conducted on the RNA samples extracted from partially purified BVDV as described above.

**Immunological detection**

After washing briefly with buffer 1 (100 mM Tris-HCl, pH 7.5 and 150 mM NaCl), filters were incubated for 30 minutes in 100 ml blocking buffer containing 1% blocking reagent (Boehringer Mannheim, Indianapolis, IN) in buffer 1. After rinsing with buffer 1, filters were incubated in 20 ml of 150 mU/ml of anti-digoxigenin (Fab fragments)-alkaline phosphatase-conjugate for 30 minutes at room temperature, followed by two washes, each for 15 min, to remove unbound conjugate with 100 ml of buffer 1. After equilibration for 2 minutes in 20 ml of buffer 2 (100 mM Tris-HCl, pH 9.5; 100 mM NaCl and 50 mM MgCl₂), the filters were incubated in 10 ml of freshly prepared color-substrate solution (45 μl of 75 mg/ml nitroblue tetrazolium (NBT), 35 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate solution and 10 ml of buffer 2) in a plastic bag in the dark overnight. The reaction was stopped
by washing the filters for 5 minutes with 50 ml of Tris-HCl buffer 3 containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

**Titration of virus in the supernatant of infected cells**

After infection of cell monolayers with cytopathic BVDV NADL, the supernatants were collected at 2 h, 4 h, 8 h, 12 h, 16 h, 24 h, 30 h, 36 h and 50 h PI. Next, 100 μl of each serial two fold dilution of the supernatants were inoculated onto BTU cell monolayers in 96 well plates in replicates of 3. After incubating for 36 h to 54 h at 37°C, cytopathic effects (CPE) were observed and the 50% endpoints quantitated as cell culture infective doses/ml (CCID<sub>50</sub>/ml) were determined.

**RESULTS**

**Strand-specificity of RNA probes**

The specificities of digoxigenin-RNA probes were determined using RNA extracted from partially purified viruses, virus infected cells and mock-infected cells in a slot-blot hybridization assay. When the negative sense RNA probe was used, a hybridization signal was detected in samples extracted from both purified viruses and virus infected cells but not in mock-infected cells (Fig. 20). This result indicated that the negative sense RNA probe reacted with positive strand BVDV RNA. When the positive sense RNA probe was used, a hybridization signal was detected only with samples extracted from virus infected cells (Fig. 20),
indicating that this probe was specific for negative strand viral RNA.

**Sensitivity of RNA probes**

In this study, the actual sensitivities of the RNA probes were not determined due to difficulty in purifying enough viral RNA. However, based on the quantity of total RNA extracted from the virus infected cells, the relative sensitivity of these two probes was determined. Positive viral RNA could be detected in 25 ng of total RNA using the negative sense RNA probe. Negative strand viral RNA in 2.5 μg of total RNA was detected by a clear hybridization signal when the positive sense RNA probe was applied (Fig. 20). In addition, viral RNA extracted from 1 ml of serum from the persistently infected heifer reacted with the negative sense RNA probe.

**Accumulation of positive sense viral RNA**

To study the accumulation of progeny positive sense RNA in infected cells, total RNA was extracted at serial time intervals PI, Northern blotted, and hybridized with negative sense, digoxigenin-labeled RNA probe. No hybridization was detected at 2 h and 4 h PI. Positive strand viral RNA was first detected at 8 h PI evident as a weak hybridization signal in the 1:1 diluted sample. After 8 h PI, positive strand viral RNA accumulated rapidly. Definite hybridization signals were detected with RNA extracted at 12 h PI and
diluted to 1:10, 16 h PI and diluted to 1:1000, and 24 h PI and diluted to 1:10000. From 24 h to 36 h PI, detection of positive strand viral RNA remained nearly constant. By 50 h PI only a weak hybridization signal was detected in the sample diluted 1:1 (Fig. 21). It should be noted that the CPE appeared 36 h PI in cell cultures. At 50 h PI, 80% of the infected cells were dead. Therefore, the decrease in positive strand RNA after the CPE appeared may have been a result of cell death.

Accumulation of negative sense viral RNA

To determine the kinetics of the accumulation of negative strand RNA in infected cells, the positive sense RNA probe was utilized with RNA extracted from infected cells at the same intervals as described previously. At 2 h and 4 h PI, the level of negative strand viral RNA was too low to be detected by hybridization. A weak hybridization signal was obtained with RNA extracted at 8 h PI and diluted 1:1. A ten fold increase in the accumulation of negative strand RNA was observed from 8 h to 12 h PI. However, in contrast to the positive viral RNA, which increased steadily and rapidly after 8 h PI, negative strand RNA maintained the same level from 12 h to 16 h PI. After 16 h PI, it increased to its highest level by 24 h PI, when a hybridization signal was detectable in the RNA sample diluted to 1:100. Similar to positive strand RNA, negative strand RNA remained almost a constant level from 24
h to 36 h PI, followed by a decrease from 36 h to 50 h PI, when only a weak hybridization signal was shown in the sample diluted 1:1 (Fig. 22).

Comparison of the synthesis of positive and negative strand viral RNA revealed that both positive and negative strand RNA became detectable 8 h PI and their accumulation patterns appeared to be similar. However, positive strand RNA accumulated much faster than the negative strand RNA and the level of the negative strand RNA was considerably lower than that of positive strand RNA. The ratio of positive to negative strand RNA at the steady phase (24 h to 36 h PI) was 100:1.

Release of infectious virus from infected cells

To investigate the relationship between the accumulation of viral RNA and the release of infectious virus from infected cells, supernatants of infected monolayers were recovered after different periods of incubation. Quantitation of viruses in the samples was made by the end-point method as appearance of CPE. The 50% endpoint quantitated as CCID$_{50}$/ml was determined. A very low virus titer was detected at 0 h and 2 h PI, which accounts for the presence of remaining virus from the inoculum. After 2 h PI, release of viruses from the infected cells could be detected and thereafter gradually increased to the highest level by 24 h PI. After 24 h PI, the virus titer remained almost constant with a little decrease
(Table 4). These results were consistent with the accumulation patterns of viral RNA in the virus infected cells.

