BOVINE HERPESVIRUS MAJOR GLYCOPROTEINS: I. ANTIGENIC DIFFERENCES OF gB, gC, AND gD BETWEEN BHV-1 AND BHV-5 II. MOLECULAR CLONING AND SEQUENCING OF BHV-5 gD GENE III. FINE MAPPING OF LINEAR NEUTRALIZING EPITOPES ON BHV-1 gD

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ABSTRACT

The BHV-5 glycoprotein D (gD) gene was cloned, sequenced and used as variant sequence to identify two highly neutralizing epitopes on the BHV-1 gD. The BHV-1-specific monoclonal antibodies (MAbs) 3402 and R54 were tested against overlapping TrpE-gD fusion proteins expressed from a series of BHV-1 gD ORF subfragments. The reactivity of the MAbs with the expressed fragments on western blots located the epitopes between amino acid 52-115 and amino acid 165-216 for 3402 and R54 MAb respectively. The precise amino acid sequences specifying the epitopes were identified on the two fragments by amino acid sequence analysis and comparison with the corresponding regions of the variant BHV-5 gD sequences. A few amino acid changes occurred in these regions of BHV-5 gD have resulted in loss of MAb reactivity with the BHV-5 gD on western blots. Based on antigenic analysis of these regions, peptides covering amino acid 92-106 (3402 epitope) and 202-213 (R54 epitope) were synthesized and examined for their epitope activity. Each peptide reduced the neutralizing ability of the corresponding MAb in a dose-dependant manner. Antisera produced in rabbits to KLH-conjugated peptides recognized the parent gD on western blot, but had low neutralizing antibody titers against BHV-1. Bovine convalescent sera with high neutralizing antibody titers against BHV-1 reacted with bacterially-expressed protein containing both of the epitopes suggesting that these neutralizing linear epitopes are important in inducing immunity to BHV-1 during natural infections.

The BHV-5 (TX89 strain) gD encoding gene was mapped to BamHI-C genomic fragment between map units 0.887-0.897. The gene encodes a predicted protein of 417 amino acids with characteristics typical of a glycoprotein. Comparison of the BHV-5 gD and BHV-1 gD predicted amino acid sequences revealed significant homology in the amino-terminal two-third of the molecule. All seven cysteine residues characteristic of alphaherpesvirus gD homologues are identically aligned between the two gD molecules. The carboxy-terminal one-third is highly variable and contains several significant insertion and deletions. These differences may be important in the differential pathogenesis of respiratory disease and neurological disease by BHV-1 and BHV-5 respectively.

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INTRODUCTION

INTRODUCTION

Bovine herpesvirus-1 is responsible for a variety of disease conditions in cattle including respiratory infections, conjunctivitis, vulvovaginitis, abortions, encephalitis and generalized systemic infection (Gibbs and Rweyemamu, 1977). BHV-1 strains responsible for these disease conditions were previously divided into three groups based on their DNA restriction profiles and pathogenic properties: the respiratory/abortigenic (IBR, BHV-1.1), the non-abortigenic genital (IPV, BHV-1.2), and the neurovirulent (BHV-1.3). The BHV-1.3 strains have genome characteristics (Brake and Studdert, 1985; Engles et al., 1986; Studdert, 1989; Bulach and Studdert, 1990) and antigenic properties (Metzler et al., 1986; Friedli and Metzler, 1987) which has lead to their classification as a separate group of viruses designated BHV-5 (Roizman, 1992).

Recovery from most herpesvirus infections, including BHV-1 is mediated by humoral as well as cellular immune responses (Rouse and Babiuk, 1978). As in other alphaherpesviruses, BHV-1 glycoproteins are the major structural components of the viral envelope and virus-infected cells. The glycoproteins play an important role in virus-cell interactions, including recognition, attachment, and penetration of herpesvirus into susceptible cells (Little et al., 1981; Highlander et al., 1987; Johnson and Ligas, 1988), viral neutralization (Glorioso et al., 1984; Lupton and Reed, 1980) and immune destruction of infected cells. Four major glycoproteins, gB (gI), gC (gIII), gD (gIV), and gE (gII), have been identified on the virus envelope and the plasma membranes of BHV-1-infected cells (Marshal et al., 1986; van Drunen Little-van den Hurk et al., 1984; van Drunen Little-van den Hurk and Babiuk, 1986). The four glycoproteins are recognized by sera from BHV-1 infected cattle (Collins et al., 1984; van Drunen Little-van den Hurk et al., 1986). Immunization with affinity-purified gB, gC, and gD individually or in combination induced protective immune response against BHV-1 infection (Babiuk et al., 1987; van Drunen Little-van den Hurk et al., 1990b). However, gD is the only glycoprotein responsible for a significant reduction in viral replication and shedding (van Drunen Little-van den Hurk et al., 1993). Glycoprotein D also induces stronger and more consistent cellular immune

response to BHV-1 than gB and gC (Hutchings et al., 1990). Monoclonal antibodies (MAbs) to gD possess the highest complement-independent neutralizing activity and have been shown to inhibit adsorption and penetration (Hughes et al., 1988; van Drunen Little-van den Hurk et al., 1990a; Dubuisson et al., 1992). These functions have been ascribed to gD which makes it one of the most important proteins of BHV-1 and an ideal candidate as a subunit vaccine.

Glycoprotein D sequence data showed the BHV-1 gD ORF to code for 417 amino acid residues (Tikoo et al., 1990) which give a molecular weight of 75K. Competitive binding assays using gD specific MAbs have been used by two separate groups of investigators to map epitopes on the gD molecule. Five epitopes, three interrelated and two independent, were reported by one group as targets of neutralizing antibodies (Marshal et al., 1988). Four antigenic domains on the protein which include conformational-dependent and conformational-independent epitopes were described by the other group (Hughes et al., 1988; van Drunen Little-van den Hurk et al., 1990a). A recent study using truncated and deleted gD-recombinant vaccinia virus proteins have located the broad amino acid regions containing these antigenic sites (Tikoo et al., 1993). The objectives of this study were:

i) Determine the antigenic relationship of the major envelope glycoproteins gB, gC, and gD between the BHV-1 and BHV-5 using MAbs and immunoassays.

ii) Identify the precise coding sequences of linear neutralizing epitopes on BHV-1 gD by using:

a) Expression of recombinant TrpE-gD fusion proteins in E.coli and testing their reactivity with type (BHV-1)-specific MAbs to locate the broad amino acid regions containing the epitopes.

b) Antigenic analyses and comparison of these amino acid regions with the corresponding regions of BHV-5 gD as a variant sequence to predict the potential precise sequences specifying these epitopes.

c) Synthesis of peptides covering these amino acid regions and evaluation of their epitope activity.

iii) Determine the structural differences between BHV-5 and BHV-1 gD genes. This

objective is achieved by:

a) Molecular cloning and sequencing of the BHV-5 gD gene.

b) Comparison and analysis of the predicted amino acid sequences between BHV-5 gD and BHV-1 gD.

LITERATURE REVIEW

LITERATURE REVIEW

General Characteristics of the Virus and Disease Conditions

Bovine herpesvirus-1 (BHV-1) is classified as a member of the family Herpesviridae and of the subfamily alphaherpesvirinae (Roizman et al., 1981). This classification is based on biological properties which include variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and the capacity to establish latent infection in sensory ganglia. The virus has a typical herpesvirus structure (Ludwig, 1983) which consists of an icosahedral nucleocapsid 95-110 nm in diameter made up of a ds DNA core and a 62-capsomere protein capsid. The nucleocapsid is surrounded by an electron-dense zone, called the tegument and a lipid envelope containing glycoprotein spikes on its surface. The mature enveloped virion is about 150-200 nm in size. BHV-1 is an economically important pathogen of cattle throughout the world (Wyler et al., 1989; Straub, 1990), causing a wide range of clinical syndromes depending on the age and immune status of the animal at the time of infection. The virus is an important etiologic agent of the bovine respiratory disease complex also known as shipping fever. The virus is responsible for infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), conjunctivitis, abortion, and fatal systemic infections.

The BHV-1 strains were previously categorized into three major groups based on their DNA restriction profiles and type of disease the cause to the animals (Engles et al., 1986; Bulach and Studdert, 1990). These groups are respiratory and abortogenic (IBR; BHV-1.1), The non-abortogenic and genital (IPV; BHV-1.2), and the neurovirulent (BHV-1.3). The BHV-1.2 and BHV-1.3 groups were further subdivided into group a and b based on restriction endonuclease analysis (Osorio et al., 1985; Engles et al., 1986), monoclonal antibody reactivity, and viral protein profiles (Metzler et al., 1985,1986; Friedli and metzler, 1987). The differences in the restriction site maps of BHV-1.1 and BHV-1.2 occurs in specific genomic regions, whereas the BHV-1.3 isolates have restriction site alteration throughout the genome (Engles et al., 1986). Cross-hybridization studies have shown the genetic homology between BHV-1.1 and BHV-1.2 to be about 95% (Seal et al., 1985; Engles et al.,

1986) and between BHV-1.3 and other BHV-1 strains to be 85%. All these findings suggested that the neuropathogenic strains (BHV-1.3) represent a distinct highly conserved genetic entity with genome characteristics (Brake and Studdert, 1985; Engles et al., 1986; Studdert, 1989; Bulach and Studdert, 1990) and antigenic properties (Metzler et al., 1986; Friedli and Metzler, 1987) which has lead to their classification as a separate group of viruses designated BHV-5 according to the recent report by the international committee for taxonomy of viruses (ICTV) (Roizman et al., 1992).

Infectious bovine rhinotracheitis (IBR) is a component of the bovine respiratory disease complex. The disease is characterized by fever, depression, ocular and nasal discharges, and coughing. More importantly the virus causes immunosuppression predisposing infected animals to secondary bacterial infections especially Pasteurella hemolytica (Yates, 1982). When secondary bacterial infection occurs, bronchopneumonia, abortion of infected pregnant cows, mastitis, and conjunctivitis may occur.

Infectious pustular vulvovaginitis (IPV): BHV-1 strains causes IPV in cows and infectious balanoposthitis (IBP) in bulls. The conditions in both males and females are characterized by the development of pustular lesions in the genital organs usually following natural service, artificial insemination, or tail contamination (Straub, 1990). Erosion of epithelia occurs in the vulva and vagina of females and penis and prepuce of males. The strains of BHV-1 causing IPV are generally less virulent than those causing IBR. Abortions don't occur usually with IPV form. IPV virus are closely related to IBR virus (Gillespie et al., 1959) and could not be distinguished by serology.

Encephalitis: BHV-5 strains (previously classified as neurotropic strains of BHV-1 or BHV-1.3) have been isolated from outbreaks of encephalitis in Argentina (Carillo et al., 1983), Australia (French et al., 1962), the united states (Eugster et al., 1974) and sporadically from other countries around the world (Bartha et al., 1969; Moretti et al., 1964). The disease predominantly found in young calves and is usually fatal. Generalized infection and clinical disease develops 10 - 11 days following

infection. Nervous symptoms include incoordination, muscular tremors, recumbency, ataxia, circling movements, blindness and eventually death. The BHV-5 strains are major cause of mortality in cattle in Argentina with more than 100 outbreaks recorded since 1981 (Schudel et al., 1986). The TX89 strain of BHV-5 was isolated in Texas from an outbreak in unvaccinated cattle (Eugster et al., 1974). The N569 strain, isolated in Australia, is considered the prototype of the BHV-5 strains (French, 1962ab; Bulach and Studdert, 1990).

Latency

Latency is defined as the silent persistence of the virus in the body not detectable by conventional virological procedures, with subsequent intermittent episodes of reexcretion. After multiplication of BHV-1 at the local site of infection the virus enters the peripheral nervous system and is transported presumably by retrograde axonal transport, mainly to the trigeminal and sacral ganglia (Narita et al., 1981). Axonal entry is required as the neuronal perikarya seem to lack virus receptors (Homan and Easterday, 1982). Macrophages (Forman et al., 1982; Geder et al., 1981), epithelial cells (Thiry et al., 1986), and parts of the brain (Rziha et al., 1986) has been studied as possible sites for latency. All BHV-1 and BHV-5 strains can establish latency, and none of the inactivated or live vaccine presently available are able to prevent completely establishment of latency by a superinfecting challenge virus. It is not clear exactly as to how the virus is maintained in the latent state, however it has been hypothesized that immune surveillance (Babiuk and Rouse, 1979), or stage of host cell differentiation determines maintenance. Reactivation may occur either spontaneously or induced by natural or artificial stimuli such as transport , parturition (Thiry et al., 1984, 1985), immunosuppressive treatment with glucocorticoid (Pastoret et al., 1980), superinfection with other viruses e.g parainfluenza-3 or microorganism.

Using in situ hybridization , BHV-1 DNA could be detected in the trigeminal (Ackermann et al., 1982) and sacral (Ackermann and Wyler, 1984) ganglia of latently infected calves following intratracheal and intravaginal inoculations respectively. However RNA hybridization studies detected viral transcripts in the nuclei of

trigeminal neurones (Rock et al., 1986, 1988). The rabbit latency model has been used to study the molecular basis of BHV-1 latency (Rock et al., 1986 and 1987). These studies have shown that the BHV-1 genome is transcriptionally active in latently infected ganglionic neurones, with transcription restricted to a region approximately 1.16 Kb in size (0.740-0.748) within HindIII D genomic fragment (Rock et al., 1987; Wirth et al., 1991, Kutish et al., 1990). Studies with HSV suggested that transcription during latency is restricted to the region of HSV-1 genome that contain the immediate-early gene ICP0 (Deatly et al., 1987; Puga and Notkins, 1987; Stevens et al., 1987) and demonstrated that the RNA is anti-sense to that of the ICP0 gene (Stevens et al., 1987). However the functional significance of these transcripts remained to be determined. A transcriptional promotor which regulates the latency-related transcript of BHV-1 has been identified and characterized (Jones et al., 1990). Transcription is extremely efficient in primary sensory ganglionic neurones, however it is less efficient in other types of cells. Results obtained from these studies indicated the LTR gene of BHV-1 is regulated by a variety of tissue-specific or species-specific transcription factors and interference of regulation of these genes may lead to latent viral reactivation.

BHV-1 Genetics

Genome Characteristics

The BHV-1 genome is a linear double stranded DNA consisting of 135-140 Kilobase pairs (Kb) with 104 Kb unique long segment, an 11 Kb unique short segment, and 12 Kb inverted repeats (Wyler et al., 1989). The BHV-1 genome exhibits the typical arrangement of group D herpesvirus DNA, resembling that of pseudorabies virus (PRV, suid herpesvirus 1), equine herpesvirus 1 and 3 (EHV-1 and 3), caprine herpesvirus 1 (CapHV-1, BHV-6), and varicella zoster virus (VZV). Group D genomes consist of a unique short sequence which inverts relative to a unique long sequence, leading to two isomeric forms of DNA which are flanked by internal and terminal repeats (Roizman et al., 1981). The genome arrangement of herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and bovine mammillitis virus (BHV-2) belong to the class E in which sequences from both termini are repeated

in an inverted orientation internally, dividing the genome into two components each consisting of unique long and short segments flanked by inverted repeats. Both components of the genome can invert relative to each other resulting in DNA populations containing four isomers (Roizman, 1990a). No significant homology was observed between BHV-1 and HSV-1 and HSV-2 by DNA:DNA hybridization (Ludwig, 1983). Complete cross hybridization occurs between BHV-1 and CapHV-1 DNAs indicating high degree of DNA sequence homology (Engles et al., 1987). Approximately 8% DNA sequence homology has been detected between BHV-1 and PRV, either dispersed throughout the genome (Bush and Pritchett, 1985) or localized in specific regions of IR and UL segments (Whetstone et al., 1987).

Temporal Control of Transcription and Expression of BHV-1 Genes

Based on comparison with other alphaherpesvirus genomes, the BHV-1 genome has the capacity to code for 68-69 genes (Wyler et al., 1989). However a total of 54 viral transcripts have been mapped both temporally and spatially (Wirth et al., 1989). More than 50 viral polypeptides have been shown to be synthesized during lytic infection (Wirth et al., 1991). The synthesis and expression of these polypeptides is temporally ordered in three kinetic classes (Ludwig and Letchworth, 1987; Misra et al., 1983) based on their expression in the presence of metabolic inhibitor or their sequence homology with HSV-1 homologues. The three classes are: i) immediate-early or α proteins which are expressed in the presence of cycloheximide i.e without prior viral protein synthesis ii) early or ß proteins which are inhibited in the presence of cycloheximide i.e dependant upon viral protein synthesis iii) late or τ proteins which are inhibited by cytosine arabinoside i.e dependent upon viral DNA replication. The temporal distribution of 54 BHV-1 transcripts was determined using northern blotting techniques. Three major α-transcripts, 21 ß-transcripts, 12 τ-transcripts, and 17 transcripts couldn't be classified unambiguously as α or τ. Three viral IE proteins were originally identified in the BHV-1-infected cells (Misra et al., 1983). The three IE transcripts were spliced and originated from two divergent transcription units with start sites located in the inverted repeat (Wirth et al., 1991). Two major BHV-1 IE proteins, 180 KD and 52-57 KD phosphoproteins are

structurally and functionally similar to HSV-1 ICP0 and ICP22 respectively (Hayes and Rock, 1990; Wirth et al., 1991). These proteins play vital roles in regulation of BHV-1 productive and latent infection by transactivating a variety of viral and cellular promoters. The BHV-1 appears to occupy an intermediate position in the evolution of alphaherpesviruses with regard to the number and layout of IE genes. Whereas PRV and EHV-1 produce a single IE transcript encoding related species of IE proteins , the human herpes viruses VZV and HSV-1 encode four or five IE proteins from different IE transcripts arranged similarly to the arrangement of BHV-1.

The early (ß) proteins reach a peak rate synthesis 5-7 hr after infection and are mainly involved in viral nucleic acid metabolism such viral DNA polymerase and thymidine kinase. The ß transcripts are dispersed throughout the genome with a cluster on the Us sequence (Wirth et al., 1989). The BHV-1 TK gene, encoding a 4.3-Kb transcript, is a representative of an early class gene. The size of the transcript is larger than the sequenced gene (Mittal and Field, 1989). This is because the BHV-1 TK is found adjacent to the gH gene and they share a 3' coterminal transcript (Bello et al 1992). BHV-1 gB (Misra et al., 1988; Lawrence et al., 1988) and gD (Tikoo et al., 1990) are early class proteins. These proteins can be detected by immunoprecipitation at 2 hr post infection (pi) but are not detected until 4 hr pi by fluorescent antibody staining and are synthesized in the presence of PAA (Ludwig and Letchworth, 1987).

The late (τ) genes code mostly for viral structural proteins and are located mainly in the unique long region (Wirth et al., 1989). Of the three major BHV-1 glycoproteins, gB and gD were classified as ß-proteins whereas gC as τ-protein (Misra et al., 1983; Ludwig and Letchworth, 1987). Synthesis of gC RNA occurs primarily in cells wherein viral DNA synthesis was not inhibited. The 1.6 Kb gC transcript was detected in BHV-1-infected cells at late times during infection cycle (Seal et al., 1992). The BHV-1 gC was detected by radioimmunoprecipitation at 8 hr pi but not when infected cells were treated with PAA (Ludwig and Letchworth, 1987). The BHV-1 gC is the homologue of HSV-1 gC which has been classified as a late gene (Zhang and Wagner, 1987).The late proteins include the major tegument

protein VP8, a protein kinase carried in the BHV-1 virion.

Several BHV-1 genes have now been identified. Thymidine kinase (TK) gene has been expressed (Kit and Qavi, 1983; Weinmaster et al., 1982), mapped to the HindIII A fragment (Bello et al., 1987) and sequenced (Mittal and Field, 1989). The BHV-1 DNA polymerase gene was mapped within a 2.4 Kb HpaI-BamHI subfragment of the HindIII G fragment using a homologous gene of HSV-1 as a probe (Owen and Field, 1988). Using similar techniques a putative DNA binding protein gene was located adjacent to the BHV-1 DNA polymerase gene (Bandyopadhyay et al., 1990). Glycoprotein B (gI) the homologue of HSV gB, was mapped in the HindIII A fragment of the BHV-1 Cooper strain genome (Lawrence et al., 1986) and sequenced (Misra et al., 1988; Whitbeck et al., 1988). BHV-1 glycoprotein C (gIII), the homologue of HSV-1 gC, has been mapped in the HindIII I fragment, cloned, and sequenced (Fitzpatrick et al., 1989). Glycoprotein D (gIV) of BHV-1, the homologue of HSV-1 gD, was mapped to the HindIII K fragment (Tikoo et al., 1990) of the unique short Us region of BHV-1 Cooper genome where a cluster of early transcripts are synthesized (Seal et al., 1991, 1992). Three IE transcripts were mapped to the HindIII C fragment (Wirth et al., 1991; Seal et al., 1991). A latency-related transcript identified in the trigeminal ganglia of rabbits was mapped to the HindIII D fragment (Rock et al., 1987), and 1.15 Kb transcript was identified that overlaps an IE and early transcriptional unit in the complementary strand (Kutish et al., 1990). Although transcription maps of other BHV-1 genes are not yet available, some of these genes, such as glycoprotein E (gII), the homologue of HSV gE (Simard et al., 1990), gE, gX, and VP4 (Wirth et al., 1989), were identified, cloned, and sequenced.

BHV-1 Glycoproteins

Up to 33 structural polypeptides , 11 of which are glycosylated, have been described for BHV-1 (Misra et al., 1981, Bolton et al., 1983). Ten of the 11 glycoproteins are located on the surface of the virus (Chang et al., 1986, Marshal et al., 1986). The use of monoclonal antibodies in immunoprecipitation, immunoblotting, immunofluorescence, and ELISA studies characterized four major envelope

glycoproteins namely, gB (gI), gC (gIII), gD (gIV), and gE (gII)(Marshal et al., 1986; van Drunen little-van den Hurk and Babiuk, 1986). The envelope glycoproteins are pivotal in the host-virus relationship since they are involved in the recognition, attachment , and penetration of herpesviruses into susceptible cells (Little et al., 1981), in viral neutralization (Glorioso et al., 1984; Lupton and Reed, 1980), in immune destruction of infected cells (Bishop et al., 1984; Carter et al., 1981; Rouse and Horohov, 1984). BHV-1 gB, gC, and gD have been extensively studied (Collins et al., 1984, 1985, Gregerson et al., 1985; Marshal et al., 1986; van Drunen little-van den Hurk et al., 1984; van Drunen little-van den Hurk and Babiuk, 1986b; Trepanier et al., 1986, Scott et al., 1988) and have been shown to induce virus-neutralizing antibodies in animals (van Drunen Little-van den Hurk et al., 1986b, 1990a), and serve as targets for antibody-dependent, and antibody-complement-mediated lysis of virus-infected cells. Glycoprotein E (gII), the homologue of HSV-1 gE received much less attention since it does not induce virus-neutralizing antibodies.

BHV-1 glycoprotein B (gB) belongs to a group of homologous glycoproteins that have been detected in all herpesviruses analyzed to date. The prototype for this family of glycoproteins is gB of HSV (Pellett et al., 1985b; Bzik et al., 1986). Glycoproteins with homology to gB have been described for human cytomegalovirus (HCMV) (Cranage et al., 1986), VZV (Keller et al., 1986), Epstein-Barr virus (EBV) (Pellett et al., 1985a), PRV (Robbins et al., 1987), EHV-1 (Whalley et al., 1989) and EHV-4 (Riggio et al., 1989). These glycoproteins show a high degree of homology at the DNA, protein, and structural level, which suggests that they play a central role in the biology of herpesviruses. This underscored by the observation that HSV gB and PRV gII are essential for viral replication (Cai et al., 1987; Rauhl et al., 1991). On the basis of the high degree of homology between the gB homologues it is likely that BHV-1 gB is an indispensable glycoprotein. A number of functions have been ascribed to this glycoprotein, among which are a role in attachment and penetration into susceptible cells (Liang et al., 1991), induction of cell-cell fusion (Fitzpatrick et al., 1988, 1990b), and the induction of neutralizing antibodies(van Drunen Little-van den Hurk and Babiuk, 1985). Immunization of cattle with affinity-purified gB confers

protection against BHV-1 challenge (van Drunen Little-van den Hurk et al., 1990a), which makes it a suitable subunit vaccine candidate. Mature gB consists of three related polypeptides that are derived from a common 105 KD primary translation product. After glycosylation the precursor is cleaved by a cellular protease into two smaller glycoproteins of 74 KD and 55 KD that are covalently linked by disulfide bonds (Marshall et al., 1986; van Drunen Little-van den Hurk et al., 1986b). The proteolytic cleavage process, however, is not complete which results in the presence of a 130 KD uncleaved glycoprotein in BHV-1 infected cells. The homologues of other herpesviruses like VZV gpII, HCMV gB, EBV gB, PRV gpII, and EHV-1 show similar proteolytic processing. The HSV gB although not processed proteolytically, nevertheless, the gB homologues are highly conserved structurally and serologically among the herpes virus family (Davison and Taylor, 1987; Misra et al., 1988).

BHV-1 gC (gIII) is a major viral glycoprotein expressed at high levels on the surface of infected cells and in the viral envelope (van Drunen Little-van den Hurk et al., 1984). It is a 95 KD to 99 KD molecule containing both N-linked and O-linked oligosaccharides which assembles into homodimers (van Drunen Little-van den Hurk and Babiuk, 1986) and a macromolecular structure visible as a thin 20 - 25 nm spikes protruding from the virion envelope (Fitzpatrick et al., 1990a). In this configuration, gC functions as a major viral attachment protein as evidenced by virus-cell binding studies (Okazaki et al., 1987, 1993) and the induction of viral neutralizing monoclonal antibodies in animals immunized with BHV-1 (Collins et al., 1984). The homologous glycoprotein of HSV (gC) has been shown to possess complement factor C3b binding activity (Eisenberg et al., 1987), however this activity may not be conserved in BHV-1 gC (Bielefeldt Ohman and Babiuk, 1988). BHV-1 gC and its homologues in HSV and PRV (gIII) have been shown to be nonessential for virus replication in vitro (Liang et al., 1991; Holland et al., 1984; Robbins et al., 1986b).

BHV-1 gD is an integral component of the virion envelope and appear to be essential for virus replication (Fehler et al., 1992). Monoclonal antibodies to gD possess the highest complement-independent neutralizing activity and have been shown to inhibit adsorption and penetration (Hughes et al., 1988; van Drunen Little-

van den Hurk et al., 1990a; Dubuisson et al., 1992). Immunization with affinity-purified gB, gC, and gD individually or in combination induced protective immune response against BHV-1 infection (Babiuk et al., 1987; van Drunen Little-van den Hurk et al., 1990b). However, gD is the only glycoprotein responsible for a significant reduction in viral replication and shedding. Glycoprotein D also induces stronger and more consistent cellular immune response to BHV-1 than gB and gC (Hutchings et al., 1990). In a recent study cattle injected with recombinant gD, were completely protected against challenge by BHV-1 and show no detectable virus shedding (van Drunen Little-van den Hurk et al., 1993). BHV-1 gD has also been implicated in virus adsorption (Hughes et al., 1988) and cell fusion (Tikoo et al., 1990). These functions of gD which makes it one of the most important proteins of BHV-1 and an ideal candidate as a subunit vaccine. Glycoprotein D sequence data showed the BHV-1 gD ORF to code for 417 amino acid residues (Tikoo et al., 1990) which give a molecular weight of 75K - 77K. The protein contains a signal sequence of 18 amino acids, which cleaved during translocation of the polypeptides in the endoplasmic reticulum; a large amino-terminal extracellular domain of 343 amino acids; a transmembrane domain of 29 amino acids; a highly charged cytoplasmic domain of 29 amino acids, (Tikoo et al., 1990 and both N- and O-linked oligosaccharides (van Drunen Little-van den Hurk and Babiuk 1985, 1986). Competitive binding assays using gD specific MAbs have been used by two separate groups of investigators to map epitopes on the gD molecule. Five epitopes, three interrelated and two independent, were reported by one group as targets of neutralizing antibodies (Marshal et al., 1988). Four antigenic domains on the protein which include conformational-dependent and conformational-independent epitopes were described by the other group (Hughes et al., 1988; van Drunen Little-van den Hurk et al., 1990a) A recent study using truncated and deleted gD-recombinant vaccinia virus proteins have located the broad amino acid regions containing these antigenic sites (Tikoo et al.,1993).

Role of Glycoproteins in Virus-Cell Interactions

Entry of virus into permissive cells constitutes the first step of virus replication.

