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Glycoprotein gp48 of bovine viral diarrhea virus is the potential pestiviral analog to the secreted segment of the flaviviral pre-M protein

Ilse U. Silva-Krott

To the Graduate Council:

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Leon N. D. Potgieter, Major Professor

We have read this dissertation and recommend its acceptance:

M. Breider, D. Brian, R. Carroll

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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David A. Brian

Accepted for the Council:

Associate Vice Chancellor
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GLYCOPROTEIN GP48 OF BOVINE VIRAL DIARRHEA VIRUS IS THE POTENTIAL PESTIVIRAL ANALOG TO THE SECRETED SEGMENT OF THE FLAVIVIRAL PRE-M PROTEIN

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

Ilse U. Silva-Krott

May 1992

This dissertation is dedicated to my father, Dr. Peter Krott, who set an example to my brothers and me to let interests shape our careers. He dedicated his life to his interest in animals and taught us to regard our work as important and thereby ensure an accomplished and fulfilled life. I also dedicate this dissertation to Dr. Robert Rausch, who made it possible for me to pursue graduate studies in the United States, for his unfailing support during the difficult adjustment period and for his inspiration to continue my education in veterinary pathology and virology.

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ABSTRACT

Total cellular and viral RNA isolated from cells infected with noncytopathic Bovine Viral Diarrhea Virus strain 2724 was used for reverse transcription of viral specific sequences encoding gp48 and its putative protein signal sequence. The cDNA template was amplified twice by the polymerase chain reaction with primers designed from nucleotide sequences of cytopathic NADL and 72 BVD viruses. The amplified product was incorporated into plasmid vector pCR1000 and subcloned into expression vector pGem-4z. Nucleotide sequence analysis of the cloned cDNA indicated it was 921 base pairs long, encoded 307 amino acid residues, had high sequence homology to other pestiviruses, and had no significant sequence homology to members of the Flaviviridae. *In vitro* expression of the cDNA yielded a 30kDa protein that was precipitated by BVDV polyclonal antiserum. The protein was glycosylated in the presence of canine microsomal membranes to give a 46kDa product and was secreted into the lumen of the microsomal vesicles. The characteristics of the putative signal peptide were consistent with signal sequences for protein translocation found in eukaryotes. A putative signal peptidase cleavage site was identified at a glycine residue at amino acid position 79. Based on signal peptidase cleavage of gp48 and lack of a protein anchor, I propose that gp48 is a glycosylated secreted protein analog of the glycosylated secreted portion of the pre-M protein of Flaviviruses.

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CHAPTER 1

GENERAL INTRODUCTION AND PURPOSE

Bovine viral diarrhea virus is classified in the genus Pestivirus of the Togaviridae.[77] However, reclassification of Pestiviruses within the Flaviviridae, or introduction of a new family, Pestiviridae, has been suggested as new knowledge of the molecular biology of this group of viruses has emerged.[27,30,193] The Pestivirus genus includes hog cholera virus (HCV), bovine viral diarrhea virus (BVDV), and Border disease virus (BDV).[204] All are important animal pathogens that lead to large economic losses in livestock worldwide every year. Two biotypes of bovine viral diarrhea virus strains exist, those that are cytopathogenic and those that are noncytopathogenic in infected cell cultures.[61] The biotype has an important role in the manifestation of disease following infection.[126,156]

The genomic structure and organization of the cytopathogenic BVDV strain NADL and two strains of HCV have been determined.[28,29,121,128] The pestivirus genome is approximately 12,500 nucleotides long and has one large open reading frame. This open reading frame encodes a polyprotein that is co- and post-translationally processed to yield mature viral proteins.[28,121,128] The organization of the genome was determined by immunoprecipitation of viral proteins from infected cell extracts by specific antisera. Antibodies to viral proteins encoded by specific portions of the genome were obtained by using bacterial fusion proteins, derived from expression of cloned viral sequences, as immunogens.[28,187] Structural proteins

of pestiviruses were encoded at the 5' third of the genome and nonstructural proteins at the 3' two-thirds of the genome. The first protein encoded by BVDV NADL genome, p20 was autocatalytic and cleaved itself from the nascent polypeptide chain.[206] Immediately downstream of p20, the NADL genome encoded a small 14 kDa peptide (Collett, pers. comm.), followed by three glycoproteins (gp48, gp25 and gp53).[28,30] The third glycosylated protein, gp53, elicited neutralizing antibodies in infected animals, and may be the major envelope glycoprotein.[38,108] Immuno-precipitation of HCV and BVDV NADL virion preparations by antibodies to bacterial fusion proteins of cloned HCV sequences suggested that the first gene product of the open reading frame, p23 was not part of the virion. A nonglycosylated protein, p14 and three glycosylated proteins (gp44/48, gp33, gp55), were precipitated in virion preparations. p14 may be the capsid protein.[193] HCV gp33 and gp55 were analogous to BVDV gp25 and gp53, respectively. Precipitation of HCV glycoproteins by monoclonal antibodies against gp44/48 and gp55 under reducing and non-reducing conditions suggested the existence of gp55 homodimers, gp55/gp33 heterodimers and gp44/48 homodimers.[193,205] The gp44 and gp48 precipitation products probably represented different glycosylated proteins with the same peptide backbone and were analogous to gp48 of BVDV.[193] The function of BVDV gp48 (HCV 44/48) is unknown. However, it may be a minor envelope protein.[39,41,128] The 5' end of the open reading frame including gp55 of HCV expressed by a vaccinia vector in swine protected them against lethal challenge inoculation with wild type HCV. The same

vaccinia vector with the HCV sequences without gp55 elicited partial protection in swine.[180]

Cloning, sequencing and *in vitro* expression of gp48 will allow speculations concerning the function of this protein, add to the knowledge of properties of pestiviral proteins and taxonomic identification of these important pathogens. Ultimately immunogenic properties of the viral protein can be assessed and its usefulness in development of a sensitive diagnostic test of infected animals and recombinant vaccine. We concentrated on a noncytopathogenic BVDV strain as noncytopathogenic viruses are the predominant biotype in nature.

CHAPTER 2

LITERATURE REVIEW

Historical Background

A transmissible disease of cattle characterized by diarrhea, mucosal erosions in mouth and esophagus, fever, leukopenia and abortions was described first by Olafson in 1946.[135] The disease occurred in several herds as one with high morbidity and low mortality and was easily reproduced experimentally by application of fecal material on the skin or by subcutaneous injection of blood and splenic emulsions of affected animals. Olafson distinguished the transmissible disease from Rinderpest and termed it Virus Diarrhea.[136]

James Baker (1954)[7] further characterized bovine virus diarrhea and collected circumstantial evidence that a viral agent was responsible for the disease. Noncytopathogenic bovine viral diarrhea virus (BVDV) strains were first isolated in 1957 and 1959 by Lee and Gillespie, respectively. The first cytopathogenic strain of BVDV was isolated by Gillespie in 1959.[60]

Another disease syndrome of cattle similar to virus diarrhea, characterized by extensive mucosal erosions, low morbidity and high mortality was first described by Ramsey (1953)[158] and Pritchard (1956)[151] and became known as mucosal disease. A viral agent was isolated from lesions of affected cattle by Underdahl in 1957.[194] Gillespie (1961)[62] determined that viral strains isolated from cattle with virus diarrhea were similar serologically to those recovered of mucosal disease.

The existence of a close serological relationship between mucosal disease virus (MDV) and hog cholera virus (HCV) was reported by Darbyshire, 1960.[33] Hog cholera (swine fever) was first described in 1833 and was reproduced by a filterable agent in 1904 (reviewed by Hutyra in 1926).[81] Hog cholera virus caused severe epidemics with high death rates in the swine population worldwide prior to eradication in the U.S.A. and western Europe. However, new outbreaks of the disease have occurred in western countries recently.

A third disease, Border disease of sheep (BD), first described by Hughes in 1959,[80] was caused by a virus related to BVDV and HCV.[1,58] Border disease was characterized by abortions, malformations and birth of "hairy shaker" lambs.

BVDV, HCV and BDV are the only known members of the genus Pestivirus. Pestiviral infections have not been identified in other domestic animals and man, however one report detailed circumstantial evidence incriminating pestivirus infection in diarrhea outbreaks in human infants.[208] The researchers used a monoclonal antibody to BVDV gp48 in a solid phase enzyme immunoassay to identify pestiviral antigen in excretions from the infants.

Recently the cause of non-A, non-B hepatitis in people was identified as hepatitis C virus. The virus had some morphologic similarities to pestiviruses and flaviviruses.[125] Hepatitis C virus had single stranded RNA of positive polarity with one long open reading frame. Charge distribution, glycosylation patterns and hydrophobicity of hepatitis C virus proteins encoded on the 5' end of the genome were similar to those of pestiviruses and members of the Flaviviridae. Also,

nucleotide sequences of Hepatitis C virus p125 coding region was similar to those of pestiviruses and flaviviruses.[88,89,125,190]

Taxonomic status of pestiviruses

Bovine viral diarrhea virus (BVDV), border disease virus (BDV) and hog cholera virus (HCV) are closely related viruses and presently constitute the pestiviruses, a genus of the Togaviridae.

Pestiviruses, rubivirus and arterivirus were the only non arthropod-borne members of the Togaviridae,[77] however, reclassification of the pestiviruses as members of the Flaviviridae has been proposed.[30] As more details about the molecular biology of pestiviruses became known, similarities and differences to both Togaviruses and Flaviviruses have become apparent, and recently establishment of a new virus family, Pestiviridae was proposed.[193]

Members of the Togaviridae family are small enveloped icosahedral viruses with a single-stranded, positive sense RNA genome.[119] Members of the alphavirus genus of the Togaviridae of which Sindbis virus is the type species have two envelope proteins. The genomic RNA has a 5' end cap and 3' poly A tail. The genome encodes non structural proteins at the 5' end and structural proteins at the 3' end. During viral replication, subgenomic messenger RNA encoding the structural proteins is produced.[87]

The Flavivirus genus with its type species Yellow fever virus, represented initially a second genus in the Togaviridae family.[119] However, an independent

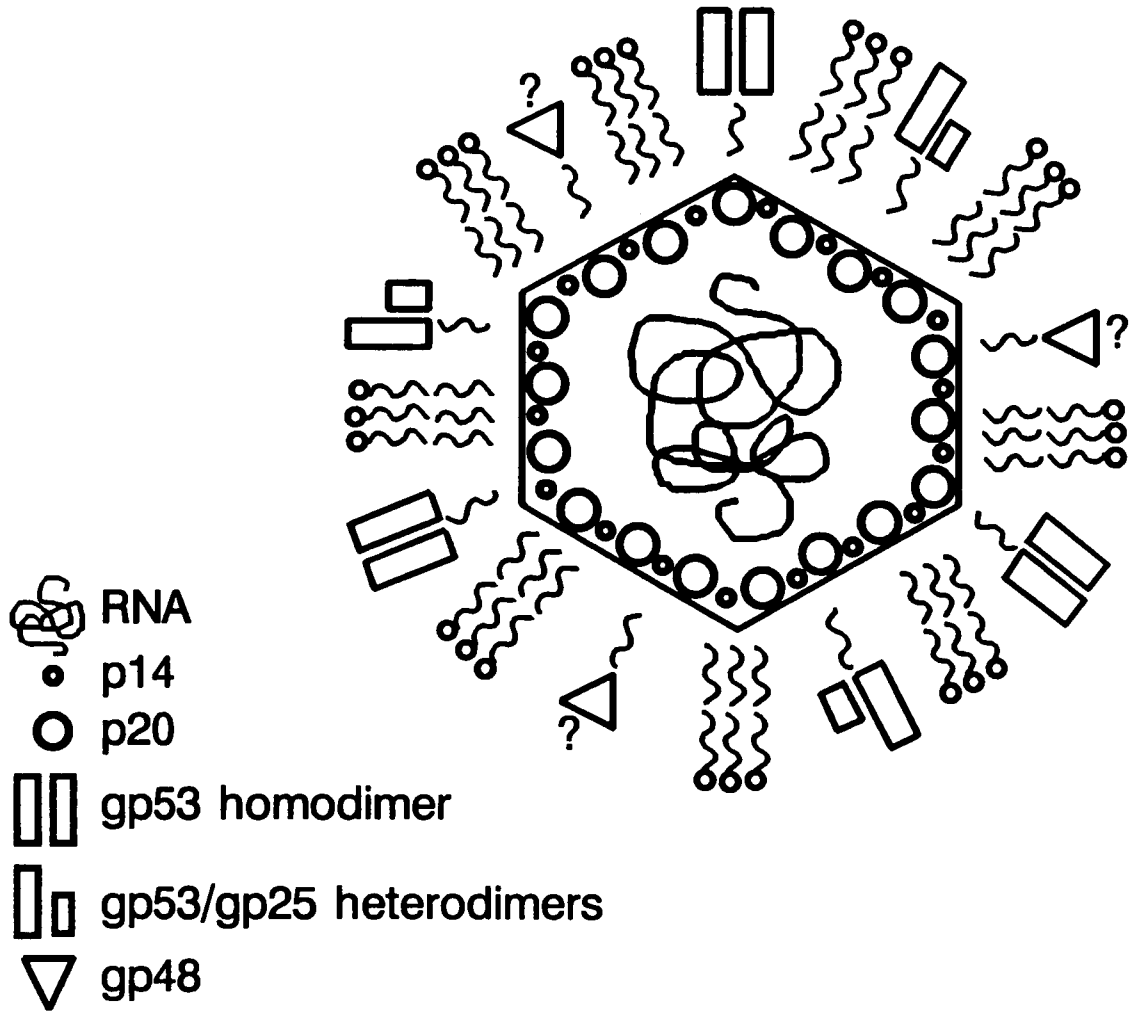
Flaviviridae family was established as more information about Flavivirus genomic structure, replication strategy and gene sequence emerged.[204] Flavivirus virions only have one envelope protein. The genomic RNA is capped at the 5' end and does not have a 3' end poly A tract. The genome encoded structural proteins at the 5' end and non structural proteins at the 3' end. A single RNA species is produced during replication.[165]

Morphology and Molecular Biology of BVDV

Members of the pestivirus genus are small (approximately 40 nm in diameter), enveloped, probably icosahedral viruses with a single-stranded, positive-sense RNA genome.[37,71,77] Figure 2.1 gives the schematic structure of a pestivirus.

Measurements by denaturing gel electrophoresis indicated the genome of BVDV strains OSLOSS[163] and NADL[29] was approximately 12.5 kb. HCV strain BRESCIA RNA measured about 12 kb.[127,128] The pestiviral RNA genome had a high degree of secondary structure since isolated RNA was resistant to mild RNase treatment of the isolated RNA. However, it was susceptible to hydrolysis by high concentrations of RNase A and thus distinguishable from true duplex (replicative intermediate) RNA.[153] BVDV RNA behaved like double-stranded RNA since it bound to CF-11 cellulose in presence of 15% ethanol[154] and was soluble in 2 M LiCl.[29] Cell-free translation of viral RNA isolated from infected cell lysates was only successful after denaturation of the RNA template.[155] The viral RNA was not poly-A tailed and therefore could not be bound to oligo(dT)-cellulose.[153,163] Circumstantial

Figure 2.1. Schematic diagram of a bovine viral diarrhea virion.


?
?
?
RNA
p14
p20
gp53 homodimer
gp53/gp25 heterodimers
gp48

evidence suggesting that a 5' end cap existed was that the RNA could not be labelled with [32P]ATP by polynucleotide kinase.[30] The cytopathic BVDV strain NADL was cloned by Collett (1988)[29] and more detailed information about the genomic structure was obtained. The cloned sequence measured 12,573 nucleotides and had several small and one large open reading frame in the virion-sense (positive polarity) sequence encoding 3988 amino acids. The 5' region of the BVDV NADL genome had a 386 nucleotide, non-coding segment with several ATG codons preceding short open reading frames. The methionine codon encoded by nucleotides 386 to 388 opened a long reading frame to nucleotide 12,349 encoding a putative 449 kDa polyprotein. The non-coding region has a GC-rich segment suggestive of a ribosomal binding site. Recent *in vitro* translation studies by Poole (1992)[143] revealed that translation of BVDV NADL was primarily initiated at ATG at nucleotides 386 to 388 and that ribosomes likely were bound internally and upstream of the functional ATG. A second cytopathic strain of BVDV, OSLOSS was cloned by Renard (1987)[163] and had 72% nucleotide sequence identity to the NADL strain. Renard (1987)[163] reported two large open reading frames for the OSLOSS strain; however, a cloning mistake in the OSLOSS sequence could have occurred and it is most likely that only one large open reading frame exists in the virus genome.[30] Two strains of HCV virus, Brescia and Alfort, have been cloned and had a nucleotide identity of approximately 68% and amino acid similarity of approximately 85% to the BVDV NADL and OSLOSS.[121,128] A major difference between BVDV OSLOSS and HCV Alfort genomes was a 210-nucleotide long nucleotide insertion encoding 90 amino

acid residues within the non-structural protein (p125) coding region of the BVDV genome. The inserted nucleotide sequence was nearly identical (2 amino acid residue difference) to the highly conserved cellular sequence ubiquitin.[121,122] The same author also identified an insertion of 270 nucleotides located at this site in the NADL BVDV strain. The insertion in the NADL strain had high homology with a bovine messenger RNA.[122]

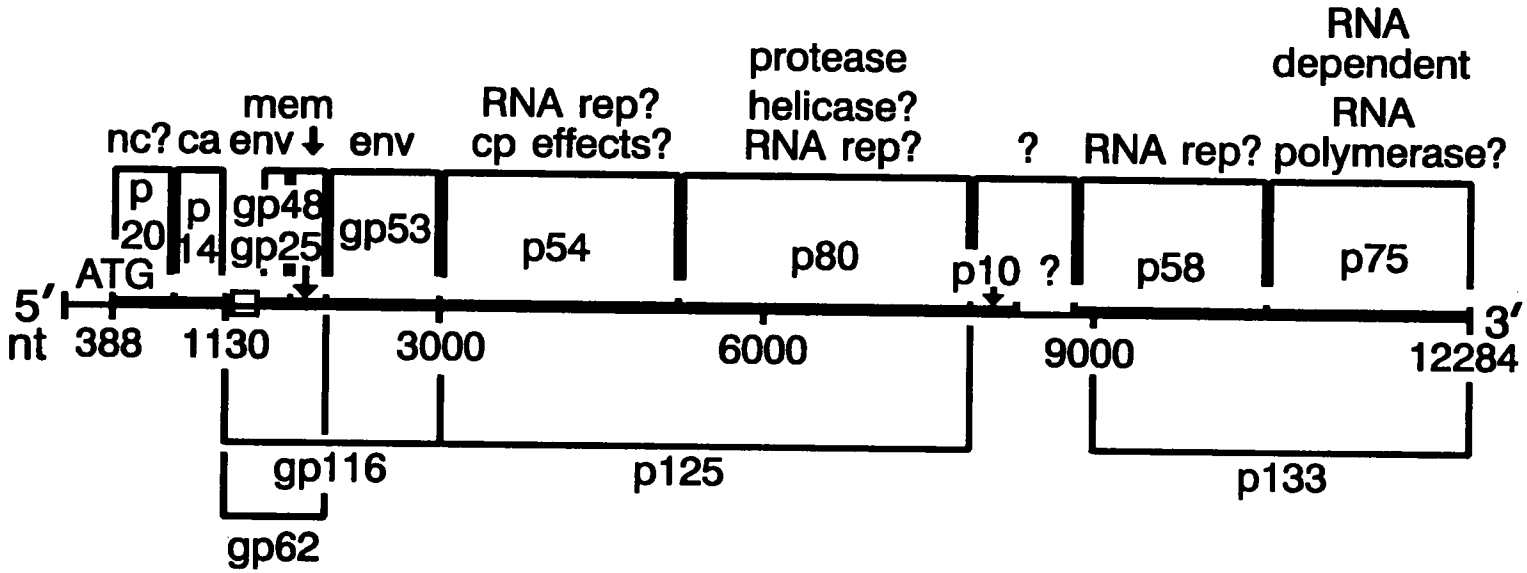
As in Flaviviruses, only one large viral RNA species was found in tissues and cells infected with pestiviruses.[28,121,128,142] The large open reading frame of the genome was translated to a polyprotein which was further co- and post-translationally processed to yield the final structural and non-structural viral proteins.[28,121,128,142]

Collett (1989) reported on the genomic organization of BVDV NADL.[30] Cloned viral sequences were expressed as fusion proteins in *E. coli*. The expressed products then were used to elicit BVDV specific antibodies in rabbits. Antisera directed against proteins encoded by specific portions of the BVDV genome were used to precipitate labelled viral proteins from infected cell lysates. Viral protein genes then were positioned on the genome. The first gene product encoded by the large open reading frame was a 20 kDa peptide followed by three glycoproteins, gp48, gp25 and gp53, respectively. The structural proteins then were followed by nonstructural proteins p54, p80, p58 and p75, respectively. Several larger proteins also were precipitated and precursor to product relationships were established by the use of specific antisera: gp116 resulted in gp62 (gp48 and pg25) and gp53, p125 produced p54 and p80 and p133 was the precursor of p58 and p75. The genomic

organization has held true for all BVDV strains and HCV strains studied so far and only one additional small protein, p14, encoded between the p20 and gp48 genomic regions has been identified in HCV Alfort and BVDV NADL strains.[193] Figure 2.2 is a schematic representation of the BVDV genome, indicating genomic positions of protein encoding regions. The exact cleavage sites and amino and carboxy termini of BVDV proteins are unknown.[29,30,206,207] The exact size of BVDV structural and non structural proteins also has not been determined and functional properties of viral proteins are largely unknown.[30] Precursor to product relationships are given in Figure 2.3.

A major problem for many workers was the low viral yield in cell culture which has not allowed detailed study of virions. Almost all studies of viral proteins were carried out by analysis of infected cell lysates which contained virions and viral precursor proteins. Table 2.1 gives molecular weights of virus specific proteins of various strains of BVDV, HCV and BD. The number of proteins and molecular weights of virion proteins identified by different workers varied and the variation likely was due to different treatments of cell lysates and use of different antisera for immunoprecipitations. Initial experiments to characterize BVDV-specific proteins done by Pritchett (1975)[152] revealed four viral components with molecular weights 93 to 110 kDa, 50 to 59 kDa, 25 kDa and 70 kDa, respectively. Coria (1983)[32] purified cytopathogenic strain Singer BVDV virions and identified four major viral proteins after gel electrophoresis and Coomassie blue staining; gp75, p66, gp54 and p26. Two of these viral proteins, gp75 and gp54, were glycosylated. New data about properties

Figure 2.2. Genomic organisation of the BVDV genome (Collett 1988). Numbers (nt) below the genome indicate nucleotide location relative to the BVDV NADL genome. Proteins encoded by the genome are marked as p20, p14 (Collett, pers. comm.), gp48, gp25, gp53, p54, p80, p10, p58, p75. Precursor proteins gp62, gp116, gp125, p133.



nc - non-coding
ca - capsid
env - envelope
mem - membrane
env - envelope
RNA rep - RNA replication
cp effects - cytopathic effects

□ - signal sequence

Figure 2.3. Predicted precursor to product relationships of BVDV proteins (redrawn from Collett 1988). Regions encoding structural and nonstructural proteins are indicated.