**DISCUSSION**

As a positive sense virus, BVDV replication shares some properties with other positive-stranded viruses. It is generally assumed that positive-stranded viruses initiate infection with the translation of parental genomic RNA to produce the viral RNA replicase, which utilizes the parental positive strand RNA as a template for the synthesis of negative strand RNA. The negative strand RNA in turn serves as a template for the synthesis of positive strand RNA and/or subgenomic RNA (Strauss and Strauss, 1983). However, the accumulation patterns of positive and negative strand RNA varied among different viruses indicating the various mechanisms by which the synthesis of positive and negative strand RNA may be regulated. Based on the results of this study, the accumulation of positive strand RNA of BVDV resembles that of other positive-stranded viruses (Sawicki and Sawicki, 1986a; Chu and Westaway, 1985; Bruton and Kennedy, 1975). Typically, synthesis, once initiated, is stable and continues throughout the replication cycle. However, the synthesis of negative strand RNA of BVDV is similar to coronaviruses (Sawicki and Sawicki, 1986a) and flaviviruses (Chu and Westaway, 1985) but different from alphaviruses (Bruton and Kennedy, 1975). In alphaviruses the synthesis of
negative strand RNA was temporally regulated (Bruton and Kennedy, 1975). From 1.5 h to 2.5 h PI, the rate of negative strand synthesis increased rapidly and thereafter, the rate fell sharply and by 4 h PI was undetectable (Bruton and Kennedy, 1975). For BVDV, the synthesis of negative strand RNA was not detected until 8 h PI. Thereafter, it accumulated continuously throughout the replication cycle.

In this study, a 0.5 moi was used to inoculate cell monolayers, which may not have allowed all cells to be simultaneously infected. Following the initial release of virus from the infected cells, secondary infections of uninfected cells may have occurred. Therefore, viral RNA accumulation patterns and virus release obtained in this study may, to a certain extent, differ from the kinetics of BVDV RNA synthesis and virus release when a moi of 1.0 or more was used.

The asymmetric synthesis of positive and negative strand RNA of BVDV was demonstrated by the ratio of 100:1 at the steady phase, which was similar to that of brome mosaic virus (BMV) (French and Ahlquist, 1987; Rao et al., 1990) and coronaviruses (Sawicki and Sawicki, 1986a). Different degrees of asymmetry between positive and negative strand RNA in infected cells have been reported for other positive-stranded RNA viruses, e.g., 10:1 for flaviviruses (Cleaves et al., 1981), 20:1 for alphaviruses (Wang et al., 1991), and 1000:1 for alfalfa mosaic virus (Nassuth and Bol, 1983),
respectively. Little is known about the mechanism by which the asymmetric synthesis of positive and negative strand RNA is regulated. It has been reported that a cis-acting element in the RNA-3 of BMV was the primary determinant of asymmetric replication of positive and negative strand viral RNA (Marsh et al., 1991). Wang et al. (1991) described that a nonstructural protein, nspl, of sindbis virus was involved in the regulation of negative strand RNA synthesis. It also has been proposed that two different RNA replicase activities were responsible for the asymmetric synthesis of positive and negative strand RNA of picornaviruses (Strauss and Strauss, 1983). One of the nonstructural proteins of BVDV was proposed to be the viral RNA replicase (Collett et al., 1989). However, no direct experimental data has been reported about its function in viral RNA replication. The primary and secondary structural analysis of the 5' and 3' end nucleotide sequence of BVDV revealed stem loop structures with different details at both ends (Deng and Brock, 1992b). Whether these structures function as the recognition signals with different efficiencies for viral positive and negative RNA replication remains to be determined.

Both RF and RI RNA species have been observed in a number of positive-stranded RNA viruses, including flaviviruses (Cleaves et al., 1981; Chu and westaway, 1985; Westaway, 1987), coronaviruses (Sawicki and Sawicki, 1986a) and alphaviruses (Bruton and Kennedy, 1975; Sawicki and Sawicki,
1986b). The negative strand RNAs detected were exclusively in the form of either RF or RI in these viruses. Neither RF nor RI RNA species have been described in BVDV infected cells (Collett et al., 1989). In this study, the negative strand RNA of BVDV was demonstrated in the BVDV infected cells by a strand specific RNA probe. It seems to be unlikely that, in contrast to other positive-stranded RNA viruses, negative strand RNA of BVDV was in the single stranded form rather than the RF or RI form. Poor replication of BVDV in cell culture (Collett et al., 1989) and considerable asymmetry of positive and negative strand RNA may account for the major reason why RF or RI RNA species have not been purified and characterized in BVDV infected cells. In addition, the presence of extensive secondary structure of the genomic RNA of BVDV may also interfere with the identification of RF and/or RI RNA species (Collett et al., 1989).

Ridpath and Bolin (1990) reported that viral polypeptide synthesis was detected with both NCP and CP BVDV by 8 h PI. Our results were in agreement with this observation. Both positive and negative strand viral RNA were detected by hybridization at 8 h PI. However, in other positive-stranded RNA viruses they could be detected as early as 1.5 h and 4 h PI (Chu and Westaway, 1985; Bruton and Kennedy, 1975; Sawicki and Sawicki, 1986a; Sawicki and Sawicki, 1986b). One explanation was that viral RNA replication of BVDV was slower than that of these positive-strand viruses at the initial
stage of the infection. However, this could not exclude the possibility of the different sensitivities of the detection methods applied.