For alphaherpesviruses, this process consists of virus attachment to host cells, via specific interactions between virus attachment proteins and cellular receptors and subsequent virus penetration events involving virus protein-mediated membrane fusion (Roizman and Sears, 1990). The mechanism of BHV-1 attachment and penetration into susceptible cells is similar to that reported for HSV and PRV (Fuller and Spear, 1987; Cai et al., 1988; Highlander et al., 1987; Johnson and Ligas, 1988; Mettenleiter et al., 1990; Johnson et al., 1990; Langeland et al., 1990; Muggeridge et al., 1990; Herold et al., 1991). BHV-1 gC mediates the initial and dominant interactions between the virus and permissive cells (Okazaki et al., 1991, 1993; Liang et al., 1993). BHV-1 gC$^-$ mutant showed impaired attachment and greater sensitivity to neutralization mediated by specific anti-gB and anti-gD MAbs. Glycoprotein B and gD are involved mainly in penetration (Fitzpatrick et al., 1990b; Hughes et al., 1988), however a minor role in attachment has been reported (Dubuisson et al., 1992). WuDunn and Spear (1989) were the first to demonstrate that the initial interaction between HSV and permissive cells involved recognition of cellular heparin-like components by the virus. The use of heparan sulfate proteoglycans as viral receptors was subsequently demonstrated for other members of alphaherpesvirus family , including BHV-1 (Okazaki et al., 1991, 1993) and PRV (Mettenleiter et al., 1990). More recently, the BHV-1 gC domains which are responsible for heparin-binding have been characterized (Liang et al., 1993). Results from these experiments identified 5 heparin binding sites between amino acid 126 and 310 of BHV-1 gC.

Attachment of virus to cell surface activates a process mediated by viral glycoproteins that cause the fusion of the viral envelope and the cell plasma membrane. Penetration may be a multi-step event involving more than one viral glycoproteins. Studies with HSV demonstrated that gB$^-$ and gD$^-$HSV-1 mutants attach to permissive cells but do not penetrate (Roizman et al., 1990). However, HSV-1 gD-expressing cells allowed attachment and endocytosis of both HSV-1 and HSV-2 (Roizman et al., 1990). These results demonstrated that both gB and gC recognize as well as attach to, cell receptors; gB and gD play an indispensable role in the fusion of the envelope with the plasma membranes; and gD sequester the cell membrane

proteins required for fusion of the viral and cellular membranes. Attachment and penetration studies using radiolabelled BHV-1 and MAbs indicated that higher concentrations of gB, gC, and gD-specific MAbs are required to inhibit viral attachment. However productive infection following attachment was prevented by lower concentrations of gD and gB-specific MAbs. Glycoprotein D-specific MAb had the same inhibitory effect whether added before or after attachment, suggesting an indispensable role in penetration (Dubuisson et al., 1992).

The knowledge of specific mechanisms of bovine herpesvirus tissue tropism and virulence is inadequate. However studies with other herpesviruses have demonstrated that neurovirulence has multigenic origin. Studies with pseudorabies virus suggested that alteration in viral glycoproteins and other genes resulted in reduced virulence. These alterations occurred due to deletions in the Us region coding for gI and gp63 (analogues of BHV-1 gE and gi respectively) (Card et al., 1992) and in the UL region coding for gIII (BHV-1 gC analogue), and the genes coded in the BamHI-4 fragment (Barbara et al., 1992). Studies with HSV have shown that a single epitope change in gC and multiple epitope changes in gB significantly reduced virulence to mice (Kumel et al., 1985). In an other study a single amino acid change in gD has affected the neuroinvasive property of HSV (Izumi and stevens, 1990).

BHV-1 Vaccines

Conventional BHV-1 vaccines with either live modified (MLV) or inactivated virus are available as monovalent or polyvalent preparations in many countries. Immunization of cattle with these vaccines may be effective in reducing clinical disease, duration of virus shedding and titers of excreted virus after an infection with field virus (Gibbs and Rweyemamu, 1977; Nettleton and Sharp, 1980). Therefore economical losses can be diminished and spread of BHV-1 controlled to a certain degree, but vaccination neither protect fully against infection nor prevent establishment of latency (Rossi and Kiessel, 1982; Kit et al., 1986).

Parenterally-administered MLV vaccines are generally attenuated by multiple passages in bovine kidney cells, by adaptation to porcine or canine cells, by

adaptation to cell cultures at 30°C, or by selection of heat stable mutants (Gibbs and Rweyemamu, 1977; Lupton and Reed, 1980). Although these types of vaccines may appear to induce protective immunity, they are associated with many drawbacks, including causing infection in vaccinated animals, ovarian lesion, fetal death, followed by abortion when vaccinating pregnant animals (van der Maaten et al., 1985; Smith et al., 1990). In addition , MLV vaccines may cause immunosuppression and therefore increase susceptibility of vaccinated individuals to bacterial infection (Gregersen and Ludwig, 1985).

MLV for intranasal administration were attenuated by serial passage in rabbit cell culture, or modified by chemical treatment or by selection of temperature sensitive mutants (Zygraich et al., 1974). These types of vaccines have been shown to be safe in pregnant cattle, and provide rapid and sound local protection by inducing prompt production of interferon and secretory IgA on mucosal surfaces (Todd et al., 1971, 1972).

Inactivated vaccines circumvented some of the problems associated with MLV vaccines such latency, recurrence, and infection. However, these vaccines are not efficient and may cause severe postvaccinal reactions (Kahrs, 1977). The inactivated vaccines produce low level of immunity which may not prevent virus shedding from vaccinated animals showing clinical signs (Straub, 1990). An additional disadvantage of the conventional vaccines result from extensive cross-reactivity between wild-type strains and both MLV as well as inactivated vaccine strains which makes differentiation by serodiagnosis between vaccinated and infected (latent carrier) animals in a herd extremely difficult to achieve. Recently, it has been shown that a subunit vaccine consisting of one or different combinations of the three major BHV-1 glycoproteins gB, gC, and gD induced higher levels of protective immunity than conferred by a killed virus vaccines (Babiuk et al., 1987; van Drunen Little-van den Hurk et al., 1990a). However gD was the only glycoprotein responsible for a significant reduction of viral replication and shedding. High levels of gD the protein were expressed in different mammalian and prokaryotic system and tested for their ability to protect cattle against BHV-1 challenge (van Drunen Little-van den Hurk

et al., 1993). With the exception of E.coli derived gD, all form of gD (baculovirus, vaccinia, and adenovirus-expressed) induced high levels of neutralizing antibodies in immunized animals and completely protected against BHV-1 challenge.

Genetic engineering is being used to improve live modified vaccines. A direct approach to increasing vaccine safety consisting of identifying and irreversibly removing "virulence factors". The vaccine virus is appropriately attenuated by deletion of virulence factors and used as vector to deliver foreign antigens from other microbial pathogens. The genetic deletion can be used as markers to differentiate vaccinated from naturally infected animals. The deleted genes could be replaced by several important bovine pathogen genes to serve as a multivalent live virus vaccine. The TK gene of BHV-1 plays a significant role in the pathogenesis of the virus evidenced by the fact that inactivation of the gene greatly attenuates the virus and reduces or eliminates its ability to induce abortion (Kit and McConnels, 1988; Miller et al., 1991). The BHV-1 gC gene is non-essential and has been deleted and replaced by Foot and Mouth disease capsid protein epitope (Kit and Kit, 1991) and PRV gIII (Kit et al., 1991). A new methodology with the potential to address many of the shortcomings of present-day vaccines involve the injections of genes encoding protective antigens directly into the host. Since its first description in 1990 (Lin et al., 1990; Wolff et al., 1990), direct gene injection and expression has been demonstrated with many genes in several species. Recently, Recombinant plasmids carrying BHV-1 gD gene injected into mice and cattle have generated a significant BHV-1 neutralizing response in both mice and calves and reduced virus shedding in calves (Cox et al., 1993). These reports indicate the potential of DNA injection as a method of vaccination, however the safety issue needs to be adequately addressed.

SHORT NOTE (Antigenic Differences of the Major Envelope Glycoproteins of BHV-1 and BHV-5)

ANTIGENIC DIFFERENCES OF THE MAJOR ENVELOPE GLYCOPROTEINS OF BHV-1 AND BHV-5

INTRODUCTION

Bovine herpesvirus-1 is responsible for a variety of disease conditions in cattle including respiratory infections, conjunctivitis, vulvovaginitis, abortions, encephalitis and generalized systemic infection (Gibbs and Rweyemamu, 1977). BHV-1 strains responsible for these disease conditions were previously divided into three groups based on their DNA restriction profiles and pathogenic properties: the respiratory /abortogenic (IBR, BHV-1.1), the non-abortogenic genital (IPV, BHV-1.2), and the neurovirulent (BHV-1.3)(Engles et al., 1986; Bulach and Studdert, 1990). The BHV-1.3 strains have distinct genome characteristics (Brake and Studdert, 1985; Engles et al., 1986; Studdert, 1989; Bulach and Studdert, 1990) and antigenic properties (Metzler et al., 1986; Friedli and Metzler, 1987) which has lead to their classification as a separate group of viruses designated BHV-5 according to the recent report by the international committee for taxonomy of viruses (Roizman et al., 1992). The BHV-5 strains are responsible for many outbreaks of fatal encephalitis in young cattle in different parts of the world including the U.S. (Carillo et al., 1983; French, 1962; Eugster et al., 1974; Bartha et al., 1969). Although all BHV-1 isolates are readily identified with polyvalent antisera directed to BHV-1 (Cooper strain)(Metzler et al., 1986; Bratanich et al., 1991). Significant antigenic differences between BHV-5 strains and other BHV-1 isolates have been found by crossed virus neutralization tests (Bagust, 1972; Metzler et al., 1986), SDS-PAGE of radiolabeled polypeptides (Schudel et al., 1986; Metzler et al., 1985, 1986), and by monoclonal antibodies (MAb) reactivity (Metzler et al., 1986; Friedli and Metzler, 1987).

In this communication we used MAb reactivity to study the antigenic differences on the major envelope glycoproteins gB, gC, and gD of the BHV-1 (Cooper strain) and the U.S isolate of BHV-5 (TX89) by cross-ELISA, cross-neutralization, cross-immunoblotting, and cross-immunofluorescence reactions.

MATERIALS AND METHODS

Monoclonal Antibodies

Two panels of murine MAbs against the BHV-1 (Cooper strain) (Abdelmagid et al., 1992) and BHV-5 (TX89 strain) respectively, were produced and characterized as described previously (Abdelmagid, 1989). MAbs specific to the three major envelope glycoproteins gB (gI), gC (gIII), and gD (gIV) were used in cross-ELISA, cross-neutralization (Abdelmagid et al., 1992), cross-immunoblotting, and cross-immunofluorescence reactions to study the antigenic variation between the BHV-1 and BHV-5.

Indirect Immunofluorescence

Indirect immunofluorescence studies were performed on Madin-Darby bovine kidney (MDBK) cells grown on 24-well tissue culture plate. The cells were infected with either BHV-1 or BHV-5 at a multiplicity of infection (m.o.i) of approximately 1. After 1 hr adsorption at 37°C, excess virus was removed, and replaced with DMEM containing 5% FBS. Twenty-four hrs later, the medium was removed and the cell monolayers were washed twice with cold PBS and fixed in cold 75% acetone. 200 μl of MAbs diluted 1:500 - 1:1000 in PBS were added to each well and incubated at 37°C for 30 min. The MAb solution was removed and the cells were then washed 3X with PBS. Fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Hyclone Lab.Inc., Logan, Utah) was diluted 1:40 in PBS and added to the wells. The plates were allowed to incubate at 37°C for 30 min. After three washes in PBS, the stained monolayers were examined under a UV epifluorescent microscope.

Western Blotting

Following electrophoresis, viral proteins were electrophoretically transferred to nitrocellulose (NC) membranes using an electrotansfer unit from Hoefer. The transfer was completed in one hr at 0.90 amp. NC membranes were washed briefly in DDH_2O, blocked in 3% nonfat milk in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) or PBS. The NC membranes were placed in plastic bags and MAb, rabbit, or bovine antisera diluted in 0.05% Tween 20 TBS (TBST) or PBS (PBST), were added to the plastic bags and incubated overnight at room temperature with shaking. anti-mouse, anti-rabbit, or anti-bovine biotinylated antibodies and avidin-peroxidase-substrate system were obtained as a kit (Vector Laboratories, Inc., Burlingame, CA)

and used following manufacturer instructions.

RESULTS

Monoclonal antibodies to BHV-5 strain TX89 were produced and their polypeptide specificities were determined by immunoprecipitation and immunoblotting (Table 1). Six MAbs recognized epitopes on different viral proteins. Two MAbs (8B1 and Q21) were specific for gC, one MAb (F83) was specific for gB, and the remainder of MAbs were specific for 160/45 KD, 75/50 KD, and 160/180 KD proteins. An other panel of MAbs to BHV-1 (Cooper strain) has been previously developed and characterized (Abdelmagid, 1989). To demonstrate antigenic variation between BHV-1 and BHV-5 strains, MAbs specific for the three major envelope glycoproteins gB (gI), gC (gIII), and gD (gIV) were used in cross-ELISA, cross-neutralization, cross-immunoblotting, and cross-immunofluorescence reactions. The results of Immunoblotting were shown in Fig. 1 and summarized with the results of ELISA and immunofluorescence in table 2. Cross-neutralization results were shown in table 3. Two out of three gB-specific and two out of three gD-specific BHV-1 MAbs reacted with BHV-5 by ELISA and immunofluorescence. Similar reactions were obtained by immunoblotting for MAbs 83, 94, and R54 (Fig. 1). However none of six gC-specific BHV-1 MAb were able to recognize BHV-5 by ELISA, IF, or immunoblotting (Fig. 1 and table 2). The two gC-specific BHV-5 MAbs 8B1 and Q21 also failed to recognized the gC of BHV-1. Reactivity with gC-specific MAbs was the most disparate of the three glycoprotein-specific panels of MAbs. According to previous MAb competition radioimmunoassay (RIA) studies (Abdelmagid, 1989) the gB, gC, and gD panels of BHV-1 MAbs represent three, two, and five distinct epitopes respectively.

BHV-1 MAbs were tested for their ability to neutralize BHV-5. As shown in table 3 only MAb 111B cross-neutralized BHV-5 with a low (1:100) titer. The neutralization results were consistent with ELISA, IF, and immunoblotting.

DISCUSSION

Antigenic differences between the major envelope glycoproteins of BHV-1 and BHV-5 were demonstrated by comparison of cross-reactivity of two panels of MAbs

with BHV-1 and BHV-5. Of the three glycoproteins, gB, gC, and gD, gC showed major antigenic differences between the two viruses. None of six gC-specific BHV-1 MAbs recognized BHV-5, and none of two gC-specific BHV-5 MAbs recognized BHV-1. The BHV-1 MAbs represent at least 5 different epitopes based on previous studies (Abdelmagid, 1989). These findings indicate that epitopes were present on gC of either BHV-1 or BHV-5 were not shared with the other. Glycoproteins gB and gD of the two viruses share common epitopes. Three each gB-specific and gD-specific BHV-1 MAbs represent three and two distinct epitopes on gB and gD respectively. Antigenic variation between BHV-1 and other herpesviruses including BHV-5 (N569 strain) has been studied previously using MAbs (Friedli and Metzler, 1987) and monospecific bovine sera (Collins et al., 1993). Results of antigenic variation studies between BHV-1 and BHV-5 conducted in our lab with the U.S isolate TX89 of BHV-5 are similar to results from previous studies for the N569 (Friedli and Metzler, 1987) and A663 strains of BHV-5 (Bratanich et al., 1991). The antigenic differences between the major envelope glycoprotein provide significant information for immunity and cross-protection between BHV-1 and BHV-5. Cross-protection studies performed by Bratanich et al., 1991 using the Argentinean strain of BHV-5 A663, have demonstrated that calves immunized with BHV-1 were protected when challenged with BHV-5. However the effectiveness and the duration of immunity is not known. The differences between the major envelope glycoproteins may play an important role in the difference in pathogenesis and tropism between the two viruses.

Glycoprotein C exhibits the greatest heterogeneity between the two viruses and it is role in attachment to susceptible cells is well documented (Okazaki et al., 1991; Liang et al., 1991, Fitzpatrick et al., 1990). Glycoprotein B and D are also important for attachment and penetration (Liang et al., 1991; Dubuisson et al, 1992). Further studies are required to determine the specific functional roles of these differences with respect to the differential pathogenesis of the two viruses, using genetic engineering techniques to introduce specific mutations in the genes.

FIGURES AND TABLES

Fig. 1. Immunoblotting analysis of BHV-1 Monoclonal antibodies. Each MAb is reacted against BHV-5 (lanes 1, 3, 5, 7, 9, and 11), BHV-1 (lanes 2, 4, 6, 8, 10, and 12) and mock-infected cell lysates (lanes M's). MAbs shown in the figure are produced against BHV-1 except MAb 8B1 which produced against BHV-5. MAbs 3 (lanes 1 & 2) and 10B (lanes 5&6) are gC specific and recognized only BHV-1. MAb B2 (lanes 3 & 4) is gC specific and recognized both strains. MAb 8B1 (lanes 7 & 8) is gC specific and recognized only BHV-5. MAbs R54 (lanes 9 & 10) and 83 (lanes 11 & 12) are specific to gD and gB respectively, and recognized only BHV-1. None of the MAbs reacted against mock-infected cells.

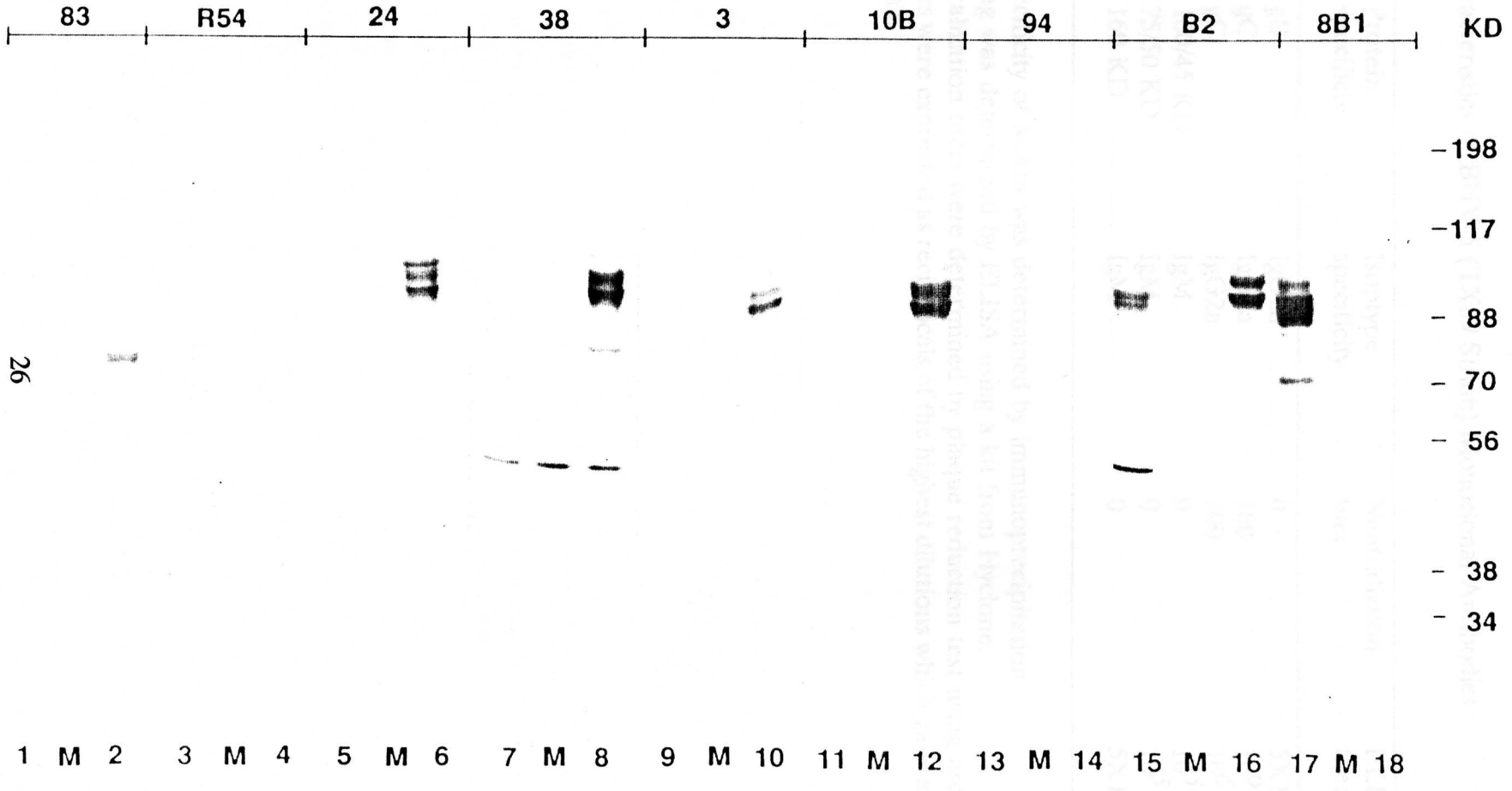

83
R54
24
38
3
10B
94
B2
8B1
KD
198
117
88
70
56
38
34
26
1 M 2 3 M 4 5 M 6 7 M 8 9 M 10 11 M 12 13 M 14 15 M 16 17 M 18

Fig. 1.

Table 1. Characteristics of BHV-5 (TX89 Strain) Monoclonal Antibodies

MAb	Protein specificty	Isoptype specificity	Neutraliztion titer	ELISA titer
F83	gB	IgG2a	0	$5X10^5$
8B1	gC	IgG2a	100	10^6
Q21	gC	IgG2a	100	10^6
L37	160/45 KD	IgM	0	10^5
M16	75/50 KD	IgM	0	10^5
L14	160 KD	IgM	0	$5X10^5$

a. Protein specificity of MAbs was determined by immunoprecipitation.
b. Subisotyping was determined by ELISA using a kit from Hyclone.
c. Virus neutralization titers were determined by plaque reduction test using ascites fluid.
d. ELISA titers were expressed as reciprocals of the highest dilutions which gave absorbance twice the blank.

Table 2. Cross reactivity of Monoclonal antibodies with BHV-5 (TX89) and BHV-1 (Cooper)

		Virus Strains					
		TX89 (BHV-5)			Cooper (BHV-1)		
MAb	gp Specificity	ELISA	IF	IB	ELISA	IF	IB
BHV-1 MAbs							
83	gB	-	-	-	+	+	+
94	gB	+	+/-	+	+	+	+
66	gB	+	+	(-)	+	+	(-)
111B	gD	+	+	(-)	+	+	(-)
12	gD	+	+	(-)	+	+	(-)
R54	gD	-	-	-	+	+	+
92	gC	-	-	(-)	+	+	(-)
24	gC	-	-	-	+	+	+
10B	gC	-	-	-	+	+	+
3	gC	-	-	-	+	+	+
38	gC	-	-	-	+	+	+
B2	gC	ND	ND	+	ND	ND	+
BHV-5 MAbs							
Q21	gC	+	+	(-)	-	-	(-)
8B1	gC	+	+	+	-	-	-

ELISA plates were coated with the same concentration of virus for different strains.
ELISA titers were calculated as the reciprocal of the highest dilution which gave 2X the absorbance of the blank.
+ = Titers more than 10^4
- = Titers less than 10^2
IF = indirect immunofluorescence
IB = immunoblotting
For IF and IB MAbs were diluted 1:500-1:1000.
+ = Strong reaction
- = No reaction
+/- = weak reaction
(-) = indicates MAbs which don't blot.

Table 3. Cross neutralization between BHV-5 (TX89) and BHV-1 (Cooper) using neutralizing monoclonal antibodies.

		Virus strains	
MAb	gp Specificity	TX89 (BHV-5)	Cooper (BHV-1)
83	gB	0	10,000
R54	gD	0	3,000
111B	gD	100	3,000
12	gD	0	100

Neutralization titers were determined by plaque reduction test.

REFERENCES

Abdelmagid, O.Y., Orten, D.J., Xue, W., Blecha, F., and Minocha, H.C. (1992). Anti-idiotypic antibodies to bovine herpesvirus-1 inhibit virus infection in cell cultures. *Arch. Virol.* 122, 163-173.

Abdelmagid, O.Y. (1989). Comparison of rabbit polyclonal antiidiotypes to bovine herpesvirus-1. *Thesis.*

Bagust, T.J, and Klarke, L. (1972). Pathogenesis of meningoencephalitis produced in calves by infectious bovine rhinotracheitis herpesvirus. *J. Comp. Pathol.* 82:375-383.

Bartha, A., Haidu, G., Aldassy, P., Paczolay, G. (1969). Occurrence of encephalomyelitis caused by infectious bovine rhinotracheitis virus in calves in Hungary. *Acta. Vet. Acad. sci. Hung.* 19, 145-151.

Brake, F., and Studdert, M.J. (1985). Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo, and caprine herpesvirus 1 and bovine encephalitis herpesvirus. *Aust. Vet. J.* 62, 331-334.

Bratanich, A.C., Sardi, S.I., Smitstaart, E.N., Schudel, A.A. (1991). Comparative studies of BHV-1 variants by in vivo and in vitro tests. *Zbl. Vet. Med. B* 38, 41-48.

Bulach, D.M., and Studdert, M.J. (1990). Comparative genome mapping of bovine encephalitis herpesvirus, bovine herpesvirus 1, and buffalo herpesvirus. *Arch. Virol.* 113, 17-34.

Carillo, B.J., Pospischil, A., and Dahme, E. (1983). Pathology of bovine necrotizing encephalitis in Argentina. *Zbl. Vet. Med.* B 30, 161-168.

Collins, J.K., Ayers, V.K., Whetstone, C.A., van Drunen Little-van den Hurk, S. (1993). Antigenic differences between the major glycoproteins of bovine herpesvirus type 1.1 and bovine herpesvirus encephalitis type 1.3. *J. Gen. Virol.* 74, 1509-17.

Dubuisson, J., Israel, B.A., and Letchworth III, G.J. (1992). Mechanisms of bovine herpesvirus type 1 neutralization by monoclonal antibodies to glycoproteins gI, gIII, and gIV. *J. Gen. Virol.* 73, 1-9.

Engles, M., Giuliani, C., Wild, P., Beck, T.M., Loepfe, E., and Wyler, R. (1986). The genome of bovine herpesvirus 1 (BHV-1) exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. *Virus Res.* 6, 57-73.

Eugster, A.K., Angulo, A.A., Jones, L.P.(1974). Herpesvirus encephalitis in range calves. In, Proceedings, 17th *Ann. Meet. Amer. Asso. Vet. Lab. Diagnostician*, pp. 267-290.

Fitzpatrick, D.R., Redmond, M.J., Attah-poku, S.K., Van drunen Little-van den hurk, S., Babiuk, L.A., and Zamb, T.J. (1990a). Mapping of 10 epitopes on bovine herpesvirus type-1 glycoproteins gI and gIII. *Virol.* 176, 145-147.

French, E.L. (1962). A specific virus encephalitis in calves: Isolation and characterizati on of the causal agent. *Aust. Vet. J.* 38, 207-215.

Friedli, K., and Metzler, A.E. (1987). Reactivity of monoclonal antibody to proteins of neurotropic bovine herpesvirus 1 (BHV-1) strain and to proteins of representative BHV-1 strains. *Arch. Virol.* 94, 109-122.

Gibbs, E.P.J., and Rweyemamu, M.M. (1977). Bovine herpesvirus. I. Bovine herpesvirus-1. *Vet. Bull. (London)* 47, 317-343.

Liang, X., Babiuk, L.A., Van Drunen Little-van den Hurk, S., Fitzpatrick, D.R., and Zamb, T.J. (1991). Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII and gIV. *J. Virol.* 65, 1124-1132.

Metzler, A.E., Matile, H., Gassman, U., Engels, M., and Wyler, R. (1985). European isolates of bovine herpesvirus 1: A comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. *Arch. Virol.* 85, 57-69.

Metzler, A.E., Schudel, A.A., and Engles, M. (1986). Bovine herpesvirus 1: Molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. *Arch. Virol.* 87, 205-217.

Okazaki, K., Matsuzaki, T., Sugahara, Y., Okada, J., Hasebe, M., Iwamura, Y., Ohnishi, M., Kanno, T., Shimizu, M., Honda, E., and Kono, Y. (1991). BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparine like moeity on the cell surface. *Virol.* 181, 666-670.

Schudel, A.A., Carillo, B.J., Wyler, R., and Metzler, A.E. (1986). Infections of calves with antigenic variants of bovine herpesvirus-1 (BHV-1) and neurological disease. *Zbl. Vet. Med. B* 33, 303-310.

Studdert, M.J. (1989). Bovine encephalitis herpesvirus. *Vet. Rec.* 125, 584.

MANUSCRIPT (Fine Mapping of Linear Neutralizing Epitopes on BHV-1 gD Using Recombinant Fusion Proteins and Type-Specific Monoclonal Antibodies)

ABSTRACT

Overlapping fragments of the BHV-1 (BHV-1.1) gD ORF were expressed as TrpE-gD fusion products in Escherichia coli to map linear neutralizing epitopes defined by BHV-1-specific monoclonal antibodies (MAbs) 3402 and R54. The reactivity of the MAbs with the protein fragments located the epitopes between amino acid 52 - 115 and amino acid 165 - 216 for 3402 and R54 MAb respectively. The precise amino acid sequences defining the epitopes were identified by amino acid sequence analysis and comparison with the corresponding regions on gD of BHV-5 (gD of BHV-5 (BHV-1.3) has been cloned and sequenced and is reported in this manuscript). Five amino acid changes occurred in each of the two regions of BHV-5 gD have resulted in loss of MAb reactivity with the parent BHV-5 gD. Based on antigenic analysis of these regions peptides covering amino acid 92 - 106 and 202 - 213 were synthesized and examined for their epitope activity. Each peptide reduced the neutralizing ability of the corresponding MAb in a dose-dependant manner indicating that they carry the epitopes. Antisera produced in rabbits to KLH-conjugated peptides recognized the parent gD on western blot, but have low neutralizing antibody titers against BHV-1. These findings support the identification of the precise amino acid sequences of two different neutralizing linear epitopes which may be important for immunity to BHV-1 in natural infections.