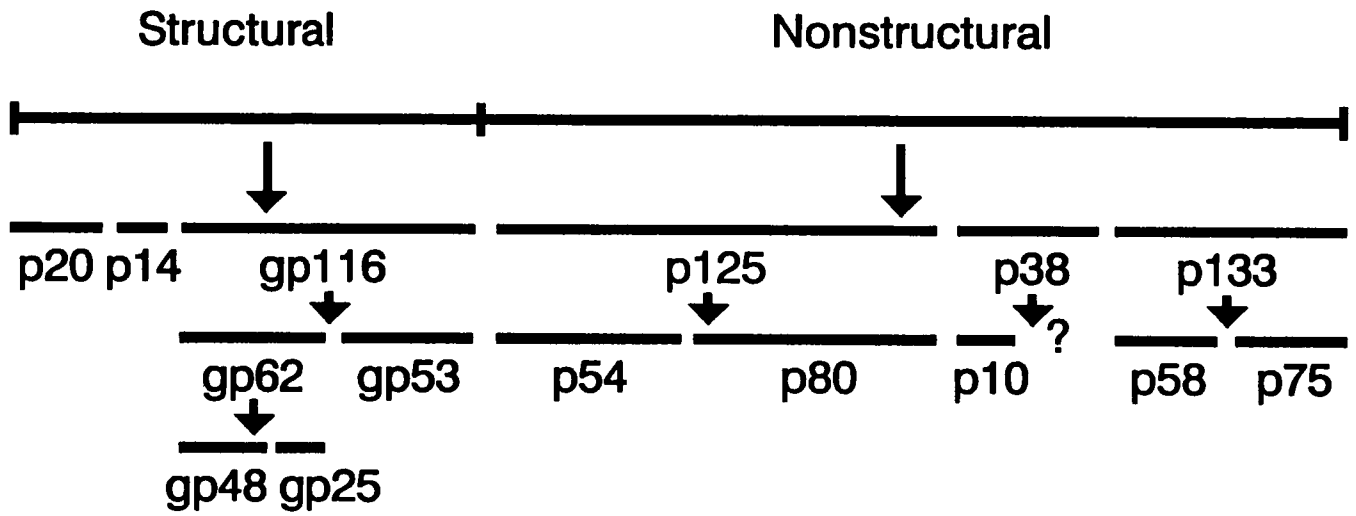

Structural
Nonstructural
p20
p14
gp116
p125
p38
p133
?
gp62
gp53
p54
p80
p10
p58
p75
gp48
gp25

Table 2.1. Pestivirus specific polypeptides in infected cell lysates[a]

Virus:		BVDV									HCV	BDV
Strain:	NADL	NADL CD4V	NADL	SINGER[b]	NADL	NADL	SINGER	SINGER	NADL	NY-1[c]	ALFORT	11,12
Polypeptides:								170		170		220
							165					165
	133						135	130		130		
	125											
		93-110			115	120	gp118[d]	118		118		118
	80				80	80	80	80	80			84
	75											
				66				60	60	60		66
	58											55
	54											53
												45
		70		gp75			gp75					37
	gp62			66		gp69	gp65					31
	gp53	50-59	gp54	gp54	gp55	gp57	gp57	53	53	53	gp55	gp55
												gp53
	gp48		gp44		45	gp49		45	45	45	gp44/48	gp45
			34		38	37		37	37	37	gp33	gp31
	gp25	23		26		33		25	25	25	23	
	20					gp23	gp25					
							19				14	
Investigator	Collett 1988	Pritchett 1975	Matthaeus 1979	Coria 1983	Purchio 1984	Pocock 1987	Donis 1987	Akkina 1991	Akkina 1991	Akkina 1991	Thiel 1991	Akkina 1991

[a] Values are molecular weight estimates in Kilodaltons
[b] virion associated polypeptides
[c] noncytopathogenic BVDV
[d] gp indicates glycoprotien

of the first protein (p20) encoded at the 5' end of the genome have emerged from *in vitro* translation and expression of cloned BVDV sequences in mammalian cells.[206,207] P20 was autocata-lytically cleaved off the nascent polypeptide chain, most likely at tryptophane, the amino acid residue at position 164. The proteinase did not, however, have any activity on further processing of the viral polyprotein.[206] Autoproteolytic cleavage of p20 also occurred in cytopathogenic BVDV 72 replication.[143] This phenomena had been identified in alphaviruses where autoproteolysis of the first structural protein precursor (the capsid protein) occurred.[67] The first protein encoded by the BVDV genome (p20) may also be the capsid protein because of its basic amino acid composition and because its genomic position is similar to that of structural proteins of Flaviviruses and Alphaviruses.[206] Thiel (1991),[193] however, found that a smaller protein, p14, encoded immediately downstream from p20 was present in BVDV NADL and HCV Alfort virions whereas p20 was not. He precipitated radiolabelled purified virion suspensions by specific p14 antisera. The same author speculated that P14 was encoded by the genomic region between tryptophane at position 164 and alanine at position 250. Downstream from alanine, the amino acid residue at position 250, the genome encoded a very hydrophilic stretch of 20 amino acids suggestive of a signal sequence. The putative signal sequence is followed by a region encoding a glycoprotein precursor (gp 116) which is ultimately cleaved to yield the three glycoproteins gp48, gp25 and gp53.[28] The functions of the glycoproteins are unclear; however, polyclonal antisera have consistently precipitated gp48, gp25 and gp53 in infected cell lysates[32,118,152,154] and

gp53 induced neutralizing antibodies.[108] In addition, gp53 and gp25 were coprecipitated by a neutralizing monoclonal antibody and then separated under denaturing conditions after disruption of disulfide bonds.[205] Cloned cDNA encoding gp53 of non cytopathogenic BVDV 2724 expressed *in vitro* remained anchored in microsomal membranes.[93] These two lines of evidence suggested that gp53 is the major envelope protein and gp25 a second membrane protein of the BVDV virion. Little data has been reported on gp48. Its non- glycosylated molecular weight was close to 27 kDa which suggested that 8 N-linked glycan sites were functional assuming an average molecular weight change of 2.6 kDa each.[28]

Thiel (1991)[193] detected homodimers of HCV Alfort gp44/48 by immuno-precipitation with a monoclonal antibody and suggested gp44/48 could be a minor envelope protein. This would be consistent with properties of Alphavirus which generally have two envelope proteins and at variance with characteristics of members of the Flaviviridae which have only one envelope protein.[172]

The non-structural proteins p80 (80-87 kDa) and p125 (93-120 kDa) were consistently detected by radioimmunoprecipitation of labelled viral proteins from lysates of cells infected with cytopathogenic strains of BVDV (NADL, Singer). Several additional higher molecular weight products were found by hypertonic translation initiation blockage prior to immunoprecipitation.[2,39] However, p80 was not precipitated by polyclonal antisera from lysates of cells infected with non-cytopathogenic BVDV strains.[40,108,142] This difference between cytopathogenic and non-cytopathogenic strains of BVDV may have been due to a difference in post-

translational processing of p125 between the two biotypes, as p80 is a product of p125. Also, insertion of nucleotide sequences in the BVDV genome of cytopathogenic strains OSLOSS and NADL occurred at the putative cleavage site of p125 into p54 and p80 (Clarke, as quoted by Brownlie, 1990).[18] Partial proteolytic peptide mapping revealed clear differences between p125 of cytopathogenic BVDV and that of noncytopathogenic BVDV strains.[2] Amino acid sequence analysis suggested the existence of RNA binding activity of the aminoterminal portion (p54 in cytopathogenic strains) and trypsin-like serine protease activity of the carboxyterminal portion (p80 in cytopathogenic strains of BVDV) of p125.[64] The aminoterminal region of p80 of BVDV NADL had trypsin-like proteolytic activity and was involved in processing of the viral polyprotein.[207] Nonstructural protein p133 which is processed to p58 and p57 may encode the viral replicase.[30]

Antigenic Variability

Numerous serologic studies have been done to assess strain relationship among pestiviruses. Initially only polyclonal antisera were available and generally, serologic cross-reactivity between cytopathogenic and non-cytopathogenic BVDV strains, HCV strains and BDV strains occurred.[33,65,120,141] Immunofluorescence with antisera raised against any one pestivirus reacted with BVD viruses as well as HCV and BDV.[192] Strain divergence among pestiviruses was recognized as a result of cross-neutralization tests.[202] Sixteen pestivirus strains were grouped into four groups; HCV viruses, BVDV and BDV viruses that also could infect swine and pestivirus

strains that had a closer serologic relationship to BVDV and BDV than to HCV. Antigenic variation among pestiviral isolates was recognized also when monoclonal antibodies with neutralizing activity were used.[16,46,140,199-201] However, division of pestiviral isolates into distinct groups based on their reaction with monoclonal antibodies was not possible with all viral strains tested. Most neutralizing pestivirus monoclonal antibodies reacted with gp53, and neutralized most BVDV strains suggestive of conservation of gp53 antigenic epitopes among BVDV strains.[34] However, some monoclonal antibodies that neutralized one BVDV strain bound to other BVDV strains without neutralizing them.[34] Ten neutralizing epitopes have been identified on the major glycoprotein (gp53) of BVDV;[126] eight of these epitopes may be clustered in one domain. Wensvoort (1989), as quoted by Moennig (1990), identified three major antigenic domains containing a total of eight neutralizing epitopes on the major glycoprotein (gp55) of hog cholera virus.[126] One monoclonal antibody directed against gp48 of BVDV cross-reacted with several BVDV isolates and one HCV strain.[30] A monoclonal antibody directed against BVDV gp48 reacted with pestiviral-like antigen in feces of infants with diarrhea of unknown cause.[208] Thirty-three pestivirus strains were characterized by immunofluorescence in cell cultures with 42 monoclonal antibodies raised against BVDV, 19 monoclonal antibodies raised against BDV and 19 monoclonal antibodies raised against HCV. Generally, monoclonal antibodies were broadly cross-reactive between BVDV and BDV strains, whereas HVC strains were serologically distinguishable.[23] The most conserved antigenic epitopes of pestiviruses occurred in the p80/p125 proteins[34,126]

consistent with high homology of genomic sequence data encoding these proteins.[30,128]

Disease Associated with BVDV Infection

Olafson (1946, 1947)[135,136] initially characterized the disease caused by BVDV infection as one with fever, leukopenia, depression, nasal discharge, anorexia, dehydration, diarrhea, mucosal erosions in the gastrointestinal tract and occasional abortions.

As our knowledge about the virus and BVDV infections improved, several disease syndromes were attributed to BVDV infection. Central to the understanding of disease caused by BVDV infection was the recognition of two biotype classes of BVDV based on their cytopathogenicity in cell culture.[7,61,194] The majority of BVDV strains endemic in cattle population were non-cytopathogenic and caused transient mild disease in adult cattle leading to seroconversion.[15] Prevalence of seropositive animals was 80-100% in adult cattle.[156] Economic losses were primarily due to abortions, deaths, suboptimal performance and secondary infections.[44,156]

Postnatal Infection

Acute bovine virus diarrhea in adult cattle is due to primary post natal infection by non-cytopathogenic or cytopathogenic strains of BVDV. The source of BVDV infecting susceptible animals has included acutely or persistently-infected animals and contaminated BVDV vaccines.[15,156] Transmission occurred by direct and indirect contact between infected animals and susceptible cattle.[150] The virus was

found in high titers in mucosal membranes and the reticuloendothelial system of cattle after infection.[21,109] BVDV had marked tropism for lymphoid cells, keratinized cells and mucosal epithelial cells.[134] Adult cattle may or may not have clinical signs after infection. Disease symptoms varied widely and consisted of transient mild diarrhea, fever, depression and leukopenia.[138,156] Experimental BVDV infections were characterized by suppression of humoral and cellular immunity.[84,111,130,148,149,162,176,178] Strain variation in pneumopathogenicity has been described.[147] Usually infected cattle produced serum neutralizing antibodies within 10 to 14 days after the infection and recovered from clinical disease. However, concomitant infections with bovine herpesvirus-1, respiratory syncytial virus, parainfluenza virus 3, reovirus type 3 and bovine adenovirus type 1 to 7 and *Pasteurella haemolytica* enhanced severity of BVDV-induced disease.[8,161,167]

Fetal Disease

Fetal infection by noncytopathogenic strains of BVDV resulted in infertility, abortion, stillbirth, malformations and birth of persistently infected calves.[156] The exact nature of BVDV disease in pregnant susceptible cows is dependent on the time of gestation when initial infection of the dam occurred.[92,171] Infection of the dam during breeding, which could be a consequence of BVDV present in the semen, has caused failure of conception.[156] Fetal infection during the first 100 days of gestation has resulted in abortion or fetal mummification.[92] Infection before and around day 125 of gestation resulted in birth of normal, but sometimes persistently-infected,

calves. These animals were specifically immunotolerant to the infecting BVDV strain and shed large amount of virus.[106] Malformations consisting of cerebellar hypoplasia, ocular abnormalities and hydranencephaly have occurred in calves infected between day 125 and 180 of gestation.[15-17] The bovine fetus was refractory to BVDV-induced disease after gestation day 180 and often developed virus-specific neutralizing antibodies.[18]

Mucosal Disease

The etiological connection between BVDV and mucosal disease was first recognized by Gillespie (1961).[62] It was described as a rare and fatal disease of cattle characterized by severe erosions of mucous membranes, anorexia, fever and diarrhea. Mucosal disease was regarded as a direct sequel to persistent BVDV infection until recently. Brownlie (1984)[19] recovered both noncytopathogenic and cytopathogenic BVDV strains from individual animals with mucosal disease. In addition the disease could be reproduced in a persistently-infected cow of the same herd by superinfection with the cytopathogenic BVDV strain isolated from the animals with mucosal disease. Isolation of both biotypes of BVDV from animal with mucosal disease has been reported also by McClurkin (1985).[106] Mucosal disease could not always be reproduced by superinfection of persistently-infected animals with cytopathogenic BVDV strains.[69] Cross neutralization tests between cytopathogenic and noncytopathogenic BVDV pairs isolated from animals with mucosal disease indicated close antigenic similarity between them. These animals

also were immunotolerant to virus strains recovered from them.[79] Howard et al. (1987),[79] suggested that the cytopathogenic BVDV strain found in animals with mucosal disease might have arisen by mutation of the noncytopathogenic strain responsible for persistent infection in these animals.

When persistently-infected animals were challenge-inoculated with a heterologous BVDV strain, an antibody response occurred and they did not develop mucosal disease.[19] The exact origin of the cytopathogenic strain isolated from animals with mucosal disease remains to be elucidated, since some genomic and antigenic differences between virus pairs found in animals with spontaneous mucosal disease have been identified.[168]

Diagnosis of BVDV Infection

Detection of Virus

The most sensitive and specific detection of BVDV infection is by viral isolation in cell cultures.[43] Virus has been isolated from feces, nasal and tracheal swabs, white blood cells and tissues from infected animals. Cytopathogenic and noncytopathogenic BVDV strains were serologically identified in cell cultures by virus neutralization, direct or indirect immunofluorescence, immunoperoxidase staining and agar gel diffusion.[9,51,53,91] Virus isolation is tedious and expensive. In addition, adventitious noncytopathogenic BVDV in cell cultures may suppress replication of the virus to be isolated and mask presence of BVDV in a sample. Virus also has been detected by direct immunofluorescence of nasal epithelial cells and naso-

pharyngeal epithelial cells collected on cotton swabs.[170,186] Direct detection of viral antigen was affected by quality of antisera used, amounts of viral antigen present in samples and potential degradation of antigen during sampling and shipping.[43] Quantitative assays for cytopathogenic and noncytopathogenic BVDV titers by end-point dilution microtitration were developed.[169] The sensitivity and specificity of BVDV detection has become an important issue as many laboratory cell lines, biological reagents and commercially-available fetal calf sera may be contaminated by BVDV.[43,49,73,94,133] The cloning and sequencing of pestiviruses allowed the application of new molecular techniques to detect viral RNA such as hybridization and polymerase chain reaction (PCR).[72,102,145] So far, an economically-feasible diagnostic technique has not been developed which consistently demonstrates BVDV in persistently-infected carrier animals, cell cultures, fetal calf sera and biologicals (Potgieter, pers. comm.). Double PCR amplification producing BVDV derived cDNA fragments proved to be a very sensitive and specific technique to demonstrate presence of viral RNA in serum and cell cultures (Al-Ansari, pers. comm.).

Detection of anti-BVDV Antibody

The standard test for BVDV antibody detection in sera has been virus neutralization.[43,156] Paired serum samples (convalescent and acute phase) are necessary to confirm recent viral infection.[43] Usually cytopathogenic BVD virus strains have been used as the indicator in cell culture systems to measure neutralizing antibodies. Great variation in neutralizing antibody titers reported by different

laboratories likely is due to antigenic differences among various indicator BVDV strains used in the test.[43] Enzyme linked immunosorbent assays (ELISA) were developed to detect BVDV antibodies in sera, however, antigenic variability of BVDV strains and variable quality of antigen preparations resulted in variable sensitivity of the test. The sensitivity was dependent on the antigenic relationship of the BVDV strain used in the antibody capture assay and the BVDV strain inducing the antibody response.[26,45,78,86,90] A highly specific competition ELISA to p80 provided data that certain p80 epitopes were conserved among BVDV isolates.[99,137]

Control of BVDV Infection

Prevention of BVDV Infection

Several investigators have argued that the spread and persistence of BVDV infection in cattle, swine and small ruminant populations is dependent on the presence of persistently-infected carrier animals.[156] Persistently-infected animals usually do not produce detectable concentrations of specific antibodies and can only be identified by viral isolation and by viral RNA or antigen detection.[43,156] A sensitive, specific and practical test for screening serum samples for viral RNA or antigens is needed (Potgieter, pers. comm.). Serologic studies have been done to identify herds with endemic viral infection and were necessary to follow BVD virus incidence and prevalence. Essential for the use of serology to monitor viral infection in a herd is the specificity of the test.[156] Persistently-infected animals produced antibodies to a unrelated BVDV strain. Therefore, it is unwise to depend on the

absence of a serologic response in an endemically-infected (or vaccinated) herd to detect persistently-infected animals. The detection of the latter is important in controlling BVD. Screening of all cattle prior to addition to the herd would lead to establishment of a BVDV-free herd.[156]

Noncytopathogenic BVDV often was present in various laboratory cell lines.[49] Fetal calf serum in medium usually was the origin of adventitious virus. In addition, fetal calf serum containing BVDV antibodies suppressed viral replication and prevented BVDV detection in contaminated cell lines until antibody-free serum was used allowing rapid viral replication.[73] These conditions allowed false negative results of virus isolation, when virus replication was inhibited by either fetal calf serum with viral antibodies or interference by adventitious virus in the cells. In addition, false positive results occurred when virus-free sample and new media with antibody-free fetal calf serum was added which allowed adventitious BVDV already present in cells to replicate.[43,73] Production of biologicals from persistently-infected cell lines has been responsible for BVDV transmission to cattle.[43] Sensitive tests to identify viral infection in cell lines and fetal calf serum are essential to prevent viral spread. Viral RNA was rendered non-infectious by gamma radiation of fetal calf serum, but its nutritional quality for cell cultures was adversely affected.[49] Treatment of fetal calf serum with β-propriolactone destroyed viral infectivity and has been used to inactivate BVDV in sera destined for use in cell cultures.[52]

Vaccines

Several modified live and inactivated cytopathogenic and noncytopathogenic BVDV vaccines are commercially available.[5,6,156] Modified live vaccines induced high levels of neutralizing antibodies, however the vaccine virus itself resulted in transient immunosuppression in vaccinated animals[177] and led to increased severity of secondary infections.[114-117] BVD-like disease occurred after vaccination with a temperature-sensitive, live virus vaccine.[103,104] Contamination of the vaccine with a wild-type BVDV strain, however, could not be ruled out in disease outbreaks after vaccinations.[11,50,175] Fetal disease similar to wild-type infections have occurred after vaccination of pregnant dams.[5,6,156,188] In addition, the spectrum of protection provided by modified live vaccines might not include all viral strains.[17] Inactivated vaccines were safe and not associated with disease, but repeated vaccinations were necessary to induce protection against infection.[11,97] Solubilized viral proteins administered to calves induced antibody levels comparable to antibody levels after administration of inactivated whole virus. A major epidemiological problem in disease management and control is that vaccinated animals cannot be distinguished from animals recovered from natural infections.

An optimal vaccine should be safe, economical and easy to administer. It should induce good and long lasting immunity.[44,70,138,146] In addition vaccinated animals should be distinguishable from animals which had experienced natural infection. Recombinant DNA technology could allow development of a highly immunogenic and safe BVDV vaccine. Highly conserved viral proteins capable of

inducing protective immunity need to be identified. Genetic alteration of an immunogenic viral peptide could allow easy serologic identification of immunized animals. Finally, the altered immunogen would need to be incorporated in an effective delivery system such as recombinant vaccinia virus or pseudorabies virus.[146]

Experiments with a genetically engineered vaccine have been done in swine. A recombinant vaccinia virus producing all structural proteins of HCV protected against lethal challenge with wild type virus.[180] Vaccination with a recombinant vaccinia vector expressing all structural proteins except the major envelope protein (gp55) alone resulted in partial immunity. The major envelope protein alone expressed by a pseudorabies recombinant virus protected vaccinated pigs against challenge inoculation with both pseudorabies virus and HCV.[210] Recombinant pseudorabies vaccine strains with deletion of glycoprotein X allowed distinction by ELISA between vaccinated and wild-type virus infected animals.[31]

CHAPTER 3

RATIONALE

BVDV is a very important pathogen of cattle that also infects swine and small ruminants. Other related pestiviruses, hog cholera virus and Border disease virus cause severe diseases in swine and sheep. BVDV causes several disease syndromes in adult cattle and bovine fetus. Non-cytopathogenic BVDV strains are more common in nature and responsible for virus persistence in herds due to the existence of persistently-infected animals which shed large amounts of virus. Primary post-natal disease in cattle and fetal disease are caused by either cytopathogenic or noncytopathogenic BVD viruses whereas animals succumbing to mucosal disease always harbor both viral biotypes. Persistently-infected dams give birth to persistently infected calves. Serum from one persistently-infected bovine fetus may contaminate large amounts of pooled commercial fetal calf serum. BVDV can infect a variety of cells in culture and is often found as a persistent infection of cell lines. Persistently-infected cell lines may alter results of BVDV isolation and indirect immuno-fluorescence assays. In addition, BVDV from persistently infected cells may contaminate vaccines and other biologicals.

BVDV infection could be controlled by identification and culling of persistently-infected animals and prevention of transplacental transmission. Serology surveys often fail to detect persistently-infected animals as the latter may produce antibodies to certain BVDV strains and therefore test false positive. Virus

neutralization assays and indirect immunofluorescence may be inaccurate due to antigenic variability of BVDV strains and persistent infection of cells used in diagnostic tests. Fetal calf serum containing anti-BVDV antibodies may inhibit viral growth in virus isolation assays. Novel approaches to develop sensitive and specific tests for presence of BVDV in live animals, commercial fetal calf serum, cell cultures, vaccines, virus stocks and other virological products are needed.

Prevention of BVDV infection by effective vaccination is another important goal in controlling this disease. Vaccines currently employed are of questionable efficacy, and live products can have many of the same sequelae such as abortion, congenital defects, and immunosuppression that occur with viral infection. Antigenic diversity among various BVDV strains may be the cause of insufficient protection against some BVDV strains in vaccinated animals. Inactivated vaccines are not practical as they have to be administered several times to elicit protective antibody levels. There is a need for a safe, highly immunogenic, multivalent vaccine that offers broad spectrum protection. However, the antigenic determinants responsible for cross protection must be identified. Neither the mechanism of immunity to BVDV in cattle nor the antigen(s) responsible for this protection are known. Genetic engineering holds the key to the evaluation of the BVDV proteins and for determining their role in the immune response to the virus. Research should emphasize noncytopathogenic BVDV strains as they occur more commonly in nature.

Less is known about the putative structural BVDV protein gp48 than any of the other proteins encoded in the 5' genomic region of the virus genome. It may be

a minor envelope protein, but detailed studies of its molecular biology have not been reported. As a putative envelope protein it could be important in eliciting protective immunity alone or in combination with other structural BVDV proteins. Proof of a second structural protein would implicate closer relationship between pestiviruses and alphaviruses. As a nonstructural protein gp48 might still be of importance immunologically and also suggest highly similar organization of the pestiviral and flaviviral genome. The work presented here entails characterization of gp48 derived from a non-cytopathogenic strain of BVDV.

New molecular techniques allow site specific transcription of viral RNA into cDNA followed by amplification of the DNA by the polymerase chain reaction. Thus, even with low viral titers in cell culture, enough virus-derived genetic material suitable for cloning can be obtained. Recombinant plasmids containing foreign cDNA can easily be propagated and large quantities of DNA in question for sequencing and further characterization can be purified. Sequence data on cDNA can confirm the presence of foreign DNA in a vector and allow prediction of the encoded amino acid sequence as well as characterization of the putative translation product by computer analysis. Sequence data of cDNA derived from the BVDV region encoding gp48 will add to our knowledge of noncytopathogenic BVDV 2724 and increase the data on the relationship among members of the pestiviruses. Comparisons with other pestiviruses and related positive stranded RNA viruses could establish conserved sequences suggestive of putative conserved antigenic domains. Direct nucleotide and amino acid sequence comparisons as well as comparisons of

hydrophobicity and secondary structure predictions of the encoded proteins among related viruses may lead to characterization of virus genera and families. Further characterization of the cDNA by *in vitro* transcription and translation provides experimental proof of a genetically functional cDNA clone. Immunoprecipitation of the translation product by anti BVDV antiserum would be final proof of the presence of a functional BVDV gene in a cloning vector. Properties of the translation product such as presence of a functional signal sequence, signal peptidase cleavage site, glycosylation sites and membrane anchor may then be assessed by *in vitro* translation in a cell free rabbit reticulocyte system with canine microsomal membranes. *In vitro* characterization may suggest possible function of gp48 and its position in the virion. If membrane anchorage occurs *in vitro*, gp48 likely would be a second BVDV envelope protein. The data will add to characterization of pestiviruses and their relationship to other positive stranded RNA viruses.

Ultimately the research will provide tools to obtain gp48 antibodies. Antibodies could be elicited in experimental animals by use of an immunogenic peptide derived from the amino acid sequence encoded by gp48 cDNA. Alternatively, further subcloning of gp48 in a baculovirus expression system would yield large amounts of pure glycosylated protein. The recombinant product then could be evaluated for its immunogenicity and its role in pathogenesis. Pure recombinant protein would allow induction of nonspecific antibodies which, in turn, would allow purification of native gp48 from infected cell lysates in order to evaluate amino- and carboxytermini of the native viral protein. Neutralizing activity of gp48

antibodies would further support the presence of gp48 on the virion surface. Cross-reactivity studies of gp48 antibodies among BVDV and other pestiviruses would provide evidence of conserved epitopes of gp48. Suitable antibodies could be used in an antigen capture ELISA test specific for BVDV gp48. Elucidation of the role of gp48 in protective immunity against viral infection could lead to development of a recombinant BVDV vaccine.

CHAPTER 4

OBJECTIVES

1. Cloning specific virally-derived sequences encoding gp48 amplified by the polymerase chain reaction in a bacterial vector.

2. Base sequence determination of the cDNA and comparison of sequence data with sequences of flaviviruses and other pestiviruses.