REFERENCES


Fig. 20. Specificity of RNA probes for positive and negative strand RNA of BVDV. RNAs extracted from virus infected cells, partially purified virions and mock infected cells were ten-fold diluted and applied on nitrocellulose membrane. (I) Negative sense RNA probe was used to detect positive strand viral RNA. (II) Positive sense RNA probe was used to detect negative strand viral RNA. Column a is the RNA sample from virus infected cells, column b is the RNA sample from purified virions and column c is the RNA sample from mock infected cells.
Fig. 21. Synthesis of positive strand RNA of BVDV after infection of cells with virus. Total RNA extracted from virus infected cells at serial time intervals postinfection (p.i.) was ten-fold diluted and applied to nitrocellulose membrane. The positive strand viral RNA was detected with a digoxigenin-labelled, negative sense RNA probe.
Fig. 22. Synthesis of negative strand RNA of BVDV after infection of cells with virus. Total RNA extracted from virus infected cells at serial time intervals postinfection (p.i.) was ten-fold diluted and slot-blotted on nitrocellulose membrane. The negative strand viral RNA was detected with a digoxigenin-labelled, positive sense RNA probe.
Table 4. Temporal Release of Viruses From Infected Cells

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GENERAL DISCUSSION

Strain SD-1 is a NCP BVDV, which naturally established a persistent infection in a heifer. The persistently infected heifer provided the virus source for these studies. The genomic sequences of both CP NADL and Osloss were determined from the viral stocks that had been adapted to and propagated in cell cultures. Both adaptation to and passage in cell culture of viruses are selection courses for the virus population. In order to eliminate the possibility of adaptive selection of virus during cell culture passage and to eventually evaluate the naturally occurring mutation rate of the viral genome in vivo, the virus obtained from a persistently infected animal without cell culture passage was utilized to clone and sequence the SD-1 genome. The cDNA sequence of SD-1, therefore, represents the genomic sequence of the virus obtained in vivo, which may be different from that of the virus obtained in vitro due to the adaptive selection of virus during cell culture passage.

The most remarkable difference between the genome sequence of NCP BVDV SD-1 and CP BVDV NADL and Osloss is that SD-1 genome has no cellular RNA insertion sequence in the p125 region whereas both NADL and Osloss have an inserted cellular
RNA sequence in that region. This result strongly supports the working hypothesis for the cytopathogenicity of BVDV proposed by Meyers et al., (1991) that the insertion of a cellular RNA sequence by RNA recombination into NCP BVDV genome was responsible for the development of CP BVDV from NCP BVDV. In addition to the obvious difference of cellular insertion, there are many base substitutions in NADL and Osloss in the N-portion of p125, which result in changes in the amino acid sequence. The p80 has been reported to be the only marker of CP BVDV (Meyers et al., 1991). Probably as a consequence of cellular RNA insertion, p125 acquires properties that can lead to the cleavage of p125, resulting in the formation of p80 and p54 in CP BVDV (Collett et al., 1991). However, this notion can not explain the observation that some CP BVDV strains lack cellular RNA insertion in the region of p125. In addition, it also has been reported that some of the CP BVDV strains had almost identical sizes of p125 with that of NCP BVDV (Akkina, 1991, Greiser-Wilke et al., 1992), implying that minor changes of amino acid sequence in p125 also could result in the release of the p80.

Comparison of NCP BVDV SD-1 nucleotide sequence with CP BVDV NADL and Osloss at the N-terminal part of p125 revealed that, in addition to the obvious difference of cellular RNA insertion, there are many base substitutions in NADL and Osloss, which result in the change of amino acid sequence, particularly in the V3 domain. Secondary structure prediction
from amino acid sequence in this region reveals that some of the amino acid changes alter the secondary structure of the N-terminal portion of p125 (data not shown). Therefore, in addition to the insertion, the base substitutions resulting in conformational change of the N-terminal part of p125 may also contribute to the release of p80 from the polyprotein. It is tempting to propose that the mutations that occurred in NCP BVDV, such as insertion, deletion, duplication and substitution, result in conformational changes in the N-terminal portion of p125. Following the conformational change, the cleavage site at the N-terminus of p80 becomes functional and the p80 is released from the viral polyprotein, resulting in the development of CP BVDV from NCP BVDV.

Distinct antigenic differences between BVDV and HoCV have been described by a number of authors using cross-neutralization test and monoclonal antibody mapping. The SD-1 nucleotide sequence data correlate with these observations. The overall nucleotide sequence homologies of SD-1 are 88.6% with BVDV NADL, 78.3% with BVDV Osloss, but 67.1% with HoCV Alfort and 67.2% with HoCV Brescia.

Comparison of both nucleotide and amino acid sequences of SD-1 with that of other pestiviruses revealed that the most variable sequences are located in the viral glycoproteins, particularly the gp53, and nonstructural proteins p54 and p58. It has been reported repeatedly that neutralizing monoclonal antibodies against BVDV bound to and immunoprecipitated
glycoprotein gp53 (Magar et al., 1988, Donis et al., 1988, Bolin et al., 1988, Xue et al., 1990, Weiland et al., 1990). Antigenic variation of this protein among BVDV isolates was demonstrated by the fact that none of the monoclonal antibodies neutralized all the BVDV isolates tested (Magar et al., 1988, Xue et al., 1990). It is, therefore, reasonable to state that the high heterogeneity of the amino acid sequences in the viral structural proteins, particularly the gp53, are responsible for most antigenic variations on the interspecies and to a lesser extent on the intraspecies level. Two hypervariable domains (V1, V2) were identified in the viral glycoprotein gp53. The hydrophobicity analysis indicated that these two domains are hydrophilic, suggesting that V1 and V2 domains are present on the outer surface of virions and may represent the protective epitopes of this protein, which are the important targets for the immune response of the host, and therefore under the high immunological pressure. The high diversity of amino acid sequence in the viral glycoproteins among BVDV and HoCV implies the difficulty in developing an effective vaccine for preventing pestivirus infections by expression of these glycoproteins.