INTRODUCTION

Bovine herpesvirus-1 is responsible for a variety of disease conditions in cattle including respiratory infections, conjunctivitis, vulvovaginitis, abortions, encephalitis and generalized systemic infection (Gibbs and Rweyemamu, 1977). BHV-1 strains responsible for these disease conditions were previously divided into three groups based on their DNA restriction profiles and pathogenic properties: the respiratory/abortogenic (IBR, BHV-1.1), the non-abortogenic genital (IPV, BHV-1.2), and the neurovirulent (BHV-1.3)(Engles et al., 1986; Bulach and Studdert, 1990). The BHV-1.3 strains have distinct genome characteristics (Brake and Studdert, 1985; Engles et al., 1986; Studdert, 1989; Bulach and Studdert, 1990) and antigenic properties (Metzler et al., 1986; Friedli and Metzler, 1987) which according to the recent report by the international committee for taxonomy of viruses (Roizman et al., 1992) has lead to their classification as a separate group of viruses designated BHV-5. The BHV-5 strains are responsible for many outbreaks of fatal encephalitis in young cattle in different parts of the world including the U.S. (Carillo et al., 1983; French, 1962; Eugster et al., 1974; Bartha et al., 1969).

Recovery from most herpesvirus infections, including BHV-1 is mediated by humoral as well as cellular immune responses (Rouse and Babiuk, 1978). As in other alphaherpesviruses, BHV-1 glycoproteins are the major structural components of the viral envelope and virus-infected cells. The glycoproteins play an important role in virus-cell interactions, including recognition, attachment, and penetration of herpesvirus into susceptible cells (Little et al., 1981; Highlander et al., 1987; Johnson and Ligas, 1988), viral neutralization (Glorioso et al., 1984; Lupton and Reed, 1980) and immune destruction of infected cells. Four major glycoproteins, gB (gI), gC (gIII), gD (gIV), and gE (gII), have been identified on the virus envelope and the plasma membranes of BHV-1-infected cells (Marshal et al., 1986; van Drunen Little-van den Hurk et al., 1984; van Drunen Little-van den Hurk and Babiuk, 1986). The four glycoproteins are recognized by sera from BHV-1 infected cattle (Collins et al., 1984; van Drunen Little-van den Hurk et al., 1986). Immunization with affinity-purified gB, gC, and gD individually or in combination induced a protective immune

response against BHV-1 infection (Babiuk et al., 1987; van Drunen Little-van den Hurk et al., 1990b). However, gD was the only glycoprotein that elicited a significant reduction in viral replication and shedding (van Drunen Little-van den Hurk et al., 1993). Glycoprotein D also induces a stronger and more consistent cellular immune response to BHV-1 than gB and gC (Hutchings et al., 1990). Monoclonal antibodies (MAbs) to gD possess the highest complement-independent neutralizing activity and have been shown to inhibit adsorption and penetration (Hughes et al., 1988; van Drunen Little-van den Hurk et al., 1990a; Dubuisson et al., 1992). Several functions have been ascribed to gD which makes it one of the most important proteins of BHV-1 and an ideal candidate as a subunit vaccine.

Glycoprotein D sequence data showed the BHV-1 gD ORF to code for a protein with 417 amino acid residues (Tikoo et al., 1990) having a molecular weight of 75K - 77K. Competitive binding assays using gD specific MAbs have been used by two separate groups of investigators to map epitopes on the gD molecule. Three interrelated and two independent epitopes, were reported by one group as targets of neutralizing antibodies (Marshal et al., 1988). Four antigenic domains on the protein which include the conformational-dependent and conformational-independent epitopes were described by the other group (Hughes et al., 1988; van Drunen Little-van den Hurk et al., 1990a). A recent study using truncated and internally deleted gD-recombinant vaccinia virus proteins have located the broad amino acid regions containing these antigenic sites (Tikoo et al., 1993).

In this communication we have identified the precise coding sequences of two different linear neutralization epitopes using type-variant sequence analysis and synthetic peptides antibody reactivity. Initially the broad amino acid regions containing these epitopes were located by expressing various portions of the BHV-1 gD ORF as TrpE fusion proteins in E.coli and testing their reactivity with BHV-1 (type)-specific MAbs. The BHV-5 gD gene was cloned, sequenced and used as a type-variant sequence to precisely determine the epitope sequences. Peptides covering the epitope regions were synthesized and tested for epitope activity. We also report the molecular cloning and sequencing of the gD glycoprotein from BHV-5

(TX89 strain) and discuss the nucleotide and amino acid sequence analyses and comparison with BHV-1 gD sequence to understand some of functional roles of the structural differences between the two viral proteins.

MATERIALS AND METHODS

Virus strains and Cell lines

The Cooper (Colorado-1) strain of BHV-1 (BHV-1.1) was obtained from the American Type Culture Collection (ATCC)(Rockville, Md). The TX-89 strain of BHV-5 (BHV-1.3) which isolated from an encephalitis outbreak in Texas and was kindly provided by Dr. d'Offay from Oklahoma State University. The two viruses were propagated and titrated in Madin-Darby Bovine Kidney (MDBK) cells grown in Dulbecco Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Monoclonal antibodies

MAbs R54 and 3402 used in this study were selected from a panel of BHV-1 gD-specific MAbs (Abdelmagid et al., 1992) because they did not cross-react with BHV-5 gD (BHV-1-specific) and they recognize the denatured gD on immunoblots. Both MAbs are IgGs and have high neutralizing titers (1:12,000) against BHV-1. MAb R54 has been erroneously characterized (Trepanier et al., 1990) and quoted (Abdelmagid et al., 1992) as gC specific, however in this study we recharacterized it as gD specific. MAb 3402 was provided by Dr. G. Letchworth III from the University of Wisconsin-Madison.

Construction of Recombinant pATH Expression Plasmids and Preparation of Fusion Proteins.

Plasmid DNA was prepared and manipulated with DNA-modifying enzymes by using standard methods (Sambrook et al., 1989). The complete coding sequence of gD from BHV-1 was excised by MaeI restriction endonuclease from a subclone of the plasmid pBH18 containing HindIII-K fragment (Mayfield et al., 1983; Lawrence et al., 1986) (Fig. 1 A and B). The plasmid pBH18 was provided by Dr. W. Lawrence from the university of Pennsylvania). The ends of the 1.3 Kb MaeI fragment were filled in with the Klenow Fragment of DNA polymerase and it was then cloned into

the SmaI site of pATH 23 to yield an in-frame TrpE-gD fusion gene (Dieckmann and Tzagoloff, 1985). The recombinant plasmid was usedto transform E. coli RR1. Insert-containing transformants were selected by colony hybridization using ^{32}P-nick-translated specific probes (Sambrook et al., 1089). Restriction endonuclease fragments encompassing different portions of the gD ORF (Fig. 3) were generated using the published gD sequence (Tikoo et al., 1990) and cloned into the appropriate pATH expression plasmids.

The synthesis of TrpE-gD hybrid proteins was induced in modified M9 medium by Tryptophan starvation and indoleacrylic acid (IAA) as described (Koerner et al., 1991). Briefly, modified M9 medium containing 0.5% casamino acids, 50 μg/ml ampicillin, 20 μg/ml Tryptophan, was inoculated with pATH-transformed cells. At midlogarithmic phase, the culture was used to inoculate as 1:10 ratio modified M9 medium without tryptophan. IAA was added to a final concentration of 10 μg/ml of culture and the culture was left to grow for an additional 4 hr. The cells were harvested by centrifugation and lysed in buffer containing 0.05 M Tris-HCl, pH 7.5, 3 mg/ml lysozyme, 0.7% Nonidet-P40. Cell lysates were then centrifuged and the pellets were solubilized in 4X sample buffer and analyzed by SDS-PAGE in a 10% polyacrylamide gel (Laemmli, 1970).

Westernblot Analysis

Following electrophoresis, viral and fusion proteins were electrophoretically transferred to nitrocellulose (NC) membranes. NC membranes were blocked in 3% nonfat milk in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) or PBS. MAbs or rabbit, or bovine antisera diluted in 0.05% Tween 20 TBS (TBST) or PBS (PBST) were added to the membranes and incubated overnight. Biotinylated secondary antibodies specific for mouse, rabbit, or bovine and peroxidase-substrate system were obtained as a kit (Vector Laboratories, Inc., Burlingame, CA) and used following manufacturer instructions.

Preparation of TrpE-gD Fusion Proteins for Immunization

TrpE-gD fusion protein expressing the N-terminal 216 amino acids was selected for rabbit immunization because it carries at least two linear epitopes. The

fusion protein antigen was prepared by elution of protein from the NC membrane after electrophoretic transfer as described previously (Szewczyk and Summers, 1988). Briefly , after preparative electrophoresis and transfer of the protein to NC membrane, membranes were washed in PBS containing 0.1% Tween 20 (PBST) to remove SDS and then air-dried. The portion containing the protein was sliced, dissolved in DMSO (Dimethylsulfoxide) and precipitated by adding an equal volume of 0.05 M sodium carbonate buffer, pH 9.6, and centrifugation. The pellet was resuspended in PBS, emulsified in Freund's complete adjuvant and used for immunization.

Cloning and Sequencing of BHV-5 gD gene

The DNA restriction site mapping and construction of BHV-5 genomic library have been described in detail (Engles et al., 1986). The 1.3 Kb MaeI-MaeI subfragment carrying the entire gD gene of BHV-1 was labeled with ^{32}P by nick-translation and used as a probe. A pUC-based plasmid library containing the BamHI genomic fragments of the BHV-5 (TX89) strain has been previously developed (unpublished data). All plasmids were amplified in E.coli strain DH5α using standard procedures (Sambrook et al., 1989). Southern hybridization of the BamHI genomic fragments with the probe localized the gD gene to the BamHI-C fragment. The BamHI-C fragment was digested with MaeI restriction endonuclease, separated by electrophoresis on 1.2% agarose electrophoresis and analyzed by hybridization. BHV-5 DNA fragments to be sequenced were mapped (Fig. 7) by double restriction endonuclease digestion, subcloned, and sequenced by the method of Maxam and Gilbert, 1977 and the dideoxy chain termination method of Sanger et al., 1977. Both strands of each fragment were sequenced twice by Sanger's method and verified once by Maxam and Gilbert method (Fig. 7).

DNA Sequence Analysis

Sequence data were assembled and analyzed using the Seqaid II sequence analysis software (Rhodes, D.D, and Roufa, D.J., Center for Basic Cancer Research, KSU, Manhattan). The amino acid sequence homology between BHV-5 and BHV-1 gD sequences was conducted by an alignment program, using a ktup value = 2,

provided in the same software. Hydropathicity analyses were performed (Kyte and Doolittle, 1982) using a nine-amino acid window. Antigenicity analysis of the epitope-bearing amino acid regions of BHV-1 gD was compared to that of the corresponding amino acid regions of BHV-5 gD using the software above.

Peptide Synthesis

Peptides were synthesized at the Kansas State University Biotech. Center, using FMOC (fluorenylmethyloxycarbonyl)-chemistry (Barany and Merrifield, 1980) on an ABI Model 431A automated peptide synthesizer (Applied Biosystem, Inc., Foster City, CA). After amino acid analysis, high performance liquid chromatography, and capillary electrophoresis, peptides were used directly for MAb competition analysis or conjugated for rabbit immunization. Peptides were conjugated to KLH (keyhole limpet hemocyanin) using SMCC (succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) as a cross-linker. An additional irrelevant cysteine was added to the C-terminus of peptide 92-106 to facilitate coupling.

Immunization of Rabbits

New Zealand white rabbits were injected intradermally at multiple sites along the back and subcutaneously in the inguinal region, with 300 μg of conjugated synthetic peptides or TrpE-gD fusion protein emulsified in Freund's complete adjuvant. Second and third injections were given intramuscularly at biweekly intervals in incomplete Freund's adjuvant. Animals were bled 1 week after each booster dose and sera were analyzed for specific antibodies by Westernblot and slotblot ELISA against the corresponding antigen and by neutralization of BHV-1 by plaque reduction assay.

Neutralization Assay

Rabbit sera or MAb ascites fluids were diluted in DMEM containing 5% FBS and incubated with 50-100 pfu of virus for 1 hr at 37°C. Antibody-virus mixtures were added to confluent MDBK monolayers in 24-well plates, allowed to adsorb for 1 hr, and overlaid with 1.2% methylcellulose in DMEM containing 2% FBS. After 2-3 days incubation, cells were fixed with 10% formaldehyde, stained with 0.35% crystal violet,

and plaques were counted. Titers were expressed as reciprocals of the highest dilutions that reduced plaques by 50%.

Inhibition of Monoclonal antibody Neutralization by Peptides

Serial five-fold dilutions of peptides in DMEM starting at a concentration of 25 μg/ml were incubated with equal volumes of a MAb ascites dilution which gave a 50% plaque neutralization. The mixtures were allowed to react for 1.5 hr at 37°C. BHV-1 was added to a final number of 100 pfu/200 μl of the mixture and incubated for an additional 1 hr at 37°C. 200 μl of the mixture was added to each of two wells of confluent MDBK monolayer in 24-well plate and the neutralization assay was continued as described above. As controls for the assay, the virus was incubated with peptide alone or MAb alone. The ability of each peptide to inhibit the neutralizing activity of the corresponding MAb was calculated by the following formula:

$$\frac{\text{Average \# of plaques (peptide \& MAb) - Average \# of plaques (MAb only)}}{\text{Average \# of plaques (peptide only) - Average \# of plaques (MAb only)}} \times 100$$

Results were recorded and represented as % virus plaques restored.

RESULTS

Expression and Immunoreactivity of TrpE-gD Fusion Proteins

To determine the expression of the recombinant TrpE-gD fusion proteins, induced culture lysates were analyzed by SDS-PAGE and stained with Coomassie brilliant blue (Fig. 2A). Control pATH plasmid expressing only the TrpE product with a molecular weight of 37K (lane 1) and the recombinant fusion proteins yielded different molecular sizes (lanes 2 to 10) depending on the length of the fused gD gene fragments.

TrpE-gD fusion plasmids expressing whole proteins (amino acid (aa) 1-417, lane 2) and the N-terminal half (1-216, lane 3) reacted on western blot with both MAbs 3402 (Fig. 2B) and R54 (Fig. 2C) indicating that the two epitopes are located within the N-terminal 216 aa. TrpE-gD recombinant plasmids expressing aa residues 128-216 (lane 8) and aa residues 165-216 (lane 10) were recognized by MAb R54 (Fig. 2C and Fig. 3) and not by MAb 3402 (Fig. 2B and Fig. 3) narrowing the location of R54 epitope to a 52 aa segment expressed by NarI-SmaI subfragment

(Fig. 3). MAb 3402 recognized overlapping TrpE-gD recombinants expressing aa residues 52-163 (lane 5) and aa residues 52-127 (lane 6). However the MAb didn't react with TrpE-gD protein (117-163, lane 9) indicating the location of this epitope between aa residues 52-115 was contained in the recombinant plasmid expressing the ApaI-BanII subfragment (Fig. 3). A summary of the immunoreactivity of the fusion proteins and the restriction endonuclease subfragments of the BHV-1 gD ORF specifying these proteins are shown in Fig. 3.

Immunogenicity of gD fusion proteins

The immunogenicity of the TrpE-gD fusion protein expressing the N-terminal 216 aa of BHV-1 gD specified by subfragment MaeI-SmaI (Fig. 3), was determined by testing sera from immunized rabbits by immunoblotting and BHV-1 neutralization. These antisera (diluted 1:500 in TBST) recognized the authentic BHV-1 gD from infected cells on immunoblots (Fig. 4A), however it didn't neutralize the virus.

The antigenic authenticity of the TrpE-gD fusion protein (1-216) was examined for reactivity with convalescent bovine sera. Fifty field bovine serum samples were tested for BHV-1 specific neutralizing antibodies at the diagnostic service laboratory of the College Veterinary Medicine. These samples were retested for their reactivity with TrpE-gD fusion protein (1-216) by western blot (Fig. 4B). All serum samples tested positive for neutralization also reacted with the fusion protein, however of 5 samples tested negative by neutralization, two samples recognized the fusion protein on Western blot indicating that these animals may have been previously exposed to the virus.

Cloning and Sequencing of BHV-5 gD gene

Southern hybridization of the BHV-5 BamHI genomic library with ^{32}P-labeled MaeI fragment containing the BHV-1 gD gene identified a 14.6 Kb (BamHI-C) fragment spanning map unit (m.u) 0.827 - 0.944 (Engles et al., 1986) (Fig. 5 and 7). Restriction site mapping, subcloning of the BamHI-C fragment, and hybridization with the same probe identified a 1.62 Kb MaeI fragment (m.u 0.886 - 0.899) with complementary DNA sequences (Fig. 6 and 7). The nucleotide sequence of the DNA fragment identified above was determined and the only ORF large enough to encode

BHV-5 gD was discerned (Fig. 8). This ORF is 1,251 nucleotides long and starts from the ATG codon at nucleotide 247 and terminates with stop codon TGA at nucleotide 1498. The gD ORF spans m.u.(0.887 - 0.897) of the BHV-5 genome and codes for 417 aa. The putative upstream regulatory sequences (CAAT and TATA boxes), typical of eukaryotic promoters (Corden et al., 1980) were located 173 and 60 nucleotides upstream of the start codon, respectively. The nucleotide sequence analysis did not extend further downstream through the stop codon and did not include location of potential consensus polyadenylation signals. The nucleotide composition of the ORF was calculated to be 14.0% A, 12.1% T, 37.6% C, and 36.3% G.

Amino acid Sequence Analysis

Hydropathy analysis of the predicted protein revealed the presence of two prominent hydrophobic peaks representing the putative signal sequence and the putative transmembrane anchor sequence. Based on empirical rules for predicting signal sequences (von Heinje, 1986), aa residues 4-18 have the position, length, relative hydrophobicity, and consensus cleavage site characteristic of a signal sequence (Fig. 9 and 8). Similarly aa residues 355 -382 have the position, length, and relative hydrophobicity characteristic of transmembrane anchor sequences (Fig. 9 and 11).

Comparison of the BHV-1 and BHV-5 gD Predicted Amino acid Sequences

The predicted gD protein sequence of BHV-5 was compared to the homologous protein (gD) of BHV-1 (Tikoo et al., 1990) using an amino acid sequence alignment program with Ktup value = 2. Results of the analysis are shown in Fig. 9. The amino terminal two-third of the protein (aa 1-282 of BHV-5 gD) are relatively well conserved without significant insertions or deletions, however the carboxyterminal one-third (283-417 of BHV-5 gD) is highly variable. This region contains the putative transmembrane segment (355-382 of BHV-5 gD) and a stretch of 72 aa residues (283-354) immediately upstream of the transmembrane segment that specifies a major hydrophilic peak in the case of BHV-1. The corresponding region on BHV-5 gD contains several insertions and deletions resulting in a series of

hydrophilic peaks (Fig. 11). In addition, a significant deletion of 9 aa long is located at position 319 of BHV-5 gD (Fig. 9). Similary BHV-1 gD (Tikoo et al., 1990), a series of negatively charged residues from aa 281-295 (corresponding to aa residue 280-292 of BHV-1) is also present in this segment of the BHV-5 gD. However BHV-1 gD exhibits several changes with respect to the BHV-1 sequence. The overall amino acid homology between the two proteins is 82%.

As in other alphaherpesvirus gD homologues, the predicted protein has seven cysteine residues, six of which are located in the putative external domain. All cysteine residues are well conserved between the two sequences and aligned identically. Two potential N-linked glycosylation sites (Kornfeld and Kornfeld, 1985) located at aa position 102 and 411 of BHV-1 gD are conserved whereas a third site at aa position 41 of BHV-1 gD is absent in BHV-5 gD.

Analysis of Predicted Amino Acid Sequences and Selection of Peptides

To determine the probable location of epitopes recognized by MAbs 3402 and R54, the aa regions containing these epitopes (aa 52 - 115 and aa 165 - 216 for MAbs 3402 and R54 respectively) (Fig. 2 and 3) were compared with the corresponding regions of BHV-5 gD. Since the two MAbs recognize specifically BHV-1 gD and not BHV-5 gD (type specific), these epitopes should be present on the regions of BHV-1 gD corresponding to the altered aa.

Antigenicity plot analysis (Fig. 10A) of aa sequence 52-115 indicated potential antigenic regions. Comparison of the aa sequence of these regions with the corresponding regions of BHV-5 gD showed 5 aa changes. Four of the five aa changes of BHV-1 gD occurred at positions 92, 97, 98, and 100 and one aa change at position 57. Due to the four aa changes, a significant antigenic peak between residues 94-101 was lost in the corresponding region of BHV-5 gD (Fig. 10A). Based on these observations, a 12mer peptide covering residues 92-106 was synthesized and analyzed further.

Antigenicity plot analysis of aa region 165-216 and comparison with the corresponding regions of BHV-5 gD showed a total of 5 aa changes (Fig. 10B). Immediately downstream of aa residue 216 we noted two additional aa changes at

position 217 and 222 which we included in this analysis. Since none of these aa changes has affected the antigenicity of that region of the protein, we decided to make two synthetic peptides covering aa residues 202-213 and 213-225 and further analyze them for epitope activity by MAb neutralization inhibition assay.

Monoclonal antibody Specificity and Antigenic Activity of Synthetic Peptides

As shown in Fig. 13, certain concentrations of peptides (92-106 and 202-213) were able to reduce the neutralizing activity of MAb 3402 and R54 respectively suggesting interaction of these peptides with the MAbs. At optimum concentrations of peptides (25 μg/ml) and MAb (1:12X10^3 ascites), the highest percentage of virus restoration occurred. However peptides covering aa residues 213-226 showed no inhibitory effect on MAb R54. Based on these observations two peptides (92-106 and 202-213) were injected into rabbits to produce antibodies for further evaluation. Antisera from these rabbits were tested for peptide and BHV-1 specificity by slotblot ELISA. Each slot was coated with 40 μg or 8 μg of peptide or virus protein indicated in Fig. 12A by 1 and 2 respectively. The amounts of bound protein was determined by Ponceau S protein staining (Sigma, St. Louis, Mo). Each anti-peptide sera was diluted 1:100 in TBST and reacted specifically with the immunizing peptide and with the virus. On Western blots, anti-peptide (202-213) sera (diluted 1:200) recognized a 30K break down product of gD from BHV-1 infected MDBK cell lysate (Fig. 12B lane 1) whereas anti-peptide (92-106) serum recognized the parent gD as well as the 30K protein band (Fig. 12B lane 3). The reaction to the whole gD is weak compared to strong reaction to the 30K band. A control MAb R54 (Fig. 12B lane 5) reacted with the gD (75K) and the 30K product. No reaction was detected by anti-peptide sera against mock-infected MDBK cells.

DISCUSSION

Several approaches have been used to identify antigenic sites on BHV-1 glycoprotein D including competition binding assays (Marshal et al., 1988; Hughes et al., 1988; van Drunen Little-van den Hurk et al., 1984) and expression of mutant forms of the protein as recombinants in a mammalian expression system (Tikoo et al., 1993). Although the latter approach has mapped epitopes on broad amino acid

regions, the precise amino acid sequences of these epitopes were not identified. The main purpose of this study is to identify the precise amino acid sequences of linear neutralization epitopes on BHV-1 gD. To achieve this goal we have adopted a different approach which combines expression of gD ORF subfrgments in E.coli, identification of amino acid regions reactive with BHV-1-specific MAbs, antigenic analysis of these regions and comparison with the corresponding regions of BHV-5 gD (as a type-variant sequence), Synthesis of peptides from regions of antigenic potential and carry mismatched amino acids between BHV-1 and BHV-5 gDs. Although overlapping synthetic peptides and monoclonal antibody resistant (Mar) mutants are efficient techniques for precise identification (fine mapping) of epitopes the two techniques are expensive and time consuming.The results obtained in this study proved the combination of type-specific MAbs and type-variant sequence analysis to be effective in precise identification of epitopes.

Previous studies located three linear (conformational-independent) epitopes (Ia, IIIa, and IV) on three separate antigenic domains (Tikoo et al., 1993; van Drunen Little-van den Hurk et al., 1984 and 1990a). Our results showed that MAb R54 which is highly neutralizing recognized the smallest TrpE-gD fusion protein (165-216) specified by restriction fragment NarI-SmaI (Fig. 2 and 3). This finding confirmed the location of Ia epitope between 165-216 as described (Tikoo et al., 1993). However antigenicity analysis and amino acid sequence comparison of the extended region 165-226 with the corresponding region of BHV-5 gD identified a total of seven amino acid changes. Three amino acid changes between 216-222 and the single amino acid change at position 205 (BHV-1 gD) corresponded to potential antigenic regions which have been diminished on BHV-5 gD (Fig. 10). Two peptides were synthesized to cover amino acid residues 202-213 and 213-225. One peptide 202-213, reduced the neutralizing activity of MAb R54 in an in vitro neutralization inhibition assay (Fig. 13) and induced antisera in rabbits which specifically recognized the parent gD molecule (Fig. 12B) and has low neutralizing (>1:4) titer against BHV-1 indicating the location of the MAb R54 epitope and most probably the Ia epitope reported previously between amino acid residues 202-213. MAb 3402 which is highly neutralizing and

block viral penetration into susceptible cells (Dubuisson et al., 1992) has been assigned previously to a separate antigenic site (V) based on competition binding assays (Marshal et al., 1988). In this study we have mapped the 3402 epitope to a new linear antigenic site between amino acid residues 92-106. MAb 3402 recognized TrpE-gD fusion protein (52-115)(Fig. 2 and 3) specified by ORF subfragment ApaI-BanII (Fig. 3). Antigenic analysis and comparison of this region 52-115 (Fig. 10A) with BHV-5 gD as discussed above, revealed five amino acid changes. Four non-conservative amino acid changes occurred between residues 94-101 and corresponded to a large antigenic peak on BHV-1 gD which was completely absent on BHV-5 gD. A synthetic peptide covering this region 92-106 reduced the neutralizing activity of MAb 3402 (Fig. 13) and induced antisera in rabbits which specifically recognized the parent gD molecule and a 30K band. The 30K band matched a 30K BHV-1 gD break down product recognized by MAb R54 (Fig. 12B). Similar observations of anti-peptide sera strongly reactive with lower molecular size bands have been reported for gD of herpes simplex virus (HSV) (Weijer et al., 1988). Anti-peptide 92-106 sera has low (1:4) neutralizing titer against BHV-1 indicating that this peptide contains the 3402 epitope.

The partial reduction of MAb neutralizing ability by the peptides and the low neutralizing titer of anti-peptide sera against BHV-1 may be explained by low MAb affinity to peptides and weak avidity of rabbit antisera to the virus. Although these peptides carry the epitopes the level of immunoreactivity is influenced by several parameters. The particular "conformational" properties of the peptide antigen may vary from the native protein structure and thus reduce antibody binding. In addition the species difference of major histocompatibility complex (MHC) influences the recognition of a particular peptide antigen by the immune system (Berzofsky, 1990).

The linear epitopes identified in this study may be very important in immunity during natural infection since they induce highly neutralizing antibodies. MAbs 3402 (Dubuission et al., 1992) and R54 (unpublished observations) have been shown to neutralize the virus efficiently before or after attachment and to prevent virus attachment in absence of complement . These observations suggest that the two

epitopes may be critical to the gD molecule with respect to functional interaction with membranes and membrane-bound molecules. Further studies are required to determine the actual roles of these epitopes in the events of virus-cell interactions. In this study the expression of BHV-1 gD subfrgments in E.coli proved to be an effective tool for the identification of linear epitopes. Rabbit antisera raised to the bacterially-expressed N-terminal half of gD (TrpE-gD 1-216), recognized the authentic gD molecule from infected cell lysate. However the antisera did not neutralize the virus. To further determine the authenticity of the fusion protein, fifty field bovine sera previously tested for BHV-1 neutralization were retested for their reactivity with the fusion protein (1-216) on Western blots. All the neutralizing sera recognized the fusion protein with intensities that were proportional to their neutralizing titers. However two sera, which were negative by neutralization reacted with the protein which suggest that these animals have been previously exposed to the virus. The non-neutralizing antibody response to the fusion protein in rabbits may be due to the harsh treatment by DMSO during antigen preparation but not due to absence of glycosylation since this fusion protein contains at least two linear neutralizing epitopes. E.coli-expressed BHV-1 gD has been shown to elicit a low neutralizing antibody response in cattle attributed to the alteration of discontinuous epitopes due to absence of glycosylation and dimerization (van Drunen Little-van den Hurk et al., 1993).