3. Prediction of amino acid sequence encoded by the DNA and characterization of the putative protein by computer analyses.

4. Characterization of putative gp48 by *in vitro* translation.

CHAPTER 5

MATERIALS AND METHODS

Cells and Virus

Non-cytopathic BVDV strain 2724, a field strain isolated in our laboratory,[149] was grown in primary bovine turbinate cells. Cells were passaged 15 to 20 times and indirect immunofluorescence was done to confirm the absence of adventitious BVDV. Cells were grown in Dulbecco's MEM supplemented with 10% fetal bovine serum. The latter was treated with Beta-propriolactone, at a 0.05% final concentration, prior to its use in medium. Cells were grown in 75 cm^2 flasks and infected with virus at a 0.1 multiplicity of infection. Medium was replaced with fresh medium 72 hours after infection and cells were collected 7 days later. Indirect immunofluorescence of cell suspensions dried on glass slides confirmed presence of virus in the cells.

Oligonucleotides

Synthetic oligonucleotides used as primers for cDNA synthesis and polymerase chain reaction (PCR) were designed according to sequence data from the published sequence of BVDV NADL genome[29] and sequence data from BVDV 72 genome.[143] Primer 1 was complementary to nucleotides 1891-1912 of the NADL genome (5'-GATGTCGATCGCAAAATTGGC - 3'). Primer 2 was identical to nucleotides 938-959 of the 72 genome (5'- GACAGATTAGAAAAAGGGAAG - 3'). The nucleotide

numbers of BVDV 72 were estimated by alignment with nucleotide sequence data from BVDV NADL, as the entire noncoding region of BVDV 72 had not been cloned and sequenced. The sequences of primers 3 and 4 were 5'-GATGACAGGATCCAGCAGCGTATGCTCCAAACCA-3' and 5'-GAAAATAAAGCTTAAAGAATCTGAGAAAGACAGT-3', respectively. Primer 3 was complementary to nucleotides 1859-1879, and primer 4 was identical to nucleotides 960-994 of the BVDV NADL genome. Primer 3 had a BamH I restriction endonuclease site (GGATCC) between nucleotides 1880 and 1889 of the genome and primer 4 had a HIND III restriction endonuclease site (AAGCTT) between nucleotides 968 and 974. Oligonucleotides were synthesized by Oligos etc. Inc. The sequences of primers for nucleotide sequencing of the internal regions of the cDNA were derived from cDNA sequence data obtained by use of pUC/M13 forward and reverse primers which bound to the vector flanking the cDNA insert. The sequences of internal primers were 5'-CAACGGGCAATTCCAAAGGG-3' and 5'-GCTGGTGCGCATTCATCTTCTT-3' corresponding to BVDV genome nucleotides 1247-1266 and 1714 to 1695, respectively. Sequencing primers were synthesized by Bio-Synthesis, Inc. and Oligos Etc., Inc.

RNA Preparation and First Strand cDNA Synthesis

Total cellular and viral RNA was extracted from cells after lysis in guanidinium isothiocyanate (GTIC) buffer (4M GTIC, 25 mM sodium citrate, pH7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), followed by selective precipitation with 2

M sodium acetate (pH 4.0), and phenol extraction and purification with chloroform/ isoamyl alcohol (49:1).[24] RNA was precipitated from the aqueous phase by isopropanol, pelleted by centrifugation at 10,000 g for 20 minutes, washed in 70% ethanol, dried briefly (Speed Vac Concentrator, Savant) and resuspended in water. In order to protect RNA from RNases, all water used was ultrapure water treated with 0.1% diethylpyrocarbonate (DEPC). All glassware was soaked in DEPC-treated water for 60 min and baked at 200 C for 4 hours. Pipette tips and microcentrifuge tubes were siliconized and then steam sterilized prior to use. Laboratory personnel wore gloves at all times.

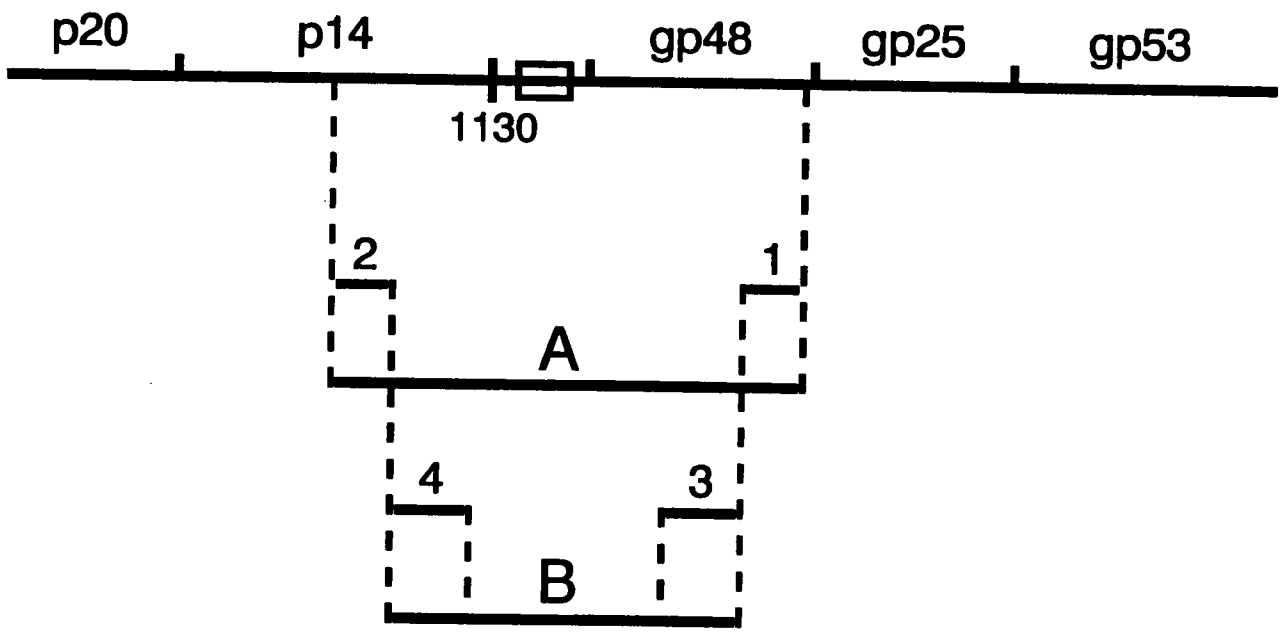
RNA and protein concentrations were quantified spectrophotometrically in a Perkin Elmer Lambda 3B spectrophotometer. The RNA was heated to 90 C for 2 minutes and cooled on ice prior to reverse transcription. First strand cDNA synthesis was done by reverse transcription with Moloney murine leukemia virus reverse transcriptase using conditions recommended by the manufacturer (Bethesda Research Laboratories). Briefly, the reaction mixture consisted of 1-3 μg RNA, 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 100 μM of each dNTP (Bethesda Research Laboratories), 10 mM dithiothreitol (DTT), 20 units of human placental RNase inhibitor (Bethesda Research Laboratories), 0.5 μM primer 1 and 200 units reverse transcriptase in a total volume of 50 μl. The reaction mixture was incubated at 37 C for 1 hour.

PCR and Cloning

Second strand cDNA synthesis and amplification was catalyzed with thermostable Taq DNA polymerase (Promega). Twenty five μl of the reverse transcriptase product was mixed with PCR buffer (15 mM Tris-HCL, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin), 0.5 μM PCR-primer (primer 2), and 2.5 units Taq polymerase in a total volume of 100 μl. The reaction mixture was heated to 94 C for 10 minutes prior to addition of enzyme, and an overlay of 70 μl light mineral oil then was added. Amplification of the cDNA template was achieved by 30 cycles, each consisting of denaturation (94 C for 1.5 minutes), annealing (50 C for 1 minute) and extension (72 C for 1.5 minutes) in an automatic thermocycler (Precision Scientific). The final cycle had an annealing time of 2 minutes and extension time of 10 minutes. The amplification product of the first PCR was subjected to a second PCR using a similar procedure as in the first reaction. A 10 μl aliquot of the first PCR product was used in the second polymerase chain reaction with primers 3 and 4. Primers 3 and 4 were designed to bind to the DNA template internally to primers 1 and 2, respectively (Figure 5.1). PCR products were evaluated by horizontal gel electrophoresis in 1% agarose gels stained with ethidium bromide (0.1%). A 1 kb DNA ladder (BRL) was loaded next to PCR products in each gel.

DNA products were purified from PCR reaction mixtures by absorption of the DNA to glass beads and subsequent elution into DEPC water according to instructions by the manufacturer (Geneclean Kit, Bio 101). Eluted DNA products were ligated into the plasmid vector pCR 1000 as recommended by the supplier (TA

Figure 5.1. Schematic representation of double amplification by PCR utilizing a nested set of primers. Proteins encoded by the genome are indicated. Primers 1,2,3 and 4 are represented by short black bars. A indicates the PCR product of the first amplification. B represents the PCR product of the second amplification.



□ = signal sequence

Cloning Kit, Invitrogen). All DNA eluted from one PCR reaction was ligated to 50 ng linearized pCR 1000 vector at 12 C overnight with 4 units T4 Ligase. Transformation of competent *E. coli* (INV F') cells was done according to instructions of the supplier (Invitrogen) with slight modifications. The cell and DNA mixture was incubated on ice for 40 minutes and heated rapidly to 37 C for 1 minute. Transformed *E. coli* (INV F') were grown on Luria broth agar plates containing kanamycin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl B-D-galactopyranoside (x-Gal) (50 μg/ml). Transformed white bacterial colonies were analyzed after 24 hours of growth for presence of recombinant plasmids by PCR using primers 3 and 4 by the protocol given by Dorfmann et al. (1987).[42] Polymerase chain reaction was done as described before.

Analysis of Recombinant Plasmids

Plasmid DNA purification from bacterial suspension cultures was done by standard molecular techniques.[110] cDNA inserts was detected after digestion with restriction endonuclease enzymes EcoR1 and HIND III (Stratagene). Restriction endonuclease digestion of 1-2 μg purified DNA was done simultaneously with both enzymes in reaction buffer 3 (Stratagene) according to instructions by the supplier. Digested plasmid DNA and c-DNA then were analyzed by agarose gel electrophoresis.

Storage of Transformed Bacteria

One and one-half ml aliquots of bacterial suspensions were mixed with 20% sterile glycerol, frozen rapidly in liquid nitrogen and stored at -70 C.

DNA Sequencing and Sequence Analysis

Nucleotide sequence analysis of recombinant plasmids was done by dideoxy chain termination according to Sanger (1977) with the Sequenase Kit 2.0 (United States Biochemical). Four to 6 μg DNA was denatured by alkali treatment and heating for each sequencing reaction. Alkali treatment consisted of addition of 4 μl of 2 M NAOH in 2 mM EDTA to the DNA suspended in 40 μl DEPC treated water. The reaction mixture was incubated at 65 C for 5 minutes followed by ethanol precipitation as previously described. The precipitated DNA was washed, dried and resuspended in 7 μl water prior to addition of 1 μl primer (0.5 pmol/μl) and 2 μl reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl2, 250 mM NaCl). The reaction mixture then was incubated in boiling water for 5 minutes and cooled on ice. Labelling and termination reactions were done according to the directions of the supplier. Separation of prematurely terminated DNA fragments was done in 0.2 mm thick, 6% polyacrylamide gels with 8 M urea on a 60 cm glass plate (vertical base runner nucleic acid sequencer, IBI). Electrophoresis was done at 2000 V for 6 hours. Gels were transferred to paper (Whatmann 3 MM) and soaked in 5% acetic acid and 5% methanol for 20 minutes prior to drying under vacuum at 90% C for 90 minutes (Biorad Gel Dryer Model 583). Radiographic film (Kodak X-omatic AR film) was

placed on dried gels for 48 hours and developed in an automatic processor (Kodak X-omatic 460 RA). Computer analysis of the nucleotide sequence was done with the sequence analysis programs of the Genetics Computer Group, version 6.0, of the University of Wisconsin.[36] Secondary structure of the deduced polypeptide was predicted by methods of Chou and Fasman (1978)[25] and Garnier-Osguthorpe-Robson (1978).[59] Hydrophilicity was calculated according to Kyte-Doolittle (1982)[96] and Hopp-Woods (1981).[76] Secondary structure predictions of the RNA transcribed from the cDNA were calculated by the program of Zuker (1981).[211] Free energy values used in Zuker's program were defined by Freier, et al. (1986).[56]

Subcloning of the cDNA into pGem-4z

The cloned viral cDNA was subcloned into the plasmid vector pGem-4z (Promega) taking advantage of restriction endonuclease sites Eco R1 and Sac 1 (Stratagene) by standard molecular techniques.[110] Briefly, 5 μg purified pcISK 48 DNA and 5 μg purified pGem-4z DNA were digested separately by restriction endonuclease SAC 1 in assay buffer 1 according to instructions by the supplier (Stratagene). The linearized DNA was purified by adsorption to glass beads followed by elution into DEPC treated water (Geneclean Kit, Bio 101). A second restriction endonuclease digestion of pcISK 48 and pGem-4z with restriction endonuclease Eco R1 was carried out in Universal Buffer following recommendations of the supplier (Stratagene). Reaction mixtures were loaded on a 1% agarose gel containing ethidium bromide (0.1%) and electrophoresed at 75 V for 45 minutes in the dark in

order to separate the cDNA from the plasmid DNA and linearized pGem-4z from the small DNA piece between Eco R1 and SAC 1 cloning sites. cDNA and vector DNA were detected under UV light. Agarose pieces containing the cDNA and vector DNA were removed from the gel and DNA was eluted from agarose gel slides according to the procedure provided by the supplier of a DNA purification kit (Geneclean, Bio 101). Six hundred ng cDNA and 200 ng vector DNA were ligated in 10 μl reaction volume at 12 C overnight with 4 units T4 ligase (Invitrogen).

Transformation competent *E. coli* JM 101 (Promega) were prepared by the procedure described by Hanahan (1983). Two μl of the ligation mixture were added to 8 μl DEPC water. Six μl of the mixture were added to 100 μl transformation competent *E. coli* JM 101 after the latter had been thawed on ice. Cells and DNA were mixed gently and held on ice for 40 minutes followed by rapid heating to 37 C for 1 minute. Five hundred μl Luria-Bertani (LB) broth (1% bacto-tryptone; 1% bacto-yeast extract; 1% NaCl), heated to 37 C, were added and the mixture was incubated for 1 hour at 37 C prior to transfer of 100 μl aliquots onto agar containing ampicillin [1% bacto-tryptone; 0.5% bacto-yeast extract; 1% NaCl; 100 μg/ml ampicillin; 40-60 μg/ml 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal); 40-60 μg/ml isopropylthio-B-D-galactoside (IPTG)]. Inoculated agar plates were incubated at 37 C overnight. White colonies, missing the Lac Z gene and ampicillin resistant, were selected and grown in "terrific broth" (TB).[110] Plasmid DNA was purified according to the "miniprep" procedure (Promega) and digested by restriction endonucleases EcoR1 and HIND III as described previously. cDNA was detected

by electrophoresis of restriction endonuclease digestion mixtures on 1% agarose gels with 0.1% ethidium bromide. Presence of specific cDNA in recombinant plasmids was confirmed by DNA base sequencing.

Expression of the cDNA

Transcription of the cDNA

Purified circular plasmid DNA was linearized by AVA 1 enzyme digestion according to recommendations of the supplier (Stratagene). Restriction endonuclease AVA 1 was used because it does not produce 3' overhangs which may result in erroneous transcription products. RNA transcripts of cDNA were produced by transcription of linearized recombinant plasmid DNA template with SP-6 RNA polymerase according to instructions of the manufacturer (Promega). cDNA template then was removed by digestion with RNase-free DNase (Promega). RNA transcripts were purified from the reaction mixture by phenol/chloroform extraction and ethanol precipitation and suspended in DEPC treated water as previously described. The RNA concentration was estimated by comparison between the brightness of the RNA band produced after electrophoresis of a 2 μl aliquot and of a DNA band produced by known DNA concentrations in a 1% agarose gel containing ethidium bromide (0.1%).

Translation of the RNA Transcript

One to 2 μg aliquots of RNA transcript derived from the cDNA were

translated in nuclease free rabbit reticulocyte lysate (Promega) in presence of 10 % [^{35}S] methionine (Amersham) using the procedure provided by the supplier (Promega).

Transmembrane Transport and Glycosylation

Transport of the translation product into the rough endoplasmic reticulum and glycosylation was evaluated by addition of microsomal membranes (Promega) to the translation reaction mixture.

Membrane Anchorage

Anchorage of translation product in microsomal membranes was assessed by separation of membrane fractions from intraluminal soluble proteins after translation according to the procedure described by Fujiki et al. (1982).[57] Translation products were labelled with 5% [^{35}S] methionine and 5% L-[U-^{14}C] Leucine (Amersham) for efficient detection of the products. Microsomal membrane vesicles were converted to open sheets by addition of 100 mM sodium carbonate (pH 11.5) to translation mixtures. All translated and secreted proteins within the lumen of membrane vesicles thus were released into the supernatant. Membranous fractions then were separated from soluble fractions by centrifugation. Proteins in the soluble fraction (supernatant) were precipitated with acetone. SDS-loading buffer (50 mM Tris-HCL, pH 6.8; 100 mM DTT; 2% SDS; 0.1% bromophenol blue; 10% glycerol; 5% 2-mercaptoethanol) was added to the membranous fraction and separately to

precipitated proteins of the soluble fractions prior to electrophoresis in SDS-polyacrylamide gels.

Sodeiumdodecylsulfate Polyacrylamide Electrophoresis (SDS PAGE)

Translation products were analyzed by vertical gel electrophoresis in 12% SDS-polyacrylamide gels under denaturing conditions.[10] ^{14}C-labelled protein molecular weight markers (Amersham) were loaded on each gel next to translation products. SDS PAGE gels were stained with Coomassie brilliant blue (10% methanol, 7% acetic acid, 2 g Coomassie brilliant blue R250/l) for 12 hours or overnight on a slowly rocking platform, followed by incubation in destaining solution (10% methanol, 7% acetic acid) for 1 hour. Gels then were placed on paper (Whatman 3 MM) and dried at 65 C under vacuum for 2 hours (Drygel Jr., Hoefer Scientific Instruments). Radioactive protein products were detected by radiography. Radiographic film (Kodak X-omatic AR film) was placed on gels for 48 hours prior to processing of the films.

Immunoprecipitation

Translated Products

Precipitation of protein translation products with polyclonal antiserum against BVDV virus was done according to the procedure described by Anderson and Blobel (1983)[12] with modifications. The polyclonal antiserum and non immune serum obtained from gnotobiotic calves were provided by K. Brock (The Ohio State

University). Briefly, Triton X-100, at a final concentration of 4% was used to solubilize proteins in the translation reaction mixture. Four volumes of dilution buffer (1.25% Triton X-100; 190 mM NaCl; 60 mM Tris-HCl; 6 mM EDTA) and 10 μl antiserum were added to the translation reaction mixture. The mixture of serum and translated proteins was held at 4 C overnight. Antibody-translation product complexes were bound to 60 μl of a 1:1 suspension of protein G-sepharose beads (Pharmacia) in wash buffer without detergent (50 mM Tris-Hcl, pH 7.5; 5 mM EDTA; 150 mM NaCl). After incubation on a rocking platform at room temperature for 3 hours, beads were pelleted by centrifugation, washed four times with wash buffer with detergent (0.1 % Triton X-100, 0.02 % SDS) and once with wash buffer without detergent. Bound protein was eluted from pelleted beads with SDS loading buffer containing urea (300 mg/ml) and 5% 2-mercaptoethanol. Samples were placed in boiling water for 4 minutes prior to separation of beads from eluted proteins by centrifugation. The supernatant with eluted proteins was loaded on SDS PAGE gels. Vertical gel electrophoresis, processing of SDS PAGE gels and radiography were done as described above.

Viral Polypeptides in Infected Cell Lysates

BTU cells were grown in 315 mm^2 culture dishes to confluency. Ten μl virus suspension (multiplicity of infection 0.001) was added to cells in culture dishes followed by incubation at 37 C for 48 hours. The medium was replaced by 2 ml medium without methionine and leucine. One hour later, 200 μCi [^{35}S] methionine

and L-[U-^{14}C] leucine were added to the medium in dishes for a 3 hours. Preparation of cell lysates followed the method given by Maniatis (1989).[110] Immunoprecipitation of viral proteins was done as described above.

CHAPTER 6

RESULTS

RNA was extracted from BVDV 2724 infected cells. The putative viral RNA sequence encoding the BVDV structural glycoprotein 48 (gp48) was used as a template for cDNA synthesis by reverse transcription followed by specific amplification by polymerase chain reaction.

A 10 μl aliquot of the PCR reaction with primers 1 and 2 did not contain enough DNA to detect in agarose gels after electrophoresis. A second PCR with DNA template produced by the first reaction using primers designed to bind the DNA adjacent and internal to the first set of primers resulted in a DNA product of approximately 900 bases in length. Additionally small amounts of DNA, approximately 400 bases long, were produced. Attempts at reverse transcription followed by double PCR amplification of RNA extracted from uninfected BTU cells did not yield any demonstrable DNA products. Figure 6.1 gives PCR products of first and second amplification of viral and cellular RNA. Figure 6.2 illustrates the predicted secondary structure of RNA with positive polarity transcribed from the amplified DNA. Secondary structure predictions were based on calculations of the minimum free energy for an RNA molecule based on published values of stacking and loop destabilizing energies. The minimal free energy of the RNA molecule derived from the BVDV cDNA was minus 208.5 kCal/mole.

The 900-base amplified PCR product was cloned into plasmid pCR 1000 by

Figure 6.1. Double amplification of BVDV 2724 cDNA by PCR. Product of the first amplification (lane 2), second amplification (lane 3), first and second amplifications of cDNA produced from RNA from uninfected cells (lanes 4 and 5), positive PCR control (lane 6), DNA ladder (lanes 1 and 7). Ten μl reaction mixture were loaded on 2% agarose gels, electrophoresed at 75V for 1.5 h and stained with ethidium bromide. Note the distinct band approximately 950 base pairs long and faint band approximately 400 base pairs long in lane 3.