With the exception of unpublished observations (Collett et al., 1988b) (Collett et al., 1989) no information has been published on the termini of pestivirus RNA to date. Although most of the genome of two strains of BVDV, NADL and Osloss, has been cloned and sequenced, the complete nucleotide
sequence of the BVDV genome covering the authentic 5’ and 3’ termini has not been established. Using RNA ligation and primer-directed amplification, the 5’ and 3’ terminal sequences of both strains NADL and SD-1 were determined. From the data obtained in this study, the 3’ terminus of the NADL BVDV genome must include an additional 5 cytosine nucleotides ("CCCCC"). This would increase the number of nucleotides in the complete genome of CP NADL to 12,578. The complete nucleotide sequence of NCP SD-1 was established to be 12,308 nucleotides in length. The size difference between CP NADL and SD-1 genome is due to a 270 base cellular RNA insertion in the CP NADL genome. Therefore, the authentic BVDV genome size, without cellular RNA insertion, should be 12,308 bases in length, which is closer to the reported genome size of HoCV (12,284 bases for strain Alfort and 12,283 bases for strain Brescia). Because the 5’ and 3’ terminal sequences of HoCV genome have not been firmly established, the slight difference between BVDV and the reported HoCV genome size could be due to the missing nucleotide sequences at the both termini of the HoCV genome.

Recently, the pestiviruses have been re-classified as members of the Flaviviridae due to similar genomic organization and replication strategies (Collett et al., 1989). Although the Flaviviridae are known to possess a terminal cap structure, to date, no cap structure has been identified for BVDV. Based on the fact that the 5’ terminus
failed to be radiolabelled with $[^{32}\text{P}]$ATP using polynucleotide kinase with or without previous phosphatase treatment, a cap structure at the 5' terminus of the BVDV genome was speculated (Collett et al., 1988a) (Collett, et al 1989). Making the assumption that the BVDV genomic RNA was capped, a decapping step was done prior to RNA ligation using TAP. However, surprisingly, identical amplification products as well as nucleotide sequence data were obtained when the RNA ligation was done without a previous decapping reaction for all three strains of BVDV (CP NADL, CP 72, and NCP SD-1). It may be argued that incomplete replicative RNA intermediates lacking a cap structure may have been present and were ligated and amplified. This was not probable due to the fact that virus from CP NADL and CP 72 were purified from supernatant fluids prior to the development of cytopathic effects. Additionally, NCP SD-1 virus was purified from serum collected from a persistently-infected animal which should not have contained incomplete RNA replicative intermediates. Although this is indirect evidence these results would suggest that BVDV genomic RNA may not possess a cap structure, which is present in all the members of Flaviviridae.

Interactions between viral template RNA and the proteins of the replication and translation complexes are presumed to be mediated by a signal sequence present in the viral RNA template. The pestivirus genome is a single-stranded, positive sense RNA molecule, and no subgenomic RNA has been detected
(Purchio et al., 1983; Renard et al., 1987a). The genomic RNA of pestivirus must serve as the template for both replication of viral genome and translation of viral polyprotein. The signals for RNA replication and polyprotein translation are presumed to be located in either the 5'-UTR or 3'-UTR of the pestivirus genome. With the completion of the 5' and 3' terminal sequences for strains CP NADL and NCP SD-1, we enabled the analysis of the primary and secondary structure for 5' and 3'-UTR of BVDV and the comparisons between BVDV and HoCV.

The characteristic feature of the primary structure of 5'-UTR of pestiviruses is the presence of multiple cryptic AUGs and several small ORFs, which is similar to the properties of 5'-UTRs of picornaviruses and hepatitis C virus, rather than a member of Flaviviridae. Because most of the small ORFs are poorly conserved among pestiviruses and no virus specific polypeptides have been detected in vivo or in vitro from this region, it was believed that these small ORFs had no coding function (Collett et al., 1989).

Compared with the consensus context of AUG start codon for efficient initiation (Kozak, 1986), the authentic AUG of pestivirus has a weak context for initiation. The weak context of the authentic AUG may be one of the reasons that pestivirus can not efficiently replicate in vitro and possibly in vivo. It has been proposed that, similar to the Shine-Dalgarno sequence in procaryotic mRNA, an oligopyrimidine
tract upstream from the authentic AUG in a number of eucaryotic mRNA and viral RNA, mediates base-pairing with the consensus sequence of 3' terminus of eucaryotic 18S rRNA and directs translation initiation (Hagenbüchle et al., 1978; Agol, 1991; Pestova et al., 1991; Pilipenko et al., 1992). Interestingly; two, rather than one, oligopyrimidine tracts were identified in 5'-UTR of pestivirus and both of these are located closely upstream to the AUGs that are conserved among all the pestiviruses. Whether either of the oligopyrimidine tracts mediate ribosome binding and direct translation initiation at these two AUG sites remains to be determined.

The functional elements of the single stranded RNA molecule of the pestivirus genome include both primary and secondary structure. Secondary structural analysis for the entire 5'-UTR of the pestivirus genome led to the establishment of a consensus model for the 5'-UTR of pestiviruses. Although the secondary structure model presented has not been demonstrated by experiments, the high conservation of the structure through a number of compensatory mutations among all the pestiviruses indicates the possibility of the existence of such a structure in vivo. Particularly, domain D, which covers two thirds of the 5'-UTR, is almost identical among all the pestiviruses, although two highly variable stretches of primary structure are located in this region.
Recent evidence suggests that picornavirus behaves unlike most other eucaryotic mRNA in its translational strategy in that it appears to initiate translation internally through the internal ribosome entry site (IRES) within 5'-UTR (Jang et al., 1988; Pelletier and Sonenberg, 1988; Jackson, 1988; Jang et al., 1989; Iizuka et al., 1991; Borman and Jackson, 1992). It also has been demonstrated that the translation initiation of hepatitis C virus (HCV) occurs by direct entry of ribosomes to the internal sequence (IRES) within 5'-UTR (Tsukiyama-Kohara et al., 1992). The features of the primary and secondary structure of 5'-UTR of pestivirus resemble that of picornaviruses and HCV. The translation initiation of pestivirus RNA is not satisfactorily accommodated by the scanning model proposed by Kozak (Kozak, 1989a). According to this model, ribosomes and associated factors initially bind to the capped 5' end of mRNA and scan the RNA molecule to reach the authentic initiator AUGs which is usually the first AUG and is in favorable sequence context, and the secondary structures upstream from the authentic AUG dramatically decrease the translation initiation efficiency (Pelletier and Sonenberg, 1985; Kozak, 1989b). Therefore, it is tempting to speculate that, similar to the translational strategy of picornavirus and HCV, an internal translational initiation mechanism could be predicted in pestivirus based on the following considerations. (I) Pestivirus RNA may lack a cap structure at the 5'end of the genome as described before,
suggesting a possible cap-independent translation initiation mechanism. (II) Pestivirus has a relatively long 5′-UTR in which multiple cryptic AUGs and several small ORFs are present upstream from the authentic AUG and it is unlikely that before reaching the authentic initiation site, which is in a weak context, the scanning ribosome could bypass 6 to 8 upstream AUGs, some of which have an equal or more favorable context for efficient initiation. (III) Since a conserved and stable secondary structure was predicted in the 5′-UTR of pestivirus, it seems unlikely that ribosomes could traverse such a great length of secondary structure by binding at the 5′end of the genome and scanning to reach the authentic translational initiation site.