Although both BHV-1 and BHV-5 are neurotropic viruses, only BHV-5 is able to cause neurological disease in calves. The BHV-1 and BHV-5 genomes share 85% DNA sequence homology (Engles et al., 1986), however restriction site maps of the two strains are markedly distinct (Mayfield et al., 1983; Engles et al., 1986; Bulach and Studdert, 1990), suggesting nucleotide sequence differences which may involve several viral genes. Previous studies with other herpesviruses have demonstrated that neurovirulence has a multigenic origen. Several glycoproteins of PRV and HSV are involved in neuropathogenesis including gD. A single aa change on gD has affected the neuroinvasiveness of the HSV-1 (Izumi and Stevens, 1990).

It has been in the interest of our laboratory to investigate the role of viral

glycoprotein genes in neuropathogenesis of BHV-5 by comparing and analyzing the molecular structure of these glycoproteins and understanding the functional relevance of their structural differences.

Our results have shown the BHV-5 gD gene to map in the short unique region (Us) of the viral genome between map units 0.887 - 0.897 which is approximately collinear and have the same orientation with respect to the genomic map as BHV-1 gD. The ORF of BHV-5 gD codes for 417 amino acid which is the same number of amino acids making the BHV-1 gD (Fig. 9). The seven cysteine residues which is a conserved feature among alphaherpesvirus gD homologues (Watson et al., 1982; Watson 1983; Petrovskis et al., 1986; Tikoo et al., 1990) aligned exactly between the BHV-1 and BHV-5 gD molecules indicating that they probably perform similar functions of stabilization and maintenance of the gD molecular structure. The N-terminal two-third (1-282 of) of the gD protein are relatively well conserved, however the C-terminal one third (283-417 of BHV-5 gD) is highly variable (Fig. 9). This region contains beside the putative transmembrane domain (355-382 of BHV-5 gD) a stretch of 72 amino acid long upstream between residue 283-354. As has been reported previously for BHV-1 gD in approximately the same region (281-295), BHV-5 gD contains a stretch of acidic amino acids plus several insertions and deletions resulting in a series of hydrophilic peaks compared to one broad peak for BHV-1 gD (Fig. 11). An other unique feature of this region on BHV-5 gD, the glycine content represent approximately 50% of the total amino acids content between residues 284-309. Proline-alanine repeats has been also described in approximately the same region for pseudorabies virus (PRV) gp50. These observations support the previous speculation (Tikoo et al., 1993) that this region may be involved in virus-host cell interactions. Further studies are in progress to define more precisely the function of these regions with respect to their role in infectivity, host-cell type susceptibility, immunity and neurovirulence.

FIGURES AND TABLES

Fig. 1. (A & B) Cloning strategy of BHV-1 gD gene. The BHV-1 gD gene was mapped to the HindIII-K fragment between map unit 0.871-0.933. A 4.35 Kb subfragment of the HindIII-K was cloned into the HindIII-XhoI digested pGEM-7Z plasmid. The 1.3 Kb MaeI subfragment containing the BHV-1 gD gene was cloned into pATH 23 plasmid for expression. The bold arrow indicate the gD ORF and the direction of transcription.

Fig. 2. SDS-PAGE and immunoblotting analysis of TrpE-gD fusion proteins. Coomassie blue-stained gel showing fusion proteins expressed by subfragments of gD ORF using pATH vector system (A) and immunoreactivity of the same proteins with MAb 3402 (B) and R54 (C). Lane 1 TrpE; lane 2 TrpE-gD (1-417); lane 3 TrpE-gD (1-216); lane 4 TrpE-gD (1-163); lane 5 TrpE-gD (52-163); lane 6 TrpE-gD (52-127); lane 7 TrpE-gD (1-50); lane 8 TrpE-gD (128-216); lane 9 TrpE-gD (117-163); and lane 10 TrpE-gD (165-216).

Fig. 3. Schematic representation of the BHV-1 gD glycoprotein and its segments which were expressed in pATH vectors. The MaeI fragment containing the gD ORF is depicted on top showing restriction endonuclease sites used for cloning into pATH plasmids. Glycoprotein D protein segments expressed are shown as solid bars identified by aa numbers on top. The aa indicate restriction sites on the gD ORF, however the actual amino acids expressed in each protein are discussed in text and legend of Fig. 2. Summary of the immunoreactivity of gD protein segments with MAb R54 and 3402 are shown on the table to the right as (+) to denote reactivity and (-) for no reactivity.

Fig. 4. Immunogenicity of TrpE-gD fusion protein expressing the N-terminal 216 amino acids. Fig. 4A shows the immunoblot reactivity of anti-TrpE-gD (1-216) rabbit sera with TrpE protein alone (lane 1), TrpE-gD (1-216) protein (lane 2), BHV-1-infected cells (lane 3), and mock-infected cells (lane 4). Fig. 4B shows the immunoblot reactivity of representative samples of field bovine sera against TrpE-gD fusion protein. BHV-1 non-neutralizing (lane 1) and neutralizing (lane 2, 3, and 4) bovine sera. A control MAb 3402 is shown in lane 5.

Fig. 5. Identification of the BamHI-C genomic fragment carrying the BHV-5 gD gene. Panel (A) is an autoradiograph of southern blot from the agarose gel on panel (B) after hybridization with ^{35}S-labelled BHV-1 gD containing probe. Lanes 1 and 6 contains HindIII restriction digestion of BHV-1 DNA, lanes 2 and 5 contains BamHI-C clone of BHV-5 (TX89 strain), and lane 3 and 4 contains BamHI restriction digestion of BHV-5 (EC-STR strain). White arrows in panel B indicate fragments which hybridized.

Fig. 6. Identification of the MaeI fragment carrying the BHV-5 gD gene. Panel (A) is an autoradiograph of southern blot from the gel on panel (B) after hybridization with ^{35}S-labelled BHV-1 gD containing probe. Panel (B) contains various restriction endonuclease digestion of the BamHI-C clone. Restriction endonucleases used are shown on top of each lane. Control 1.3 Kb MaeI fragment containing the BHV-1 gD gene is shown on lane C. Fragments hybridized are indicated by arrows.

Fig. 7. The BamHI restriction site map of BHV-5 DNA and the cloning and sequencing strategies of BHV-5 gD gene. The genomic organization of BHV-5 (TX89) strain, depicted on top, consists of unique long (U_L) and short (U_S) regions and two repeat regions (I_R and T_R). The BamHI restriction endonuclease map was originally described for the (N569) strain of BHV-5 by Engles et al 1986.The gD gene of BHV-5 was mapped to the BamHI-C (14.6 Kb) genomic fragment between m.u 0.827 and 0.944 and then to MaeI fragment (1620 bp) between m.u 0.886 and 0.899 by subcloning and hybridization with radiolabeled BHV-1 gD gene DNA probe. A restriction site map of the MaeI (1620 bp) fragment was generated and various restriction endonuclease subfragments, indicated as bold lines, were cloned and sequenced. The arrow represents the BHV-5 gD ORF which is 1,251 nucleotide long starting with ATG codon and terminating with TGA. The arrow head shows the direction of transcription.

Fig. 8. DNA sequence and predicted amino acid sequence of BHV-5 gD. The DNA sequence of the gene described in the legend of fig. 4 is shown. The putative CAAT and TATA sequences are boxed and underlined by asterisks respectively. Restriction endonuclease cleavage sites are indicated above the sequences and underlined. Below

the DNA sequence is the deduced amino acid sequence of the ORF encoding BHV-5 gD in standard three-letter code.

Fig. 9. Amino acid sequence comparison of BHV-1 and BHV-5 glycoproteins D. The predicted amino acid sequences of the BHV-1 and BHV-5 gD glycoproteins were aligned and compared by Seqaid II DNA and protein analysis software using ktup value = 2. Amino acid numbering starts at the first methionine and numbers are shown to the right. The amino acid sequences of BHV-1 gD recognized by MAb 3402 and R54 on western blot are underlined. Amino acids aligned identically between the two sequences are indicated by two dots and in case of cysteine residues by asterisks. Potential N-linked glycosylation sites are shown by ΔΔΔ. The signal sequences are indicated by broken lines and the transmembrane anchor sequences are indicated by double lines.

Fig. 10. Comparison of antigenicity plots of epitope-bearing amino acid regions of BHV-1 gD with the corresponding regions of BHV-5 gD. Antigenic analyses of amino acid regions of BHV-1 gD recognized by MAb 3402 (A) and MAb R54 (B) are shown on the bottom whereas the corresponding regions of BHV-5 gD antigenicity analyses are shown on top of each figure. The antigenicity score and amino acid sequences are shown on the vertical and horizontal axes respectively. Amino acid changes occurred on BHV-5 gD are indicated by arrows. Synthetic peptides sequences bearing-epitopes for MAb 3402 (92-106) and MAb R54 (202-213) are underlined.

Fig. 11. Comparison of the hydropathicity plots of the amino acid sequences of BHV-1 and BHV-5 glycoproteins D. The BHV-5 and BHV-1 gD amino acid sequences were analyzed for hydropathicity characteristics (Kyte and Doolittle, 1982) using a nine-amino acid window. The BHV-5 gD is shown on top and the BHV-1 gD (Tikoo et al., 1990) is shown on the bottom of the figure. The hydropathic scores and the amino acid numbers are shown on the vertical and horizontal axes respectively.

Fig. 12. Immunoreactivity of anti-peptide rabbit sera with peptides and BHV-1 proteins. Anti-peptide (92-106) and (202-213) rabbit sera were tested for BHV-1 and peptide specificities by slot blot ELISA (A). Each anti-peptide serum reacted

specifically with its immunizing peptide and with the virus. The slots were coated with 40 μg (lane 1) and 8 μg (lane 2) of peptide or purified virus protein and the bound proteins were determined by Ponceau S protein staining (Sigma). The anti-peptide sera were also tested by western blot (B) against BHV-1-infected MDBK cells lane 1 (anti-peptide 202-213) and lane 3 (anti-peptide 92-106) and against mock-infected cells (lanes 2 and 4). Positive control reaction of MAb R54 against BHV-1-infected cells is shown on lane 5.

Fig. 13. Inhibition of monoclonal antibody neutralization by peptides. Five-fold dilutions of peptides starting at a concentration of 25 μg/ml of DMEM were incubated with equal volumes of limiting MAb dilution (1:12,000) and allowed to react for 90 minutes at 37°C. BHV-1 was added to a final number of 100 pfu/200 μl of the mixture and incubated for an additional 1 hr and the neutralization assay was continued as described in materials and methods. The ability of each peptide to reduce the neutralizing activity of MAb was recorded as % virus plaques restored. Each bar represent an average of quadruplicates of readings. The legends are shown on the upper right of the figure.

Map Location and Cloning Strategy of the BHV-1.1 Glycoprotein IV Gene

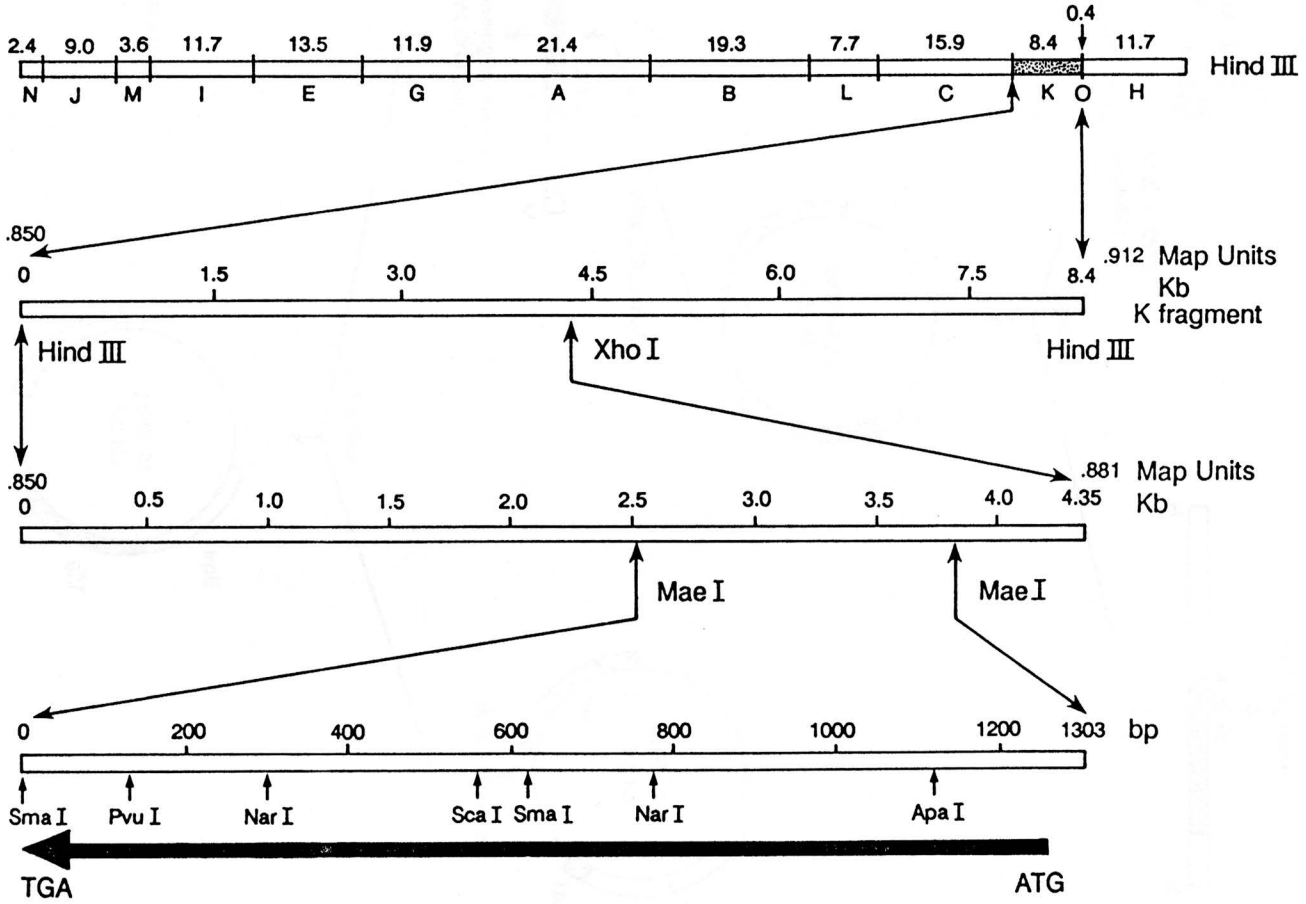


Fig. 1A.

Cloning Strategy and Expression of BHV-1.1 Glycoprotein IV Gene

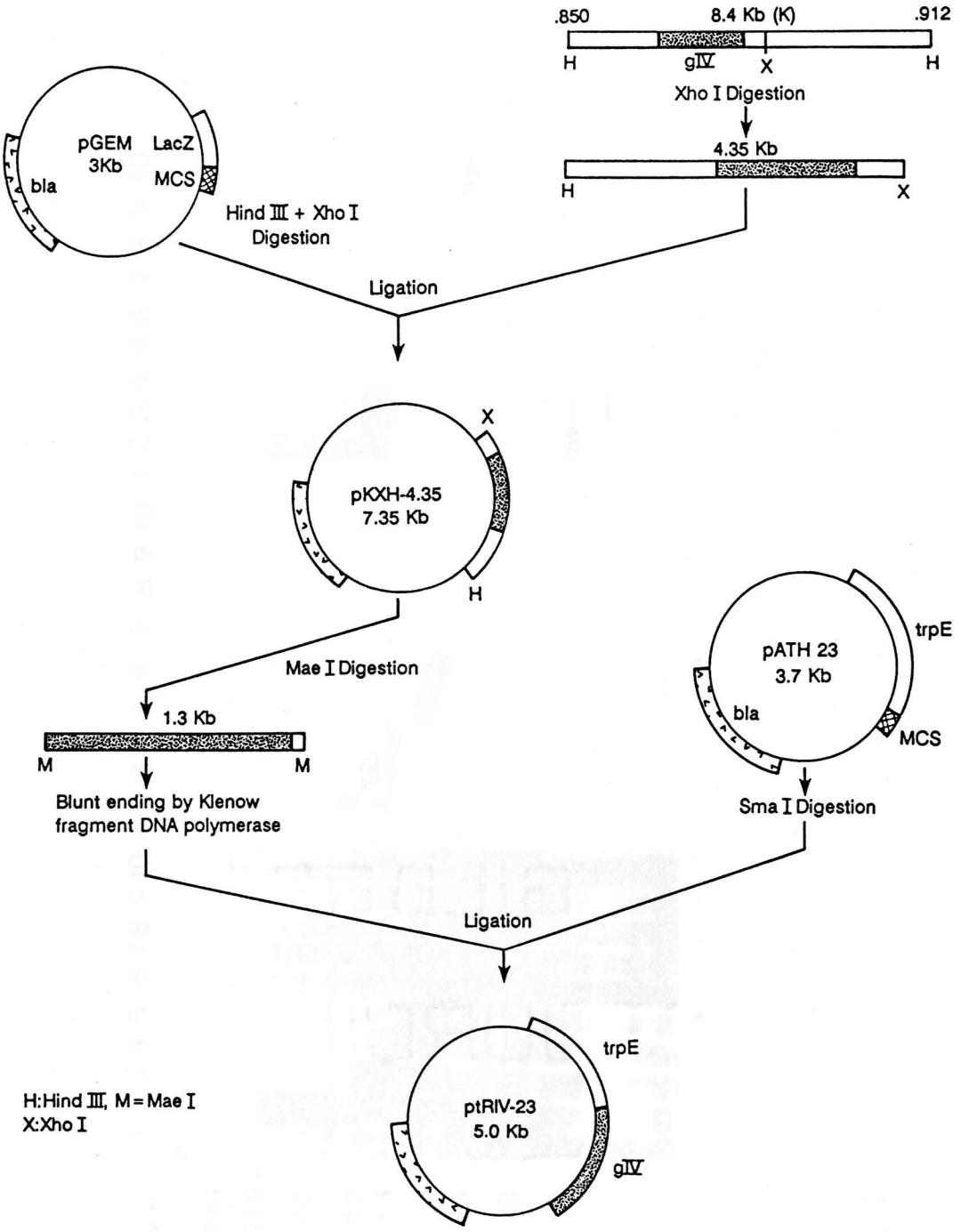


Fig. 1B.

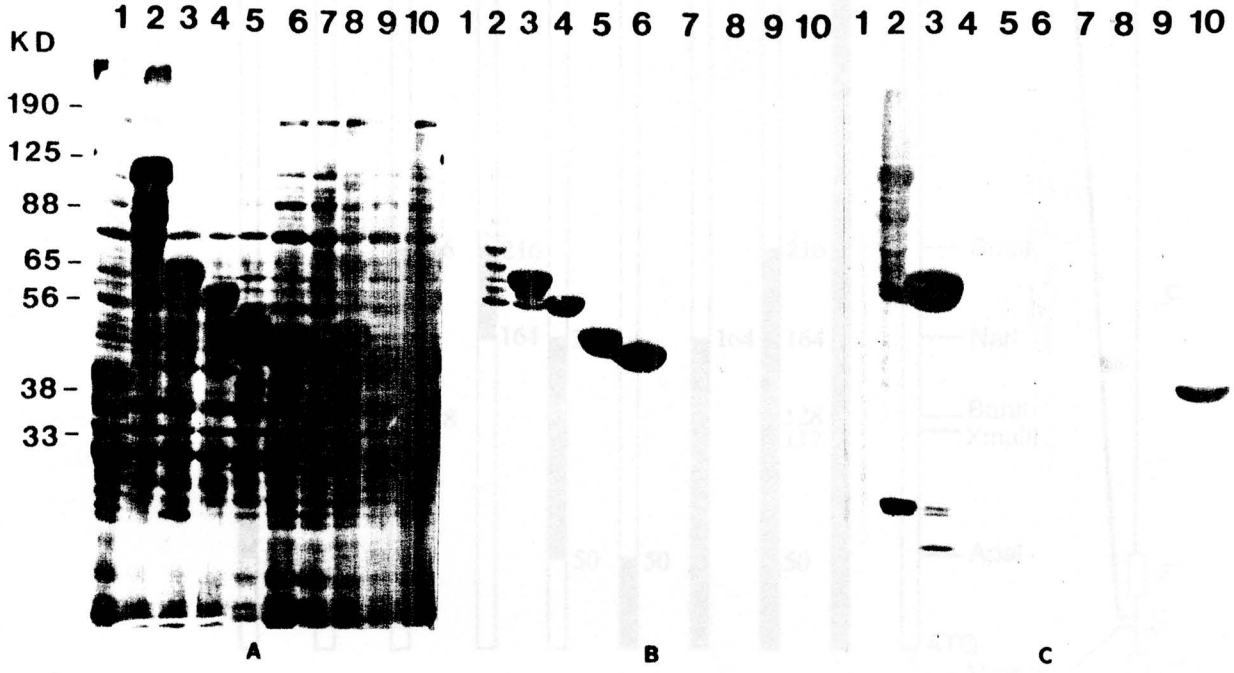
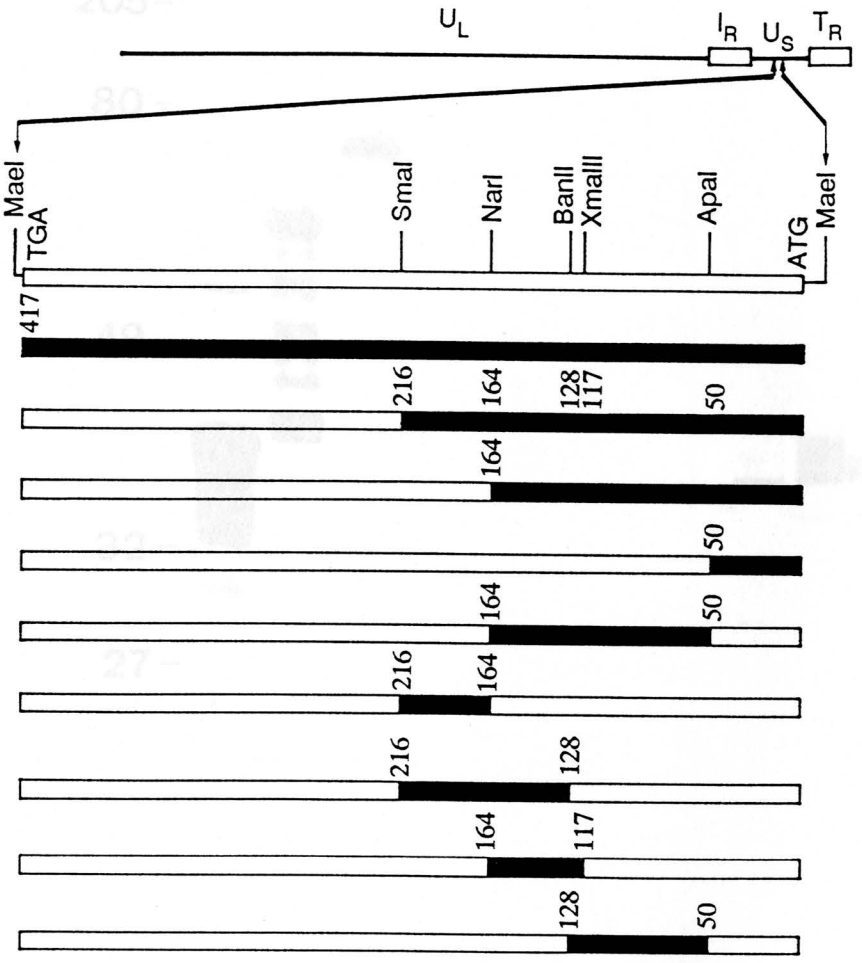

KD
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
190 -
125 -
88 -
65 -
56 -
38 -
33 -
A
B
C

Fig. 2.



R54	3402
+	+
+	+
–	+
–	–
–	+
+	–
+	–
–	–
–	+

Fig. 3.

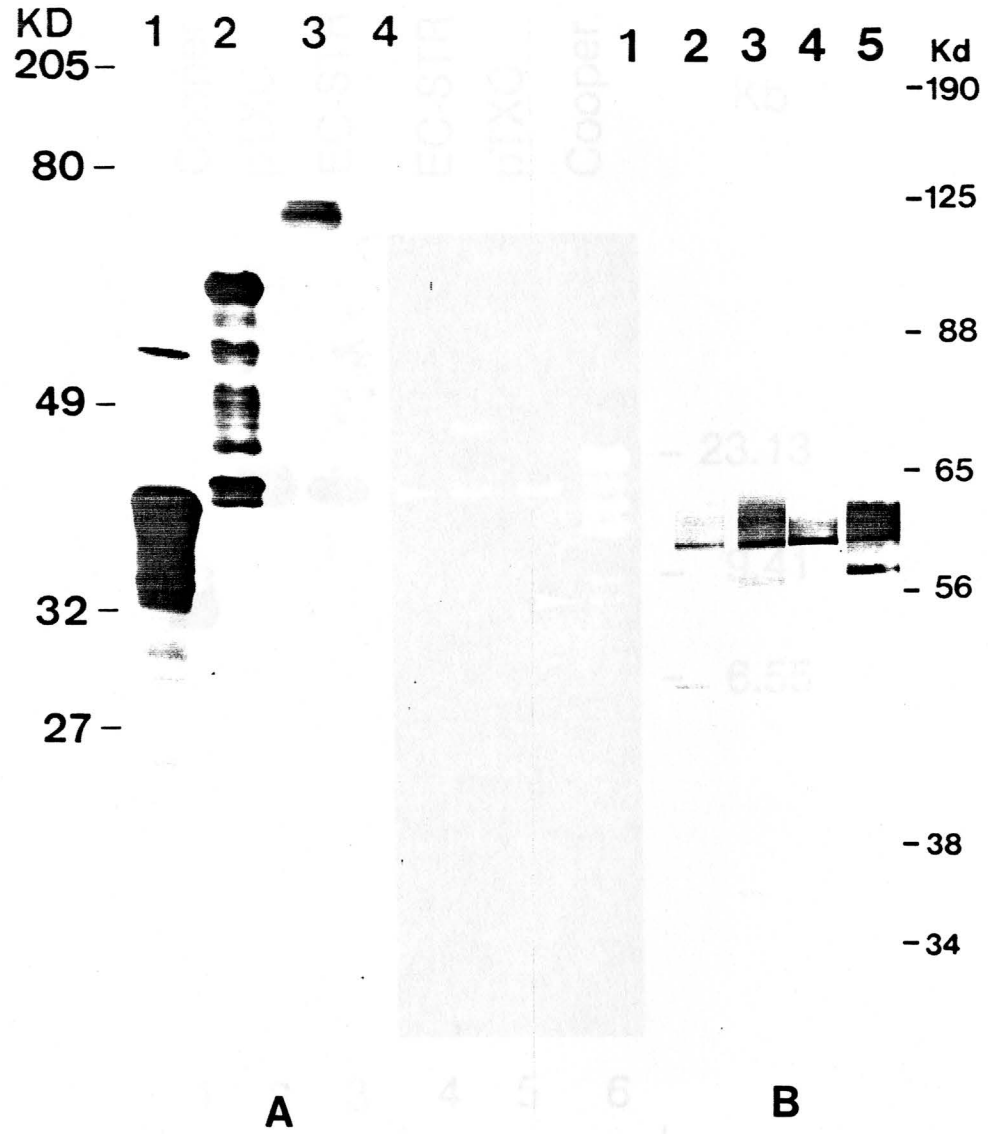


Fig. 4.