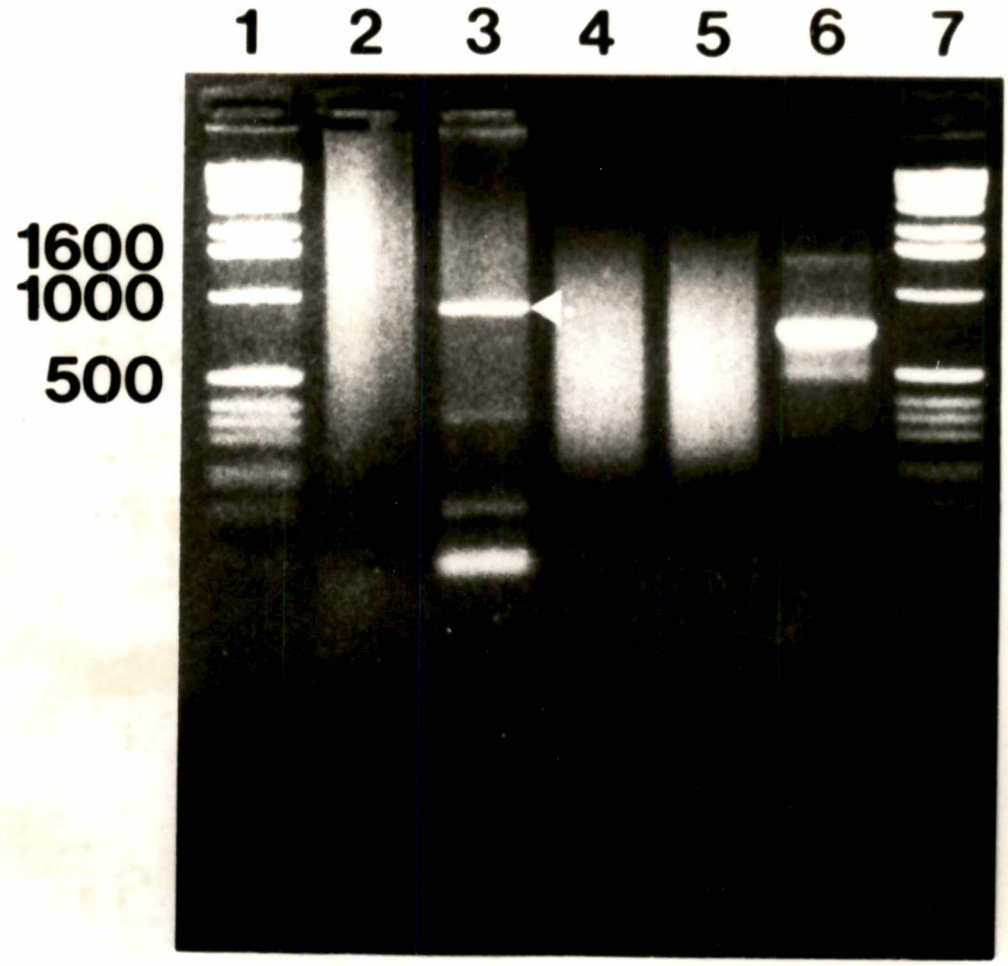
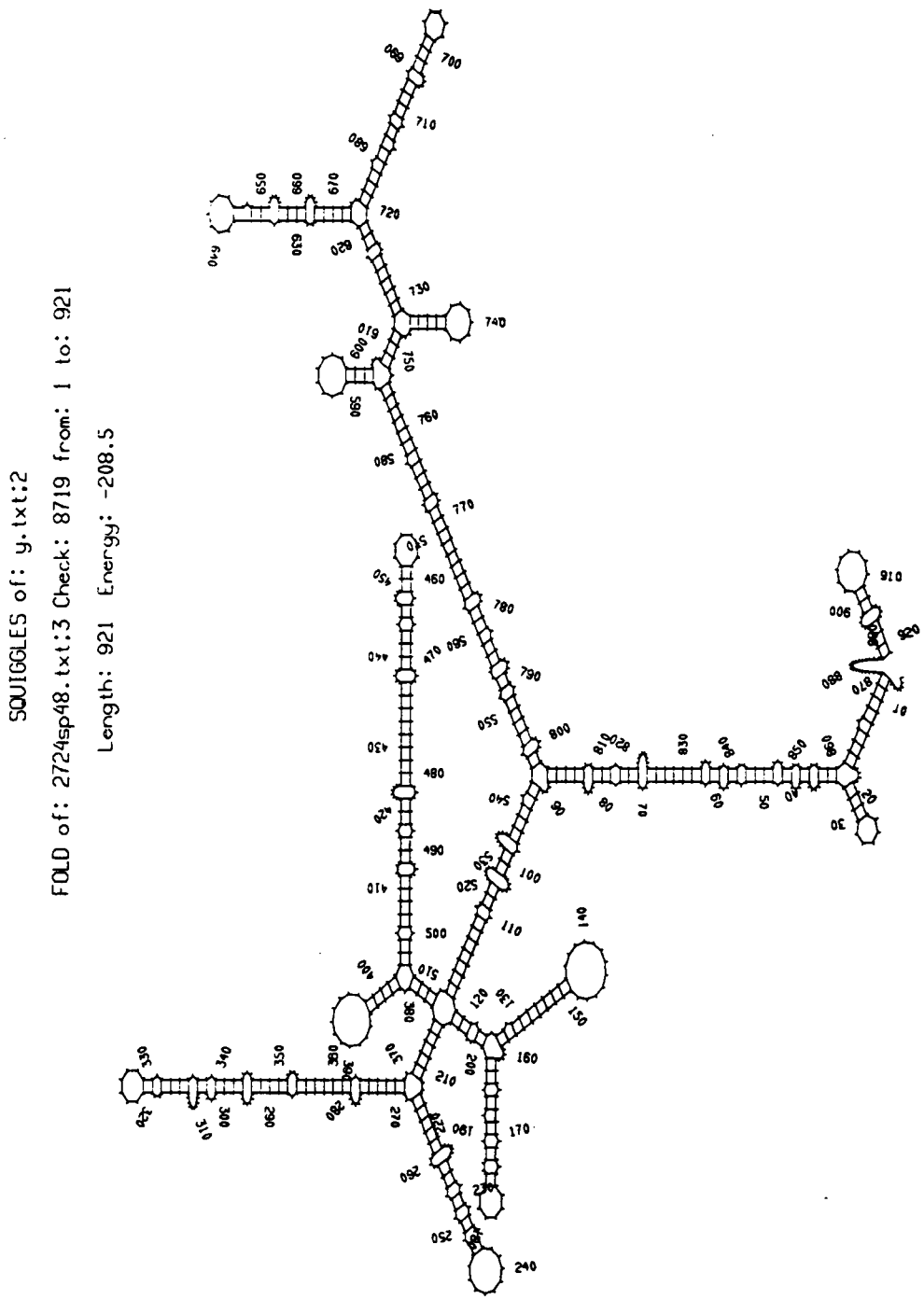

1 2 3 4 5 6 7
1600
1000
500

Figure 6.2. Secondary structure prediction of the RNA transcript from the BVDV 2724 cDNA. Structure predictions were calculated by the program of Zuker (1981) using free energy values defined by Freier (1986).


SQUIGGLES of: y.txt:2
FOLD of: 2724sp48.txt:3 Check: 8719 from: 1 to: 921
Length: 921 Energy: -208.5

taking advantage of single deoxyadenosines (dA) added to 3' ends of duplex DNA molecules by thermostable Taq-polymerase during PCR. The vector pCR 1000 had single 5' T-overhangs at the cloning site which then were linked to the 3' A-overhangs of cDNA during ligation. Best results were obtained when all DNA of one PCR reaction was ligated with 50 ng of vector DNA. Direct screening of white (transformed) bacterial colonies was done by PCR using BVDV primers 3 and 4 or pUC/13 forward and pUC/13 reverse primers. Results of PCR screening are given in Figure 6.3. Recombinant plasmids (pcISK 48) amplified by PCR with BVDV primers resulted in a DNA product approximately 900 base pairs long (Figure 6.3; lanes 2, 4, 6, 8); whereas amplification with pUC/13 primers yielded a DNA product approximately 950 base pairs long (Figure 6.3; lanes 3, 5, 7, 9). The larger size of the latter likely was due to amplification of pUC/13 sequences flanking the cDNA in the multiple cloning site of the vector. Recombinant plasmids (pcISK 48) yielding a 900 base-pair fragment by PCR were examined further by restriction endonuclease digestion of purified plasmid DNA. Digestion of pcISK 48 with restriction endonucleases Sac 1 and Eco R1 yielded a cDNA fragment approximately 900 base pairs long.

Figure 6.4 schematically illustrates pcISK 48 with flanking restriction endonuclease sites. Total calculated molecular weight of pcISK 48 was estimated at 3.9 kb.

Sequence data of pcISK 48 confirmed the presence of a 921 base-pair cDNA insert with pronounced homology to the sequence of BVDV NADL encompassed by

Figure 6.3. PCR screening of recombinant plasmids with BVDV primers (lanes 2, 6, 8) and pUC/13 forward and reverse primers (lanes 3, 5, 7, 9). DNA ladder was loaded in lane 1.

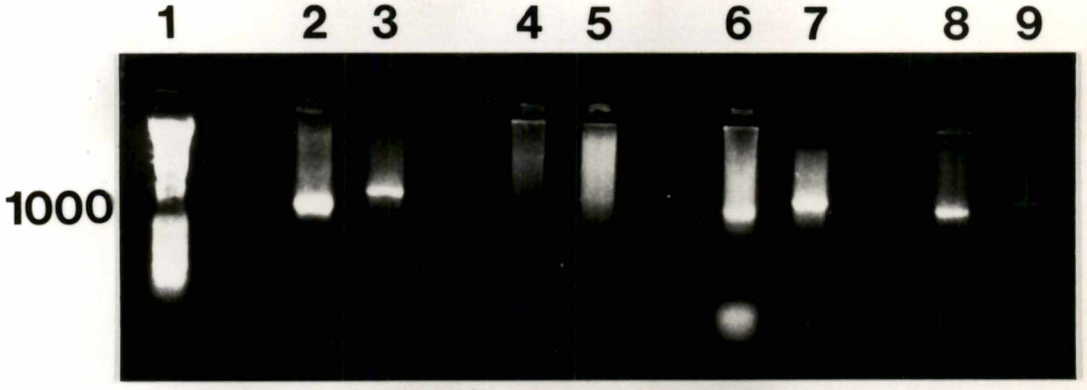
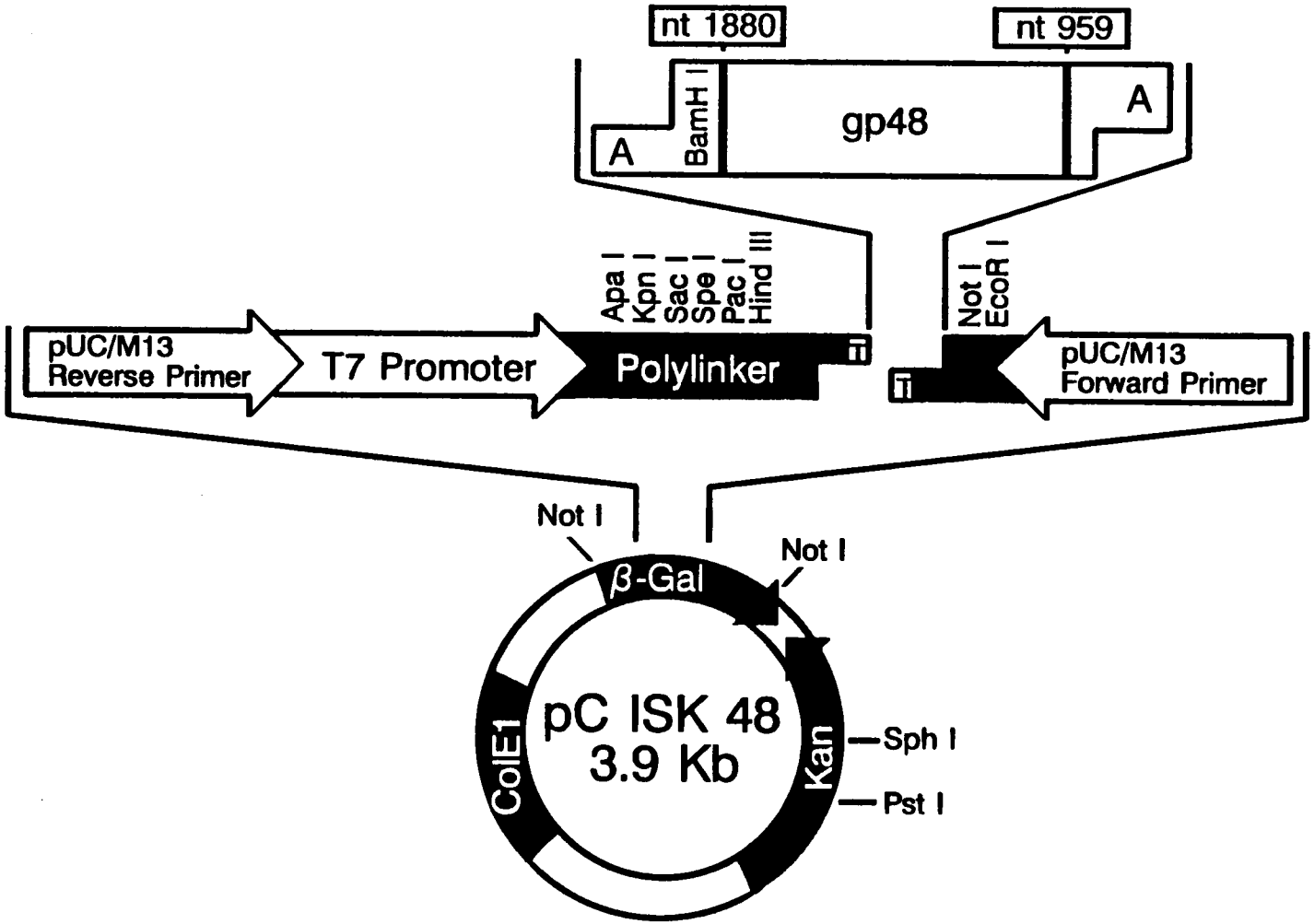

1
2
3
4
5
6
7
8
9
1000

Figure 6.4. Schematic representation of pcISK 48. The PCR product of BVDV 2724 gp48 was inserted into the polylinker site of pCR1000 by linkage of single 3'A of the insert to single 5'T of the vector sequence. Polylinker restriction endonuclease sites, pUC/M13 reverse and forward primer sequences and the T7 promoter sequence of the vector are marked. Black boxes of the vector denote the beta galactosidase gene (β-Gal), kanamycin resistance gene (Kan) and origin of replication sequence (ColE1). Nucleotide numbers (nt) of the insert are relative to genomic sequence of BVDV NADL.


nt 1880
nt 959
BamH I
A
gp48
A
Apa I
Kpn I
Sac I
Spe I
Pac I
Hind III
Not I
EcoR I
pUC/M13
Reverse Primer
T7 Promoter
Polylinker
pUC/M13
Forward Primer
Not I
Not I
β-Gal
pC ISK 48
3.9 Kb
ColE1
Kan
Sph I
Pst I

nucleotides 959 and 1880 (Figures 6.5, 6.6, 6.7; Table 6.1). It was determined also that the 5'- HIND III restriction site encoded by primer 4, had not been incorporated in the cloned DNA fragment.

The cloned cDNA encoded a polypeptide of 307 amino acids beginning with a methionine residue corresponding to methionine at amino acid residue 192 encoded by the BVDV NADL genome and ending with an alanine residue corresponding to alanine 499 of BVDV NADL (Figure 6.5). We hypothesized that the approximate aminoterminus of gp48 was at amino acid residue 265 encoded by the NADL genome[28] which would correspond to isoleucine at amino acid residue 74 of the cDNA derived from BVDV 2724. The protein predicted from the cDNA nucleotide sequence had a putative molecular weight of 34,543 Daltons and an average isoelectric point of 6.22. The polypeptide contained 32% acidic amino acids (asparagine and glutamine), 43% basic amino acids (arginine and lysine) and 28% hydrophobic amino acids (isoleucine, leucine, methionine, valine, phenylalanine, tryptophan and tyrosine). Hydrophobic amino acids were clustered between amino acid 62 and amino acid 80 of the predicted polypeptide (Figure 6.5, 6.6). There were 8 potential N-linked glycosylation sites and 9 cysteine residues on the carboxy-terminal side of the hydrophobic-rich amino acid region (i.e. putative signal sequence) (Figure 6.6, 6.7).

Secondary structure predictions of the putative protein encoded by the cDNA were made (Figure 6.7). The hydrophobic region formed a strong alpha helix compatible with a transmembrane region of a protein. The remainder of the protein

Figure 6.5 Nucleotide sequence of the cDNA and its encoded amino acids. The putative signal sequence is indicated by a box. N-linked glycosylation sites are marked by stars below the sequence. The proposed signal peptidase cleavage site is marked by the arrow between glycine 79 and glutamine 80.

```
ATG AAA ATA GTG CCC AAA GAG TCT GAG AAA GAC AGC AAG ACT AAA CCG CCA GAT GCT ACG
Met Lys Ile Val Pro Lys Glu Ser Glu Lys Asp Ser Lys Thr Lys Pro Pro Asp Ala Thr

ATA GTG GTA GAT GGA GTT AAA TAC CAG GTA AAG AAG AAG GGA AAA GTC AAG AGT AAA AAT
Ile Val Val Asp Gly Val Lys Tyr Gln Val Lys Lys Lys Gly Lys Val Lys Ser Lys Asn

ACG CAG GAC GGT TTA TAT CAT AAC AAA AAT AAG CCG CCA GAA TCA CGC AAG AAA CTA GAG
Thr Gln Asp Gly Leu Tyr His Asn Lys Asn Lys Pro Pro Glu Ser Arg Lys Lys Leu Glu

AAA GCA TTA TTG GCA TGG GCA ATA TTG GCT GCA GTC TTG ATT CAA GTT ACA ATG GGT GAA
Lys Ala Leu Leu Ala Trp Ala Ile Leu Ala Ala Val Leu Ile Gln Val Thr Met Gly Glu

AAT ATA ACA CAG TGG AAC CTA CAG GAT AAT GGG ACA GAA GGG ATA CAA CGG GCA ATG TTC
Asn Ile Thr Gln Trp Asn Leu Gln Asp Asn Gly Thr Glu Gly Ile Gln Arg Ala Met Phe
 *

CAA AGG GGG GTG AAC AGA AGT CTA CAC GGG ATC TGG CCA GGA AAA ATC TGT ACA GGT GTC
Gln Arg Gly Val Asn Arg Ser Leu His Gly Ile Trp Pro Gly Lys Ile Cys Thr Gly Val
                 *

CCT TCC CAT CTA GCC ACC GAT ATG GAA CTA AAA ACG ATC CAT GGT ATG ATG GAT GCA AGT
Pro Ser His Leu Ala Thr Asp Met Glu Leu Lys Thr Ile His Gly Met Met Asp Ala Ser

GAA AAG ACC AAC TAT ACG TGT TGC AGA CTT CAA CGC CAT GAG TGG AAC AAG CAT GGT TGG
Glu Lys Thr Asn Tyr Thr Cys Cys Arg Leu Gln Arg His Glu Trp Asn Lys His Gly Trp
              *

TGC AAC TGG TAC AAT ATT GAA CCT TGG ATT TTA CTC ATG AAT AGA ACC CAA GCC AAT CTT
Cys Asn Trp Tyr Asn Ile Glu Pro Trp Ile Leu Leu Met Asn Arg Thr Gln Ala Asn Leu
                                                 *                   *

ACT GAG GGT CAA CCA CCA AGA GAA TGC GCG GTC ACT TGT AGG TAT GAT AGA GAT AGT GAT
Thr Glu Gly Gln Pro Pro Arg Glu Cys Ala Val Thr Cys Arg Tyr Asp Arg Asp Ser Asp

CTG AAT GTG GTA ACA CAA GCT AGA GAT AGC CCC ACA CCA TTG ACA GGC TGT AAG AAA GGG
Leu Asn Val Val Thr Gln Ala Arg Asp Ser Pro Thr Pro Leu Thr Gly Cys Lys Lys Gly

CAA AAT TTT TCT TTT GCA GGC ATA TTG ATG CGG GGT CCC TGC AAC TTC GAA ATA GCT GCG
Gln Asn Phe Ser Phe Ala Gly Ile Leu Met Arg Gly Pro Cys Asn Phe Glu Ile Ala Ala
                                                 *

AGC GAT GTG TTG TTC AAA GAA GAT GAA TGC ACC AGC ATG TTT CAG GAT ACT GCG CAT TAC
Ser Asp Val Leu Phe Lys Glu Asp Glu Cys Thr Ser Met Phe Gln Asp Thr Ala His Tyr

CTC GTT GAC GGG ATG ACC AAT TCC CTG GAA AGT GCC AGA CAA GGG ACC GCT AAA CTG ACG
Leu Val Asp Gly Met Thr Asn Ser Leu Glu Ser Ala Arg Gln Gly Thr Ala Lys Leu Thr

ACC TGG TTA GGC AAG CAG CTT GGG ATA CTA GGA AAA AAA TTG GAA AAC AAG AGC AAG ACA
Thr Trp Leu Gly Lys Gln Leu Gly Ile Leu Gly Lys Lys Leu Glu Asn Lys Ser Lys Thr
                                                          *

TGG TTT GGA GCA TAC GCT GCT
Trp Phe Gly Ala Tyr Ala Ala
```

Figure 6.6. Characteristics of the protein encoded by the 2724 gp48 cDNA in pcISK 48. Numbers above and below panels indicate amino acid residues starting with the first methionine codon as number 1. Basic and acidic properties of amino acid residues (A); distribution of cysteine residues (B); distribution of N-linked glycosylation sites (C); hydrophilicity calculated according to Kyte-Doolittle (D); secondary structure calculated according to Chou-Fasman. Upper panel: Probability of alpha helix formation is indicated by a solid line, probability of beta sheet formation is indicated by a hashed line. Lower panel: Probability of turns (E); antigenicity of amino acid residues calculated according to Jameson-Wolf (F).

pc ISK 48

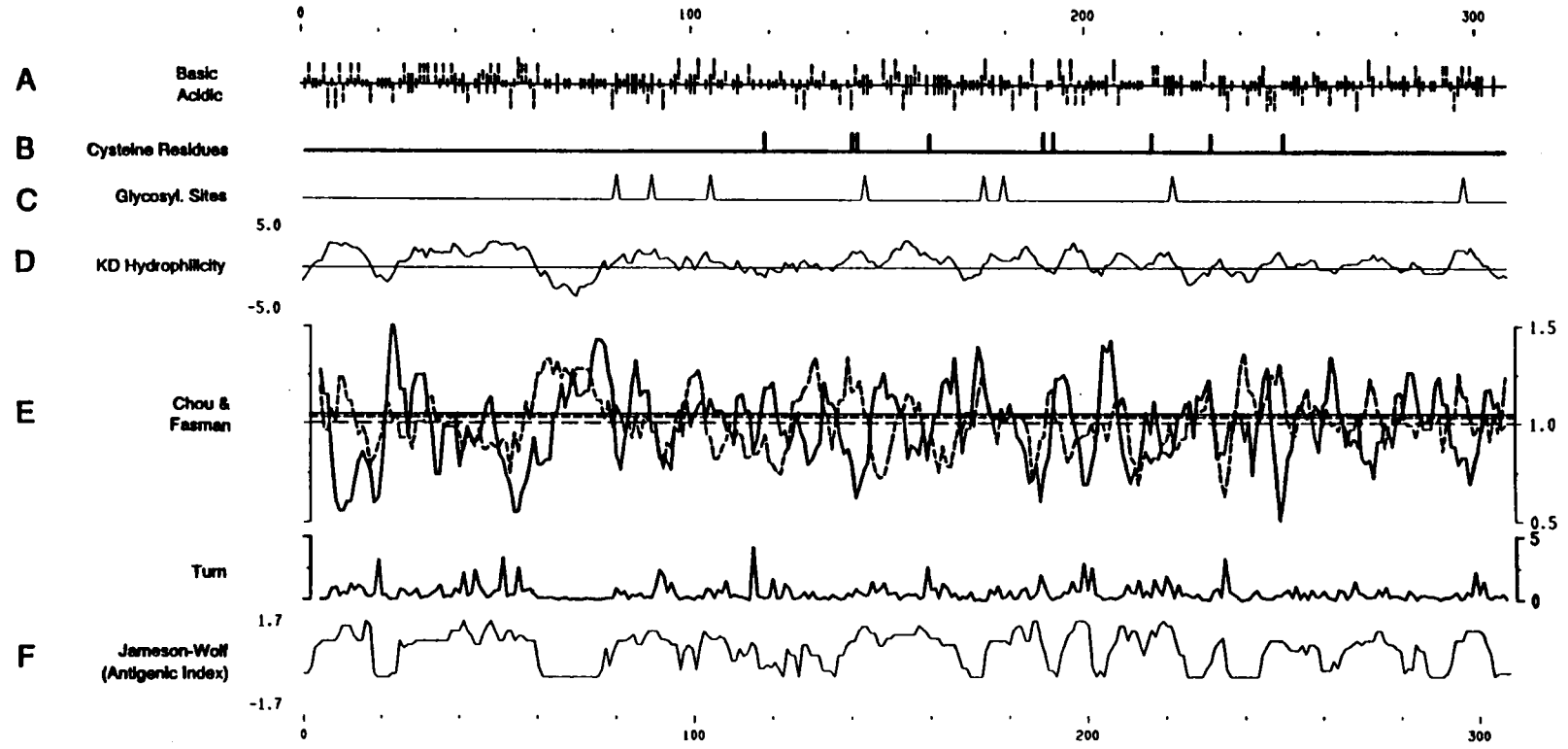

0
100
200
300
A
Basic
Acidic
B
Cysteine Residues
C
Glycosyl. Sites
5.0
D
KD Hydrophilicity
-5.0
1.5
E
Chou &
Fasman
1.0
0.5
5
Turn
0
1.7
F
Jameson-Wolf
(Antigenic Index)
-1.7
0
100
200
300

Figure 6.7. Secondary structure prediction, according to Chou-Fasman, of the putative protein encoded by the BVDV 2724 cDNA. Hydrophobicity of amino acids is denoted by large diamonds. Small hexagonal symbols indicate putative N-linked glycosylation sites. Note the high hydrophobicity of amino acid residues at position 60 to 80 just aminoterminal to the first N-linked glycosylation site. Hydrophobic amino acid residues in this region also were predicted to form a strong alpha helix. The putative N-terminus of gp48 is at glycine 79.