Nucleotide sequence comparisons between 5′-UTR and 3′-UTR of pestivirus did not show any significant similarity of primary structure. However, secondary structure prediction led to the identification of a conserved stem-loop structure (domain A) in the extreme 5′end of the genome among BVDV strains and a conserved stem-loop structure (stem-loop I) in the extreme 3′ end of the genome among all the pestiviruses. These stem loop structures may be responsible for specific template recognition by the viral RNA-dependent RNA polymerase. The different structural details between domain A and stem-loop I may reflect the different efficiency of template activity for positive and negative strand synthesis of viral RNA.
Although the accumulation patterns for both positive and negative strand RNAs of BVDV during the virus replication cycle in the cell cultures are similar, the positive strand RNA synthesis was the dominant event throughout the virus replication cycle and its level was significantly higher than that of the negative strand RNA. The ratio of positive: negative strand RNA at the steady phase was 100:1. The mechanism by which the asymmetric synthesis of positive and negative strand RNA of BVDV was regulated remains to be investigated.
APPENDIX

List of NCP BVDV strain SD-1 cDNA clones and subclones

1. ST7-866 clones

ST7-866 clones: ST7866-1, ST7866-2, ST7866-3, ST7866-4, ST7866-5, ST7866-6, ST7866-7, ST7866-9, ST7866-10. ST7866-1 clone was used to verify the sequence errors IDENTIFIED in the clones ST7-1586 A-13 and B-15. Therefore, its sequence was not completely determined.

2. ST7-1586 clones

a. Original cDNA clones:
   PCR reaction A: A-1, A-2, A-3, A-4 (these clones may be the contaminated ST7-866 clones based on the restriction enzyme mapping); A-8, A-13, A-14 (the insert in these clone was confirmed based on enzyme digestion results); A-18, A-22, A-26, A-27, A-28, A-31, A-32 (unidentified). ST7-1586 A-13 clone was used to sequence.
   PCR reaction B: B-1, B-4, B-5, B-6, B-8, B-12, B-13, B-15, B-16, B-17, B-18 (the insert in these clones was confirmed). ST7-1586 B-15 clone was used to determine the sequence

b. Subclones of ST7-1586 A-13 clone:
   Pst I sites were utilized to subclone.
   250 bp band: B-1, B-2, B-4, B-5, W-1, W-2, W-5, W-6. W-1 clone was sequenced.
   280 bp band: W-3, W-9. W-9 clone was sequenced
   400 bp band: 4-1, 4-2, 4-3, 4-4, 4-6, 4-7, 4-8. 4-1 clone was sequenced.
   811 bp band: 8-1, 8-2, 8-3, 8-4, 8-5, 8-7, 8-8. 8-1 clone was sequenced.

   The nucleotide (nt) sequence of ST7-1586 A-13 clone starts with NADL primer sequence (nts 11 to 41) and ends at SD-1 nts 1585 followed by the NADL primer sequence (nts 1586-1605). One nucleotide error is present in this clone at nts
Subclones of ST7-1586 B-15 clone:
Pst I sites were utilized to subclone.
250 bp band: B-1, B-2, B-3, B-4, W-1, W-2, W-3, W-4. W-2 clone was sequenced.
280 bp band: W-1, B-1, B-2. B-2 clone was sequenced.
400 bp band: 4-1, 4-3, 4-4, 4-6, 4-7, 4-8. 4-3 clone was sequenced.
811 bp band: 8-1, 8-2, 8-4, 8-6, 8-8. 8-2 clone was sequenced.
The sequence of ST7-1586 B-15 clone starts with T7 promoter sequence and a NADL primer sequence (nts 1 to 41) and ends at SD-1 nts 1585 followed by a NADL primer sequence (nts 1586-1605). Four nucleotide errors are present in this clone at nts 359 (G should be A), 600 (T should be G), 737 (G should be A) and 760 (T should be C).

3. S1392-2978 clones

a. Original cDNA clones:
PCR reaction A: S13A-2, S13A-3, S13A-8, S13A-9, S13A-10, S13A-13, S13A-14, S13A-27. S13A-3 was used to subclone and sequence.
PCR reaction B: S13B-1, S13B-2, S13B-6, S13B-8. S13B-6 clone was used to subclone and sequence.
PCR reaction C: S13C-4, S13C-5, S13C-6, S13C-7, S13C-8, S13C-9. S13C-6 clone was used to subclone and sequence.

b. Subclones of S13A-3 clone:
BamH I and Pst I sites were utilized to subclone.
BP band 1 (480 bp): BP-1, BP-5, BP-6. BP-6 clone was used to sequence.
BP band 2 (450 bp): BP-2, BP-3, BP-4, BP-7, BP-8. BP-7 clone was used to sequence.
Pst I band (750 bp): P-750-1, P-750-2, P-750-3, P-750-4. P-750-2 clone was used to sequence.
S13A-3 clone was used to verify the error sequences in S13B-6 and S13C-6 clones. Therefore, its sequence was not completely determined.