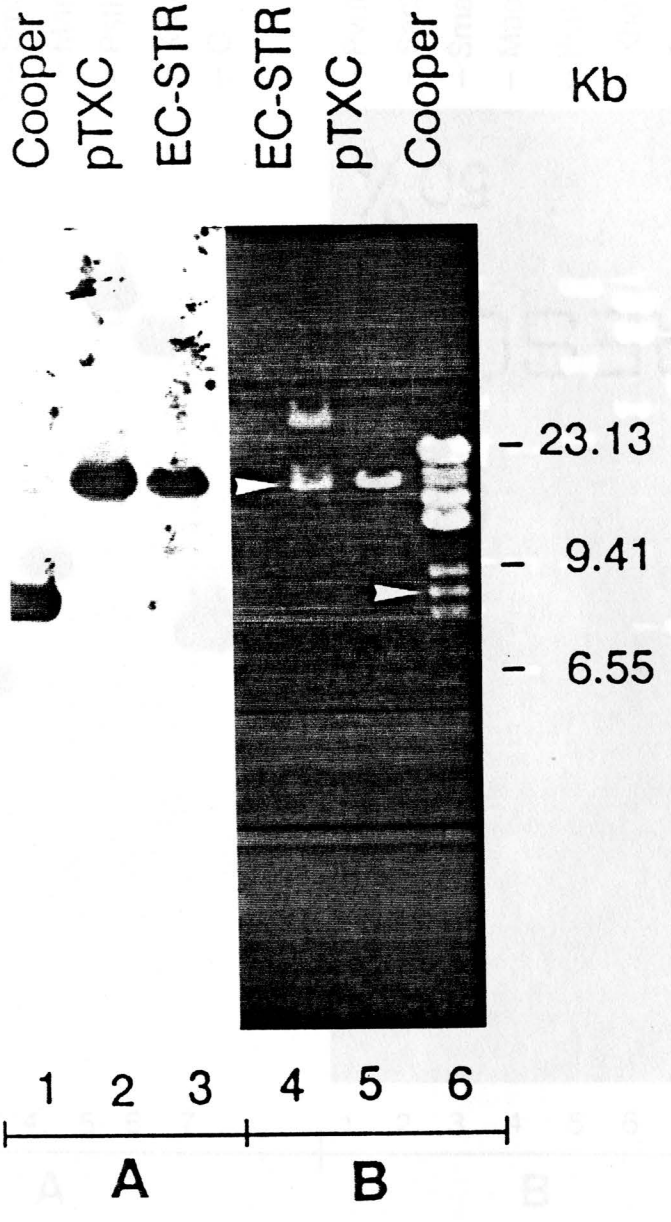
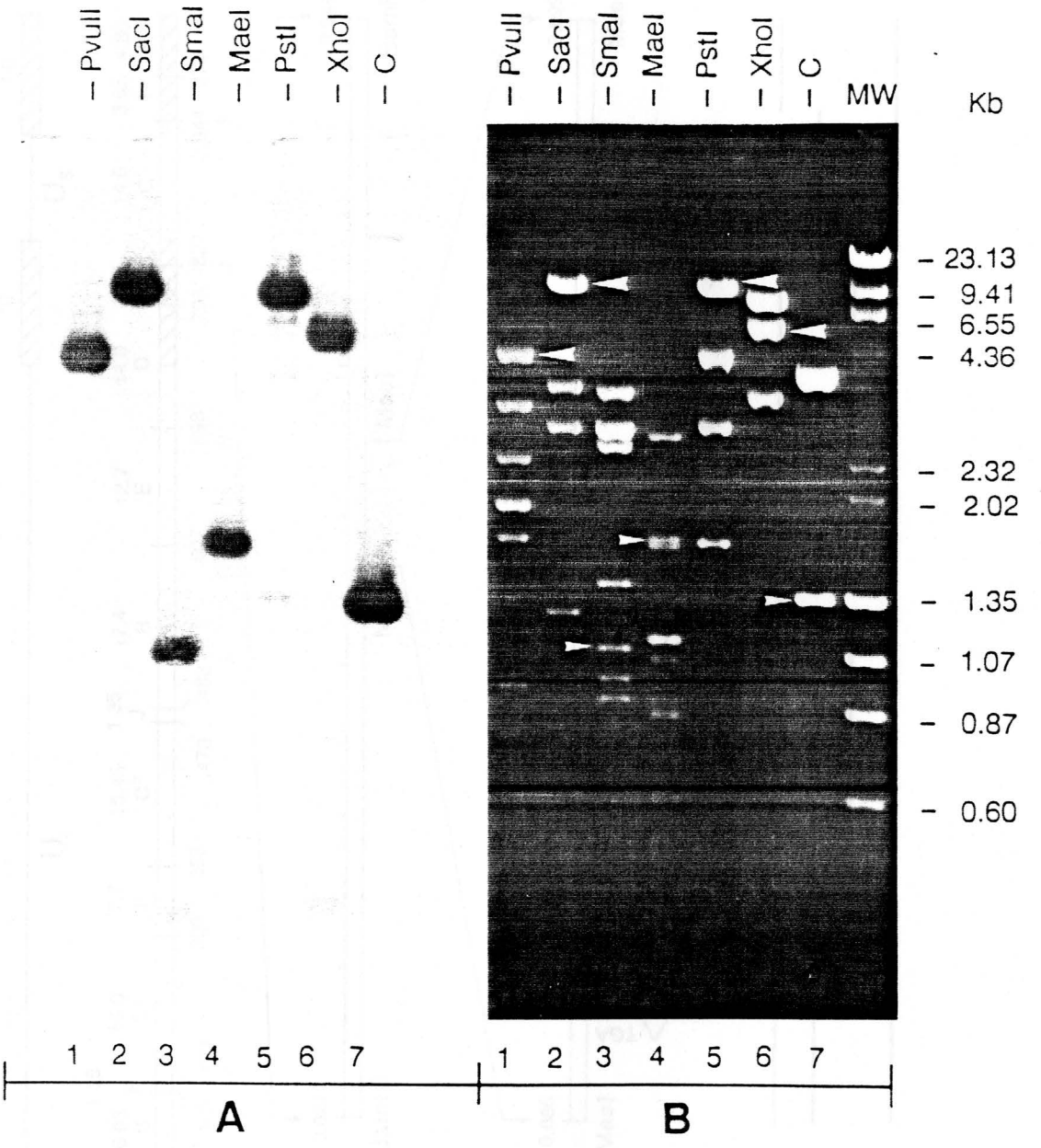

Cooper
pTXC
EC-STR
EC-STR
pTXC
Cooper
Kb
23.13
9.41
6.55
1 2 3 4 5 6
A
B

Fig. 5.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– SacI
– SmaI
– MaeI
– PstI
– XhoI
– C
– PvuII
– SacI
– SmaI
– MaeI
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– XhoI
– C
MW
Kb
– 23.13
– 9.41
– 6.55
– 4.36
– 2.32
– 2.02
– 1.35
– 1.07
– 0.87
– 0.60
1 2 3 4 5 6 7
A
1 2 3 4 5 6 7
B

Fig. 6.

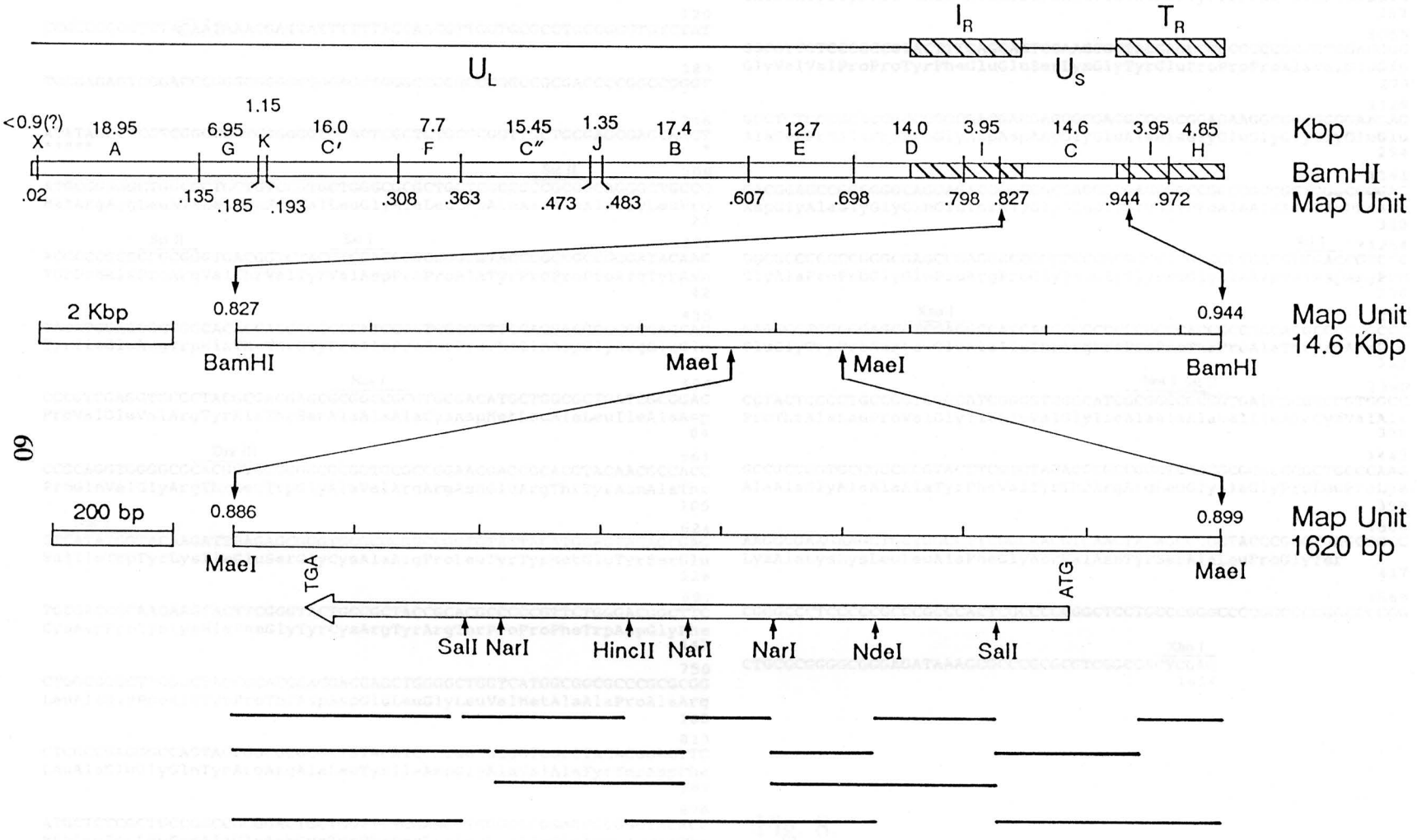
I_R
T_R
U_L
U_S
<0.9(?) X
18.95 A
1.15
6.95 G
K
16.0 C′
7.7 F
15.45 C″
1.35 J
17.4 B
12.7 E
14.0 D
3.95 I
14.6 C
3.95 I
4.85 H
Kbp
BamHI Map Unit
.02
.135
.185
.193
.308
.363
.473
.483
.607
.698
.798
.827
.944
.972
2 Kbp
0.827
0.944
Map Unit 14.6 Kbp
BamHI
MaeI
MaeI
BamHI
200 bp
0.886
0.899
Map Unit 1620 bp
MaeI
MaeI
TGA
ATG
SalI
NarI
HincII
NarI
NarI
NdeI
SalI

Fig. 7.

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                                                                      57
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                                                                     120
CCGCCCGCGTTTACAATAAACGATTATTTTTTACCAACGTTGGTGCGCCTGCCGCGTGTCTAT

                                                                     183
TGCGAGAGTCGGACCCGGGCGGGGCGGGAGCTGGGCCGGGCCGGGCCGCGACCCCGGCCGGGT

                                                                     246
ATATAGCCTCGTCGGGCGGTCGGGGGTGCACTCGCTCTGCGCGGTTGCTGCGAGCGAGCAACT
*****                                                             *

                                                  Sst II             309
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MetArgArgLeuAlaLeuLeuSerValLeuGlyAlaLeuLeuAlaAlaAlaAlaGlyLeuPro
                                                                      21
          Sst II                        Sal I                       372
ACGCCCGCGCCGCGGGTGACGGTCTACGTCGACCCGCCGGCGTACCCGCCGCCGCGATACAAC
ThrProAlaProArgValThrValTyrValAspProProAlaTyrProProProArgTyrAsn
                                                                      42
                                                                     435
TACATCGAGCGCTGGCACACCACCGGGCCCATCCCGTCGCCCTTCCAGGACGGCCGCGAGCAG
TyrIleGluArgTrpHisThrThrGlyProIleProSerProPheGlnAspGlyArgGluGln
                                                                      63
                                  Not I                              498
CCCGTCGAGGTGCGCTACGCGACGAGCGCGGCCGCGTGCGACATGCTGGCGCTGATCGCGGAC
ProValGluValArgTyrAlaThrSerAlaAlaAlaCysAspMetLeuAlaLeuIleAlaAsp
                                                                      84
                    Dra III                                          561
CCGCAGGTGGGGCGCACGCTGTGGGGCGCGGTGCGCCGGAACGAGCGCACGTACAACGCCACG
ProGlnValGlyArgThrLeuTrpGlyAlaValArgArgAsnGluArgThrTyrAsnAlaThr
                                                                     105
  Ndc I                                                              624
GTCATATGGTACAAGATTGAGAGCGGGTGCGCCCGGCCGCTGTACTACATGGAGTACAGCGAG
ValIleTrpTyrLysIleGluSerGlyCysAlaArgProLeuTyrTyrMetGluTyrSerGlu
                                                                     126
                                                                     687
TGCGACCCCAAGAAGCACTTCGGGTACTGCCGCTACCGCACGCCCCCGTTCTGGGACGGCTTC
CysAspProLysLysHisPheGlyTyrCysArgTyrArgThrProProPheTrpAspGlyPhe
                                                                     147
                                                                     750
CTGGCGGGCTTCGCCTACCCCACGGACGACGAGCTGGGGCTGGTCATGGCGGCGCCCGCGCGG
LeuAlaGlyPheAlaTyrProThrAspAspGluLeuGlyLeuValMetAlaAlaProAlaArg
                                                                     168
                                                                     813
CTCGCCGAGGGCCAGTACCGGCGCGCGCTGTACATCGACGGCGCGGTCGCCTACACGGACTTC
LeuAlaGluGlyGlnTyrArgArgAlaLeuTyrIleAspGlyAlaValAlaTyrThrAspPhe
                                                                     189
                                                                     876
ATGCTCTCGCTGCCGGCCGGGGACTGCTGGTTCTCGAAGCTCGGCGCGGAGCGCGGGTACACC
MetLeuSerLeuProAlaGlyAspCysTrpPheSerLysLeuGlyAlaGluArgGlyTyrThr
                                                                     210
                                                                     939
TTCGGCGCCTGCTTCCCGATCCAGGACTACGAGCAGGGCAAGGTCCTGCGCCTGACGTACCTC
PheGlyAlaCysPheProIleGlnAspTyrGluGlnGlyLysValLeuArgLeuThrTyrLeu
                                                                     231
```

```
  Sca I                                                             1002
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                                                                     252
                                                                    1065
GGCGTCGTCCCGCCGTACTTTGAGGAGTCGAAGGGCTACGAGCCGCCGCCCGCCGTCGAGGGG
GlyValValProProTyrPheGluGluSerLysGlyTyrGluProProProAlaValGluGly
                                                                     273
                                                                    1128
GCCTCTCCCGCACCGCCCGGCGACGACGACGGCGAGGCCCACGGAGAAGGCGGCGGGGAAGAG
AlaSerProAlaProProGlyAspAspAspGlyGluAlaHisGlyGluGlyGlyGlyGluGlu
                                                                     294
                                                                    1191
GACGGAGCCGGCGGGCAGGAGACCGGCGGCGAGGGGGAGGGCCCGGCCGCCGCTGGGCCCGAC
AspGlyAlaGlyGlyGlnGluThrGlyGlyGluGlyGluGlyProAlaAlaAlaGlyProAsp
                                                                     315
                                                  Sal I              1254
GGCGCCCCCCCGGGCGAGCCGAGGCCCGGCCCCGGCGGCCCCGGCGCGGACGTCGACCGCCCC
GlyAlaProProGlyGluProArgProGlyProGlyGlyProGlyAlaAspValAspArgPro
                                                                     336
                  Xho I                                             1317
GAGGGCTGGCCGAGCCTCGAGGCCATCACGCGGCCCCCGCCCACCCCCGCCACGCCCGCGCCC
GluGlyTrpProSerLeuGluAlaIleThrArgProProProThrProAlaThrProAlaPro
                                                                     357
                                  Not I Sst II                      1380
CCTACTGCCCTGCCGGTCGGCATCGGGGTCGGCATCGCGGCCGCGGTGATCGCGTGCGTGGCC
ProThrAlaLeuProValGlyIleGlyValGlyIleAlaAlaAlaValIleAlaCysValAla
                                                                     378
                                                                    1443
GCCGCCGGTGCCGCCGCGTACTTCGTCTACACGCGCCGGCTCGGCGCGGGCCCGCTGCCCAAG
AlaAlaGlyAlaAlaAlaTyrPheValTyrThrArgArgLeuGlyAlaGlyProLeuProLys
                                                                     399
                                                                    1506
AAGGCGAAGAAGCTGCTGGCCTTCGGCAACGTCAACTACAGCGCGCTACCCGGGTGAGCGGCC
LysAlaLysLysLeuLeuAlaPheGlyAsnValAsnTyrSerAlaLeuProGlyTer
                                                                     417
                                                                    1569
CGGGCCCTCCCCCGCCCGCCCACTCGCCCCCGGCTCCTGCCCGGGCCCCGGCCCCGGCCCCGG

                                                  Xho I
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                                                                    1614
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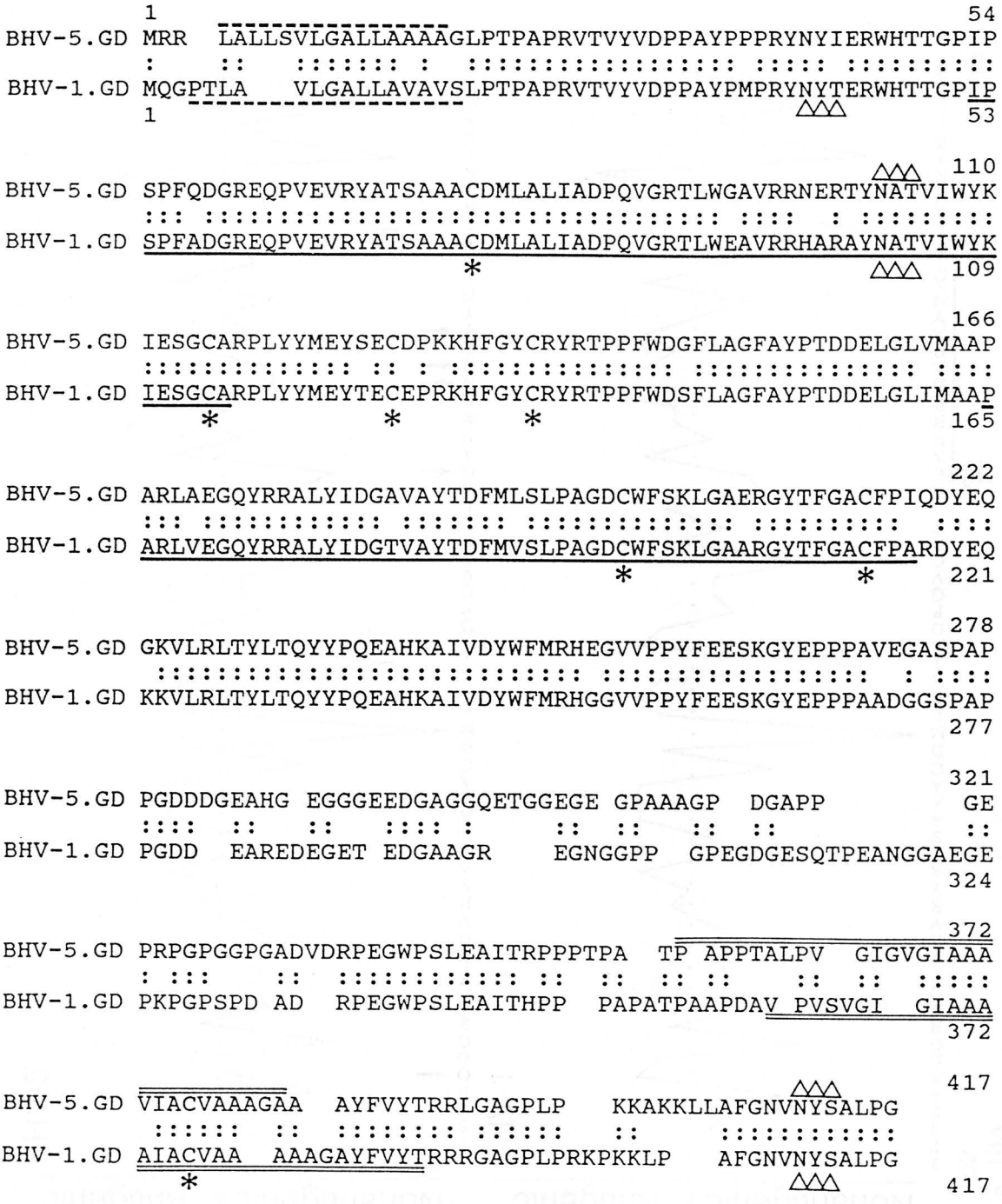
Fig. 8.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BHV-1.GD MQGPTLA VLGALLAVAVSLPTPAPRVTVYVDPPAYPMPRYNYTERWHTTGPIP
BHV-5.GD SPFQDGREQPVEVRYATSAAACDMLALIADPQVGRTLWGAVRRNERTYNATVIWYK
BHV-1.GD SPFADGREQPVEVRYATSAAACDMLALIADPQVGRTLWEAVRRHARAYNATVIWYK
BHV-5.GD IESGCARPLYYMEYSECDPKKHFGYCRYRTPPFWDGFLAGFAYPTDDELGLVMAAP
BHV-1.GD IESGCARPLYYMEYTECEPRKHFGYCRYRTPPFWDSFLAGFAYPTDDELGLIMAAP
BHV-5.GD ARLAEGQYRRALYIDGAVAYTDFMLSLPAGDCWFSKLGAERGYTFGACFPIQDYEQ
BHV-1.GD ARLVEGQYRRALYIDGTVAYTDFMVSLPAGDCWFSKLGAARGYTFGACFPARDYEQ
BHV-5.GD GKVLRLTYLTQYYPQEAHKAIVDYWFMRHEGVVPPYFEESKGYEPPPAVEGASPAP
BHV-1.GD KKVLRLTYLTQYYPQEAHKAIVDYWFMRHGGVVPPYFEESKGYEPPPAADGGSPAP
BHV-5.GD PGDDDGEAHG EGGGEEDGAGGQETGGEGE GPAAAGP DGAPP GE
BHV-1.GD PGDD EAREDEGET EDGAAGR EGNGGPP GPEGDGESQTPEANGGAEGE
BHV-5.GD PRPGPGGPGADVDRPEGWPSLEAITRPPPTPA TP APPTALPV GIGVGIAAA
BHV-1.GD PKPGPSPD AD RPEGWPSLEAITHPP PAPATPAAPDAV PVSVGI GIAAA
BHV-5.GD VIACVAAAGAA AYFVYTRRLGAGPLP KKAKKLLAFGNVNYSALPG
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Fig. 9.

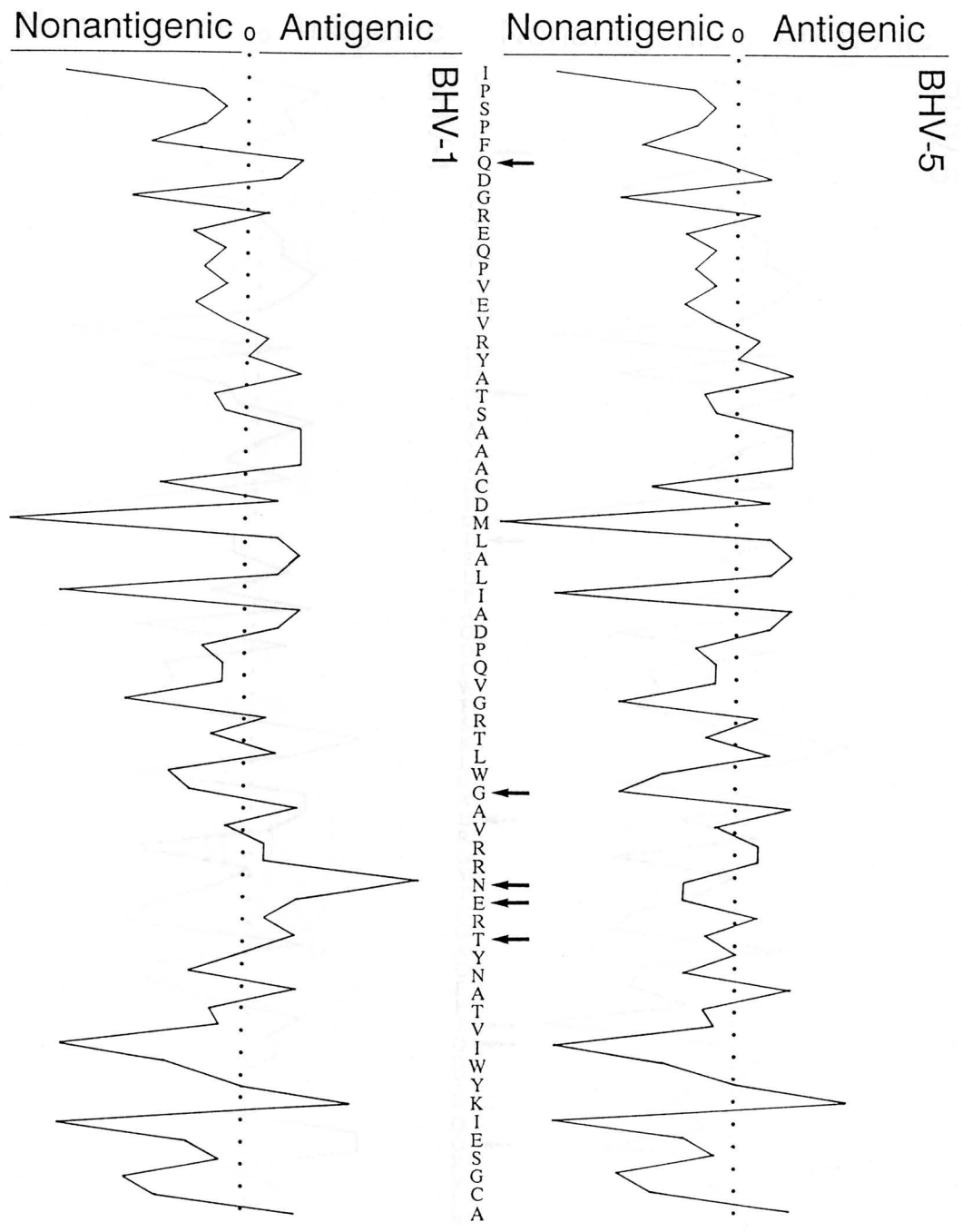
Nonantigenic
0
Antigenic
BHV-5
BHV-1
I P S P F Q D G R E Q P V E V R Y A T S A A A C D M L A L I A D P Q V G R T L W G A V R R N E R T Y N A T V I W Y K I E S G C A
I P S P F A D G R E Q P V E V R Y A T S A A A C D M L A L I A D P Q V G R T L W E A V R R H A R A Y N A T V I W Y K I E S G C A
52
92
106
115

Fig. 10A.

Nonantigenic
0
Antigenic
BHV-5
P A R L A E G Q Y R R A L Y I D G A V A Y T D F M L S L P A G D C W F S K L G A E R G Y T F G A C F P I Q D Y E Q G K V L R
Nonantigenic
0
Antigenic
BHV-1
P A R L V E G Q Y R R A L Y I D G T V A Y T D F M V S L P A G D C W F S K L G A A R G Y T F G A C F P A R D Y E Q K K V L R
165
202
213
226

Fig. 10B.

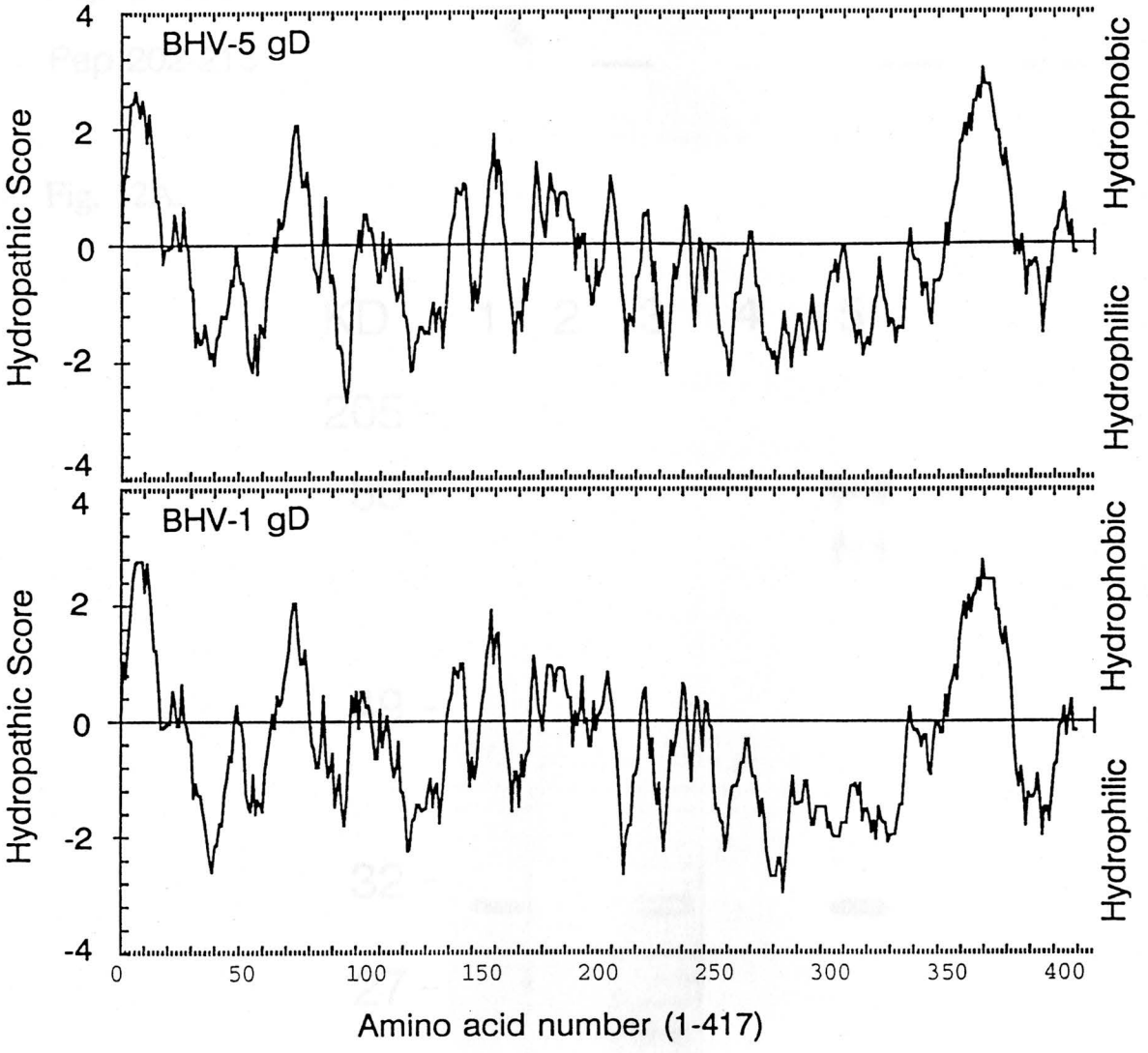
BHV-5 gD
BHV-1 gD
Hydropathic Score
Hydrophobic
Hydrophilic
4
2
0
-2
-4
0
50
100
150
200
250
300
350
400
Amino acid number (1-417)

Fig. 11.