BVDV 2724 ISK 48

PLOTSTRUCTURE of: 2724SP48.PEP;1 ck: 2918

TRANSLATE of: 2724sp48.txt check: 8719 from: 1 to: 921

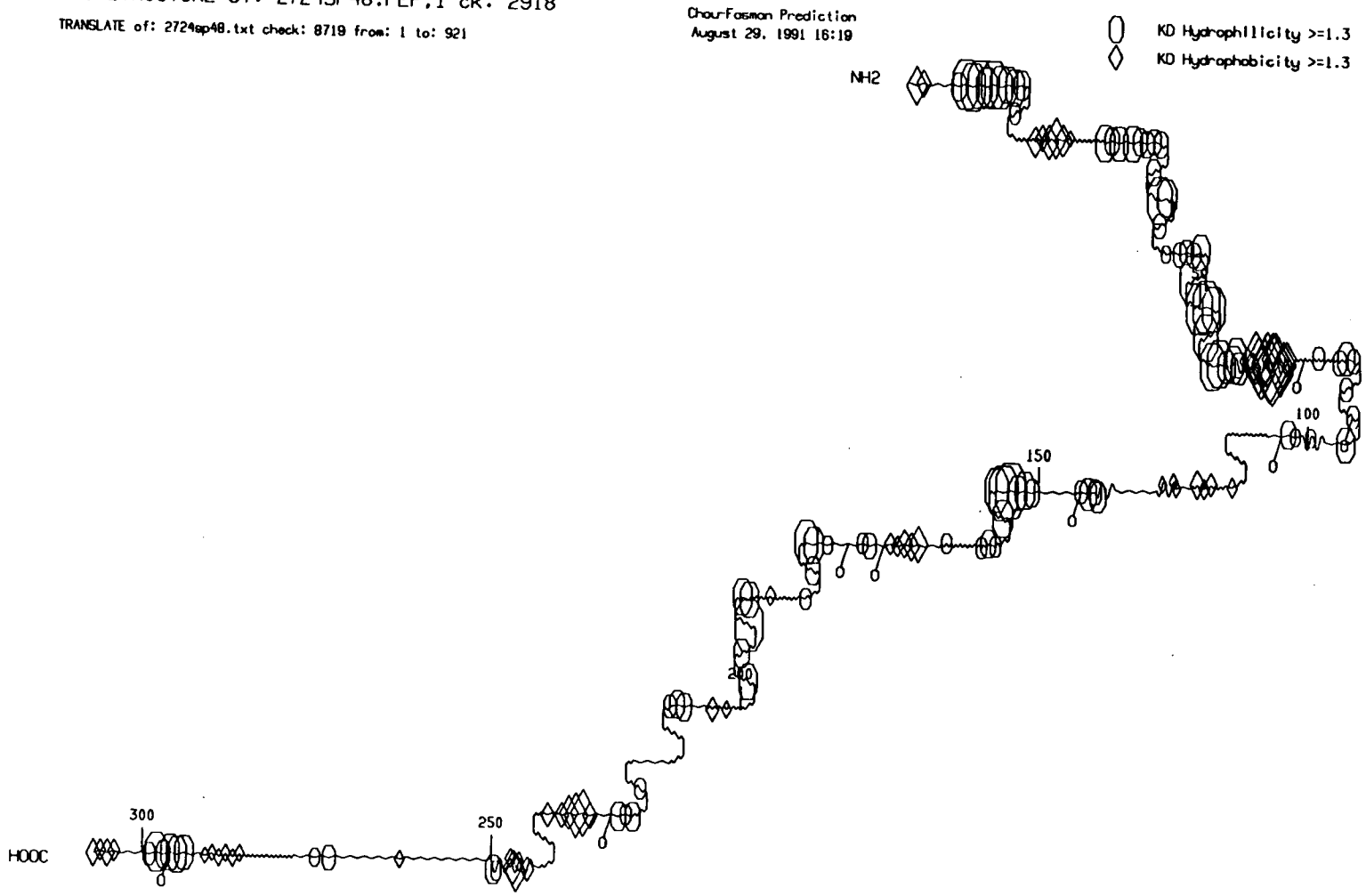


Table 6.1 Comparison of gp48 nucleotide and amino acid sequences of BVDV 2724 to those of members of pestiviruses and Flaviviridae and Hepatitis -C virus.

		Nucleotide % Identity	Amino Acid % Identity	% Similarity	Cysteine Residues	Glycosylation Sites
BVDV NADL[a]		88.8	96.6	(98.8)	9/9	8/8
BVDV Osloss[b]		81.9	89.9	(95.7)	9/9	7/8
HCV GSA[c] (Alfort)		68.6	75.8	(87.5)	9/9	7/8
HCV G3PE[d] (Brescia)		68.6	74.8	(86.6)	9/9	5/8
Yellow Fewer[e]	pre-M[i]	39.7	13.3	(39.9)	2/9	2/8
(17 DVaccine strain)	E[j]	40.7	2.17	(46.7)	1/9	0/8
Dengue Type II[f]	pre-M[i]	41.8	11.5	(42.7)	1/9	0/8
	E[j]	41.9	16.6	(45.5)	0/9	1/8
Kunjin[g]	pre-M[i]	38.9	15.1	(39.6)	0/9	1/8
	E[j]	38.0	16.3	(40.4)	0/9	0/8
Hepatitis C[h]		36.5	40.7	(17.8)	4/9	3/8

a: NADL Sequence data from Collet, M.S. et al. 1988.
b: Osloss Sequence data from Renard, A. et al. 1987.
c: HCV GSA Sequence data from Meyers, G. et al. 1989.
d: HCV Brescia Sequence data from Moormann, R.J.M. 1990.
e: YF Sequence data from Rice, C.M. et al. 1985.
f: D Sequence data from Hahn, Y.S. et al. 1988.
g: Ku Sequence data from Coia, G et al. 1988.
h: HPc Sequence data from Kato, N et al, 1990.
i: Nucleotide comparison of gp48 with flavivirus pre-M protein
j: Nucleotide comparison of gp48 with flavivirus envelope protein

did not have any additional strong alpha helices or beta-pleated sheets with pronounced hydrophobicity suggestive of membrane association (Figure 6.6, 6.7) cDNA nucleotide sequence and amino acid composition of the putative encoded protein was compared with analogous sequences of other pestiviruses; the pre-M and E proteins of yellow fever, dengue and Kunjin viruses; and Hepatitis-C virus (Table 6.1). There were pronounced homologies between the BVDV 2724 cDNA and corresponding genomic regions of BVDV NADL, BVDV OSLOSS, HCV Alfort and HCV Brescia; however, no significant homologies with Flaviviruses and Hepatitis-C virus were identified. Number and position of cysteine residues and N-linked glycosylation sites were nearly identical among all pestiviruses compared (Table 6.1; Figure 6.8A). Hydrophilicity plots and distribution of acidic and basic amino acid residues of the putative protein encoded by the BVDV 2724 cDNA and corresponding proteins of published pestiviral sequences were very similar (Figure 6.8A, B).

The cDNA was subcloned into pGem-4z by taking advantage of restriction endonuclease sites Sac 1 and Eco R1 resulting in a 3.7 kb long recombinant plasmid (pgISK 48) containing a 985 base pair cDNA insert (Figure 6.9). Nucleotide sequencing of the recombinant plasmid (pgISK 48) established that the cDNA insert had its first ATG codon downstream to the SP6 promoter of the vector. A schematic of pgISK 48 is given in Figure 6.10.

pgISK 48 was linearized by digestion with restriction endonuclease AVA 1, and run off RNA transcripts were produced with SP6 RNA polymerase (Figure 6.11). The cDNA derived RNA was translated *in vitro* with rabbit reticulocyte lysate in the

Figure 6.8. Comparison of hydrophobicity graphs of the protein encoded by 2724 cDNA and corresponding regions of BVDV NADL and OSLOSS and HCV strains GSA (Alfort) and 3PE (Brescia). A. Hydrophobic regions are areas below the centerline. Potential N-linked glycosylation sites are marked by arrows. The putative signal peptide is between amino acid residues at positions 60 and 80. The putative N-terminus of gp48 is immediately aminoterminal to the first N-linked glycosylation site.

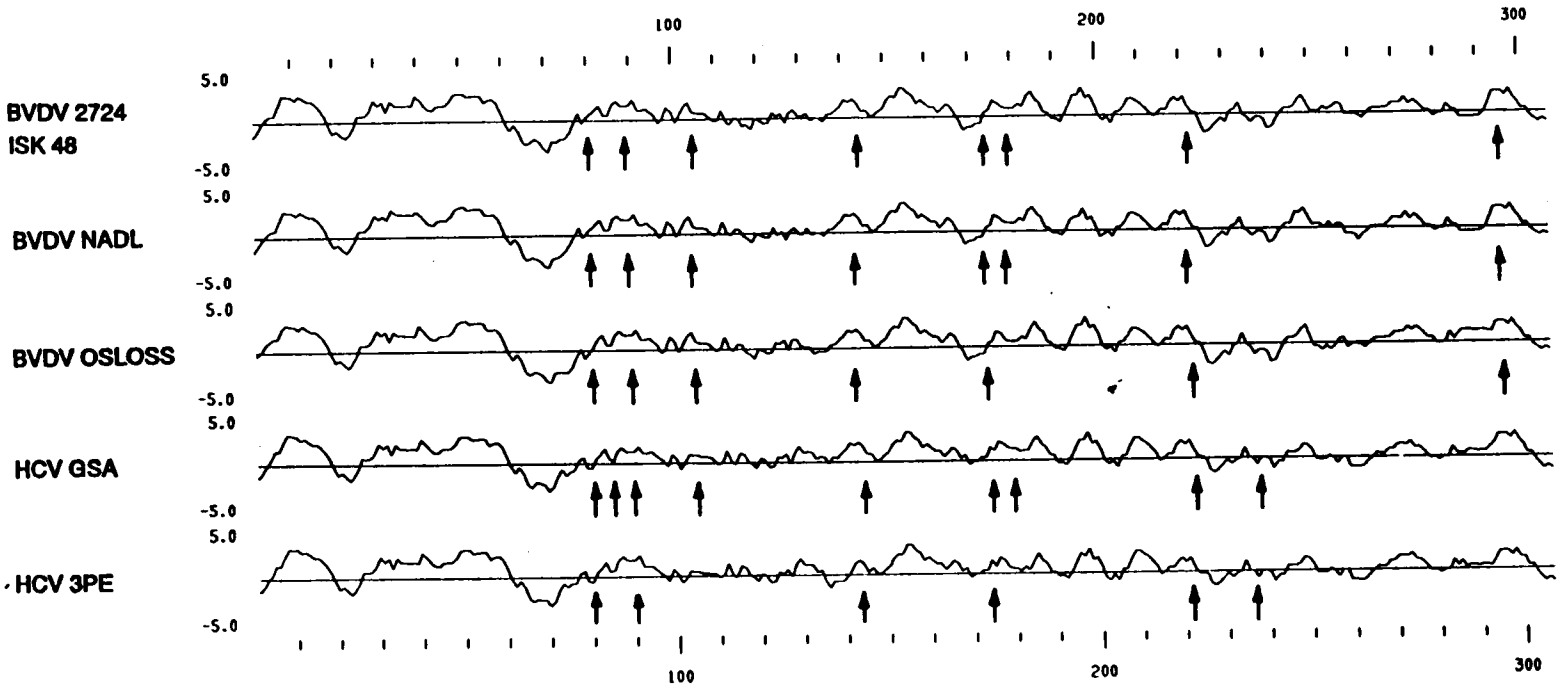

100
200
300
5.0
BVDV 2724
ISK 48
-5.0
5.0
BVDV NADL
-5.0
5.0
BVDV OSLOSS
-5.0
5.0
HCV GSA
-5.0
5.0
HCV 3PE
-5.0
100
200
300

Figure 6.8 (continued). B. Comparison of acidic and basic amino acid residues encoded by 2724 cDNA with distribution of charged amino acid residues of corresponding regions of BVDV NADL and OSLOSS and HCV strains GSA (Alfort) and 3PE (Brescia).

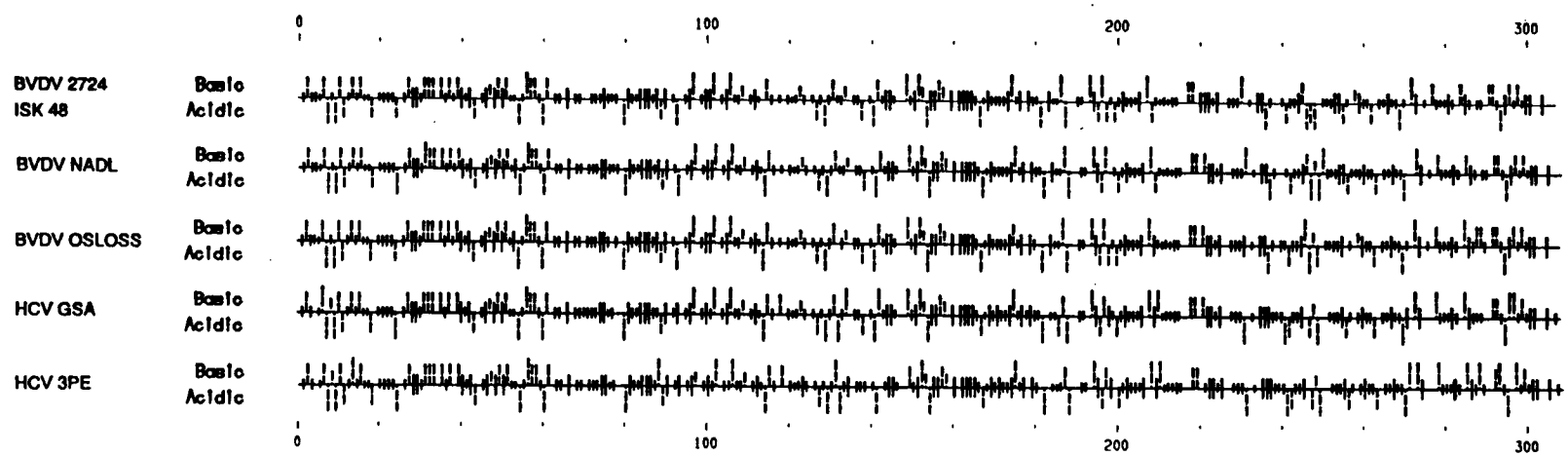

0
100
200
300
BVDV 2724
ISK 48
Basic
Acidic
BVDV NADL
Basic
Acidic
BVDV OSLOSS
Basic
Acidic
HCV GSA
Basic
Acidic
HCV 3PE
Basic
Acidic
0
100
200
300

Figure 6.9. Restriction endonuclease digestion of purified plasmid DNA with Eco R1 and HIND III. Recombinant plasmid DNA (pgISK 48) with cDNA approximately 1000 base pairs long (lanes 1 and 2); undigested plasmid DNA (lanes 3 and 4); DNA ladder with 75, 150, 200, 400, 500, 1000 and 1600 kB fragments (lane 5); undigested pGem-4z (lane 6); pGem-4z DNA digested with Eco R1 only (lane 7); pGem-4z DNA digested with HIND III only (lane 8).

Note the cDNA (lanes 1 and 2) migrating parallel to the 1000 kB band of the DNA ladder.

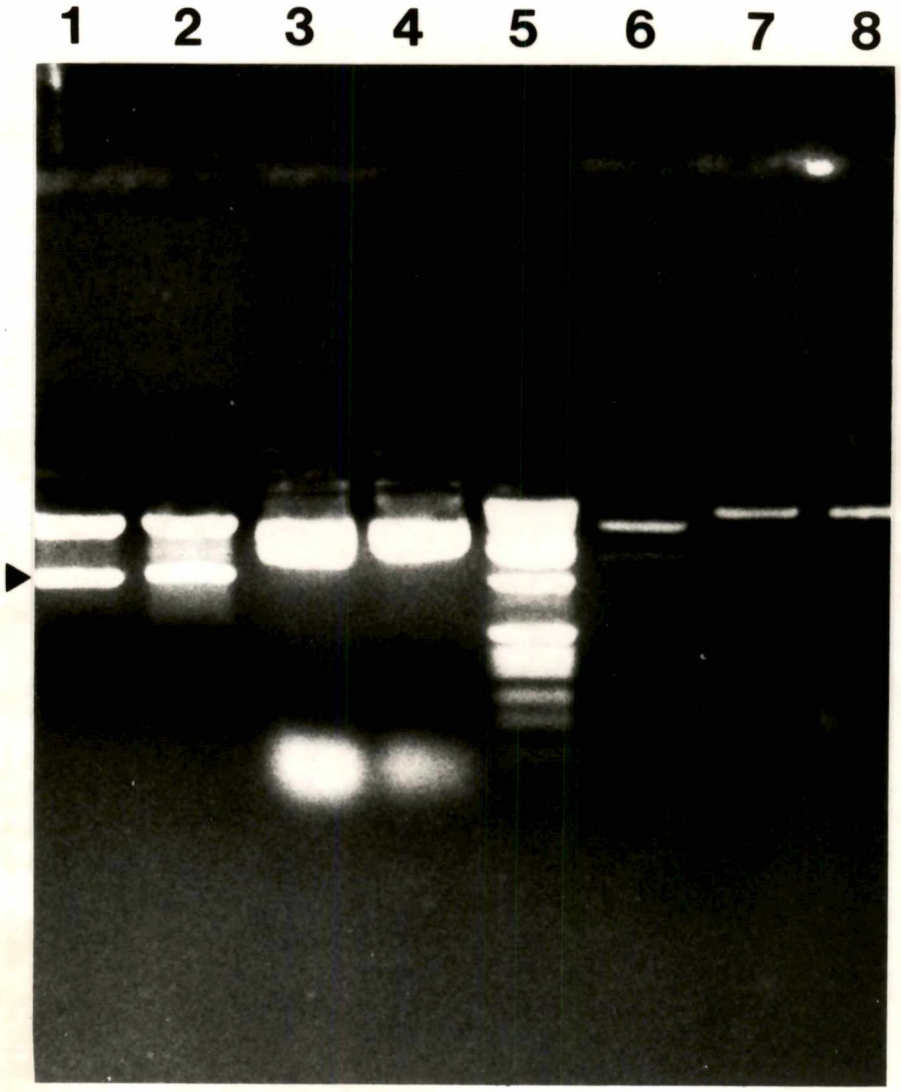

1
2
3
4
5
6
7
8

Figure 6.10. Schematic representation of pgISK 48. 2724 gp48 was inserted in pGem-4z at restriction endonuclease sites EcoR1 and Sac1. Polylinker restriction endonuclease sites and SP6 and T7 promoter sequences are marked. Black boxes of the vector denote the beta galactosidase gene (lacZ) and ampicillin resistance gene (Ampr). The sequence of origin of replication is marked ori. Nucleotide numbers (nt) of the insert are relative to the genomic sequence of BVDV NADL.

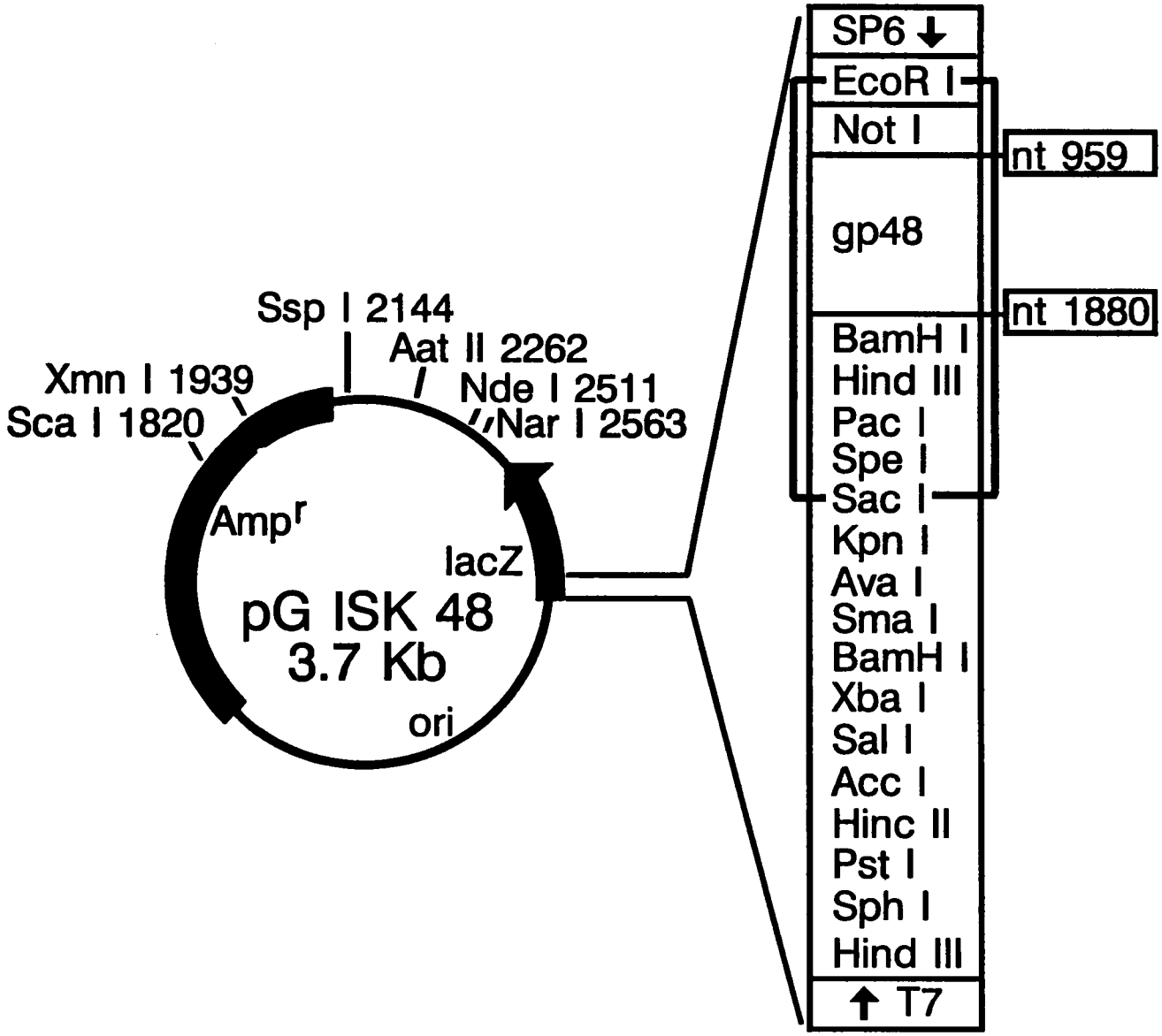
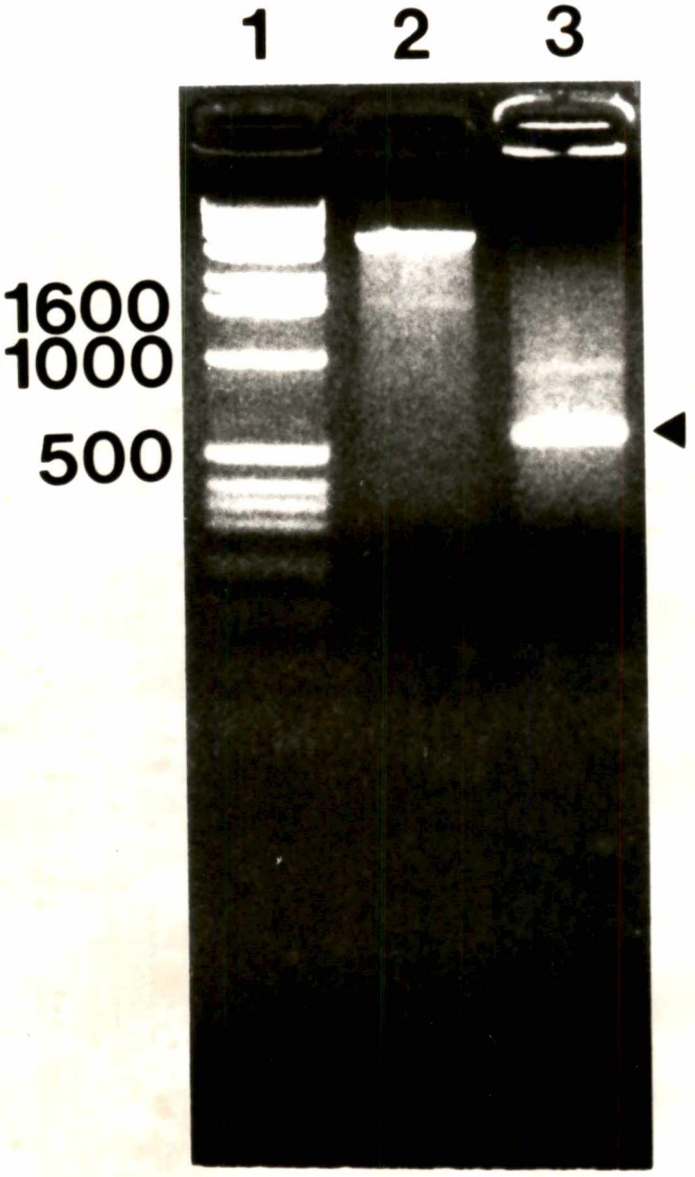

Ssp I 2144
Aat II 2262
Xmn I 1939
Nde I 2511
Sca I 1820
Nar I 2563
Amp^r
lacZ
pG ISK 48
3.7 Kb
ori
SP6
EcoR I
Not I
nt 959
gp48
nt 1880
BamH I
Hind III
Pac I
Spe I
Sac I
Kpn I
Ava I
Sma I
BamH I
Xba I
Sal I
Acc I
Hinc II
Pst I
Sph I
Hind III
T7

Figure 6.11. RNA run off transcripts of linearized pgISK 48. DNA ladder (lane 1); linearized pgISK 48 (lane 2); transcribed RNA (lane 3). Approximately 500 μg DNA or RNA were loaded on 2% agarose gels, electrophoresed at 75V for 1.5 h and stained with ethidium bromide.


1
2
3
1600
1000
500

presence of [35C]-methionine (Figure 6.12; lane 1). The translation product migrated during electrophoresis as a 30 kDa protein. A second, higher molecular weight protein of approximately 35 kDa, was present also in some translation reactions. Attempts at translation of RNA transcribed from linearized and non-linearized non-recombinant plasmid did not yield any products demonstrable by SDS-PAGE (Figure 6.12; lanes 2 and 3). The *in vitro*-translated 30 kDa polypeptide encoded by the cDNA was immunoprecipitated by BVDV polyclonal antiserum (Figure 6.12; lanes 6 and 8). The same antiserum precipitated viral polypeptides in cell lysates infected by BVDV 2724 (Figure 6.13; lane 2). Several viral precursor proteins and viral polypeptides; gp55, gp48 and gp25; were precipitated. Gp55 migrated as a double band consistent with findings reported by Kennedy (1991).[93] Gp48 migrated close to the 46 kDa molecular weight marker. Proteins in lysates of non-infected cells were not precipitated by BVDV antisera (Figure 6.13; lane 3). The faint band migrating during SDS-PAGE at 46 kd may have been due to the presence of cellular actin. Actin is commonly precipitated in infected and non infected cell lysates by BVDV antisera (Bolin, pers. comm.). Nonimmune serum did not precipitate BVDV specific polypeptides in lysates of infected cells (Figure 6.13; lane 4) and also did not precipitate the *in vitro* translated 30 kDa polypeptide (Figure 6.12; lanes 7, 9).