c. Subclones of S13B-6 clone:
BamH I and Pst I sites were utilized to subclone.
BP band 1 (480 bp): BP-1, BP-2, BP-3, BP-4, BP-5, BP-6, BP-9. BP-3 clone was used to sequence.
BP band 2 (450 bp): BP-7, BP-8. BP-7 clone was used to sequence.
Pst I band (750 bp): P-750-1, P-750-2, P-750-3, P-750-4. P-750-1 clone was used to sequence.
The sequence of S13B-6 clone starts at SD-1 nucleotides
1392 and ends at SD-1 nucleotides 2999. Five nucleotide errors are present in this clone at nts 1698 (T should be A), 1732 (C should be T), 1746 (T should be A), 2183 (G should be A) and 2547 (C should be T).

d. Subclones of S13C-6 clone:
BamH I and Pst I sites were utilized to subclone.
BP band 1 (480 bp): BP-1, BP-2, BP-3, BP-6, BP-7, BP-8. BP-2 clone was used to sequence.
BP band 2 (450 bp): BP-4, BP-5, BP-9. BP-4 clone was used to sequence.
Pst I band (750 bp): P-750-1, P-750-2, P-750-3, P-750-4. P-750-2 clone was used to sequence.
The sequence of S13C-6 clone starts at SD-1 nts 1392 and ends at SD-1 nts 2999. No nucleotide error is present in this clone.

4. S2817-4285 clones

a. Original cDNA clones:
PCR reaction B: S28B-1, S28B-3, S28B-4, S28B-5, S28B-6, S28B-7, S28B-21, S28B-22, S28B-24, S28B-26, S28B-27. S28B-4 clone was used to subclone and sequence.

b. Subclones of S28A-7 clone:
Mbo I sites were used to subclone.
105 bp band: 1#, 2#, 3#, 4#, 5#, 6#. 1# clone was used to sequence.
210 bp band: 1#, 2#, 3#, 4#, 5#, 6#. 6# clone was used to sequence.
320 bp band: 1#, 2#, 3#, 4#, 5#. 3# clone was used to sequence.
340 bp band: 1#, 2#, 3#. 1# clone was used to sequence.
470 bp band: 1#, 2#. 2# clone was used to sequence.
The sequence of S28A-7 clone starts with a NADL primer sequence (nts 2817 to 2834), followed by SD-1 sequence (nts 2835 to 4284) and ends with a NADL primer sequence (nts 4285 to 4310). Three nucleotide errors are present in this clone at nts 3295 (G should be C), 3398 (G should be A) and 3588 (T should be A).

c. Subclones of S28B-4 clone:
Mbo I sites were used to subclone.
105 bp band: 1#, 2#, 3#, 4#, 5#, 6#. 4# clone was used to sequence.
210 bp band: 1#, 2#, 3#, 4#, 5#, 6#. 2# clone was used to sequence.
320 bp band: 1#, 2#, 3#. 1# clone was used to sequence.
340 bp band: 1#, 2#, 3#, 4#. 2# clone was used to sequence.
470 bp band: 1#, 2#, 3#, 4#. 2# clone was used to sequence.

The sequence of S28A-4 clone starts with a NADL primer sequence (nts 2817 to 2834), followed by SD-1 sequence (nts 2835 to 4284) and ends with a NADL primer sequence (nts 4285 to 4310). One nucleotide error is present in this clone at nucleotides 4175 (G should be A).

d. S28A-28 and S28B-5 clones were utilized to verify the sequence errors identified in the clones S28A-7 and S28B-4. Therefore, the sequences of S28A-28 and S28B-5 clones were not completely determined.

5. S4193-5862 clones

a. Original cDNA clones:
   PCR reaction A: S41A-1, S41A-11, S41A-17, S41A-25. S41A-1 clone was used to subclone and sequence.
   PCR reaction B: S41B-17. This clone was used to subclone and sequence.
   PCR reaction C: S41C-1, S41C-2, S41C-3, S41C-5, S41C-6. S41C-1 clone was used to subclone and sequence.

b. Subclones of S41A-1 clone:
   Hind III and Kpn I sites were used to subclone.
   HK band 1 (750 bp): HK-1, HK-2, HK-3, HK-4, HK-5, HK-6, HK-7, HK-8, HK-9. HK-3 clone was used to sequence.
   HK band 2 (730 bp): HK-10, HK-12, HK-13, HK-14, HK-15, HK-16, HK-17, HK-18. HK-10 clone was used to sequence.
   S41A-1 clone was used to verify the nucleotide errors found in the clones S41B-17 and S41C-1. Therefore, its sequence was not completely determined.

c. Subclones of S41B-17 clone:
   EcoR I, Acc I and Kpn I sites were utilized to subclone.
   KE band 1 (692 bp): KE-5, KE-6, KE-7, KE-8. KE-6 clone was used to sequence.
   KE band 2 (727 bp): KE-1, KE-2, KE-3, KE-4. KE-1 clone was used to sequence.
   KA band (471 bp): KA-2, KA-3, KA-5, KA-6. KA-2 clone was used to sequence.

   The sequence of S41B-17 clone starts with a NADL primer sequence (nts 4193 to 4211), followed by SD-1 sequence (from nts 4212 to 5861) and ends with a NADL primer sequence (nts 5862-5879). Two nucleotide errors are present in this clone at nts 4356 (C should be T) and 4949 (G should A).
d. Subclones of S41C-1 clone:
EcoR I, Acc I and Kpn I sites were utilized to subclone.
KE band 1 (692 bp): KE-1, KE-5, KE-6, KE-7, KE-8. KE-5 clone was used to sequence.
KE band 2 (727 bp): KE-2, KE-3, KE-4. KE-2 clone was used to sequence.
KA band (471 bp): KA-1, KA-2, KA-3, KA-4, KA-5. KA-1 clone was used to sequence.
The sequence of S41C-1 clone starts with a NADL primer sequence (nts 4193 to 4211), followed by SD-1 sequence (from nts 4212 to 5861) and ends with a NADL primer sequence (nts 5862-5879). No nucleotide error is present in this clone.