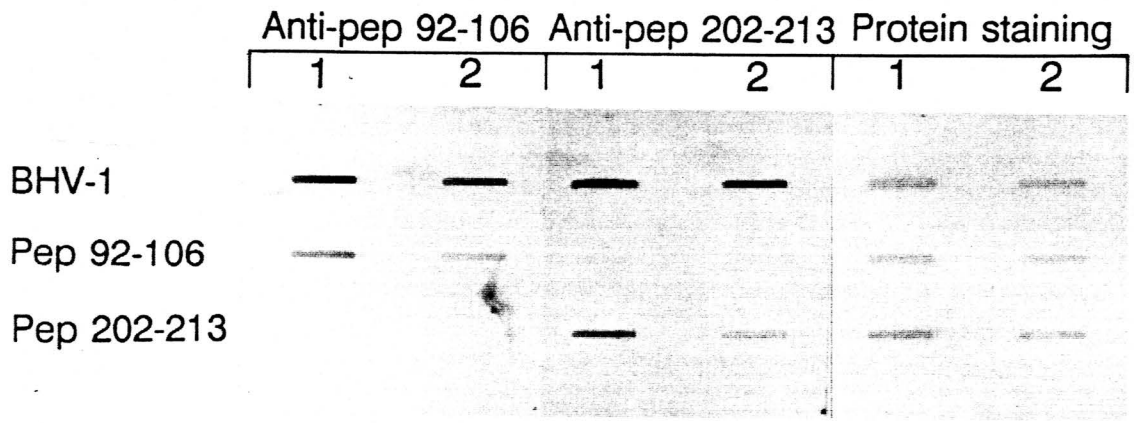

Fig. 12A.

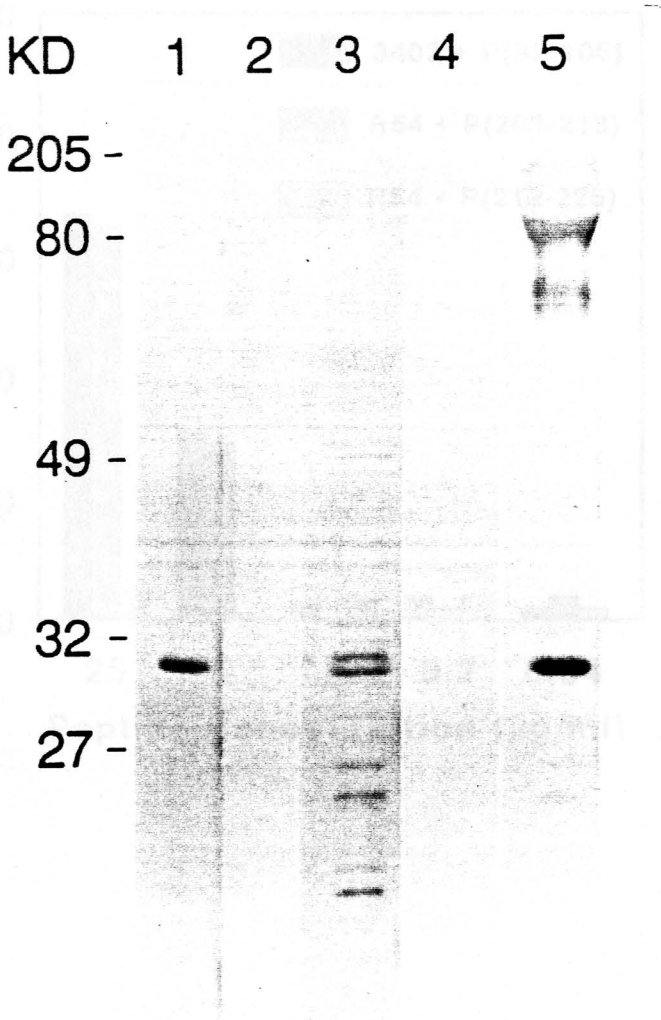

Fig. 12B.

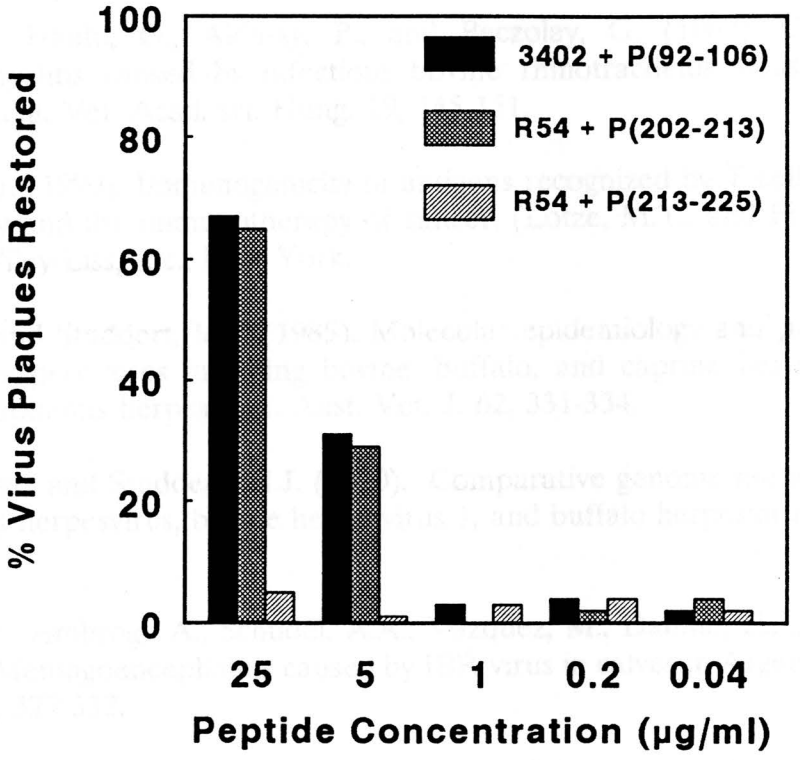
100
80
60
40
20
0
% Virus Plaques Restored
3402 + P(92-106)
R54 + P(202-213)
R54 + P(213-225)
25
5
1
0.2
0.04
Peptide Concentration (µg/ml)

Fig. 13.

REFERENCES

Abdelmagid, O.Y., Orten, D.J., Xue, W., Blecha, F., and Minocha, H.C. (1992). Anti-idiotypic antibodies to bovine herpesvirus-1 inhibit virus infection in cell cultures. Arch. Virol. 122, 163-173.

Babiuk, L.A., L'italien, J., van Drunen Little-van Den Hurk, S., Zamb, T.J., Lawman M.P.J, Hughes, G., and Gifford, G.A. (1987). Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual glycoproteins. Virol. 159, 57-66.

Barany, G., and Merrifield, R.B. (1980). Solid-phase peptide synthesis. In, The peptides, (E.Gross and J. Meienhofer, Eds.), Vol.1, pp. 1-284. Academic Press, New York.

Bartha, A., Haidu, G., Aldassy, P., and Paczolay, G. (1969). Occurrence of encephalomyelitis caused by infectious bovine rhinotracheitis virus in calves in Hungary. Acta. Vet. Acad. sci. Hung. 19, 145-151.

Berzofsky, J. (1990). Immunogenicity of antigens recognized by T-cells. In, Cellular immunology and the immunotherapy of cancer, (Lotze, M.T., and Finn, O.J., eds), pp. 1-22. Wiley-Liss, Inc., New York.

Brake, F., and Studdert, M.J. (1985). Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo, and caprine herpesvirus 1 and bovine encephalitis herpesvirus. Aust. Vet. J. 62, 331-334.

Bulach, D.M., and Studdert, M.J. (1990). Comparative genome mapping of bovine encephalitis herpesvirus, bovine herpesvirus 1, and buffalo herpesvirus. Arch. Virol. 113, 17-34.

Carillo, B.J., Ambrogi, A., Schudel, A.A., Vazquez, M., Dahme, E., and Pospischil, A. (1983). Meningoencephalitis caused by IBR virus in calves in Argentina. Zbl. Vet. Med. B 30, 327-332.

Carillo, B.J., Pospischil, A., and Dahme, E. (1983). Pathology of bovine necrotizing encephalitis in Argentina. Zbl. Vet. Med. B 30, 161-168.

Collins, J.K., Butcher, A.C., Riegel, C.A., McGrane, V., Blair, C.D., Taramoto, Y.A., and Winston, S. (1984). Neutralizing determinant defined by monoclonal antibodies on polypeptides specified by bovine herpesvirus 1. J. Virol. 52, 403-409.

Dieckmann, C.L.,and Tzagoloff, A. (1985). Assembly of mitochondrial membrane system. CBP6, a yeast nuclear gene necessary for synthesis of cytochrome b. J. Biol.

Chem. 260, 1513-1520.

Dubuisson, J., Israel, B.A., and Letchworth III, G.J. (1992). Mechanisms of bovine herpesvirus type 1 neutralization by monoclonal antibodies to glycoproteins gI, gIII, and gIV. J. Gen. Virol. 73, 1-9.

Engles, M., Giuliani, C., Wild, P., Beck, T.M., Loepfe, E., and Wyler, R. (1986). The genome of bovine herpesvirus 1 (BHV-1) exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. Virus Research 6, 57-73.

Eugster, A.K., Angulo, A.A., and Jones, L.P. (1974). Herpesvirus encephalitis in range calves. In, Proceedings, 17th Ann. Meet. Amer. Asso. Vet. Lab. Diagnostician, pp. 267-290.

French, E.L. (1962). A specific virus encephalitis in range calves: Isolation and characterization of the causal agent. Aust. Vet. J. 38, 555-556.

Friedli, K., and Metzler, A.E. (1987). Reactivity of monoclonal antibody to proteins of neurotropic bovine herpesvirus 1 (BHV-1) strain and to proteins of representative BHV-1 strains. Arch. Virol. 94, 109-122.

Gibbs, E.P.J., and Rweyemamu, M.M. (1977). Bovine herpesvirus. I. Bovine herpesvirus-1. Vet. Bull. (London) 47, 317-343.

Glorioso, J., Schroder, C.H., Kimel, G., Szczesiul, M., and Levine, M. (1984). Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. J. Virol. 50, 805-812.

Highlander, S.L, Sutherland, S.I., Gage, P.J., Johnson, D.C., Levine, M., and Glorioso, J.C. (1987). Neutralizing monoclonal antibodies for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. 61, 3356-3364.

Hughes, G., Babiuk, L.A., and van Drunen Little-van den Hurk, S. (1988). Functional and topographical analysis of epitopes on bovine herpesvirus type 1 glycoprotein IV. Arch. Virol. 103, 47-60.

Hutchings, D.L., van Drunen Little-van den Hurk, S., and Babiuk, L.A. (1990). Lymphocyte proliferative responses to separate bovine herpesvirus-1 proteins in immune cattle. J. Virol. 64, 5114-5122.

Izumi, K.M., and Stevens, J.G. (1990). Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. J. Exp. Med. 172, 487-496.

Johnson, D.C., and Ligas, M.W. (1988). Herpes simplex virus lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. 62, 4605-4712.

Koerner, T.J., Hill, J.E., Myers, A.M., and Tzagoloff, A. (1991). High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. Methods in Enzymology. 194, 477-490.

Kornfeld, R., and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54, 631-664.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of protein. J. Mol. Biol. 157, 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.

Lawrence, W.C., D'urso, R.C., Kundel, C.A., Whitbeck, J.C., and Bello, L.J. (1986). Map location of the gene for a 130,000-dalton glycoprotein of bovine herpesvirus 1. J. Virol. 60, 405-414.

Little, S.P., Jofre, J.T., Courtney, R.J., and Schaffer, P.A. (1981). A virion-associated glycoprotein essential for infectivity of herpes simplex type 1. Virol. 115, 149-160.

Lupton, H.W., and Reed, D.E. (1980). Evaluation of experimental subunit vaccines for infectious bovine rhinotracheitis. Am. J. Vet. Res. 41, 383-390.

Marshal, R.L., Israel, B.A., and Letchworh III, G.J. (1988). Monoclonal antibody analysis of bovine herpesvirus-1 glycoprotein antigenic areas relevant to natural infection. Virol. 165, 338-347.

Marshal, R.L., Rodriguez, L.L., and Letchworth III, G.J. (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus 1) by biochemical and immunological methods. J. Virol. 57, 745-753.

Maxam, A.M., and Gilbert, W. (1977). Chemical sequencing of DNA. Proc. Natl. Acad. Sci. USA 74, 560-564.

Mayfield, J.E., Good, P.J., Van Oort, H.J., Campbell, A.R., and Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesviruses 1 (Cooper strain. J. Virol. 47:259-264.

Metzler, A.E., Matile, H., Gassman, U., Engels, M., and Wyler, R. (1985). European isolates of bovine herpesvirus 1: A comparison of restriction endonuclease sites,

polypeptides, and reactivity with monoclonal antibodies. Arch. Virol. 85, 57-69.

Metzler, A.E., Schudel, A.A., and Engles, M. (1986). Bovine herpesvirus 1: Molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. Arch. Virol. 87, 205-217.

Petrovskis, E.A., Timmins, J.G., Armentrout, M.A., Marchioli, C.C., Yancey, Jr., R.J., and Post, L.A. (1986). DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. J. Virol. 59, 216-223.

Roizman, B., Desrosiers, R.C., Fleckenstein, B., Lopez, C., Minson, A.C., and Studdert, M.J. (1992). The family herpesviridae: an update. Arch. Virol. 123, 425-449.

Rouse, B.T., and Babiuk, L.A. (1978). Mechanisms of recovery from herpesvirus infections-A review. Canad. J. Comp. Med. 42, 415-427.

Sambrook J., Fritsch, E., and Maniatis, T. (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor laboratories, Cold Spring Harbor, N.Y.

Sanger, F., Nicklen, S.,and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. (USA) 74, 5463-5467.

Schudel, A.A., Carillo, B.J., Wyler, R., and Metzler, A.E. (1986). Infections of calves with antigenic variants of bovine herpesvirus-1 (BHV-1) and neurological disease. Zbl. Vet. Med. B 33, 303-310.

Studdert, M.J. (1989). Bovine encephalitis herpesvirus.Vet. Rec. 125, 584.

Szewczyk, B., and Summers, D.F. (1988). Preparative elution of proteins blotted to immobilon membranes. Anal. Biochem. 168, 48-53.

Tikoo, S.K., Zamb, T.J., and Babiuk, L.A. (1993). Analysis of bovine herpesvirus 1 glycoprotein gIV truncation and deletions expressed by recombinant vaccinia viruses. J. Virol. 67, 2103-2109.

Tikoo, S.K., Fitzpatrick, D.R., Babiuk, L.A., and Zamb, T.J. (1990). Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. J. Virol. 64, 5132-5142.

Trepanier, P., Minocha, H.C., Bastien, Y., Nadon, F., Seguin, C., Lussier, G., and Trudel, M. (1986). Bovine herpesvirus 1: Strain comparison of polypeptides and identification of a neutralization epitope on the 90-kilodalton hemagglutinin. J. Virol. 60, 302-306.

Van Drunen Little-van den Hurk, S., Parker, M.D., Massie, B., van den Hurk, J.V., Harland, R., Babiuk, L.A., and Zamb, T.J. (1993). Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 11, 25-35

Van Drunen Little-van den Hurk, S., van den Hurk, J.V., Gilchrist, J.E., Misra, V., and Babiuk, L.A. (1984). Interactions of monoclonal antibodies and bovine herpesvirus type 1 (BHV-1) glycoproteins. Virol. 135, 466-479.

Van Drunen Little-van den Hurk, S., Gifford, G.A., and Babiuk, L.A. (1990b). Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. Vaccine 8, 358-368.

Van Drunen Little-van den Hurk, S., Hughes, G., and Babiuk, L.A. (1990a). The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus type-1 glycoproteins gI and gIV. J. Gen. Virol. 71, 2053-2063.

Van Drunen Little-van den Hurk, S., and Babiuk, L.A. (1986). Synthesis and processing of bovine herpesvirus type 1 glycoproteins. J. Virol. 59, 401-410.

von Heinje, G. (1986). A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14, 4683-4690.

Weijer, W.J., Drijfhout, J.W., Geerligs, H.J., Bloemhoff, W., Feijlbrief, M., Bos, C.A., Hoogerhout, P., Kerlin, K.E.T., Popken-boer, T., Slopsema, K., Wilterdink, J.B., Welling, G.W., and Welling-Wester, S. (1988). Antibodies against synthetic peptides of herpes simplex virus type 1 glycoprotein D and their capability to neutralize viral infectivity in vitro. J. Virol. 62, 501-510.

Watson, R.J. (1983). DNA sequence of the herpes simplex virus type 2 glycoprotein D gene. Gene 26, 307-312.

Watson, R.J., Weis, J.H., Salstrom, J.S., and Enquist, L.W. (1982). Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in Escherichia coli. Science 218, 381-384.

APPENDIX

DETAILED METHODS

Virus DNA Isolation

Confluent MDBK cells grown in large tissue culture flasks were infected with virus (BHV-1, BHV-5) at moi of 1 and supplemented with DMEM containing 2 - 5% FBS. When the CPE was complete, cell-free supernatant media containing virus were collected and used for virus concentration through 30% sucrose cushion as described (Abdelmagid et al., 1989). Concentrated virus pellet was resuspended in 1.8 ml TE buffer, transferred to 30 ml-corex tubes and then 200 μl of 10% SDS and 20 μl of 10 mg/ml RNase A, were added. After 20 min incubation at 60°C, 50-100 μl of 10 mg/ml proteinase K, were added and allowed to react for 30 min at 60°C. Two ml of TE buffer were added to the mixture and the mixture was extracted by adding 2 ml, each of phenol and chloroform/isoamylalchohol and mixed gently by vortexing. The phases were then separated by centrifugation at 4000 rpm for 10 min (CRU-5000 centrifuge, IEC). The upper (aqueous) phase containing DNA was transferred to a fresh corex and 0.5 ml TE buffer tube was added to the lower phase and reextracted as described above. The upper phase was extracted once more with phenol/chloroform and the residual phenol was removed by ether extraction. The DNA was precipitated by addition of one-tenth the volume 3 M Sodium acetate, pH 5.2, 2 volumes of cold 100% ethanol, and freezing at -70°C for 1/2 h. The DNA was pelleted by centrifugation at 8000 rpm for 1/2 hr in a Sorvall SS-34 rotor, washed once with 80% ethanol, dried and resuspended in 100 - 250 μl TE buffer. The integrity of the isolated virus DNA was evaluated by agarose gel electrophoresis.

Southern Hybridization

Southern Blotting

Transfer and immobilization of DNA to nylon membranes were performed according to the technique described by (Southern 1975). DNA samples were digested by the appropriate restriction enzymes and the restriction fragments were separated by agarose electrophoresis. After agarose gel electrophoresis was complete, the gel was removed and placed on a UV illuminator for 15 min in order to introduce nicks into the DNA fragments. The DNA fragments were then denatured by soaking

the gel in denaturation buffer with gentle shaking for 1 hr. The gel was then washed with tap water and transferred to neutralization buffer and allowed to wash gently for an other 1 hr. Meanwhile the transfer apparatus was prepared by soaking two large pieces of whatman filter paper in 10X SSC buffer and placed on the platform in such away that the two sides of the paper were immersed in the buffer. The gel was removed from the neutralization buffer and placed on the platform. The nylon membrane and two whatman filter papers were cut exactly the size of the gel and soaked in 2X SSC. The nylon membrane was placed on top of the gel followed by the two whatman papers, a stack of paper towels was placed and held in position by putting weight on top. DNA fragments were transferred from the gel to the nylon membrane by diffusion. After 12 - 18 hrs the system was disassembled and the membrane was removed and soaked in 6X SSC for 5 min and baked for 2 hrs at 80°C.

Nick Translation

All DNA probes used in this study were prepared by the nick translation method described by (Kelly et al., 1970). Nicks introduced into the double stranded DNA by DNase serve as primers to extend the DNA chain incorporating radioactive nucleotides at the 3' end by E.coli DNA polymerase I. Unlabeled DNA strands are removed by the 5' to 3' exonucleolytic activity.

Forty μCi of ^{35}S-deoxycytidine 5'-(alpha-thio)-triphosphate (^{35}S-dCTP) [1000-1500 Ci/mmole] or ^{32}P-deoxycytidine 5'-triphosphate (^{32}P-dCTP) [3000 Ci/mmole] was transferred to a small eppendorf tube to label one probe. After lyophilizing the radioactive solution using a speed vac centrifuge, the nick translation reaction was set by adding the following into the tube:

1 μl 10X nick translation buffer

1 μg of DNA sample (less than 7 μl)

1.2 μl 0.5 mM deoxynucleotide mixture (dATP, dGTP, dTTP)

DD H_2O to 10 μl

The reaction mixture was chilled on ice and 0.5 μl each of 10 ng/ml DNase and E.coli polymerase I (5 U) was added and mixed by gentle tapping of the tube. After

incubation at 16°C for 1 hr, 0.4 μl of 0.5 mM dCTP was added and incubated for an additional 15 min The reaction was stopped by adding 10 μl of stop buffer and the volume was adjusted to 100 μl by adding TE buffer. Unincorporated nucleotides were removed by centrifugation at 1500 g through sephadex G50 - G75 column. The flowthrough containing the radioactive probe was collected and used for hybridization.

Hybridization

Membranes prepared by southern blotting were incubated overnight with 5-10 ml of prehybridization mix in a sealed plastic bag at 42°C in a shaking water-bath. The radioactive DNA probe and Herring fish sperm DNA were denatured by boiling for 10 min and added to hybridization buffer. The hybridization mix was added to the blots in the plastic bag and incubated for 24 hr as described above. The blots were removed from the bag and washed first for 20 min in two changes of wash buffer 1 at room temperature.Then washed for 1 hr in two changes of wash buffer 2, and finally washed for 1 hr at 60°C in two changes of wash buffer 3. The blots were then dried at 37°C and exposed to X-ray film (Kodak X-omat, KR5, Sigma). Films were developed and examined.

Molecular Cloning Techniques

Large-Scale Preparation of Plasmid DNA

Five ml of transformed-E.coli (strain DH5α) cells overnight broth culture were used to inoculate 250 ml LB broth supplemented with ampicillin at 50 μg/ml final concentration. The bacterial cells were grown overnight with shaking at 37°C. The cells were pelleted by centrifugation at 6000 rpm for 10 min in a Sorvall GSA rotor, resuspended in 12 ml STE buffer, and transferred to a conical flask. The cells were lysed by the addition of 2 ml of 10 mg/ml lysozyme, 3 ml of 25% Triton-X100, and left on ice for 30 to 60 min The contents of the flask were heated to boiling over bunsenburner flame, immediately transferred to a boiling water bath for 40 seconds, and chilled on ice. The white-creamy colored contents were centrifuged at 15,000-16,000 rpm for 1 hr in a Sorvall SS 34 rotor. The clear supernatant containing the DNA was transferred to a fresh tube to which an equal volume of ice-cold 100%

isopropanol was added and frozen at -70°C for 1/2 an hr or longer. The DNA was precipitated by centrifugation at 8000 rpm for 20 min in a Sorvall SS 34 rotor and resuspended in 1.8 ml of TE buffer. The DNA suspension was treated with 30 μl of 10 mg/ml RNase A for 30 min at 60°C followed by 60 μl of 10 mg/ml proteinase K for an other 30 min at 60°C. Plasmid DNA in the lysate was analyzed by loading a small sample on agarose electrophoresis to determine its concentration. The lysate was transferred to a fresh corex tube and its weight was adjusted to 2.4 gm by adding TE buffer and its density was brought to 1.8 gm/ml by adding 4.2 gm of cesium chloride and 0.4 ml of 10 mg/ml ethidium bromide. The contents of the tube was mixed gently by vortexing and centrifuged at 3,100 rpm for 1 hr at room temperature (CRU-5000 centrifuge, IEC) [or 8,000 rpm for 5 min in a Sorvall SS34 rotor] to remove debris layered on surface. The clear red liquid containing the DNA was carefully pipetted out with a pasteur pipet and added to quick seal tube already containing 8 ml of cesium chloride solution with a density of 1.47 gm/ml. The mixture in the tubes were then centrifuged at 55,000 rpm for 15 hrs in a Ti 70.1 rotor (Beckman ultracentrifuge) at 20°C. The gradient centrifugation separated the DNA into two distinct bands, located in the center of the gradient, which were visible under UV light. The covalently closed circular plasmid DNA making the lower band was collected by the needle method and transferred to a fresh corex tube. Ethidium bromide was removed by extraction with cesium chloride-saturated isopropanol. Equal volume of CsCl-saturated isopropanol was added to the tube mixed gently by vortexing, allowed to sit for few seconds until phases separate, then the upper phase containing the ethidium bromide was discarded. The process was repeated 3-4 times until upper phase is clear. The lower phase containing the DNA was diluted 5X with TE buffer and precipitated by the addition of cold 100% isopropanol, washed with 80% ethanol, dried, and resuspended in 200-250 μl TE buffer.

Restriction Enzyme Digestion of DNA

Restriction endonuclease digestion of DNA for all purposes was performed according to manufacturer's recommendations of temperature and buffer conditions. When DNA was to be cleaved with two or more restriction enzymes which have

different requirements a universal R buffer system was used. The DNA was digested first with the enzyme that work best in R buffer of lower ionic strength. The appropriate amount of NaCl and the second enzyme were then added and incubation continued. All digestion reactions were carried out in sterile 0.5 ml Eppendorf tubes with reaction volumes varying between 10-100 μl. Reactions were stopped by addition of one-tenth of the volume stop buffer or by heating to 65°C for 5 min.

Agarose Gel Electrophoresis

The proper amount of powdered agarose was added to a measured quantity of 1X TBE electrophoresis buffer and dissolved by microwaving for 1 - 2 min. When agarose gels were intended for Southern blotting 0.5X TBE was used. The agarose solution was cooled to 60°C and ethidium bromide was added (from a stock solution of 10 mg/ml in water) to a final concentration of 0.5 μg/ml. The agarose gel was poured into the mould and the DNA samples to be analyzed were prepared by adding load mix. Depending on the sizes of the DNA samples agarose gel concentrations varied from 0.6% - 2%. Forty to eighty constant voltage was applied and the gel was run for the desired length of time. The intercalation of ethidium bromide into the DNA allowed visualization of DNA fragment under UV light.

Recovery of DNA by Electroelution

After preparative agarose electrophoresis, the DNA band of interest was located and the agarose slice containing that fragment was cut out using a sharp sterile scalpel blade. A dialysis bag was closed from one end with a dialysis clip and filled with 1 ml of 0.1X TBE buffer. The agarose slice containing the DNA band was transferred to the buffer-filled dialysis membrane, closed by a clip, and immersed into a shallow layer of 1X TBE buffer in an electrophoresis tank. The optimum voltage was applied and the DNA was electroeluted out of the gel onto the inner wall of the bag. Polarity was reversed for one min to release DNA from the wall of the bag into the buffer. The bag was then opened and all the buffer was transferred into a fresh Eppendorf tube and the DNA was precipitated by adding an equal volume of cold 100% isopropanol, washed and resuspended as described.

Blunting of DNA ends

Blunting of 3' recessed termini

Blunting the termini of DNA in many occasions is necessary for molecular cloning. Double stranded DNA with 3' recessed termini was blunt ended by filling the ends with klenow fragment of E.coli DNA polymerase I. The klenow fragment of E.coli DNA polymerase I has polymerase activity and lacks 5'- 3' exonucleolytic activity. After restriction enzyme cleavage which leaves 5' protruding termini, the DNA was extracted once with phenol/chloroform, precipitated, and resuspended in 10 - 15 μl of DD H_2O and the reaction was carried out by adding the following: 10 - 15 μl (1 μg) of DNA to be blunted; 1 μl of 2 mM deoxynucleotide mixture (dNTP); 2.5 μl 10X nick translation buffer; complete with H_2O to 25 μl; 0.5 μl (2.5 U) klenow fragment DNA polymerase. The reaction mixture was incubated at room temperature for 30 min. The reaction volume was adjusted to 100 μl with TE buffer and extracted with phenol/chloroform, precipitated and resuspended as described.