Analysis of the putative amino acid sequence encoded by the cloned cDNA revealed a stretch of hydrophobic amino acids suggestive of a signal sequence between amino acid residues 60 and 80 (Figure 6.5, 6.6). To assess the function of this putative signal peptide we translated RNA in presence of microsomal

Figure 6.12. *In vitro* translation and immunoprecipitation of RNA transcripts of pgISK 48. 30 kDa (gp48) translation product (lane 1); translation of linearized and non linearized pGem (lanes 2 and 3), no RNA (lane 4); polypeptide ladder with 30 kDa, 46 kDa, 69 kDa, 92 kDa and 200 kDa markers (lane 5); immunoprecipitation of gp48 by BVDV polyclonal antiserum (field serum) (lane 6); immunoprecipiation by negative antiserum (lane 7); immunoprecipitation by gnotobiotic calf serum with BVDV polyclonal antibodies (lane 8); immunoprecipitation by negative antiserum (lane 9).

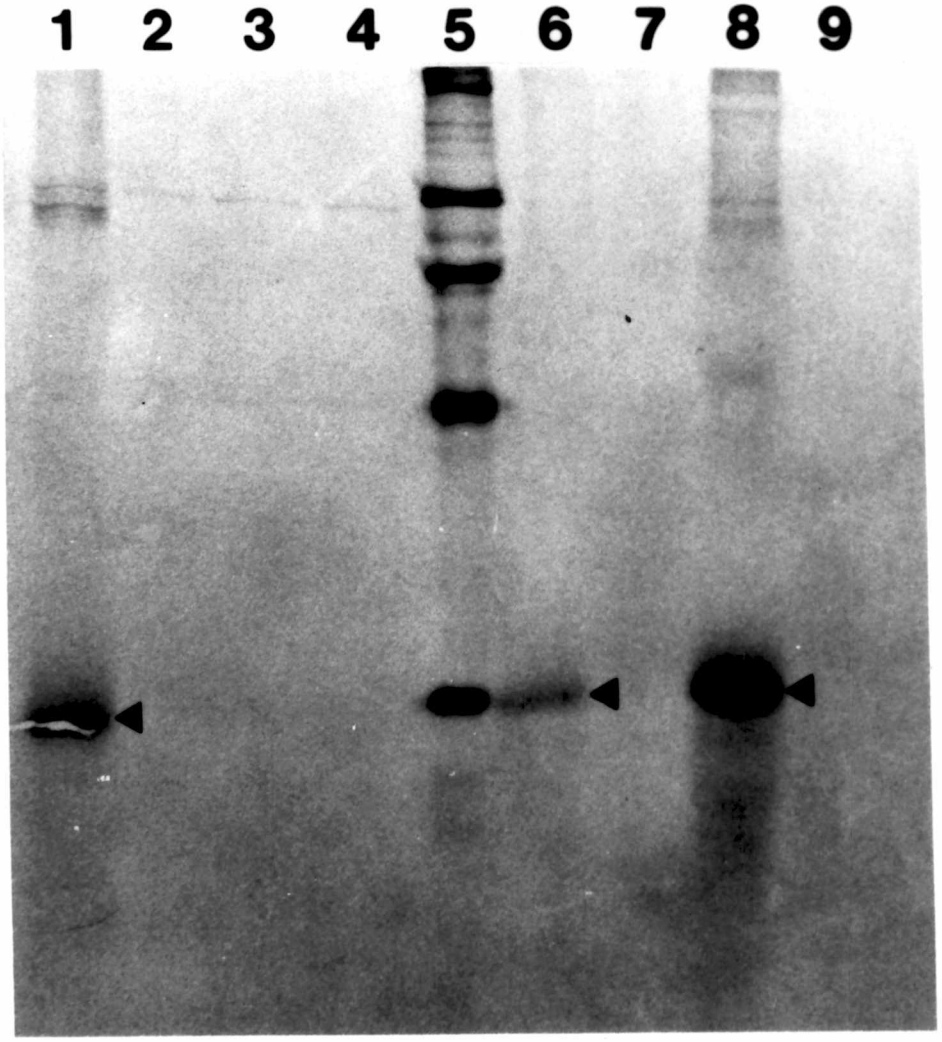
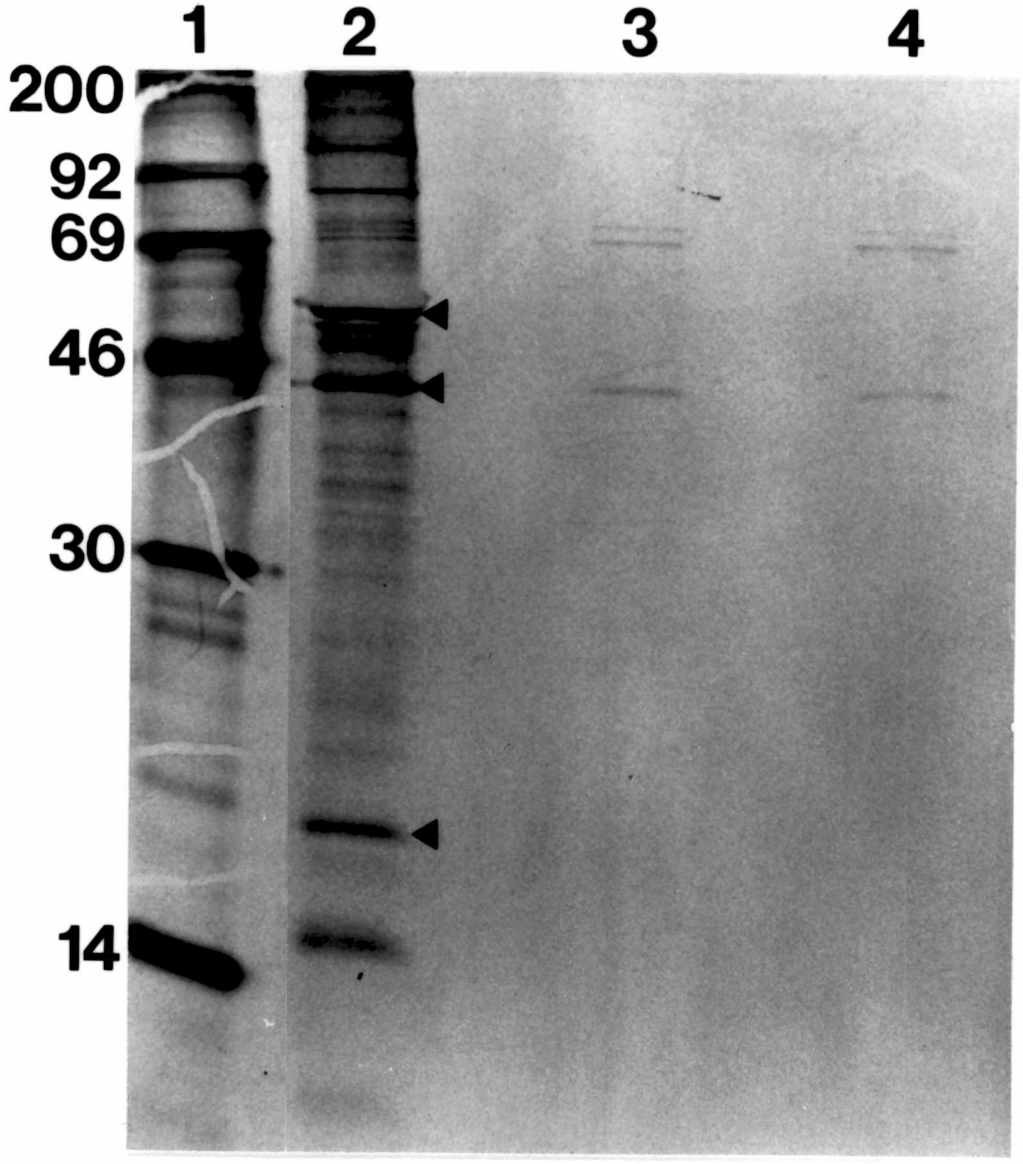

1 2 3 4 5 6 7 8 9

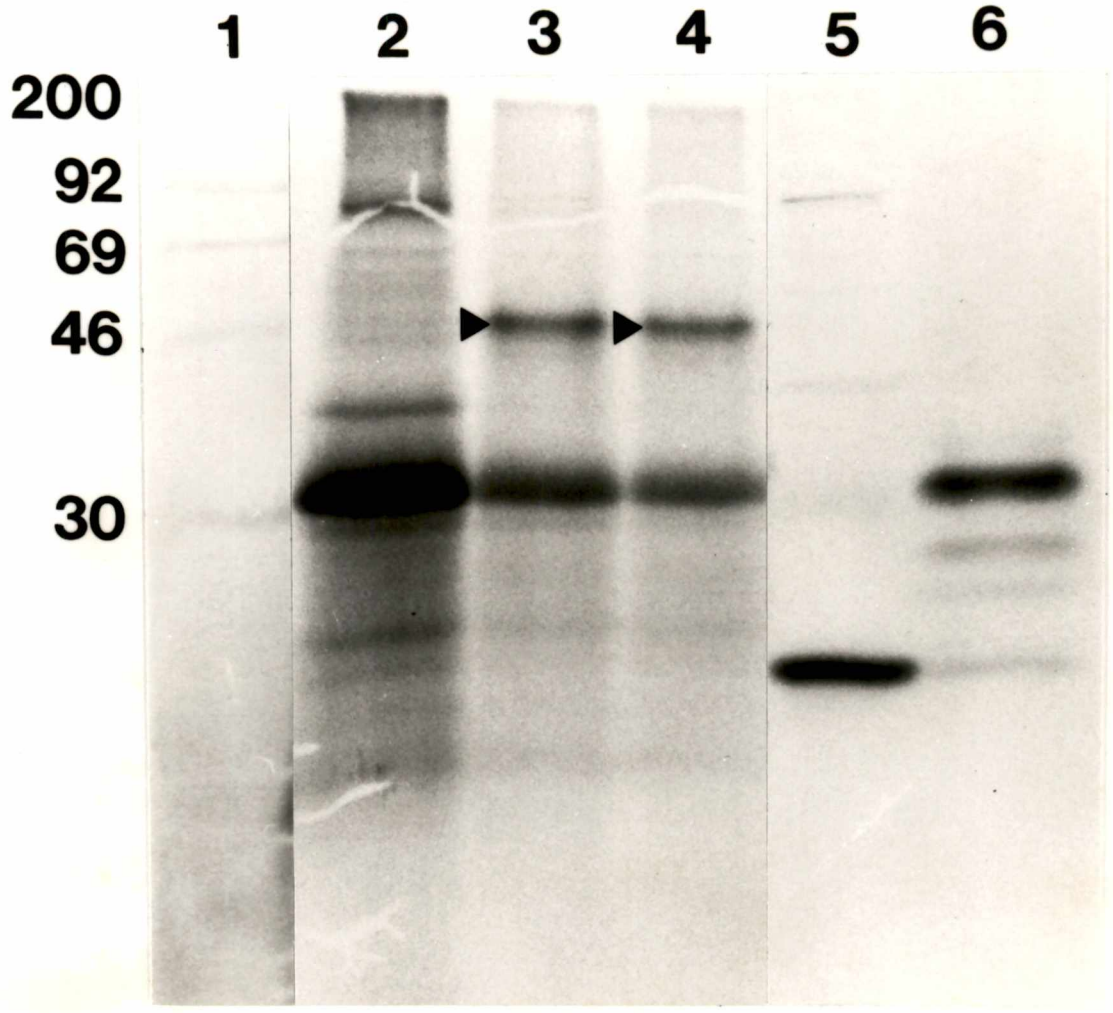
Figure 6.13. Immunoprecipitation of 35S methionine and L-[U-14-C] leucine labelled viral polypeptides from cell lysates. Polypeptide ladder with 14 kDa, 30 kDa, 46 kDa, 69 kDa, 92 kDa and 200 kDa markers (lane 1); viral polypeptides precipitated by BVDV antiserum (lane 2); peptides precipitated by BVDV antiserum in non-infected cell lysates (lane 3); peptides precipitated by negative serum in BVDV 2724 infected cell lysates (lane 4). Note viral peptides migrating at 25 kDa, 46 kDa and the double band at 53 kDA in lane 2.


1
2
3
4
200
92
69
46
30
14

membranes. SDS-PAGE of processed and glycosylated translation products resulted in an additional polypeptide band near the 46 kDa marker (Figure 6.14; lanes 3, 4) and decrease in radiographic density of the 35 kDa band. The 16 kDa increase in molecular weight of a protein from 30 kDa to 46 kDa in presence of microsomal membranes was consistent with the estimated size increase of a polypeptide when glycosylation of 8 N-linked glycosylation sites occurs. RNA encoding the alpha factor of *S. cerevisiae* was used as a control in the reaction. Molecular weight of the alpha factor RNA translation and glycosylation products were 18.6 kDa and 30 kDa, respectively (Figure 6.14; lanes 5 and 6) and consistent with established molecular weights of non-glycosylated and glycosylated products, respectively.

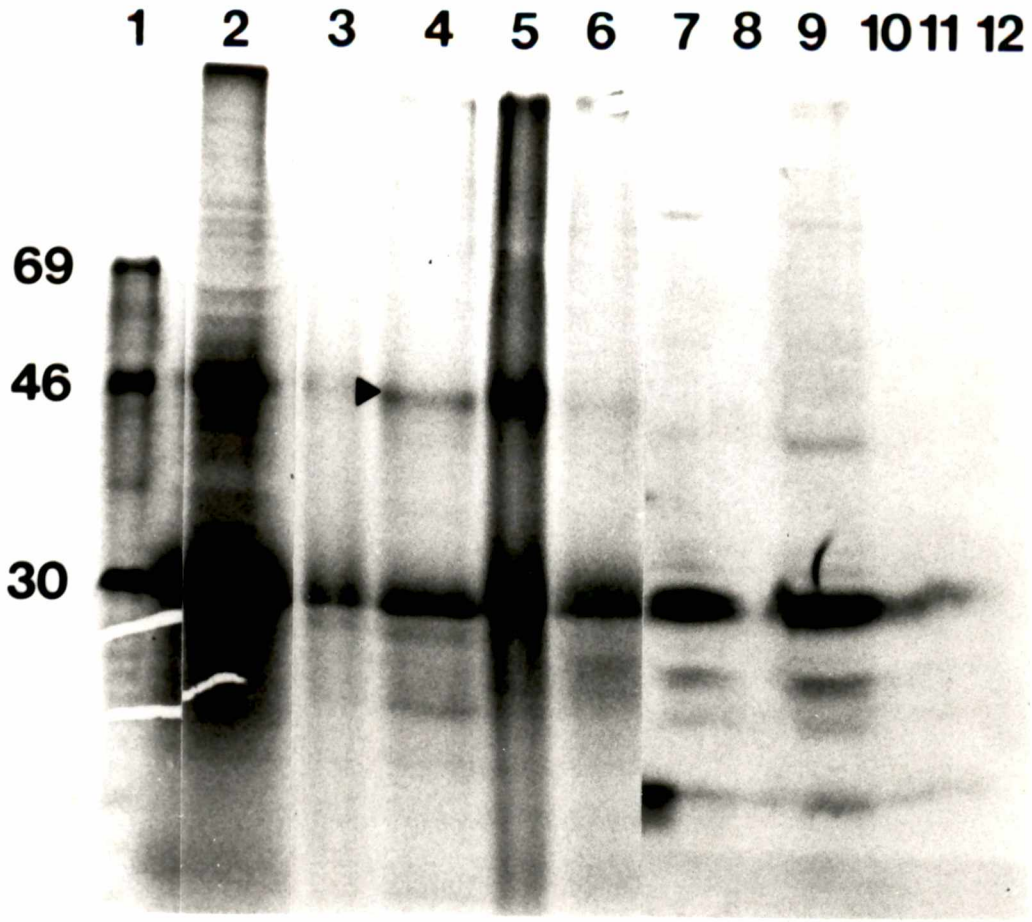
Typically, signal peptidases in microsomal vesicles cleave translocating polypeptides at a site 4 to 10 amino acids beyond the hydrophobic core of the signal sequence thereby releasing the amino terminus of polypeptides into the lumen of the microsomal vesicle. However, the translocating polypeptide will remain associated with microsomal membranes, if this cleavage does not occur and the signal sequence serves as an anchor or if an additional hydrophobic region exists on the carboxyterminal side of the cleavage site capable of anchoring the protein in the membrane. When closed microsomal membrane vesicles are converted to open sheets, secreted polypeptides are released into the soluble fraction of the translation mixture, whereas anchored polypeptides remain with the microsomal membrane fraction. In order to assess signal peptidase cleavage and anchorage of the cDNA encoded polypeptide, we added microsomal membranes to the translation reaction mixture followed by

Figure 6.14. *In vitro* translation and glycosylation of gp48. Polypeptide ladder (lane 1); translation product (lane 2); glycosylated product (lanes 3 and 4); alpha factor (lane 5), glycosylated alpha factor (lane 6). Note the glycosylated protein migrating near the 46 kDa marker and decrease in the non-glycosylated protein product in lanes 3 and 4.


1
2
3
4
5
6
200
92
69
46
30

conversion of closed microsomal vesicles to open sheets. The soluble and membrane fractions then were separated and analyzed by electrophoresis. The 46 kDa glycosylation product was present in the soluble fraction suggesting it had been secreted (Figure 6.15; lane 4). The faint 46 kDa band in the membrane fraction likely was due to residual soluble fraction in the preparation (Figure 6.15; lane 3). Alpha factor peptide, a secreted protein, was present in the soluble fraction (Figure 6.15; lane 9).

Figure 6.15. *In vitro* translation, glycosylation and secretion of gp48. Polypeptide ladder (lane 1); glycosylated 46 kDa product (gp48) (lane 2); membrane fraction treated with sodium carbonate (lane 3), supernatant fraction treated with sodium carbonate (lane 4); membrane fraction treated with control buffer (lane 5); supernatant fraction treated with control buffer (lane 6); glycosylated alpha factor (lane 7); membrane fraction (alpha factor) treated with sodium bicarbonate (lane 8); supernatant fraction (alpha factor) treated with sodium bicarbonate (lane 9); membrane fraction (alpha factor) treated with control buffer (lane 10); supernatant fraction (alpha factor) treated with control buffer (lane 11); no RNA (lane 12). Note the secreted glycosylated protein product at 46 kDa in lane 4.


1
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12
69
46
30

CHAPTER 7

DISCUSSION

Primers designed from nucleotide sequences of cytopathogenic BVDV strains NADL and 72 could be used to amplify a nucleotide sequence encoding a putative specific structural protein of a non-cytopathogenic BVDV. Specific cDNA was produced by reverse transcription of viral RNA followed by amplification by PCR with a set of primers encompassing the putative signal peptide linked to the gp48-encoding region. Single PCR-amplification did not yield enough specific product to be detected by agarose gel electrophoresis. Low yield might have been due to considerable nonspecific priming resulting in a heterogenous mixture of PCR products. Changes in annealing temperature in the PCR reaction did not result in improved specific amplification. The product of the first amplification was reamplified by PCR using a second set of primers that bound to the cDNA immediately internally to the 3' ends of the first set of primers. This double amplification with a nested set of primers increases the specificity of the reaction[129] and yielded sufficient amounts of viral cDNA to clone.

Secondary structure predictions of the RNA molecule transcribed from the cDNA resulted in a complex structure with several stem and loop structures and a minimum free energy of -208.5 KCal/mode (Figure 6.2). The very low free energy value suggested high stability of the RNA molecule existed. Even though the RNA was denatured prior to cDNA synthesis and the cDNA was denatured prior to

annealing of primers in each PCR cycle, remaining internal stem and loop structures could have allowed production of a shorter cDNA during cDNA synthesis either by the reverse transcriptase or Taq-polymerase (Figure 6.1: 400 base pair piece). RNA stem and loop structures are common in ribosomal, transfer and messenger RNAs *in vivo* and are important in RNA function.[101] DNA stem and loop structures occur rarely as DNA is usually double stranded. During PCR amplification, DNA is denatured in each cycle and may fold into stem and loop structures during the primer annealing phase.

The second amplified DNA produced was cloned into pCR1000 resulting in recombinant plasmid pcISK 48. However, sequence analysis of the cDNA revealed that the HIND III restriction endonuclease site of primer 4 was not present in the cDNA. Instead nucleotides AGTGCC were incorporated in the cDNA corresponding to the 5' terminus of primer 4 and were identical to nucleotides 964 to 970 of the sequence of BVDV NADL. Thus, the 5' terminus of the cDNA was identical to the 5' terminus of primer 4 (nucleotide 959 of BVDV NADL) and did not correspond to the 5' terminus of primer 2 which argues against the possibility that the product of the first amplification had been cloned.

Analysis of the putative amino acid sequence encoded by the cDNA identified a stretch of amino acids with high hydrophobicity between amino acid residues 62 and 80 of the cDNA. It had been speculated that a signal peptide responsible for translocation of the nascent polyprotein into the endoplasmic reticulum (ER) was

encoded by this region of the BVDV genome, as its genomic location is immediately prior to that of the glycosylated proteins.[28]

Translocation of polypeptides into the endoplasmic reticulum has been extensively studied and several mechanisms of translocation have been proposed. The signal hypothesis holds that a signal peptide in a nascent polypeptide chain binds to the ER membrane and facilitates noncovalent binding of ribosomes to the ER. It is speculated that a pore in the membrane is formed allowing transport of the signal peptide and nascent polypeptide. Transport energy is provided by elongation of the polypeptide chain.[12]

If the polypeptide contains a "stop-transfer" signal, transport is arrested and the polypeptide is anchored in the membrane. This anchorage orients the protein with its aminoterminus in the lumen of the ER and carboxyterminus in the cytosol (Type 1 membrane protein). If a stop-transfer signal is absent, the polypeptide is transported into the lumen of the ER and either secreted or membrane-anchored by the signal peptide itself. Membrane proteins anchored by this mechanism are classified as type 2 membrane proteins. Proteins anchored by the signal sequence have the carboxyterminus in the ER-lumen and the aminoterminus in the cytosol.[12,113] Parts of the cellular components mediating binding of signal peptides to the ER membrane, such as the signal recognition particle and its membrane receptor have been identified and characterized.[63,113,174] The putative transport of BVDV gp48 in the endosplasmic reticulum is facilitated by the aminoterminally located signal sequence. Gp48 could be anchored by the signal sequence (Type 2 membrane

protein), if there is no enzymatic cleavage of the signal peptide in the lumen of the endoplasmic reticulum.

Pore formation in the ER membrane was not required in a direct transfer model proposed by Randall et al. (1983),[159] as it was speculated that elongation of the peptide provides enough energy for direct translocation across the membrane. Membrane translocation would occur spontaneously and primarily post-translationally rather than cotranslationally dependent upon protein folding.[159] Other trans-membrane protein-transport theories did not include the existence of additional proteins for transport such as the signal recognition particle and its receptor but proposed that secondary and tertiary structure of the nascent polypeptide mediates translocation. The helical hairpin theory suggested that two anti-parallel helices are formed, one by the signal sequence and the other by polar amino acid residues carboxyterminal to the signal peptide. The amphophilic signal peptide helix inserted spontanously into the membrane and mediated transfer of the amphipathic polar helix.[48] All theories agree that specific sequences at the aminoterminus of a protein, such as a signal peptide, are necessary for transmembrane transport.