6. S5596-7452 clones
a. Original cDNA clones:
PCR reaction B: S55B-2, S55B-3, S55B-4, S55B-6, S55B-9, S55B-10, S55B-11, S55B-12, S55B-13, S55B-16, S55B-17, S55B-18. Based on the restriction enzyme site mapping, all these clones are the contaminated clones. Therefore, no sequence was determined from any of these clones.
PCR reaction C: S5596C-1, S5596C-2, S5596C-3, S5596C-4, S5596C-5, S5596C-6. S5596C-1 clone was used to subclone and sequence.

b. Subclones of S55A-3 clone:
BamH I and Pst I sites were utilized to subclone.
BP band (1150 bp): BP-1, BP-2, BP-3, BP-4, BP-5, BP-6. BP-2 clone was used to sequence.
The sequence of S55A-3 clone starts at SD-1 nts 5596 and ends at SD-1 nts 7451, followed by a NADL primer sequence (nts 7452 to 7474). No nucleotide error is present in this clone.

c. Subclones of S5596C-1 clone:
BamH I and Pst I sites were utilized to subclone.
BP band (1150 bp): BP-1, BP-2, BP-3, BP-4, BP-5, BP-6. BP-1 clone was used to sequence.
The sequence of S55A-3 clone starts at SD-1 nts 5596 and ends at SD-1 nts 7451, followed by a NADL primer sequence (nts 7452 to 7474). No nucleotide error is present in this clone.

7. S6857-8380 clones
a. Original cDNA clones:
PCR reaction A: A-12. No full length clone was obtained.
PCR reaction B: B-1, B-2, B-3, B-4, B-5, B-8, B-9. No full length clone was obtained.
PCR reaction C: S6857C-1, S6857C-2, S6857C-3, S6857C-4, S6857C-5, S6857C-6, S6857C-7, S6857C-8, S6857C-9. No full length clone was obtained.
PCR reaction D: S6857D-3, S6857D-4, S6857D-5, S6857D-6, S6857D-7, S6857D-9, S6857D-11, S6857D-12, S6857D-20. Only S6857D-7 and S6857D-20 clones have full length of insert. S6857D-7 clone was used to subclone and sequence.
PCR reaction E: No full length clone was obtained. The clones were not saved.
PCR reaction F: S6857F-13, S6857F-14, S6857F-15, S6857F-17, S6857F-18, S6857F-19, S6857F-20, S6857F-21, S6857F-22, S6857F-23, S6857F-24. S6857F-14 and S6857F-22 clones have the full length of insert. S6857F-14 clone was used to subclone and sequence.

b. Subclones of S6857D-7 clone:
Nhe I and Pst I sites were used to subclone.
NP small band: NPS-1, NPS-2, NPS-3, NPS-4, NPS-5, NPS-6. NPS-4 clone was used to sequence.
NP large band: NPL-1, NPL-2, NPL-3, NPL-4. NPL-2 was used to sequence.
The sequence of S6857D-7 clone starts with a NADL primer sequence (nts 6857 to 6890), followed by SD-1 sequence (nts 6891 to 8379) and ends with a NADL primer sequence (nts 8380 to 8399). Three nucleotide errors are present in this clone at nts 7626 (C should be T), 7749 (T should be A) and 8152 (C should be G).

c. Subclones of S6857F-14 clone:
Nhe I and Pst I sites were utilized to subclone.
NP large band: NPL-1, NPL-2, NPL-3, NPL-4, NPL-5, NPL-6. NPL-3 clone was used to sequence.
NP small band: NPS-1, NPS-2, NPS-3, NPS-4. NPS-2 clone was used to sequence.
The sequence of S6857D-7 clone starts with a NADL primer sequence (nts 6857 to 6890), followed by SD-1 sequence (nts 6891 to 8379) and ends with a NADL primer sequence (nts 8380 to 8399). Five nucleotide errors are present in this clone at nts 7220 (T should be C), 7535 (T should be A), 7884 (G should be A), 8118 (G should be A) and 8260 (T should be C).
d. Subclones of S6857D-20 clone:
Nhe I and Pst I sites were used to subclone.
NP small band: NPS-1, NPS-2, NPS-3, NPS-4, NPS-5, NPS-6. NPS-5 clone was used to sequence.
NP large band: NPL-1, NPL-3, NPL-4, NPL-5. NPL-3 clone was
S6857D-20 and S6857F-22 clones were used to verify the nucleotide error found in the clones S6857D-7 and S6857F-14. Therefore, their sequences were not completely determined.

8. S8300-9944 clones

a. Original cDNA clones:

   PCR reaction B: S83B-1, S83B-2, S83B-3, S83B-4, S83B-5, S83B-6, S83B-7, S83B-8, S83B-9. S83B-8 clone was used to subclone and sequence.

   PCR reaction C: S83C-23, S83C-24, S83C-25, S83C-27, S83C-28, S83C-30, S83C-32, S83C-33, S83C-35. S83C-28 clone was used to subclone and sequence.

b. Subclones of S83A-5 clone:
   Hind III, Nhe I and Pst I sites were utilized to subclone. HN band (500 bp): HN-1, HN-2, HN-3, HN-4, HN-5, HN-6. HN-3 clone was used to sequence.

   PN band (600 bp): PN-1, PN-2, PN-3, PN-4, PN-5, PN-6. PN-2 clone was used to sequence.

   HP band (600 bp): HP-1, HP-2, HP-3, HP-4, HP-5, HP-6. HP-2 clone was used to sequence.

   The sequence of S83A-5 clone starts at nts 8300 and ends at nts 9943, followed by a NADL primer sequence (nts 9944 to 9963). Five nucleotide errors are present in this clone at nts 8709 (A should be G), 8831 (G should be A), 8856 (C should be T), 9162 (C should be T) and 9328 (C should be T).

c. Subclones of S83B-8 clone:
   Hind III, Nhe I and Pst I sites were utilized to subclone. HN large band (600 bp): HNL-1, HNL-2, HNL-3, HNL-4, HNL-5, HNL-6. HNL-3 clone was used to sequence.