Blunting of 3' protruding termini

Double stranded DNA with protruding 3'termini was blunt ended by T4 DNA polymerase. The 3' - 5' exonucleolytic activity of this enzyme will remove the single stranded protruding 3' end and the high concentration dNTPs (deoxynucleotides triphosphate) will protect the duplex DNA resulting in blunt ends. The T4 DNA polymerase function in most of the restriction enzyme conditions. After restriction enzyme digestion was complete dNTP mixture was added to final concentration of 100 μM, T4 DNA polymerase was added (5 U per 1 μg of DNA), 10X restriction enzyme buffer to 5 μl, complete with H_2O to 50 μl volume. The reaction mixture was incubated for 5 min at 37°C and the reaction was stopped as described.

DNA Ligation

Ligation of DNA fragments to linearized plasmids was catalyzed by T4 DNA ligase. The ligation reaction was set up by adding 1 μl 10X ligation buffer; 0.5 μl 10 mM ATP; 0.5 μl (2.5 U) T4 DNA ligase; plasmid DNA, Insert DNA, and H_2O were added to complete the reaction volume to 10 μl. The ratio of insert DNA to plasmid DNA determined by the nature of the DNA ends to be ligated. The reaction mixture

was incubated at 16°C overnight and stopped by phenol/chloroform extraction, precipitated and resuspended in DDH_2O.

Transformation of Bacterial Cells

Ligated DNA was used to transform bacterial (DH5α or RR1) cell by electroporation using Bio-Rad gene pulser. Electrocompetent bacterial cells were prepared following Bio-Rad instructions and stored in 0.5 ml Eppendorf tubes as 40 µl aliquots at -70°C. Prior to electroporation one tube of electrocompetent cells was thawed at room temperature and placed immediately on ice. Sterile cuvettes were removed from the bags and chilled on ice. The gene pulser was set at 25 µF, 2.5 volts, and the pulse controller at 200 Ω. 1 - 2 µl ligated DNA containing approximately 10 - 25 ng, was added to the competent cells in the Eppendorf tube, mixed by gentle tapping and placed in the bottom of the cold cuvette. The cuvette was blotted dry from moisture and seated between the contacts in the base of the chamber. After a single pulse, 1 ml of LB broth was immediately added to the cells in the cuvette and the cells were transferred to a plastic tube and incubated at 37°C in a shaking water bath for 1 hr. An appropriate volume of transformed competent cells was transferred onto LB agar plate containing ampicillin (50 µg/ml) and 5% X-Gal (30 µl). Using a bent glass rod the transformed cells were spread over the plate surface, and the plates were incubated inverted at 37°C overnight for the development of colonies. Insert containing transformant (ampicillin-resistant or colorless) colonies were selected for further screening.

Rapid Screening of Plasmid DNA

Bacterial colonies selected based on antibiotic resistance and insertional inactivation of ß-gal gene were replicated by transferring a segment of the colony as 1 X 1 cm batch on a master plate and incubated overnight. A portion of the patchy growth from the master plate was transferred by a pipette tip and dissolved in 8 µl of protoblast buffer in an Eppendorf tube. Meanwhile 3 µl of lysis buffer was loaded into large slots (2 mm X 3 mm) of agarose gel. Five µl of the viscous bacterial lysate in the Eppendorf tube was loaded onto the slots and low voltage (10 - 20) was applied for 20 min. After sufficient interaction between the bacterial lysate and the

lysis buffer has taken place in the slots, the voltage was increased and the gel was analyzed under UV light. The sizes of the plasmids from each colony was compared with control plasmid and molecular weight markers. Colonies containing the proper size plasmids were selected and further analyzed by restriction digestion.

Colony Hybridization

This procedure was used when there is no white-blue selection on the plate and especially when the number of colonies to be screened was large. Bacterial colonies on the plate were evenly dispersed on the agar surface. Nitrocellulose nylon membrane disc were placed carefully on the surface of agar plate and left for 2 - 3 min. The membrane and agar surface were marked by stabbing needle holes on the edges. Using blunt-ended forcep the membrane was carefully removed and placed colony side up on a precut paraffin film containing 0.85 ml denaturation buffer and left for 2 - 3 min. The membrane was then removed and laid colony side up on a filter paper and the denaturation process was repeated once more. After denaturation the membrane was neutralized twice following the same technique and finally dried on a filter paper colony side up for 2 - 3 hr at room temperature. The membrane was sandwiched between two filter papers and baked at 80 for 1 - 2 hr. Hybridization using ^{32}P labeled probes was continued as described.

Small-Scale Preparation of Plasmid DNA (Miniprep)

A single plasmid-transformed bacterial colony was inoculated in LB broth containing the appropriate antibiotic in a tube. The culture was incubated overnight at 37°C with vigorous shaking. Bacterial cells from 2 ml culture were pelleted in an eppendorf tube at 12,000g for 30 seconds at 4°C. After decanting the supernatant, the pellets were resuspended in 425 μl of buffer mix containing 300 μl STE buffer, 75 μl 25% Triton-X100, and 50 μl of 10 mg/ml lysozyme. The contents of the tube were mixed by gentle vortexing, left on ice for 10-30 min, placed on a boiling-water bath for 1 min, and immediately chilled on ice. The bacterial lysate was centrifuged at 12,000g, the supernatant was transferred to a fresh eppendorf tube and the DNA was precipitated by addition of an equal volume of cold 100% isopropanol as described. The pelleted DNA was resuspended in 200 μl of TE buffer, treated with 5 μl and 10

μl of RNase I and proteinase K, respectively, at 60°C for 15 min each. After three phenol:chloroform and ether extractions the DNA was precipitated by addition of 2.5 volumes 100% ethanol and one tenth the volume 3 M sodium acetate. After freezing at -70°C, pelleting and washing by 80% ethanol, the DNA was resuspended in 20 μl of H_2O.

pATH Plasmid Expression System

The pATH vectors are series of E.coli plasmids designed for the production of proteins from any cloned DNA sequence that contains an open reading frame. The cloned DNA sequences are fused in-frame to the TrpE gene of E.coli, thus the hybrid protein produced contains the amino-terminal aa region of the TrpE protein followed by the translation product specified by the cloned DNA (Dieckmann and Tzagoloff, 1985). Transcription is controlled by the E.coli Trp operon promotor, which is transcribed at high levels under induction conditions. The fusion proteins usually are produced in E.coli in an insoluble form, which greatly facilitates purification

Induction and Extraction of pATH-expressed TrpE-gD Fusion proteins

pATH plasmids were always propagated in media containing tryptophan to prevent expression of the TrpE gene which may be lethal to the bacterial cells.

A loopful of freshly plated pATH-transformed RR1 cells was inoculated into a 10 ml of modified M9 media in a flask. The culture was grown at 37°C with shaking for 2 - 4 hr to midlogarithmic phase (A_{600}0.2 - 0.4). The entire 10 ml culture was then poured into a 100 ml modified M9 without Tryptophan in a 2-liter flask for aeration. The culture was grown at 37°C with vigorous shaking for 1 hr and then induced by adding 0.5 ml indoleacrylic acid (IAA) stock solution. Growth of the IAA-treated culture was continued for an additional 4 hr and stored at 4°C for overnight.

The induced cultures were harvested by centrifugation at 5000 rpm for 5 min in a Sorvall GSA rotor, resuspended in 10 mM Tris-HCl, pH 7.5, and pelleted again. The cell pellet from each 100 ml culture was resuspended in 20 ml of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 3 mg/ml lysozyme and kept on ice for two hrs. 1.4 ml of 5 M NaCl was added and the cells were mixed by inversion. Cell lysis was completed by addition of 1.5 ml of 10% Non-idet 40 and mixed again by inversion to avoid air

bubbles which are detrimental to the protein at this stage. The resulting lysate was viscous owing to the release of chromosomal DNA. The viscosity of the lysate was reduced by sonicating at 350 W until the solution was able to drop freely through a pasteur pipette. The sonicated lysate was centrifuged at 10000 rpm in a Sorvall SS 34 rotor. The soluble supernatant was discarded and the insoluble pellet containing the fusion protein was washed once in 20 ml of 10 mM Tris-HCl, 1 M NaCl pH 7.5, and once in 10 mM Tris-HCl, Ph 7.5. The pellet from 100 ml culture was resuspended in 1 ml of 10 mM Tris-HCl, pH 7.5 and dissolved by homogenization using a plastic syringe plunger. The homogenized fusion protein was diluted in 4X SDS-PAGE loading buffer to give final concentration 1X load buffer and then analyzed by SDS-PAGE.

Preparation of TrpE-gD Fusion Proteins Antigens

TrpE-gD fusion protein expressing the N-terminal 216 amino acids was selected for rabbit immunization because it carries at least two linear neutralizing epitopes. The fusion protein antigen was prepared by elution of protein from NC membrane after electrophoretic transfer from polyacrylamide gel as described previously (Szewczyk and Summers, 1988). After preparative electrophoresis and transfer of the protein to NC membrane, the NC membranes were washed in PBS containing 0.1% Tween 20 (PBST) to remove SDS and air-dried, processed immediately or stored ar 4°C. Edges of the membrane were cut and stained by Ponceau S protein staining to locate the protein band to be eluted. Using a sharp blade the portion containing the protein was sliced out, cut into small 2 X 3 mm pieces, and dissolved in 5 ml DMSO (Dimethylsulfoxide) for 1 hr. The NC membrane particles containing the protein were precipitated by adding an equal volume (5 ml) of 0.05 M sodium carbonate buffer, pH 9.6 drop-wise while vortexing. After centrifugation in a microfuge for 10 min the pellet was washed once in PBS, resuspended in PBS, emulsified in Freund's complete adjuvant and used for immunization.

DNA Sequencing of The BHV-5 gD Gene

Subclones from the 1.62 Kb MaeI fragment carrying the BHV-5 gD gene were

generated and sequenced by the dideoxy chain termination method (Sangers et al., 1979) and verified by the chemical method (Maxam and Gilbert, 1977;1980).

DNA Sequencing by the Dideoxy Chain Termination Method

The chain termination technique capitalizes on two properties of DNA polymerase. First, its ability to synthesizes faithfully a complementary copy of a single stranded DNA template. Second its ability to use 2',3'-dideoxy triphosphate as substrates. Incorporation of these analogues will terminate further extension of the growing chain. DNA synthesis is carried out in the presence of specific primers, four deoxynucleotides, one of which is labelled with ^{32}P in four separate incubation mixes containing one of the four dideoxynucleotides.

DNA sequence of the 1.62 Kb MaeI fragment was determined by Sanger's method at the biotechnology laboratory of the college of veterinary medicine using a taqdyedeoxyterminator cycle sequencing kit and analyzed on an Applied Biosystem model 373A DNA sequencing system following manufacturer's protocols. The DNA sequencing reactions were performed on DNA templates using universal M 13 primers. The reaction mixture includes: ds DNA template; dNTP mix; dye-labelled dideoxy nucleotides (dyedeoxy terminators); Taq polymerase; universal primers; 7% DMSO and water. Total reaction volume was 20 μl overlaid with a drop of mineral oil ready for thermal cycler. The DNA sequencing reaction was carried out on a Perkin-Elmer model Gene Amp 9600 (Emeryville, CA) thermal cycler using the following parameters: Denaturation at 98°C for 30 sec; annealing at 50°C for 5 sec; extension at 60°C for 4 min. After 30 cycles the PCR products were purified and analyzed.

DNA Sequencing by the Chemical Method

The principle of Maxam and Gilbert sequencing method is the degradation of original end-labeled DNA. In this procedure a fragment of DNA radiolabelled at one end is partially cleaved in five separate chemical reactions, each of which is specific for a particular base (Maxam and Gilbert, 1977, 1980, Chowdhury et al., 1990).

The specific cleavage reactions are carried out in two stages. In the first stage, specific bases (or types of bases) undergo chemical modification; in the second stage,

the modified base is removed from its sugar and the phosphodiester bonds 5' and 3' to the modified base are cleaved. This generates five populations of radiolabelled molecules that extend from the radiolabeled terminus to the site of chemical cleavage. These population are then resolved by polyacrylamide gel electrophoresis, and the end-labeled molecule are detected by autoradiography.

Preparation of ^{32}P Labelled 5' protruding termini of DNA fragments

DNA fragments for sequencing by the chemical method were prepared by CsCl gradient centrifugation as described above. Recombinant plasmid DNA (10 - 15 μg) were digested to completion with the appropriate restriction enzyme in 50 - 100 μl volume. One-tenth of the volume (5 - 10 μl) of 0.5 M Tris, pH 8.0, and 1 U of alkaline phosphatase were added and incubated at 37°C for 30 min. One unit (1 U) of alkaline phosphatase was added again and incubated for an other 30 min at 37°C. The reaction was stopped by adding one-tenth of the volume (5.5 - 11 μl) of 0.5 M EDTA and by heating for 5 min at 65°C. The mixture was extracted once with phenol/chloroform, twice with ether, and precipitated in ethanol and 3 M Sodium acetate, pH 5.2 as described. The dephosphorylated DNA was washed once in 80% ethanol and 100 - 200 μCi of τ^{32}P-ATP (50 - 100 pmol ATP) was added to the tube and dried using speed vac. The lyophilized DNA and radionucleotides were resuspended in the following reaction mixture:

2 μl 10X PNK buffer; 2 μl 10 mM Spermidine; 1 μl (20 U) PNK; 15 μl DDH_2O.

The reaction mixture was incubated at 37°C for 30 min and repeated once more by adding 1 μl (20 U) of PNK and incubated as described above. The radiolabelled DNA fragments were precipitated by adding 20 μl of 5 M Ammonium acetate, 120 μl cold ethanol, 1 μl of 5 mg/ml t-RNA and freezing at -70°C for 30 min. After centrifugation the radiolabelled DNA was washed twice with 80% ethanol and resuspended in 25 μl TE buffer and the second restriction digest was set up in 50 - 100 volume and incubated at 37°C overnight. Next day, after the restriction enzyme digestion was completed the correct DNA fragments were separated by preparative agarose electrophoresis and electroeluted. To the eluted radiolabelled DNA, 15 - 25 μg of Herring fish sperm DNA was added and precipitated by adding and equal

volume of 100% cold isopropanol, one-tenth of the volume 3 M sodium acetate, pH 5.2. After freezing at -70°C for 30 min the DNA was pelleted by centrifugation, washed twice with 80% ethanol, and resuspended in 105 μl of DDH_2O. The radiolabelled DNA was distributed in 5 colored 1.5 ml Eppendorf tubes, red, blue, green, black, and violet. Each tube received 20 μl except the green which received 25 μl. The radiolabelled DNA in the tubes was lyophilized in speed vac centrifuge and frozen at -20°C until used.

Sequence Reactions

Purine (G+A)-specific Cleavage
"Blue colored Eppendorf tube"

Lyophilized end-labelled DNA was resuspended in 30 μl of freshly prepared APU mix at room temperature and mixed by vortexing. The mixture was incubated at 20°C for 5 min. The reaction was stopped by adding 45 μl DDH_2O and 500 μl ether. The contents were immediately mixed by vortexing and centrifuged at 14000 rpm for 3 min in a microfuge. Ether was discarded and the sample was ether extracted twice. Residual ether was allowed to evaporate and the sample was frozen in solid-dry ice, lyophilized without heat for 2 - 3 hr, and stored at -20°C.

Pyrimidine (C+T)-specific Cleavage
"Green colored Eppendorf tube"

Lyophilized end labelled DNA was resuspended in 50 μl of freshly prepared PY mix, mixed by vortexing and incubated at 20°C for 7.5 min. The reaction was stopped by adding 200 μl of Acetate-RNA (hydrazine stop) mix, 850 μl cold ethanol, and freezing at -70 dry ice-methanol bath.

Cytosine (C)-specific Cleavage
"Black-marked Eppendorf tune"

Lyophilized end-labelled DNA was resuspended in 50 μl of freshly prepared C-mix, mixed by vortexing and incubated for 10 min at 20°C. The reaction was stopped by adding 200 μl of Acetate-RNA (hydrazine stop) mix, 850 μl cold ethanol, and freezing at -70 dry ice-methanol bath.

Guanine (G)-specific Cleavage
"Red colored Eppendorf tube"

Lyophilized end-labelled DNA was resuspended in 200 μl of a freshly prepared solution made by mixing 5 μl 100% DMS with 1 ml of DMS buffer. The contents of the tube were mixed by vortexing and incubated at 20°C for 5 min. The reaction was stopped by adding 50 μl DMS stop mix, 850 μl cold ethanol, and freezing at -70 dry ice-methanol bath.

Adenine and Cytosine (A>C)-specific Cleavage
"Violet colored Eppendorf tube"

Lyophilized end-labelled DNA was resuspended in 5 μl DDH_2O and mixed by vortexing at RT. 100 μl of a freshly prepared AC mix was added to the tube, mixed by vortexing, and incubated for at 90°C for 5 min. Tube caps were securely fixed in a rack to prevent opening. The reaction was stopped by adding 155 μl AC-stop mix, 750 μl 100% cold ethanol, and freezing at -70 in a dry-ice/methanol bath. All reaction tubes except the blue tube (green, black, red, and violet) were left to freeze at -70°C in a dry-ice/methanol bath for 5 - 10 min. The DNA was precipitated by centrifugation at 14000 rpm for 5 min. Supernatants from DMS and hydrazine reactions were discarded in 5 M NaOH and 3 M Ferric Chloride respectively. The pelleted DNA from all reactions was resuspended in 300 μl of 0.3 M Sodium acetate pH 7.0, mixed by vortexing. 850 μl cold 100% ethanol was added, mixed by vortexing and the tube was frozen at -70 in a dry-ice/methanol bath. The process of precipitation was repeated total 3X. Finally the pelleted DNA was washed by adding

1 ml of 80% cold ethanol to each tube, freezing at -70°C for 2 min and centrifugation for 3 min. The process of washing was repeated total 3X and the DNA was lyophilized in speed vac centrifuge.

Strand Scission Reaction by Piperidine

Piperidine is used to cleave the sugar-phosphate chain of DNA at the site of chemically modified bases. The cleavage was performed for all five base-specific reactions. All lyophilized DNA samples were resuspended in 100 μl of freshly prepared 10% (v/v) piperidine in water solution. Tube caps were sealed properly and the tubes were incubated at 100°C for 1 hr. The samples were then briefly frozen in dry ice for 15 min and lyophilized in a Savant speed vac. The piperidine was removed by resuspending the samples each time in 50 μl and 20 μl of DDH_2O respectively, followed by lyophilization afterwards. Each sample was then resuspended in 10 μl of DNA sequencing gel-load buffer, aliquoted and stored at -20°C.

Sequencing Gels (Polyacrylamide Gel Electrophoresis)

The radiolabelled DNA fragments generated by chemical reactions were separated by denaturing polyacrylamide gel electrophoresis. Three concentrations of polyacrylamide gels, 20%, 8%, 5% in the presence of 50% urea were used read up to 350 nucleotides under optimum conditions. The LKB macrophore gel electrophoresis unit (Pharmacia) were used.

Casting of Sequencing Gels

The thermostatic plate was thoroughly cleaned, dried, and placed on the macromould. Five ml of Repel-saline was carefully spread over its surface and left to dry for few min. The plate surface was then polished neatly with lint-free paper wipes, rinsed with 70% ethanol, polished, and dried again. A clean notched glass plate was placed on two supports and using lint-free paper wipes, the bind-saline solution was spread over the surface. The glass plate was allowed to dry for few min, polished, rinsed with 70% ethanol, and polished again. Appropriate spacers (0.4 mm or 0.2 mm) were fixed in positions with metal clamps on the thermostatic plate. The macromould was tilted to form a slope and the notched glass plate was placed on the thermostatic plate with treated surface facing downward. The polyacrylamide gel

solution was poured from a side arm flask just in front of the notched glass plate while sliding it up the slope. Slight pressure was applied while sliding to avoid air bubbles. When the notches are 1 cm from the upper edge of the thermostatic plate, the macromould table was leveled, and the two plates were clamped together. The comb was carefully inserted, clamped, and the gel was left to polymerize for 30 - 40 min. After polymerization the gel was attached to the buffer chambers of the electrophoresis unit and the thermostatic plate was connected to a thermostatic pump of a water bath adjusted to 50°C. The two buffer chambers were filled with 1/2 TBE buffer and the combs were removed carefully. The wells were washed with 1/2 TBE buffer very well and filled with 50% urea in 1/2 TBE. The gel was pre-electrophoresed for 1 - 2 hr at 2500 volts. After pre-electrophoresis the gel wells were washed with 1/2 TBE to remove the urea solution and the sequencing samples (1 - 1.5 μl) were loaded in the wells with a special needle. Electrophoresis of the samples was carried out at 2500 volts the length of the run was determined by the gel concentration as follows:

1. The first gel is a 20% gel (0.4 mm thick) was stopped when the bromophenol blue dye has travelled to one-third. This gel will read the first 20 nucleotides closest to the radiolabeled end.
2. The second gel is a an 8% gel (0.2 mm thick) was stopped when the bromophenol blue dye has just reached the lower margin of the gel.
3. The third gel is a 5% gel (0.2 mm thick) was stopped after 4 hr.

After completion of the run the buffer chamber was emptied and the gel sandwich was placed on the macromould table. The sandwich was disassembled by inserting a spatula into the bevelling area near the lower edge of the notched plate applying pressure away from the thermostatic plate. The notched glass plate was then lifted carefully, covered with plastic wrap in case of 20% gel, or fixed in fixative bath for 10 min. The fixed gel was carefully rinsed with DDH_2O and dried overnight at 37°C. The dried gel was exposed to X-ray film at -70°C for a period of time depending on the amount of radioactivity. The films were developed and analyzed.

REAGENTS AND BUFFERS

Virus DNA Isolation

1 M Tris-HCl (pH 7.2, 7.4, 7.5, 8.0, and 8.3)
Tris base (Hydroxymethyl-aminomethane) 12.1 gm qs 100 ml H_2O. Solution was adjusted to the required pH with HCl.

0.5 M EDTA (pH 8.0)
Ethylenediaminetetraacetic acid (EDTA.2 H_2O) 18.61 gm qs 100 ml H_2O. Add approximately 2 gm of NaOH pellets to adjust to pH 8.0. Note EDTA will not dissolve completely until pH attained.

TE Buffer
10 ml of 1 M Tris-HCl (pH 8.3), 0.2 ml of 0.5 M EDTA (pH 8.0), qs 1000 ml H_2O.

30% (w/v) Sucrose in TE buffer
30 gm of sucrose qs 100 ml TE buffer

10% SDS
10 gm of SDS (Sodium dodecylsulfate) qs 100 ml H_2O.

0.5 M Tris base
6.055 gm of Tris base (Hydroxymethyl-aminomethane) qs 100 ml H_2O.

Phenol Solution
80 ml of 100% Phenol, 20 ml of 0.5 M Trsi base, 0.1 gm of 8 Hydroxychinolin. Aliquota and store at -20°C.

Chloroform/Isoamylalcohol (24:1)
21 ml of Isoamylalcohol, 483 ml Chloroform

Proteinase K (10 mg/ml)
10 mg of Proteinase K dissolved in 1 ml of H_2O. Aliquots of 400 μl and store at -20°C.

RNase A (10 mg/ml)
10 mg of Pancreatic RNase A dissolved in 1 ml H_2O, boiled for 10 min to sterilize and aliquoted (400 μl), and stored at -20°C.

3 M Soduim acetate (pH 5.2)
40.81 gm of Soduim acetate.3H_2O were dissolved initially in 80 ml of H_2O, pH adjusted to 5.2 with glacial acetic acid and qs 100 ml of H_2O.

Ethiduim bromide (10 mg/ml)
1 gm of ethiduim bromide in 100 ml of H_2O. Stir to dissolve and store at 4°C in a dark bottle.

5M NaCl
29.22 gm of NaCl, qs to 100 ml H_2O

1.47 gm cesuim chloride (w/w)
43.53 gm of cesuim chloride qs to 100 ml H_2O

Restriction Endonuclease Cleavage and Agarose Gel Electrophoresis

2 M Tris-HCl (pH 7.6)
BSA (50 mg/ml)
1 M Dithiothreitol (DTT)
1 M $MgCl_2$

10X R Buffer
0.25 ml of 2 M Tris-HCl (pH 7.6), 0.25 ml of 1 M Dithiothreitol (DTT), 0.5 ml of 1 M $MgCl_2$, 0.1 ml 50 mg/ml BSA, 3.9 ml H_2O. Aliqots (1 ml) were stored at 4°C.

1 M KCl
0.5 M NaCl

20X TBE (Tris-Borate EDTA) Buffer (pH 8.3)
444.8 gm of Boric acid, 872 gm of Tris, 66.4 gm of EDTA.$2H_2O$, qs to 4000 ml H_2O. No need to adjust PH. If necessary adjust pH with boric acid or Tris.

0.7% Agarose Gel in 1X TBE
0.7 gm of agarose powder qs to 100 ml 1X TBE, mix and microwave for 2 min until dissolved. Add 7-8 μl of 10 mg/ml ethidium bromide solution. Store at 60°C.

DNA load mix (stop buffer)
25 gm of sucrose, 1.86 gm of EDTA.$2H_2O$, 5 ml of 2 M Tris-HCl (pH 7.6), 0.1 gm of Bromophenol blue, qs 100 ml H_2O.

Southern Blotting and DNA:DNA Hybridization

50% Dextran Sulfate
25 gm of Dextran Sulfate dissolved in 50 ml by gentle heating

Prehybridization Stock Buffer
1 gm of bovine serum albumin BSA, 1 gm polyvinylpyrrolidone (PVP, M.W 40000), 1 gm ficoll (M.W. 400,000), 0.5 gm Sodium pyrophosphate (839 mg.hydrate). Place

the mixture in 100 ml graduated cylinder and add 25 ml 1 M Tris-HCl (pH 7.5), 50 ml 10% SDS. Qs 100 ml H_2O. Seal the cylinder, mix by hand until dissolved, and add to 250 ml deionized formamide. Stir to mix, aliquots (10 ml), and store in -20°C.

Prehybridization Mix
Warm up 7 ml of prhybridization stock buffer at room temperature, add 2 ml of 50% dextran sulfate, mix the tube by inversion in 42°C water bath for 10 min, and then add 0.58 gm of NaCl to the tube and mix as above until dissolved. Boil Herring fish sperm DNA (1 mg/ml) for 10 min, add to the prewarmed prehybridization mix , mix briefly by inversion, and add to the blot into the bag.

Hybridization Mix
2.34 ml of prehybridization stock buffer, and 0.66 ml of TE buffer in a tube were mixed by inversion and heated to 42°C in awater bath for 10 min. 333 μl of Herring sperm DNA (1 mg/ml) and the radiolabelled DNA probe (50-100) μlwere boiled for 10 min, mixed with prewarmed hybridization mix by inversion and loaded into the bag with the blot.

Hybridization Wash Buffer (10X) (3 M NaCl, 0.6 M Tris-HCl pH 8.0, 0.02 M EDTA pH 8.0). 175.32 gm of NaCl, 600 ml of 1 M Tris-HCl pH 8.0, 40 ml 0.5 M EDTA pH 8.0, qs 1000 ml H_2O.

Wash Buffer 1
50 ml of 10X wash buffer, qs to 500 ml H_2O to make 1X wash buffer.

Wash Buffer 2 (1X wash buffer, 1% SDS)
50 ml of 10X wash buffer, 50 ml 10% SDS, qs to 500 ml H_2O.

Wash Buffer 3 (0.1X wash buffer)
5 ml of 10X wash buffer qs to 500 ml H_2O.

Denaturation Buffer (2X)(1 M NaOH, 3 M NaCl)
80 gm of NaOH, 350.6 gm of NaCl, qs 2 liters H_2O. Dilute 1:1 for southern blots.

Neutralization Buffer (2X) (1 M Tris, 3 M NaCl)
242.2 gm of Tris, 350.6 gm of NaCl, dissolve in 1.5 liter of H_2O. Titrate to pH 7.5 with HCl, qs 2 liters H_2O Dilute 1:1 for southern blots.

20X SSC (3 M NaCl, 3 M Citrate,pH 7.0)
701.28 gmof NaCl, 352.92 gm of sodium citrate trisodium salt, qs 4 liters H_2O. Adjust pH to 7.0 with few drops of HCl or 5 M NaOH. Filter sterlize.

10X SSC
6X SSC

2 M Tris-HCl (pH 7.2)
12.1 gm of Tris base qs to 50 ml H_2O, and adjust pH to 7.2 with HCl.

1 M $MgSO_4$
24.65 gm of $MgSO_4.7H_2O$ qs 100 ml H_2O.

0.1 M Dithiothreitol
1 ml of 1 M Dithiothreitol qs 10 ml H_2O.

10X Nick Translation Buffer
2 ml of 2 M Tris-HCl (pH 7.2), 0.8 ml of 1 M $MgSO_4$, 0.8 ml of 0.1 M Dithiothreitol, 0.08 ml of BSA (50 mg/ml), 4.32 ml H_2O.

Herring Fish Sperm DNA (5 mg/ml)
Dissolve Herring sperm DNA at concentration of 1 mg/ml in water and then sonicate (6 40-seconds bursts at 475-W setting. Add NaCl to final concentration of 0.15 M, and purify by sequential extraction with phenol:chloroform and ether. Precipitate the DNA with ethanol as described previously and wash the tube extensively with 70% ethanol to remove salts and resuspend in small volume of water. Dilute or concentrate the DNA solution with H_2O until the $O.D_{260}$=20 to give 1 mg/ml or $O.D_{260}$=100 to give 5 mg/ml.