General characteristics of eukaryotic signal peptides include a positively charged amino-terminal domain, a hydrophobic core region with a minimum length of 7 amino acid residues, and a polar carboxyterminal region immediately adjacent to the signal peptidase cleavage site.[131] In a study of 39 signal peptides of prokaryotic and eukaryotic sources, lysine or arginine residues usually preceded the hydrophobic core and alanine residues were highly conserved in positions plus 4 and

plus 6 relative to the start site of the hydrophobic core. Signal peptides were between 9 and 14 amino acid residues long and were predicted to form either strong alpha helices or beta pleated sheets.[139] Amino acid residues 50 to 60 encoded by the BVDV gp48 cDNA had a highly positive charge with an average of 2.683. Lysine at amino acid residue 61 preceded the potential hydrophobic core region of 16 amino acid residues; alanine residues were present at positions plus 1, plus 4, plus 6, plus 9 and plus 10 relative to the core region. The amino acid composition of the core region encoded by the BVDV cDNA was 27% leucine, 23% alanine, 18% valine, 13% isoleucine, 10% tryptophan and 7.5% glutamine which is similar to other hydrophobic core regions of signal peptides.[139]

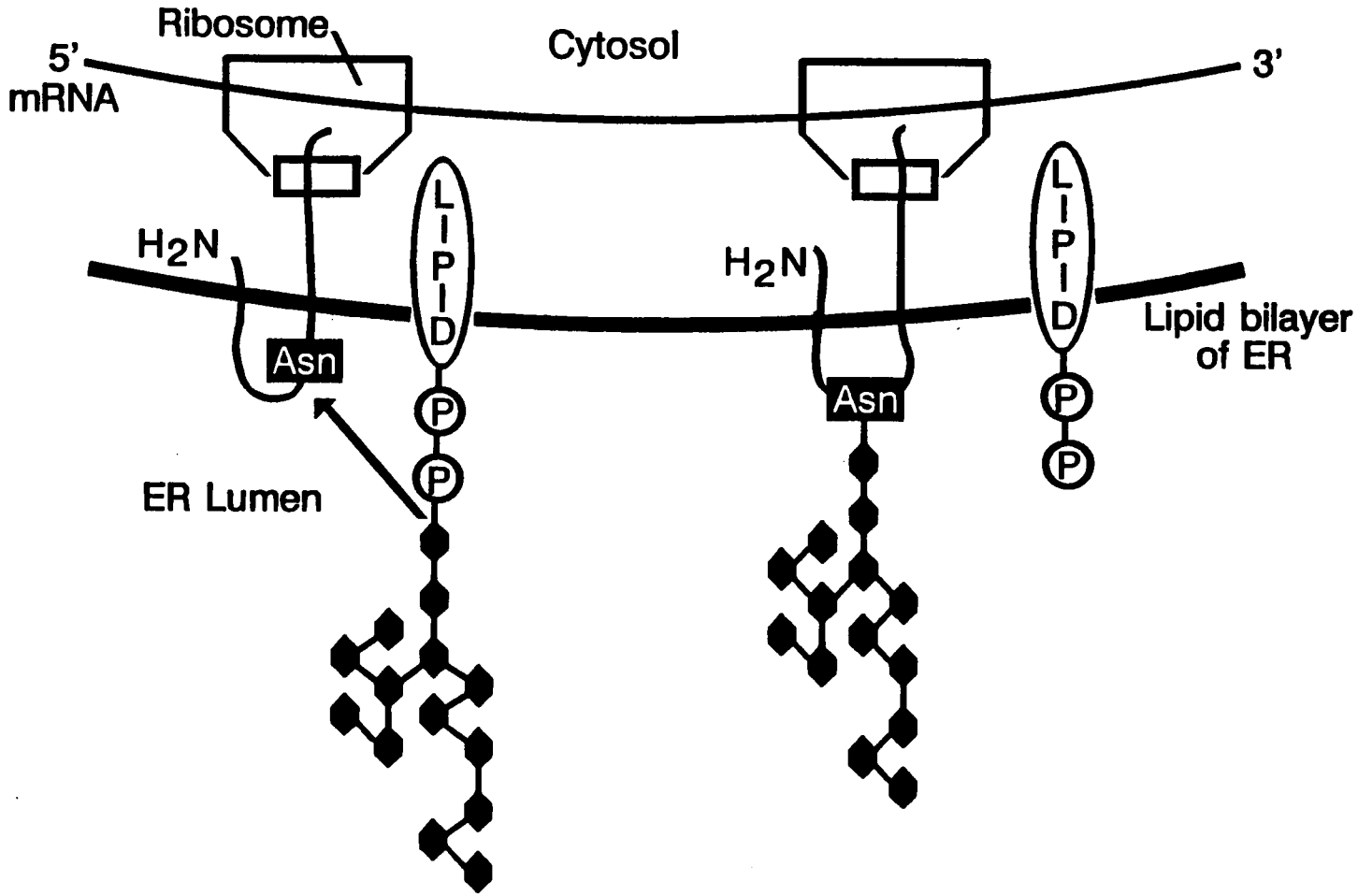
Usually the signal sequence is cleaved by signal peptidases in the ER and the aminoterminus of the translocated protein is released into the lumen. However, cleavage does not occur universally and some proteins are anchored in membranes by the signal peptide but leaving the translocated carboxyterminus of the protein free in the ER lumen. The first protein recognized as anchored at its aminoterminus was the neuraminidase molecule of influenza virus.[13] Aminoterminal anchorage of neuraminidase of various paramyxoviruses has been reported also.[14,47,74,85]

Van Hejne (1984, 1986)[195,196] studied extensively the characteristics of signal peptidase-specific cleavage sites. Cleavage specificity was maintained by conserved structural conformation of the region around the cleavage site. The "minus 3, minus 1" rule of cleavage site structure suggested by these authors stated that small neutral amino acid residues existed at the -1 and -3 positions and rarely in the -2 position.

Based on Van Hejne's principles, cleavage of the signal peptide encoded by the BVDV cDNA likely would occur between amino acid residues glycine at position 79 and glutamine at position 80. Glycine at amino acid residue 79 would be in position -1 and methionine and threonine in positions -2 and -3, respectively. The glycine at amino acid residue 79 at the putative cleavage site may interrupt the secondary structure of the protein and thus expose the cleavage site to the signal peptidase in the lumen of the endoplasmic reticulum.

Analysis of the remaining 227 amino acid residues encoded by the BVDV cDNA revealed 8 putative N-linked glycosylation sites (Figures 6.5, 6.6) consistent with the number of N-linked glycosylation sites predicted for gp48 of BVDV NADL. Glycosylation of proteins in the endoplasmic reticulum may occur co-translationally and prior to protein folding (Figure 7.1). A preformed precursor oligosaccharide with 14 sugar residues consisting of N-acetyl-glucosamine, mannose, and glucose is transferred from dolichol to every appropriate asparagine residue of the protein. Asparagine residues followed by any amino acid residue exept proline and then followed by serine or threonine residues are targets of N-linked glycosylation. Sugar transfer to target asparagine residues is mediated by an ER membrane-bound enzyme. Diversity of N-linked sugar structures occurs as a result of remodelling of the original precursor molecule in the ER and also the Golgi apparatus (reviewed by Hirschberg, 1987).[75] Three major types of asparagine-linked oligosaccharides are recognized; high mannose, complex and hybrid structures (reviewed by Kornfeld, 1985).[95]

Figure 7.1. Schematic representation of N-linked glycosylation of proteins translocating into the endoplasmic reticulum (ER) lumen. The sugar moiety is symbolized by solid hexagonal shapes. The membrane bound dolichol is denoted by "LIPID - P - P". H2N indicates the aminoterminus of the translocated protein.


Ribosome
Cytosol
5'
mRNA
3'
H_2N
LIPID
Asn
P
P
ER Lumen
H_2N
LIPID
Asn
P
P
Lipid bilayer
of ER

The putative BVDV 2724 gp48 protein also contained 9 cysteine residues (3.96% of total amino acids). The high cysteine content may allow a significant number of intramolecular or intermolecular disulfide bonds to occur in that polypeptide. Translocated and glycosylated proteins in the lumen of the ER fold as a result of intra- and intermolecular disulfide bonds between suitable cysteine residues. Folding into the conformation with the lowest free energy values, which is the most stable conformation, is facilitated by protein disulfide isomerase which cleaves unstable disulfide bonds.[55]

In vitro translation of RNA transcribed from the BVDV derived plasmid cDNA insert yielded a 30 kDa protein that was immunoprecipitated by BVDV polyclonal antisera, one of which was a field serum (Figure 6.12). The field serum precipitated BVDV 2724 specific polypeptides, including gp48 in labelled BVDV infected cell lysates (Figure 6.13). Serologic crossreactivity of antisera among different BVDV isolates is well documented.[62]

The putative signal sequence between amino acid residues 60 and 80 of the cDNA was functional when canine microsomal membranes were added to the translation reaction. Glycosylation of all 8 putative N-linked glycosylation sites likely occurred resulting in a product of approximately 46 kDa. This shift in molecular weight of approximately 16 kDa suggested an average molecular weight increase per glycosylation site of 2.6 kDa. The molecular weight shift during glycosylation was similar to data on gp48 of BVDV NADL.[28] Immunoprecipitation of BVDV NADL glycoproteins from infected cell lysates before and after endoglycosidase H and F

treatment resulted in a molecular weight shift of gp48 from 48 kDa to 27 kDa, consistent with removal of 8 N-glycan side chains, each with a molecular weight of 2.6 kD.[28] Beta-endo-N-acetylglucosaminidase H cleaves N-linked high mannose oligosaccharide side chains between the first and second N-acetyl-glucosamine. N-linked bi-antennary high mannose chains, bi-antennary hybrid chains and bi-antennary complex carbohydrate side chains are cleaved between the first and second N-acetylglucosamine by endoglycosidase F.

Other than the putative aminoterminal signal sequence, computer analysis of the region encoding gp48 did not identify any hydrophobic amino acid encoding sequence suggestive of a transmembrane region. Signal peptidases within microsomal vesicles cleaved the translocated portion of the 2724 cDNA translation product and gp48 was secreted into the lumen (Figure 6.15). The carboxyterminus of gp48 may be close to amino acid residue 520 of BVDV strain NADL considering the deglycosylated molecular weight (27 kDa) and an aminoterminus of gp48 at amino acid residue at position 79 of the genome. Analysis of the BVDV NADL amino acid sequence[28,29] revealed the next downstream hydrophobic region and putative transmembrane region encoded by the genome occurred between amino acid residues 552 and 570. However, if this region is included in gp48 and if membrane anchorage of gp48 occurred at this site, the molecular weight of non glycosylated gp48 would be 33 kDa and one more putative N-linked glycosylation site would be added suggesting its glycosylated molecular weight then would be 55/56 kDa. In addition, the glycoprotein encoded by the BVDV genome on the carboxyterminus side of gp48,

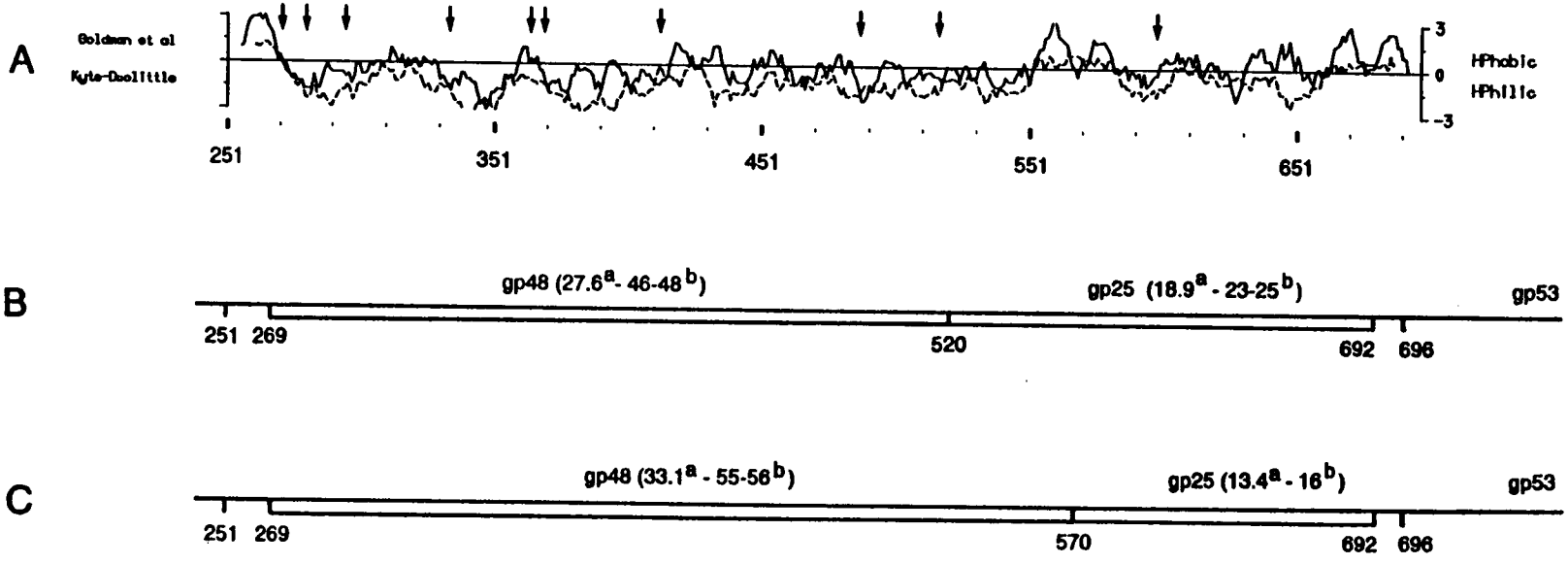
membrane glycoprotein (gp25), then only would be 122 amino acid residues long and have a calculated molecular weight of 13.5 kDa. Also gp25 would have one fewer N-linked glycosylation site and have an estimated glycosylated weight of 16 kDa instead of approximately 25 kDa. These calculations are based upon the hypothesis that the aminoterminus of gp53 is at or close to amino acid residue 692 of the BVDV genome.[93] The exact sizes of the BVDV structural proteins are not known, however, immunoprecipitations of gp48 and gp25 of several strains of BVDV have led to the conclusion that the molecular weights of the former is between 44 and 49 kDa and that of the latter is 23 to 26 kDa.[39,41,118,142,154] Therefore, the calculated molecular weight of gp48 of 55/56 kDa, if the potential transmembrane sequence downstream of the first potential anchor sequence is included in the protein as well as the calculated molecular weight of the consequently shortened gp25 (16 kDa) are not compatible with experimental findings of molecular weights of these BVDV glycoproteins.

Figure 7.2, panel A gives a hydrophobicity plot of BVDV NADL extending from leucine 251 to asparagine 696. Proposed bounderies of gp48, gp25 and gp53 and their respective molecular weights are indicated in panel B. Alternative cleavage sites and molecular weights of the resulting proteins, if the second putative transmembrane region at amino acids 552 to 570 encoded by BVDV is included in gp48 processing, are given in panel C.

Although gp48 likely is not bound to a membrane by a protein anchor, other types of membrane anchorage by this protein need to be considered. The major

Figure 7.2. Molecular weight changes of BVDV NADL gp48 and gp25 proteins, if gp48 would include the next putative transmembrane region between amino acid residues at position 551 and position 525. Hydrophobicity plot of proteins encoded by BVDV NADL genome between amino acid residues at position 251 and postion 701. Note the hydrophobic regions between amino acid residues at positions 251 to 292 and amino acid residues at positions 551 to 571. Arrows above the graph indicate potential N-linked glycosylation sites (A). Gp48 and gp25 encoding regions (Collett 1988) (B). Gp48 including the next putative transmembrane sequence and the small gp25 (C). Open boxes indicate protein regions. Numbers below the genome indicate positions of putative amino- and carboxyterminal amino acid residues of respective proteins on the genome.

BVDV NADL



[a] non-glycosylated molecular weight in Kilodaltons

[b] glycosylated molecular weight in Kilodaltons

envelope protein of retroviruses is linked to an intramembranous protein anchor by disulfide bonds.[98] Figure 7.3A illustrates possible attachment of gp48 to the virion surface by disulfide linkage to the major envelope protein (gp53) or to the intramembranous glycoprotein (gp25). Heterodimers of gp48 with gp53 or gp25 have not been detected.[32,39-41,154,205] Conceivably, weak disulfide bonds between gp48 and gp53 or gp25 could break during preparation of cell lysates or during the immunoprecipitation procedures. Homodimers of hog cholera virus gp44/48 may exist.[193] The same authors also speculated that gp48 may be a "surface protein" of BVDV.

Another mechanism of glycoprotein membrane attachment is direct linkage to the phospholipid bilayer by either covalent acylation or by glycosyl-phosphatidylinositol (GPI) structures (reviewed by Schmidt).[183,184] Envelope proteins of many enveloped RNA viruses are acylated. Usually, however, proteins with large intramembranous segments were covalently linked to fatty acids. Precursor proteins of both envelope proteins of Semliki forest virus and Sindbis virus were acylated postranslationally and prior to cleavage. The exact acylation sites of viral glycoproteins were probably hydroxyamino acids close to the membrane-spanning fragment. It was proposed that protein acylation contributes to membrane anchorage. Other putative roles of acylation were involvement in virus to cell fusion during infection and also in transport of viral proteins from the Golgi (site of acylation) to the cell surface (Schmidt, 1989).[184]

GPI-anchorage of glycoproteins has been reviewed by Ferguson (1988)[54] and

Figure 7.3. Proposed mechanisms of attachment of gp48 to the bovine viral diarrhea virion. A. Gp48 is attached to gp53 and gp25 by disulfide bonds.

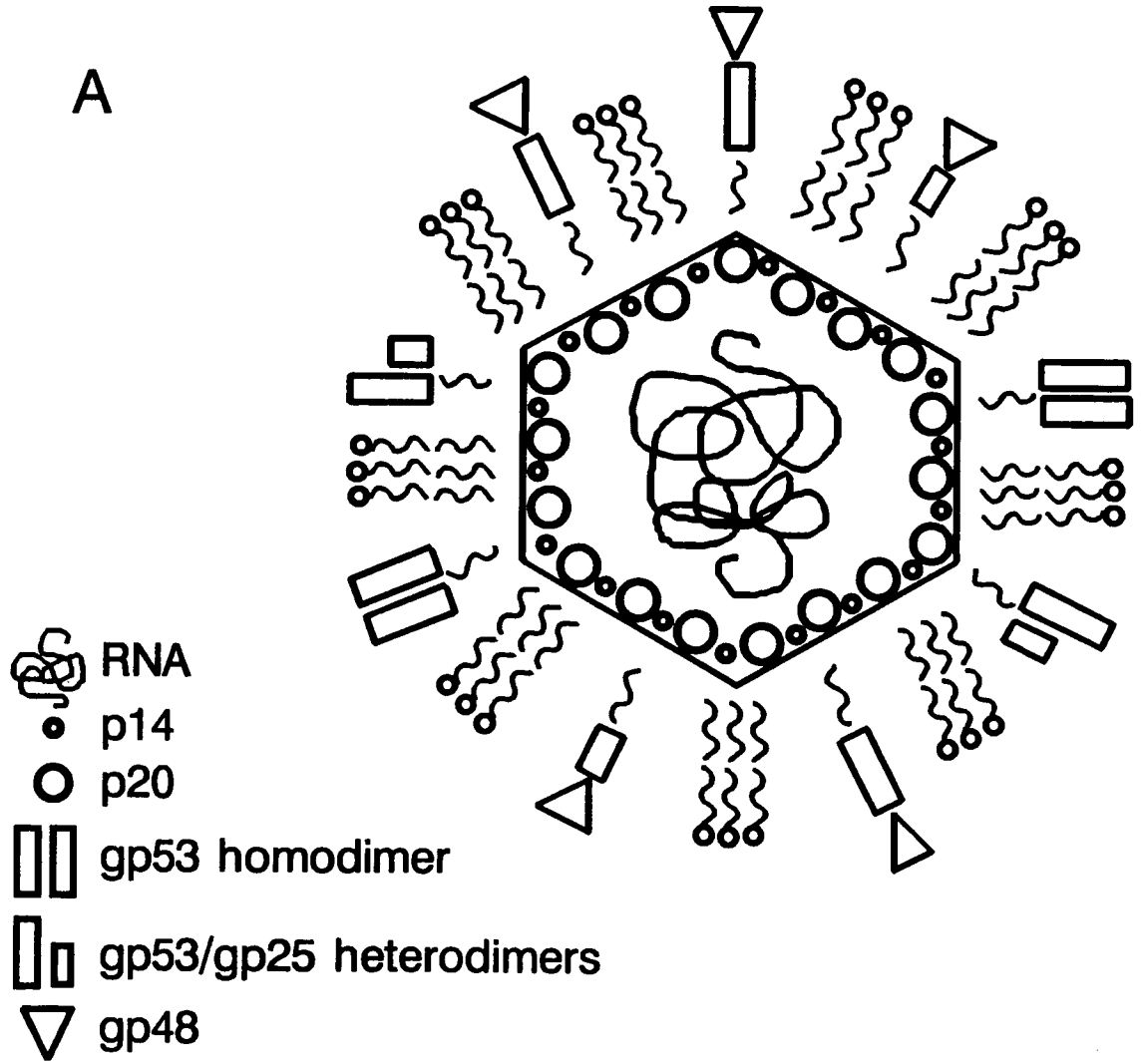

A
RNA
p14
p20
gp53 homodimer
gp53/gp25 heterodimers
gp48

Figure 7.3 (continued). Proposed mechanisms of attachment of gp48 to the bovine viral diarrhea virion. B. Gp48 is directly attached to the lipid bilayer of the envelope by acylation.

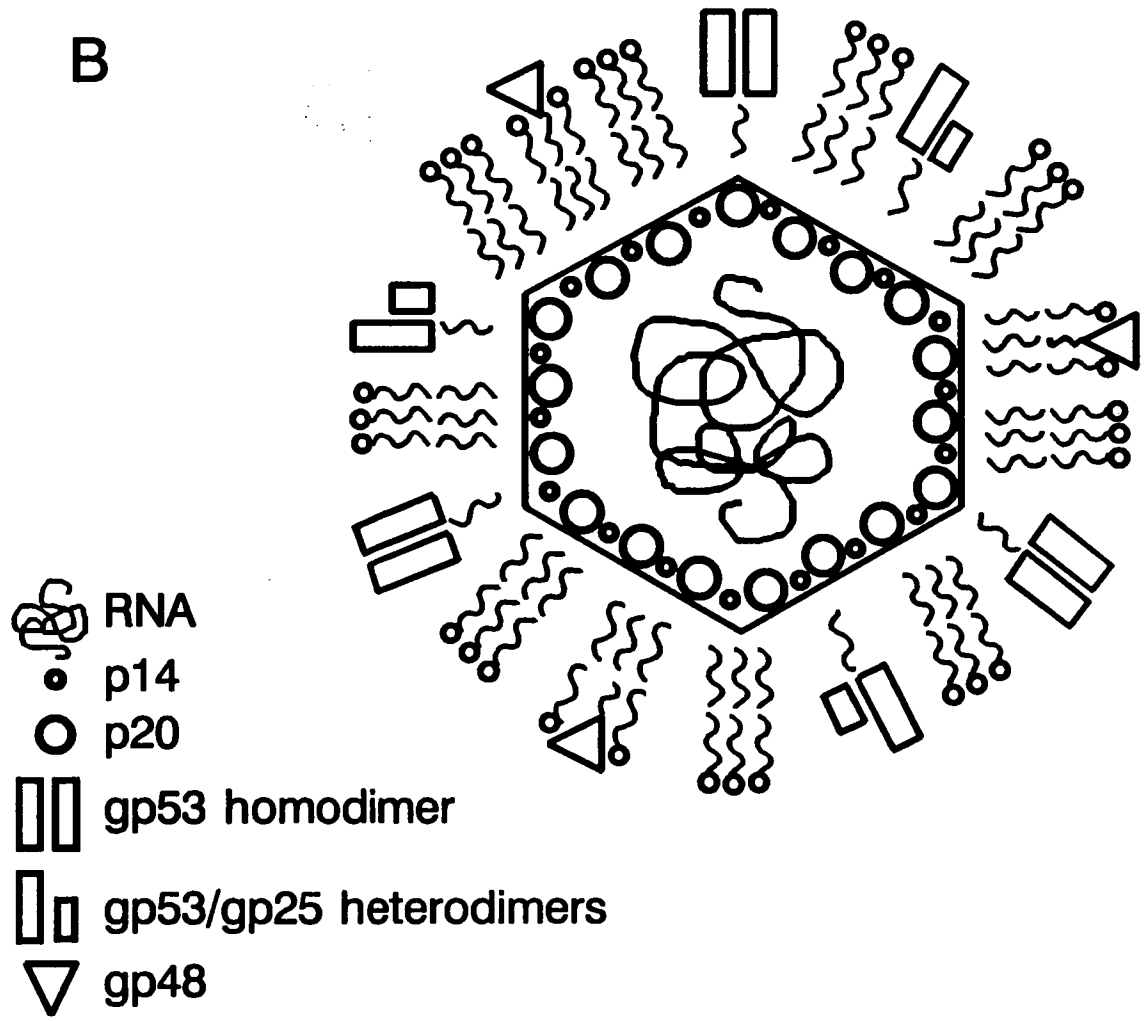
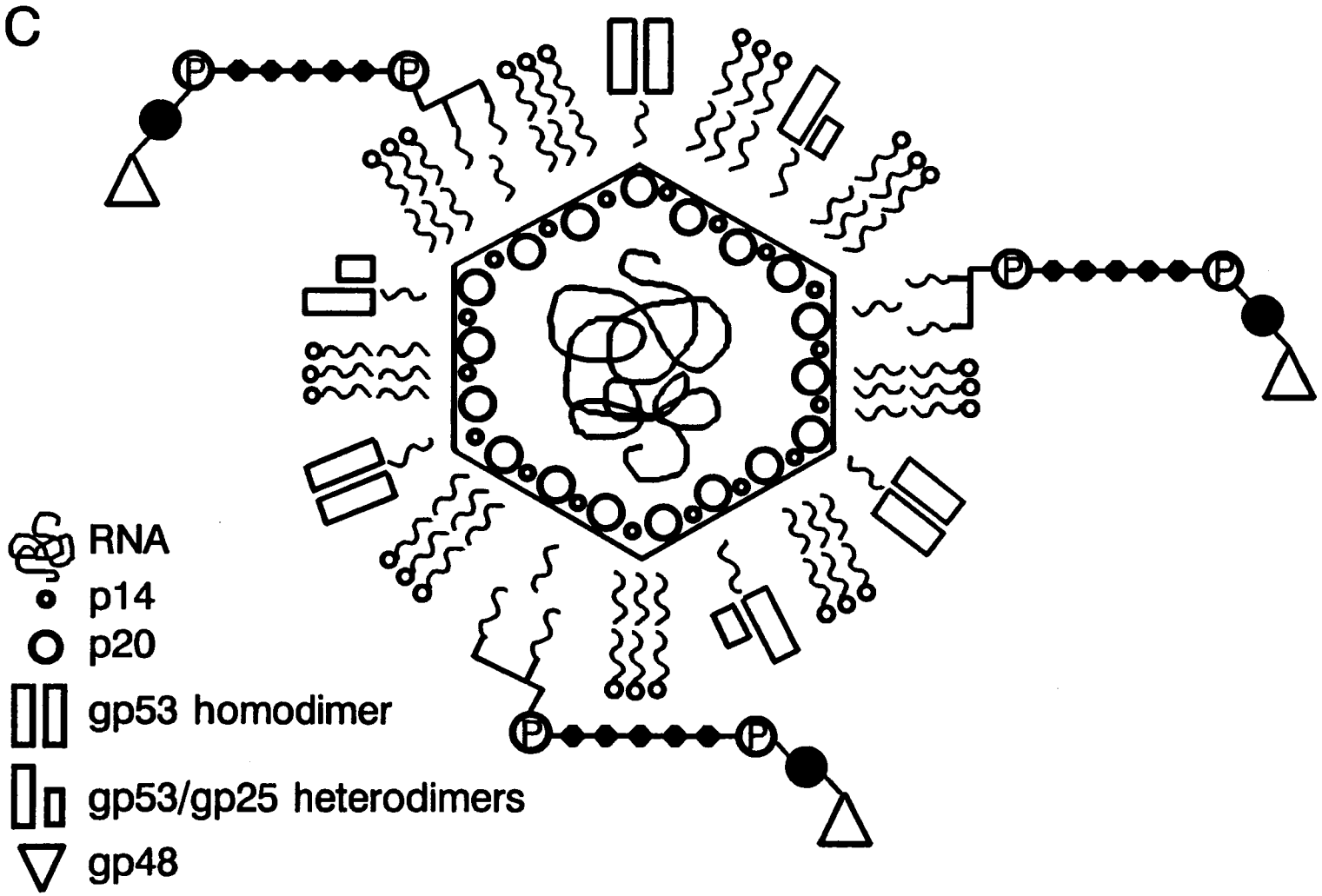

B
RNA
p14
p20
gp53 homodimer
gp53/gp25 heterodimers
gp48

Figure 7.3 (continued) Proposed mechanisms of attachment of gp48 to the bovine viral diarrhea virion. C. Gp48 is attached to the lipid bilayer of the envelope by a phosphatidyl-inositol anchor (PTI).