   HN small band (500 bp): HNS-1, HNS-3, HNS-4, HNS-5, HNS-6. HNS-1 clone was used to sequence.

   PH band (600 bp): PH-1, PH-2, PH-3, PH-4, PH-5, PH-6. PH-4 clone was used to sequence.

   The sequence of S83A-5 clone starts at nts 8300 and ends at nts 9943, followed by a NADL primer sequence (nts 9944 to 9963). Two nucleotide errors are present in this clone at nts 9292 (A should be G) and 9328 (C should be T).

d. Subclones of S83C-28 clone:
   Hind III, Nhe I and Pst I sites were utilized to subclone. HN large band (600 bp): HNL-2, HNL-3, HNL-4. HNL-3 clone was used to sequence.
HN small band (500 bp): HNS-1, HNS-2, HNS-3. HNS-1 clone was used to sequence.

HP band (600 bp): HP-1, HP-2, HP-3, HP-4. HP-1 clone was used to sequence.

S83C-28 clone was used to verify the sequence error found in the clones S83A-5 and S83B-8. Therefore, its sequence was not completely determined.

9. S9824 clones

a. Original cDNA clones:

   PCR reaction B: S9824B-2, S9824B-3, S9824B-9, S9824B-10, S9824B-16. S9824B-2 clone was used to subclone and sequence.

   PCR reaction C: S9824C-1, S9824C-4, S9824C-7, S9824C-9. S9824C-4 clone was used to subclone and sequence.

b. Subclones of S9824A-8 clone:
   Kpn I, Hind III and EcoR I sites were utilized to subclone.
   KE band: KE-1, KE-2, KE-3, KE-4. KE-1 clone was used to sequence.
   KH small band: KHS-1, KHS-2. KHS-2 clone was used to sequence.
   KH large band: KHL-1, KHL-2, KHL-3, KHL-5, KHL-6. KHL-3 clone was used to sequence.

   The sequence of S9824A-8 clone starts at nts 9824 and ends at nts 10713, followed by 9824 primer sequence (nts 9824 to 9843). No nucleotide error is present in this clone.

c. Subclone of S9824B-2 clone:
   Kpn I and Pst I sites were used to subclone.
   KP large band (559 bp): KP-2, KP-4, KP-5. KP-2 clone was used to sequence.
   KP small band (536 bp): KP-1, KP-3. KP-1 clone was used to sequence.

   The sequence of S9824B-2 clone starts at nts 9824 and ends at nts 10920. This clone was used to verify the sequence errors found in other clones. Therefore, its sequence was not completely determined.

d. The sequence of S9824A-11 clone starts with a SD-1 primer sequence (nts 9824 to 9843), followed by a 13 base unknown sequence, then the SD-1 sequence (nts 11025 to 11516) and ends with a NADL primer sequence (nts 11517 to 11536). Two nucleotide errors are present in this clone at nts 11182 (G
should be A) and 11487 (C should be T).

e. The sequence of S9824C-4 clone starts at nts 9824 and ends at nts 10920. This clone was used to verify the sequence errors found in other clones. Therefore, its sequence was not completely determined.

10. S10528-11517 clones

a. Original cDNA clones:
   PCR reaction A: S10528A-1, S10528A-2, S10528A-3, S10528A-7. S10528A-1 clone was used to subclone and sequence.
   PCR reaction B: S10528B-1, S10528B-3, S10528B-4, S10528B-5, S10528B-6. S10528B-5 clone was used to subclone and sequence.

b. Subclones of S10528A-1 clone:
   Pst I and Hind III sites were utilized to subclone.
   HP large band: HP-1, HP-2, HP-3, HP-4. HP-4 clone was used to sequence.
   The sequence of S10528A-1 clone starts at nts 10528 and ends at nts 11516, followed by a NADL primer sequence (nts 11517 to 11536). One nucleotide error is present in this clone where 7 As should be 6As.

c. Subclone of S10528B-5 clone:
   Pst I and Hind III sites were utilized to subclone.
   HP large band: HP-1, HP-2, HP-3, HP-4. HP-3 clone was used to sequence.
   The sequence of S10528B-5 clone starts at nts 10528 and ends at nts 11516, followed by a NADL primer sequence (nts 11517 to 11536). No nucleotide error is present in this clone.

11. S11353-end clones

a. Original cDNA clones:
   PCR reaction A: No positive clone was obtained from reaction A.
   PCR reaction B: S11353B-1, S11353B-2, S11353B-4, S11353B-5, S11353B-7. S11353B-7 clone was used to subclone and sequence.
   PCR reaction C: S11353C-1, S11353C-2, S11353C-3, S11353C-6, S11353C-8. S11353C-6 clone was used to subclone and sequence.

b. Subclones of S11353B-7 clone:
   Kpn I and Pst I sites were utilized to subclone.
   KP large band: KPL-1, KPL-2, KPL-3, KPL-4, KPL-5. KPL-1 clone was used to sequence.
   KP small band: KPS-1, KPS-2, KPS-3, KPS-4, KPS-5. KPS-2
c. Subclones of S11353B-7 clone:
   Kpn I and Pst I sites were utilized to subclone.
   KP large band: KPL-1, KPL-5. KPL-5 clone was used to sequence.
   KP small band: KPS-1, KPS-2, KPS-3, KPS-4. KPS-3 clone was used to sequence.
   Pst I band: P-1, P-2, P-4. P-4 clone was used to sequence.
   The sequence of S11353B-7 clone starts at nts 11353 and ends at nts 12573. Two nucleotide errors are present in this clone at nts 11866 (C should be T) and 12510 (G should be A).

Note: * All the nucleotide positions given in the list were based on the NADL sequence. The corresponding nucleotide positions in the SD-1 genome after nucleotides 4992, where a 270 nucleotide cellular RNA sequence was inserted into the genome of NADL, should be the given numbers subtracted by 270.
LIST OF REFERENCES