C
P
P
P
P
P
P
RNA
p14
p20
gp53 homodimer
gp53/gp25 heterodimers
gp48

Low (1989).[105] Membrane-bound enzymes such as alkaline phosphatase, acetyl-cholinesterase and 5'-nucleotidase; variant surface glycoproteins of *Trypanosoma brucei*, eukaryotic adhesion molecules; the Scrapie prion protein and *Schistosoma mansoni* antigen are bound to cell surfaces by a GPI-anchor. The GPI anchor was attached at the carboxyterminus of glycoproteins through ethanolamine. GPI-anchorage of viral proteins has not been reported. Figures 7.3B and 7.3C illustrate putative anchorage of gp48 to the virion by direct acylation and GPI-anchorage, respectively.

Speculations about functions of pestiviral proteins often were based upon comparison of genomic location of pestiviral polypeptides to the genomic location of respective flaviviral polypeptides (Table 2.2). Flaviviruses have been under intensive study as several are major human pathogens such as yellow fever virus, dengue virus and numerous other important viruses worldwide. Members of the flavivirus genus are arboviruses and are transmitted by insects.[165] The flavivirus genome consists of a single strand RNA, approximately 11 kilobases long with positive orientation.[14,203] The viral RNA has a 5' cap and is not polyadenylated at its 3' end.[14] The genome has one large open reading frame encoding structural proteins at the 5' end and nonstructural proteins at the 3' end. The RNA is translated into a polyprotein during viral replication. The large precursor molecule is co-translationally and post-translationally cleaved into the mature viral proteins.[203] The polyprotein contains three structural viral proteins consisting of the aminoterminal capsid protein (C) followed by the membrane protein (M) and envelope glycoprotein (E). Non-

structural proteins NS3 and NS5 facilitate RNA replication.[166] A negative strand RNA template is produced early during replication followed by transcription of the positive strand genome. The full length genomic RNA also functions as messenger RNA in translation of the viral polyprotein precursor.[14]

Figure 7.4 gives the genomic organisation and precursor to protein relationships of flaviviruses. Table 7.1 lists functions of flaviviral proteins. Table 7.2 gives comparison between flaviviral and pestiviral proteins according to hydrophylicity and sequence similarities.[27] Sequence similarities are limited to segments of pestiviral nonstructural proteins p125 and p133 and the analagous flaviviral proteins, NS3 and NS5, respectively.[27]

The structural protein precursor of West Nile Virus consisted of a stretch of basic amino acids residues (C protein), followed by 14 hydrophobic amino acid residues (signal peptide), a 166 amino acid molecule for the membrane protein (pre-M) and 496 amino acid envelope protein.[132] An identical genomic order encoding structural proteins was identified in yellow fever virus, dengue 2 virus, Kunjin virus and other flaviviruses studied.[10,165] The capsid protein (C) of West Nile virus was membrane anchored at its carboxyterminus early in the replication cycle.[132] The signal sequence following the C protein of dengue virus mediated translocation of pre-M and E proteins into microsomal membranes during *in vitro* expression of viral RNA.[112] The same authors also presented data that cleavage occurred between the signal peptide and pre-M and between pre-M and E proteins by signalases in the endoplasmic reticulum. Signal peptidases cleaved the West Nile virus precursor of

Figure 7.4. Predicted precursor to product relationships of proteins encoded by the flavivirus genome. Structural proteins C, M, E and nonstructural proteins nonM, NS1, ns2b, NS3, ns4a, ns4b and NS5 are indicated. Note that pre-M post-translationally is cleaved to non-structural nonM and structural M protein.

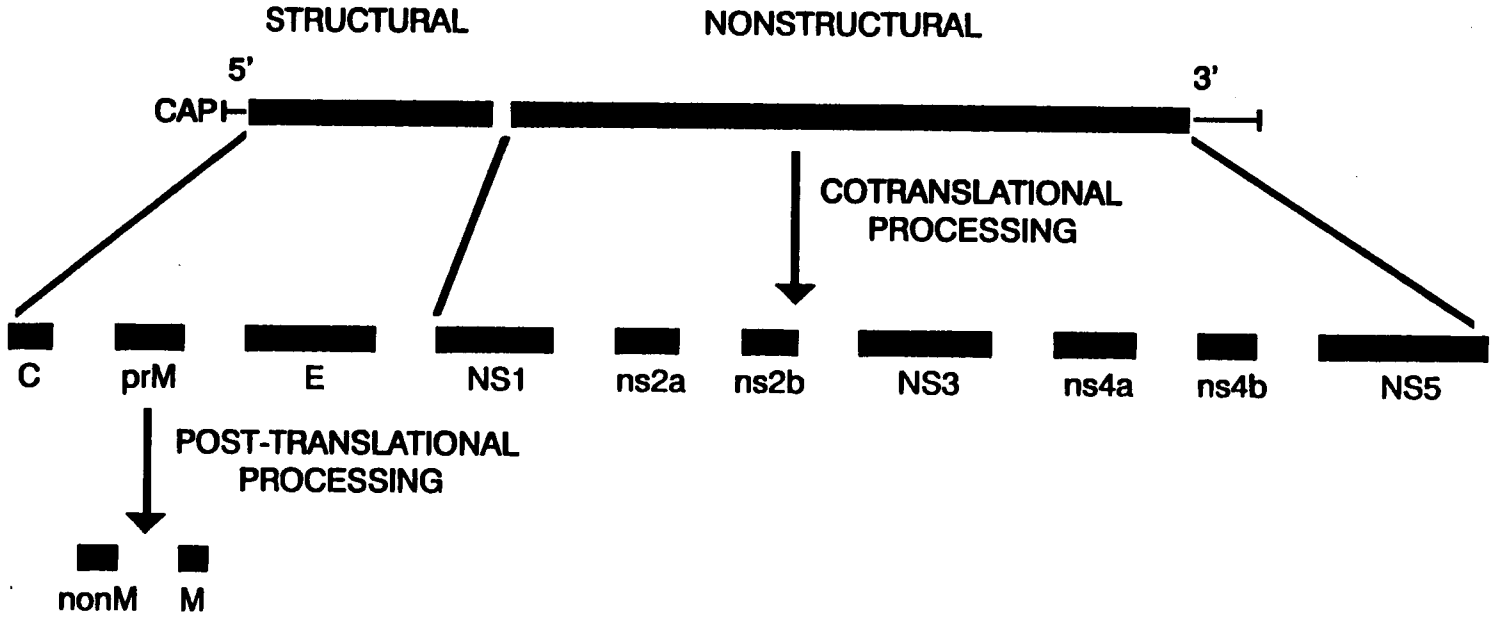

STRUCTURAL
NONSTRUCTURAL
5'
CAP
3'
COTRANSLATIONAL
PROCESSING
C
prM
E
NS1
ns2a
ns2b
NS3
ns4a
ns4b
NS5
POST-TRANSLATIONAL
PROCESSING
nonM
M

Table 7.1. Flavivirus specific polypeptides and their suggested function[a]

Protein	Function
C	Nucleocapsid protein
prM	Precursor to M protein
non M	Secreted portion of M protein
M	Membrane protein
E	Major virion envelope protein
NS1	Soluble complement-fixing antigen
ns2a	Hydrophobic protein, function unknown
ns2B	Hydrophobic protein, function unknown
NS3	Replicase component?
ns4a	Hydrophobic protein, function unknown
ns4b	Hydrophobics protein, function unknown
NS5	Replicase component?

[a]Rice et al. 1986.

Table 7.2. Comparison of pestivirus and flavivirus specific polypeptides[a]

Pestivirus	Flavivirus
p20	C + pre-M
gp62 (gp48 + gp25)	E
gp53	NS1
(30 K)	ns2a
p125 (p54 + p80)	n2b + NS3 + ns4a + ns4b
(35 K) + p133 (p58)	NS5
p133 (p75)	

[a]Collett, 1988.

C, pre-M and E proteins into mature proteins during *in vitro* expression.[132] Pre-M protein of all flaviviruses studied was cleaved during viral maturation into an aminoterminal segment, not associated with the mature virion (nonM), and carboxyterminal segment, the membrane protein (M).[22,166,185,203] The surface of cell-associated West Nile virions consisted of E + pre-M heterodimers which were destroyed and reorganized by proteolytic cleavage into individual E and M proteins during virion release.[198] However, some pre-M molecules remained intact. The pre-M cleavage occurred late in the replication cycle and was inhibited by acidotropic amines in infected cells suggesting that proteolytic cleavage occurred in the acidic post-Golgi vesicles.[160] Virions containing pre-M protein also had a low specific infectivity rate.[160] The peptide region of nonM adjacent to the cleavage site between nonM and M is highly conserved among flaviviruses and contains five cysteine residues. In addition, a conserved cysteine linked to tryptophane in this region could be an active site of a thiol protease. The same author suggested, that the carboxyterminal portion of nonM could participate in proteolytic cleavage of the viral polyprotein precursor. Little is known about the immune responses elicited by nonM protein. However, neutralizing antibodies against V1 (pre-M, M) have been identified, but it is not known whether the antibodies bound to the aminoterminal secreted segment or the carboxyterminal membrane bound portion of pre-M protein.[191] Specific dengue pre-M protein monoclonal antibodies protected mice against subsequent challenge inoculation with wild type Dengue. V1 (pre-M) isolated from Banzivirus virions added to mosquito cells prevented infection with wild type

Banzivirus.[160] The author speculated V1 was important in attachment of virions to mosquito cells. E protein is the flaviviral envelope glycoprotein which elicites neutralizing antibodies and induces hemagglutination.[166] Glycoprotein NS1 is the first nonstructural protein encoded by the flavivirus genome. Antibodies against NS1 induced complement-mediated lysis of infected cells.[165] Immunity against viral infections is dependent on antibodies to the envelope protein and also NS1 protein.[172]

Table 6.1 gives nucleotide and amino acid sequence comparisons between the 2724 cDNA or encoded amino acids and respective sequences of BVDV NADL and BVDV OSLOSS, HCV Alfort, HCV Brescia, yellow fever virus, dengue type 2 virus, Kunjin virus and hepatitis C virus. In addition, putative N-linked glycosylation sites and conserved cysteine residues are identified. Comparison of the BVDV 2724 gp48 cDNA and its encoded protein to other pestiviruses revealed a high degree of homology in nucleic acid sequence, amino acid sequence and glycosylation sites. However this degree of homology did not extent to either the pre-M and envelope proteins of flaviviruses and or to the envelope protein of hepatitis C virus (Table 6.1). Hepatitis C virus is not classified as a pestivirus, although certain of its properties resemble those of the latter.[164] Figure 7.5, panels A and B gives acidic and basic amino acid residue composition as well as hydrophobicity plots of 2724 gp48 and the aminoterminal (secreted) portion of flaviviral pre-membrane protein (nonM). No significant relationship between BVDV gp48 and flaviviral nonM was identified, but functional similarities cannot be excluded. The relationship of pestiviral and analagous flaviviral structural proteins are compared in Table 7.3.

Figure 7.5. Comparisons of distribution of acidic and basic amino acid residues (A) and hydrophobicity plots (B) of BVDV 2724 gp48 and the secreted portion (nonM) of pre-M of yellow fever, dengue and kunjin virus.

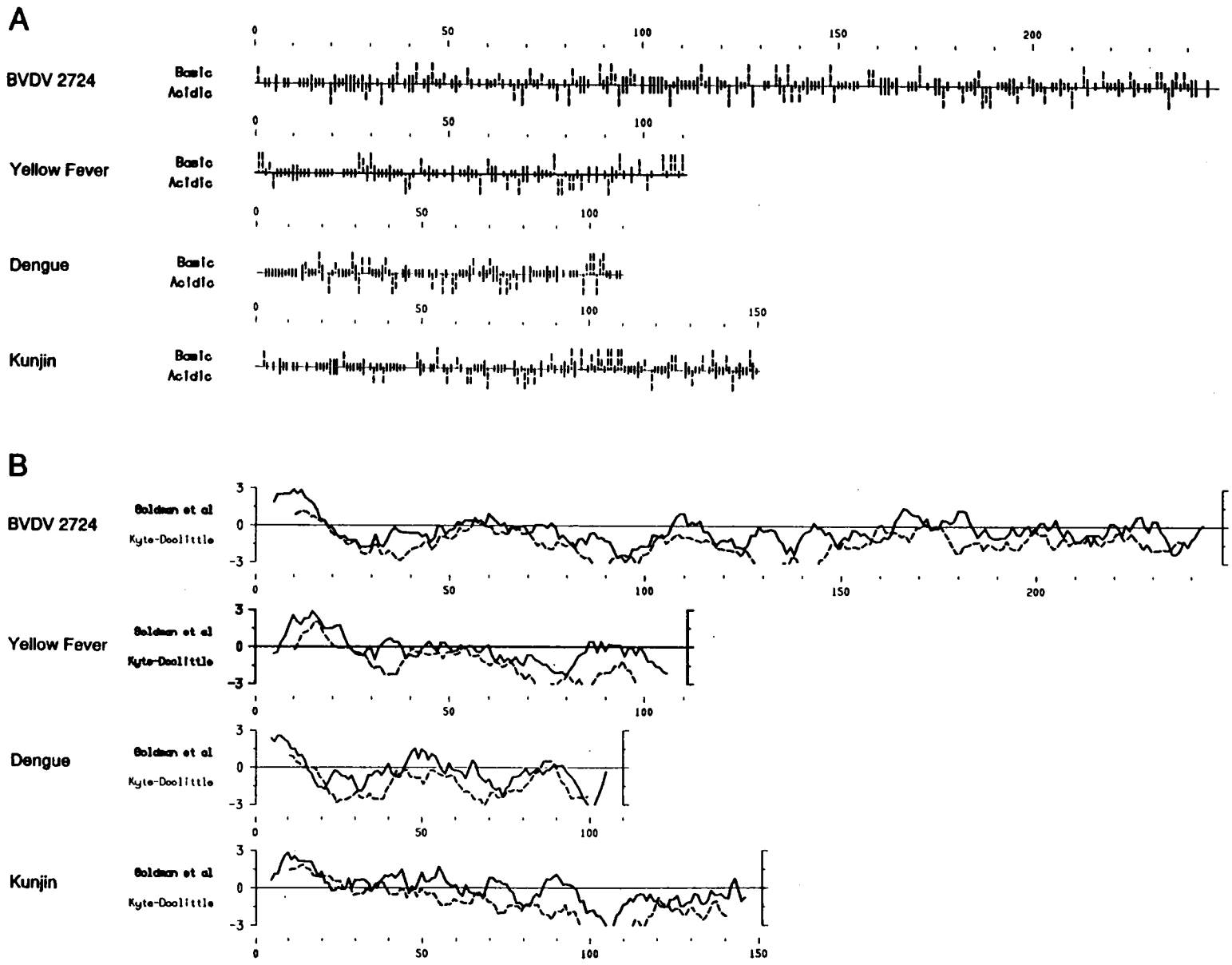

A
BVDV 2724
Basic
Acidic
Yellow Fever
Basic
Acidic
Dengue
Basic
Acidic
Kunjin
Basic
Acidic
B
BVDV 2724
Goldman et al
Kyte-Doolittle
Yellow Fever
Goldman et al
Kyte-Doolittle
Dengue
Goldman et al
Kyte-Doolittle
Kunjin
Goldman et al
Kyte-Doolittle

Table 7.3. Comparison of structural proteins of pestivirus and flavivirus polypeptides according to function.

Pestivirus	Flavivirus
p20	
C14	C (capsid)
gp62: gp48	prM: secreted portion (non M)
gp25	M (membrane)
gp53	E (envelope)

According to one research group, a recently identified pestiviral protein, p14 may be a capsid protein.[193] The putative signal peptide of BVDV 2724 on the carboxyterminal side of putative p14 and aminoterminal to gp48 was functional, mediated translocation of gp48 and was cleaved by signal peptidases consistent with characteristics of the flavivirus signal peptide. Pestiviral gp62, the precursor of gp48 and gp25, has a hydrophobic amino acid segment at its carboxyterminus which, after maturation, becomes gp25 and is analogous to the flaviviral M protein. The aminoterminal portion of gp62, gp48 likely corresponds to the nonM flavivirus protein. Like flaviviral nonM, it is the first glycosylated product encoded by the genome and does not contain a protein membrane anchor.

BVDV 2724 gp48 had 9 cysteine residues, all of which were strictly conserved among pestiviral isolates. Antibodies to BVDV gp48 and HCV gp44/48 develop in convalescent animals. A neutralizing monoclonal antibody to BVDV gp48 has been produced. In addition, this monoclonal antibody consistently reacted with all pestiviral isolates tested (Donis, per. comm.). We do not know at which point of viral replication cleavage of BVDV gp62 into gp48 and gp25 occurs or if gp62 occurs on the virion surface. The role of gp48 or gp25 in viral infections is unclear. Gp25, if functionally analogous to flaviviral M protein, might be involved in viral attachment to host cells and membrane fusion. BVDV gp53 (HCV gp55) may be the major envelope protein and induces neutralizing antibodies in infected animals and likely is analagous to flavivirus envelope protein E. The flaviviral envelope protein

E has a hydrophobic amino acid segment at its carboxyterminus that may anchor the protein in the membrane in a manner reminiscent of BVDV 2724 gp53.[93]

In conclusion, cDNA representing nucleotides 959 to 1880 of noncytopathogenic BVDV 2724 RNA was synthesized and cloned. It encoded the first signal peptide present in the BVDV polyprotein and at least the major portion of gp48. *In vitro* expression resulted in production of a serologically functional protein. The signal peptide mediated translocation of gp48 when microsomal membranes were added. In the presence of the latter, gp48 was glycosylated and secreted. Pestiviruses are clearly distinct from flaviviruses based on gp48 genomic sequence comparisons. However, both virus groups have similar genomic organization and mechanism of viral replication. Functional homologies of pestiviral and flaviviral polypeptides likely exist. Our experimental evidence suggests the functions of BVDV genomic sequences encoding the putative viral signal peptide and gp48 are similar to the flaviviral signal peptide and flaviviral nonM.

Future Research

Several questions have to be adressed in order to determine conclusively what the function of BVDV gp48 is. The existence of disulfide linkages between gp48 and gp53 or gp25 and acylation of gp48 should be experimentally assessed. The exact amino- and carboxytermini of native gp48 should be determined. In addition, presence or absence of gp48 in virions will ultimately disclose if the glycoprotein is

a structural of non structural viral protein. But specific gp48 antibodies are essential in order to purify native gp48.

In vitro expression studies may determine whether disulfide linkages between gp48 and viral glycoproteins gp25 or gp53 exist. BVDV 2724 gp53 has been cloned and expressed *in vitro*.[93] Expression in the presence of microsomal membranes of gp48 RNA and gp53 RNA, simultanously followed by SDS-PAGE without protein denaturation could suggest whether heterodimers between the two proteins exist. A similar experiment should be done with gp25. BVDV 2724 gp25 cDNA has not been cloned yet, but, experiments to clone this are planned.

Membrane linkage of gp48 by acylation may be assessed by addition of a radioisotope labelled fatty acid, usually 3H-palmitic acid or 3H-myristic acid, to virus-infected cell cultures followed by cell lysis and immunoprecipitation of viral polypeptides. Labelled viral proteins could be detected by SDS-PAGE and radiography of immunoprecipitates.

A priority should be given to production of enough recombinant gp48 to raise antibodies against this glycoprotein in experimental animals. An appropriate expression system may be the baculovirus vector. The hemagglutinin gene of influenza virus and envelope glycoprotein gene of Rift Valley fever virus have been expressed in this system.[144,182] Expressed proteins were similar antigenically to the wild type and could not be distinguished from the latter by gel electrophoresis analysis. The envelope glycoprotein of Japanese encephalitis virus and structural glycoproteins of dengue-2 virus were expressed by baculovirus and induced protective

immunitiy in laboratory animals.[35,209] An experiment to try is insertion of the gp48 cDNA into an appropriate vector, followed by transfer of the cDNA into baculovirus by recombination and then infect insect cells.[189] Baculovirus infected insect cells should produce large amounts of pure glycosylated protein. Secreted proteins can be harvested from the cell culture supernatant and used to immunize rabbits.

An alternative method of obtaining anti-gp48 antibodies entails design and production of a synthetic peptide consisting of highly antigenic domains of the protein encoded by the cDNA. Regions with high antigenicity were determined by computer analysis and were between amino acids 130 to 170 and 200 to 230 (Figure 6.6). The antigenicity index is based upon hydrophylicity and secondary structure and was calculated according to Jameson and Wolf (1988).[36] Synthesized peptides then may be used to immunize rabbits. Synthetic peptides derived from the deduced sequence of the E-glycoprotein of Murray Valley encephalitis virus induced E protein antibodies in mice. One peptide elicited low-level neutralizing antibody.[173]

Antibodies against gp48 will allow purification of native viral protein from infected cell cultures by affinity chromatography and thereafter allow determination of amino acid sequences of the amino- and carboxyterminus. The presence of gp48 or gp62 on the virion could be confirmed by detection of virion associated gp48 in purified viral pellets by immunogold labelling. A precedent to this approach exists. Immunogold electron microscopy with antibodies elicited by a synthetic peptide representing the surface portion of matrix 2 (M2) protein of influenza confirmed the presence of the protein in virions.[82] Gp48 in virus pellets obtained from infected cell

cultures would be labelled by gp48 antibody and then bound to a secondary antibody conjugated to colloidal gold. Labelled pellets would either be fixed in glutaraldehyde, enbedded in agar and processed for transmission electron microscopy or viewed directly as described by Jackson (1991).[82] Membrane bound gp48 would be associated with the viral envelope.

The capacity of gp48 to elicit an immune response may be evaluatd in part by testing the neutralizing activity of anti-gp48 serum against BVDV 2724. Cross-reactivity with other pestiviruses may be examined and reveal information about conserved epitopes of gp48.

Structural proteins derived from a noncytopathogenic BVDV strain may be useful in a recombinant vaccine. Each structural protein should be assessed separately and in various combinations for capacity to induce protective immunity in the natural host. Dengue virus premembrane and membrane proteins elicited a protective immune response in mice immunized with recombinant vaccinia virus that expressed these dengue proteins.[19] The authors reported also that pre-M protein in combination with E expressed by the same vector resulted in complete protection of immunized mice. Recombinant vaccinia virus with sequences encoding HCV Alfort structural proteins protected swine against wild type virus challenge. If part of the envelope gene was deleted from the recombinant, partial immunity occurred.[180] Recombinant pseudorabies virus with sequences encoding the envelope protein of HCV Alfort protected swine against pseudorabies as well as hog cholera.[210] Sequences encoding gp48 and gp53 could be inserted into suitable vectors such as

vaccinia virus or pseudorabies virus for delivery of the immunogen. Multivalent vaccine vectors expressing several different immunogenic proteins will provide the tool to efficent vaccination of animals.

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VITA

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