Nick Translation Stop Buffer
0.2 ml of 0.5 M EDTA, 2.0 ml of Herring fish sperm DNA (5 mg/ml), 0.1 ml of 10% SDS, 2.4 ml H_2O, aliquots stored at -20°C.

0.5 mM dATP, dGTP, dTTP Mix
5 µl 10 mM dATP, 5 µl 10 mM dGTP, 5 µl 10 mM dTTP, 85 µl H_2O, stored at -20°C.

0.5 M dCTP
5 µl 10 mM dCTP, 95 µl H_2O, stored at -20°C.

DNase I (2 mg/ml)
20 mg od DNase I, 30 µl of 5M NaCl, 5 ml glycerol, qs 10 ml H_2O. Diluted 1:2X10^5 for nick translation.

E.coli DNA Polymerase I (Promega)

Blunt End Production

2 mM dATP, dGTP, dTTP, dCTP Mix
20 µl 10 mM dATP, 20 µl 10 mM dGTP, 20 µl 10 mM dTTP, 20 µl dCTP, 20 µl H_2O, stored at -20°C.

10X Nick Translation Buffer
T4 DNA polymerase I (Promega)
Klenow fragment DNA polymerase I (Promega)

Ligation Reaction

10 mM ATP
10 mg of Adenosine triphosphate dissolved in 1.65 ml of H_2O. stored at -20°C.

2 M Tris-HCl (pH 7.5)

10X Ligation Buffer
350 μl of 2 M Tris-HCl (pH 7.5), 70 μl of 1 M $MgCl_2$, 70 μl of 10 mM ATP, 510 μl of H_2O.

T4 DNA Ligase (Boehringer)

Plasmid DNA Preparation

25% Triton X-100
25 ml gm qs 100 H_2O.

Lysozyme (10 mg/ml)
0.1 gm of egg-white lysozyme in 10 ml of H_2O, Filter, aliquot, and store at -20°C.

STE (Sucrose-Tris-EDTA) Buffer
400 ml of 25% sucrose, 25 ml of 2 M Tris-HCl (pH 7.5), 100 ml of 0.5 M EDTA, qs 1000 ml of H_2O.

LB Broth (difco-bacto)
25 gm of LB powder stir to dissolve in 1 liter of H_2O, autoclave for 15 min at 15 lb pressure (121°C).

Cacium phosphate Transformation

(100 mM $CaCl_2$, 10 mM Tris-HCl pH 8.0)

LB Agar Plates
40 gm of LB agar stir to dissolve in 1 liter of H_2O, autoclave for 15 min at 15 lb pressure (121°C), allow to cool to 55°C, add 1 ml of 50 mg /ml Ampicillin and pour on plates.

5% X-Gal
500 mg of X-Gal dissolved in 10 ml of Dimethyl-formamide, and stored at -20°C in

a dark bottle. 30 μl of 5% X-Gal was spread on the plate and allowed to dry at 37°C for 20 min before inoculation.

50 mg/ml Ampicillin
0.5 gm Ampicillin powder dissolved in 10 ml of H_2O, filter sterlize through 0.22 μM, and store in aliqots of 0.5 ml at -20°C.

DNA Sequencing

Preparation of Labelled End DNA Fragments

0.5 M Tris pH 8.0

5 mg/ml t-RNA
50 mg of t-RNA dissolved in 10 ml of H_2O, aliqoted, and stored at -20°C.

5 M Ammonium acetate
38.54 gm of Ammonium acetate, qs to 100 ml of H_2O.

T4 Polynucleotide Kinase (PNK) 20,000-50,000 U/ml from USB
Calf Intestinal Alkaline Phosphatase (New England BioLab)
Gamma ^{32}P-τ-ATP (DuPont)

10 mM Spermidine
25.46 mg of spermidine.3HCl dissolved in 10 ml H_2O, aliquoted and stored at -20°C.

10X PNK Buffer from New England BioLab

3 M Soduim acetate pH 7.0
40.81 gm of Soduim acetate.$3H_2O$ qs 100 ml of H_2O, pH was adjusted to 7.0 with acetic acid. Aliquots atored at -20°C.

0.3 M Soduim acetate pH 7.0
10 ml of 3 M Soduim acetate pH 7.0 qs 100 ml of H_2O.

Sequencing Reactions

1.2 M NaOH
4.8 gm of NaOH pellets qs 100 ml H_2O.

1 N Glacial Acetid Acid
5.75 ml of glacial acetic acid, 95 ml H_2O

1 M Sodium Cacodylate (pH 7.0)

21.4 gm of sodium Cacodylate, qs 100 ml H_2O, pH adjusted to 7.0.

APU Mix
100 mg of Diphenylamine (2% w/v) dissolved in 3.3 ml of 99% Formic acid, and 1.7 ml of H_2O was added drop by drop while vortexing, 10 μl of 0.5 M EDTA (pH 8.0). The mix was freshly prepared just before use.

PY Mix
582 μl of 98% Hydrazine (60% v/v), 368 μl of H_2O. Freshly prepared before use.

C-Mix
613 μl of 98% Hydrazine (60% v/v), 300 μl of 5 M NaCl, 87 μl of H_2O. Freshly prepared before use.

Acetate RNA (Hydrazine Stop) Mix
1 ml of 3 M sodium acetate (pH 7.0), 2 μl of 0.5 M EDTA (pH 8.0), 200 μl of 5 mg/ml t-RNA, qs 10 ml H_2O. Aliquots stored at -20°C.

DMS Buffer
2.5 ml of 1 M soduim cacodylate pH 7.0, 0.5 ml of 1 M $MgCl_2$, 0.1 ml of o.5 M EDTA pH 8.0, qs 50 ml H_2O.

DMS Stop Solution
5 ml of 3 M sodium acetate (pH 7.0), 0.69 ml of ß-mercaptoethanol, 200 μl of 5 mg/ml t-RNA, qs 10 ml H_2O. Aliquots atored at -20°C.

AC-Mix
1 ml of 1.2 M NaOH, 2 μl of 0.5 M EDTA pH 8.0. Freshly prepared before use.

AC Stop Mix
600 μl of 1 M acetid acid freshly prepared, 20 of μl 5 mg/ml t-RNA. Freshly prepared before use.

10% Piperidine
1 ml of 99% Piperidine, 9 ml of H_2O. Freshly prepared before use.

3 M Ferric Chloride
810 gm of $FeCl_3.6H_2O$ qs 1 liter H_2O. Used to detoxify hydrazine.

5 M NaOH
200 gm of NaOH qs 1 liter H_2O. Used to detoxify sodium cacodylate in DMS reaction.

DNA Sequencing Load Mix

0.05% Bromophenol blue, 0.05% Xylene cyanol, 95% Formamide. Aliquots stored at -20°C.

Sequencing Gels

Fixative Bath
100 ml of Methanol, 100 ml of glacial acetic acid, qs 1liter??

Repel-Saline is a 2% solution of dimethyldichlorosilane in 1,1,1-trichloroethanol.??

Bind-Saline Solution
25 ml of absolute ethanol, 75 μl of bind-saline, 75 μl of glacial acetic acid. Freshly prepared before use.

10% Ammonuim persulfate
TEMED

Polyacrylamide gel preparation

Gel Percent	20%	8%	5%
Acrylamide (gm)	19.3	7.6	4.75
Bis-Acrylamide	0.68	0.4	0.25
10X TBE	5 ml	5 ml	5 ml
Urea (gm)	50	50	50
10% AP	0.7 ml	0.8 ml	0.8 ml
TEMED	40 μl	80 μl	0.8 μl

The ployacrylamide, bis-acrylamide, urea, TBE buffer, were dissolved in a total of 100 ml, filtered through whatman paper, and degased for 10 min. After degasing 10% AP and TEMED were added just before pouring. No deionizing is required. All polyacrylamide reagents were obtained from BioRad. The polyacrylamide gels were prepared fresh.

1M NaOH
Required to detach old dried gels from glass plates

Quick Screening of Plasmid DNA

Protoblsat Buffer
300 μl of 1 M Tris-HCl pH 8.0, 100 μl of 0.5 M EDTA pH 8.0, 100 μl of 5 M NaCl, 8 ml of 25% sucrose, 1 ml of 1 mg/ml RNase I, 70 μl of 10 mg/ml lysozyme, 430 μl of H_2O.
Aliquots srored at -20°C.

Lysis Buffer
4 ml of 10% SDS, 2.8 ml of 25% sucrose, 1 ml of 10X TBE, 200 μl of 2.5% bromophenol blue, 2 ml of H_2O.

Induction and Extraction of Recombinant Fusion Proteins

E. coli Strain RR1: *proA2 leuB6 galK2 xyl-5 mtl-1 ara-14 rpsL20 supE44 hsdS* λ^-
E.coli Strain DH5α: *supE*44Δ*lac*U169(Φ80*lacZ*ΔM15)*hsdR*17*rec*A1*end*A1*gyr*A96*thi*-1*rel*A1

10X M9 Salts Stock Solution
60 gm of $Na_2HPO_4.H_2O$, 30 gm ofKH_2PO_4, 5 gm of NaCl, 10 gm of NH_4Cl, qs to 1 liter of H_2O. Autoclave and stre at room temperature.

Tryptophane (10 mg/ml)500X
0.1 gm of Tryptophane dissoved in 10 ml of H_2O by vortexing, filtered through 0.22 μM, and stored protected from light at 4°C. This solution is good for 14 days.

1 M $MgSO_4$
0.5 M $CaCl_2$
Thiamine B1 (10 mg/ml)
10% Non-idet 40
5 M NaCl
4X SDS Sample Buffer

Indole Acrylic Acid (IAA) (2 mg/ml)
4 mg of IAA dissolved in 2 ml of 95% ethanol. Prepared fresh before use.

40% Dextrose
40 gm og dextrose qs to 100 ml of H_2O, Filter sterelized.

Modified M9 Media
100 ml of 10X M9 salts, 5 gm of casamino acids, qs to 1000 ml. Mix, autoclave, and add 1 ml of 1 M $MgSO_4$, 200 μl of 0.5 M $CaCl_2$, 5 ml of 40% dextrose, 1 ml of 10 mg/ml thiamine B, 1 ml of 50 mg/ml Ampicillin. Tryptophan was added as 20 μg/ml final concentration in modified M9 medium. When inducing expression of the TrpE-protein do not add Tryptophane

10 mM Tris-HCl, pH 7.5
10 ml of 1 M Tris-HCl, pH 7.5, qs 1 liter, autoclave and store at 4°C

10 mM Tris-HCl, pH 7.5, 1 M NaCl
5 ml of 1 M Tris-HCl pH 7.5, 100 ml of 5 M NaCl, adjust pH to 7.5 and qs 500 ml.

50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 3 mg/ml lysozyme

25 ml of 1 M tris base, 5 ml of 0.5 M EDTA pH 8.0, 450 ml of H_2O, adjust pH to 7.5 and qs 500 ml H_2O. 3 mg/ml lysozyme was added fresh before use.

10X Stock 0.5 M Soduim carbonate (pH 9.6)

15.9 gm of Na_2CO_3, 29.3 gm of $NaHCO_3$, dissolve in 900 ml of H_2O, adjust pH to 9.6 with NaOH pellets, qs to 1 liter of H_2O, sterilize by filteration throigh 0.22 μM, and store at 4°C.

The buffer is diluted to 1X and used for elution of protein frm NC membranes.

Borate Buffer, pH 8.0

0.01 M soduim borate, 0.15 M NaCl, pH 8.0

REAGENTS AND SOURCES

ABTS [2,2 azino-di-(3-ethylbenzthiazoline sulphonic acid)]	Sigma Chemical Co., St.Louis, MO
Acrylamide and Bis-acrylamide	Bio-Rad Richmond, CA
Agarose	Promega Madison, WI
Ampicillin (Sodium salt)	Boehringer Manheim Indianapolis,IN
Anti-bovine IgGs (goat) biotinylated	KPL Inc., Gaithersburg, MD
Anti-mouse IgGs (goat) FITC, HRPO	Hyclone Lab. Logan, UT
Anti-rabbit IgGs (goat) biotinylated	KPL Inc., Gaithersburg, MD
ATP (Adenosine triphosphate)	Boehringer Manheim Indianapolis,IN
Autoradiography intensifying screen	DuPont Wilmington, DE
Avertin (2,2,2-Tribromoethanol)	Aldrich Chemical Milwaki, WI
Bovine herpesvirus-5 (TX89)	Dr. J. d'Offay Stillwater, OK
Bovine herpesvirus-1 (Cooper)	NVSL Ames, IA
Carboxymethyl cellulose (high viscosity Na salts)	Sigma chemical co., St. Louis, MO
Coomasie blue (fast stain)	Zoion Research Inc., Allston, MA
dATP, dCTP, dGTP, dTTP	Boehringer Manheim Indianapolis,IN
Dimethylsulfate	Aldrich chemical co., Milwaki, WI
Diphenylamine	Sigma chemical co., St. Louis, MO
DNA polymerase I (Klenow) fragment (5 U/μl)	Promega Madison, WI
EDTA (2Na.2H^{2}O)	Sigma chemical co., St. Louis, MO
ELISA microtiter plates	Dynatech Chantilly, Virginia

Fetal bovine serum	Hyclone Lab. Logan, UT
Films, X-Omat AR-5	Easterman Kodak Co, Rochester, NY
Formic acid 99%	Sigma chemical co., St. Louis, MO
Freunds adjuvants	Sigma chemical co., St. Louis, MO
Gelatin (EIA grade)	Bio-Rad Richmond, CA
Hunter's titer max adjuvant	Sigma chemical co., St. Louis, MO
Hydrogen peroxide (30% H_2O_2 solution)	Sigma chemical co., St. Louis, MO
Immunopure-4CN	Pierce Rockford, IL
LB agar, Miller	Difco Lab Detroit, MI
LB broth, Miller	Difco Lab Detroit, MI
Lysozyme (chicken egg white)	Sigma chemical co., St. Louis, MO
MDBK cells	ATCC Rockville, MD
Proteinase K	Sigma chemical co., St. Louis, MO
Ribi adjuvant	RibiImmunochem Research., Hamilton, MT
RNase A	Sigma chemical co., St. Louis, MO
Sephadex G-50 (DNA grade)	Pharmacia Piscata, NJ
Sequencing primers (Reversed & Universal M13)	United States Biochemicals (USB) Cleveland, OH
Spermidine	Sigma chemical co., St. Louis, MO
Subisotyping Kit for Mouse MAbs	Hyclone Lab. Logan, UT

T4 DNA ligase (5 U/μl)	Boehringer Manheim Indianapolis,IN
T4 polynucleotide kinase (20-50 U/μl)	United States Biochemicals (USB) Cleveland, OH
Vectastain ABC kit (peroxidase mouse IgG)	Vector Lab Inc., Burlingame, CA
α-^{32}P-dCTP (3000 Ci/mmole)	DuPont chicago, IL
α-^{35}S-dCTP (3000 Ci/mmole)	DuPont chicago, IL
τ-^{32}P-ATP (3000 Ci/mmole)	DuPont chicago, IL

ABBREVIATIONS

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aa	Amino acid
ATP	Adenosine 5'triphosphate
BHV-1	Bovine herpesvirus 1
BHV-5	Bovine herpesvirus 5
bp	Base pair
CPE	Cytopathic effect
dATP	Deoxyribo-adenosine-triphosphate
DMEM	Dulbecco modified eagle medium
DMS	Dimethylsulfate
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleotide triphosphate mixture (dTTP, dGTP, dATP, dCTP)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid (bisodium salt)
FBS	Fetal bovine serum
FITC	Fluorescen isothiocyanate
gB, C, D	Glycoprotein B, C, D
hr(s)	Hour(s)
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
IBR	Infectious bovine rhinotracheitis
IF	Immunofluorescence
IPV	Infectious pustular vulvovaginitis
I_R	Inverted repeat
Kb	Kilo base pair
KD	Kilo dalton
LB	Nutrient medium for growing bacteria
LR	Latency related
LTR	Latency related transcription

m.u.	Map unit
MAb	Monoclonal antibody
MDBK	Madin Darby bovine kidney cells
min	Minute
MW	Molecular weight marker
NC	Nitrocellulose
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide electrophoresis
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PRV	Pseudorabies virus
PY	Pyrimidine
R Buffer	Restriction Buffer
RIA	Radioimmunoassay
RPM	Revolution per minute
RT	Room temperature
SDS	Sodium dodecylsulfate
SSC	Standard sodium citrate
TBS	Tris buffered saline
Ter	Terminator codon
T_R	Terminal repeat
U_L	Unique long
U_S	Unique short
UV	Ultraviolet light
V/V	Volume/volume
VZV	Varicella zoster virus
W/W	Weight/weight
W/V	Weight/volume

BIBLIOGRAPHY

REFERENCES

Abdelmagid, O.Y., Orten, D.J., Xue, W., Blecha, F., and Minocha, H.C. (1992). Anti-idiotypic antibodies to bovine herpesvirus-1 inhibit virus infection in cell cultures. *Arch. Virol.* 122, 163-173.

Abdelmagid, O.Y. (1989). Comparison of rabbit polyclonal antiidiotypes to bovine herpesvirus-1. Thesis.

Ackerman, M.E., Peterhand, E., and Wyler, R. (1982). DNA of bovine herpesvirus type 1 in the trigeminal ganglia of latently infected calves. *Am. J. Vet. Res.* 43, 36-40.

Ackerman, M.E, and Wyler, R. (1984). The DNA of an IPV strain of bovid herpesvirus 1 in sacral ganglia during latency after intervaginal infection. *Vet. Microbiol.* 9, 53-63.

Babiuk, L.A., L'italien, J., van Drunen Little-van Den Hurk, S., Zamb, T.J., Lawman M.P.J, Hughes, G., and Gifford, G.A. (1987). Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual glycoproteins. *Virol.* 159, 57-66.

Bagust, T.J, and Klarke, L. (1972). Pathogenesis of meningoencephalitis produced in calves by infectious bovine rhinotracheitis herpesvirus. *J. Comp. Pathol.* 82:375-383.

Bandyopadhyay, S.K., Mittal, S.K., and Field, H.J. (1990). Identification of the gene homologous to HSV major DNA binding protein in the BHV-1 genome. *Vet. Microbiol.* 22, 203-212.

Barany, G., and Merrifield, R.B. (1980). Solid-phase peptide synthesis. In, The peptides, (E.Gross and J. Meienhofer, Eds.), Vol.1, pp. 1-284. Academic Press, New York.

Barbara, G.K., Kern, H., and Mettenleiter, T.C. (1992). The virulence determining BamHI fragment 4 of pseudorabies virus contains genes corresponding to the UL 15 (partial), UL 18, UL 19, UL 20, and UL 21 genes of herpes simplex virus and a putative origin of replication. *Virol.* 191, 900-908.

Bartha, A., Haidu, G., Aldassy, P., Paczolay, G. (1969). Occurrence of encephalomyelitis caused by infectious bovine rhinotracheitis virus in calves in Hungary. *Acta. Vet. Acad. sci. Hung.* 19, 145-151.

Bello, L.J., Whitbeck, J.C., and Lawrence, W.C. (1992). Bovine herpesvirus 1 as a live

virus vector for expression of foreign genes. *Virol.* 190, 666-673.

Bello, L.J., Whitbeck, J.C., and Lawrence, W.C. (1987). Map location of the thymidine kinase gene of bovine herpesvirus 1. *J. Virol.* 61, 4023-4025.

Berzofsky, J. (1990). Immunogenicity of antigens recognized by T-cells. In, Cellular immunology and the immunotherapy of cancer, (Lotze, M.T., and Finn, O.J., eds), pp. 1-22. Wiley-Liss, Inc., New York.

Bielefeldt Ohmann, H., and Babiuk, L.A. (1988). Induction of receptors for complement and immunoglobulins by herpesviruses of various species. *Virus Res.* 9, 335-342.

Bishop, G.A., Marlin, S.D., Schwartz, S.A., and Glorioso, J.C. (1984). Human natural killer cell recognition of herpes simplex virus type 1 glycoproteins: Specificity analysis with the use of monoclonal antibodies and antigenic variants. *J. Immunol.* 133, 2206-2214.

Bolton, D.C., Zee, Y.C., and Ardans, A.A. (1983). Identification of envelope and nucleocapsid proteins of infectious bovine rhinotracheitis virus by SDS-polyacrylamide gel electrophoresis. *Vet. Microbiol.* 8, 57-68.

Brake, F., and Studdert, M.J. (1985). Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo, and caprine herpesvirus 1 and bovine encephalitis herpesvirus. *Aust. Vet. J.* 62, 331-334.

Bratanich, A.C., Sardi, S.I., Smitstaart, E.N., Schudel, A.A. (1991). Comparative studies of BHV-1 variants by in vivo and in vitro tests. *Zbl. Vet. Med. B* 38, 41-48.

Bulach, D.M., and Studdert, M.J. (1990). Comparative genome mapping of bovine encephalitis herpesvirus, bovine herpesvirus 1, and buffalo herpesvirus. *Arch. Virol.* 113, 17-34.

Bush, C.E., and Prichett, R.F. (1986). Immunological comparison of the proteins of pseudorabies (Aujeszky's disease) virus and bovine herpesvirus-1. *Am. J. Vet. Res.* 47, 1708-1712.

Bzik, D.J., Debroy, C., Fox, B., Pederson, N.E., and Person, S. (1986). The nucleotide sequence of the gB glycoprotein gene of HSV-2 and comparison with the corresponding gene of HSV-1. *Virol.* 155, 322-333.

Cai, W., Gu, B., and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* 62, 2596-2604.

Cai, W., Person, S., Warner, S.C., Zhou, J., and Deluca, N.A. (1987). Linker insertion, nonsense, and restriction site deletion mutations of the gB glycoprotein gene of herpes simplex virus-1. *J. Virol.* 61, 714-721.

Card, J.P., Whealy, M.E., Robbins, A.K., and Enquist L.W. (1992). Pseudorabies virus envelope glycoprotein gI infleunces both neurotropism and virulence during infection of the rat visual system. *J. Virol.* 66, 3032-3041.

Carillo, B.J., Pospischil, A., and Dahme, E. (1983). Pathology of bovine necrotizing encephalitis in Argentina. *Zbl. Vet. Med.* B 30, 161-168.

Carillo, B.J., Ambrogi, A., Schudel, A.A., Vazquez, M., Dahme, E., and Pospischil, A. (1983). Meningoencephalitis caused by IBR virus in calves in Argentina. *Zbl. Vet. Med. B* 30, 327-332.

Carter, V.C., Schaffer, P.A., and Tevethia, S.S. (1981). The involvement of herpes simplex type 1 glycoproteins in cell-mediated immunity. *J. Immunol.* 126, 1655-1660.

Chang, L.W.S, Zee, Y.C., and Ardans, A.A. (1986). Neutralizing monoclonal antibodies directed to infectious bovine rhinotracheitis virus. *Arch. Virol.* 88, 203-215.

Chowdhury, S.I., Ludwig, H., and Buhk., H.J. (1988). Molecular biological characterization of equine herpesvirus type 1 (EHV-1) isolated from ruminant hosts. *Virus Res.* 11, 127-139.

Chowdhury, S.I., Buhk., H.J., Ludwig, H., and Hammerschmidt, W. (1990). Genomic termini of equine herpesvirus 1. *J. Virol.* 64, 873-880.

Collins, J.K., Butcher, A.C., Riegel, C.A., McCrane, V., Blair, C.D., Teramoto, Y.A., and Winston, S. (1984). Neutralizing determinant defined by monoclonal antibodies on polypeptides specified by bovine herpesvirus 1. *J. Virol.* 52, 403-409.

Collins, J.K., Ayers, V.K., Whetstone, C.A., van Drunen Little-van den Hurk, S. (1993). Antigenic differences between the major glycoproteins of bovine herpesvirus type 1.1 and bovine herpesvirus encephalitis type 1.3. *J. Gen. Virol.* 74, 1509-17.

Collins J.K., Butcher, A.C., and Riegel., C.A. (1985). Immune response to bovine herpesvirus type 1 infections: Virus-specific antibodies in sera from infected animals. *J. Clinical Micobiol.* 21, 456-552.

Cox, G.J.M., Zamb, T.J., and Babiuk, L.A. (1993). Bovine herpesvirus 1:Immune response in mice and cattle injected with plasmid DNA. *J. Virol.* 67, 5664-5667.

Cranage, M.P., Kouzarides, T., Bankier, A.T., Satchwell, S., Weston, K., Tomlinson, P., Barrell, B., Hart, H., Bell, S.E. Minson, A.C., and Smith, G.L. (1986). Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. *EMBO J.* 5, 3057-3062.

Davison, A.J., and Taylor, P. (1987). Genetic relations between varicella zoster virus and Epstein-Barr virus. *J. Gen. Virol.* 68, 1067-1079.

Deatly, A.M., Spivak, J.G., Lavi, E., and Fraser, N.W. (1987). RNA from an immediate early region of the type 1 herpes simplex virus genome is presentin the trigeminal ganglia of latently infected mice. *Proc. Natl. Acad. Sci. USA* 84, 3204-3208.

Dieckmann, C.L., and Tzagoloff, A. (1985). Assembly of mitochondrial membrane system. CBP6, a yeast nuclear gene necessary for synthesis of cytochrome b. *J. Biol. Chem.* 260, 1513-1520.

Dubuisson, J., Israel, B.A., and Letchworth III, G.J. (1992). Mechanisms of bovine herpesvirus type 1 neutralization by monoclonal antibodies to glycoproteins gI, gIII, and gIV. *J. Gen. Virol.* 73, 1-9.

Eisenberg, R.J., Ponce de leon, M., Friedman, H.M., Fries, L.F., Frank, M.M., Hastings, J.C., and Cohen, G.H. (1987). Complement component C3b binds directly to purified glycoprotein C of herpes virus simplex virus types 1 and 2. *Microb. Pathogen.* 3, 423-435.

Engles, M., Giuliani, C., Wild, P., Beck, T.M., Loepfe, E., and Wyler, R. (1987). The genome of bovine herpesvirus 1 (BHV-1) exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. *Virus Res.* 6, 57-73.

Engles, M., Loepfe, E., Wild, P., Scharner, E., and Wyler R. (1987). The genome of caprine herpesvirus 1: Genome structure and relatedness to bovine herpesvirus 1. *J. Gen. Virol.* 68, 2019-2023.

Eugster, A.K., Angulo, A.A., Jones, L.P.(1974). Herpesvirus encephalitis in range calves. In, Proceedings, 17th Ann. Meet. Amer. Asso. Vet. Lab. Diagnostician, pp. 267-290.

Fehler, F., Herrmann, J.M., Saalmuller, A., Mettenleiter, T.C., Keil, G.M. (1992).

Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. *J. Virol.* 66, 831-9

Field, H.J., and Owen, L. (1988). The problem of virus drug resistance in antiviral drug development. In: Antiviral drug development: A multidisciplinary approach (E. De Clercq ed.). pp. 203-236 New York and London. Plenum press.

Fitzpatrick, D.R., Redmond, M.J., Attah-poku, S.K., Van drunen Little-van den hurk, S., Babiuk, L.A., and Zamb, T.J. (1990a). Mapping of 10 epitopes on bovine herpesvirus type-1 glycoproteins gI and gIII. *Virol.* 176, 145-147.

Fitzpatrick, D.R., Babiuk, L.A., and Zamb, T.J. (1989). Nucleotide sequence of bovine herpesvirus type 1 glycoprotein gIII, a structural model for gIII as a new member of the immunoglobulin superfamily, and implications for the homologous glycoproteins of other herpesviruses. *Virol.* 173, 46-57.

Fitzpatrick, D.R., Zamb, T.J., and Babiuk, L.A. (1990b). Expression of bovine herpesvirus type 1 glycoprotein gI in transfected cells induces spontaneous cell fusion. *J. Gen. Virol.* 71, 145-157.

Fitzpatrick, D.R., Zamb, T., Parker, M.D., Van drunen Little-van den hurk, S., Babiuk, L.A., and Lawman, M.J.P. (1988). Expression of bovine herpesvirus-1 glycoproteins gI and gIII in transfected murine cells. *J. Virol.* 62, 4239-4248.

Forman, A.J., Babiuk, L.A., Misra, V., and Baldwin, F. (1982). Susceptibility of bovine macrophages to infectious bovine rhinotracheitis virus infection. *Infect. Immun.* 35, 1048-1057.

Forman, A.J., and Babiuk, L.A. (1982). Effect of infectious bovine rhinotracheitis virus infection on bovine alveolar macrophage function. *Infect. Immun.* 35, 1041-1047.

French, E.L. (1962a). A specific virus encephalitis in calves: Isolation and characterization of the causal agent. *Aust. Vet. J.* 38, 207-215.

French, E.L. (1962b). Relationship between infectious bovine rhinotracheitis virus and a virus isolated from calves with encephalitis. *Aust. Vet. J.* 38, 477-478.

Friedli, K., and Metzler, A.E. (1987). Reactivity of monoclonal antibody to proteins of neurotropic bovine herpesvirus 1 (BHV-1) strain and to proteins of representative BHV-1 strains. *Arch. Virol.* 94, 109-122.

Fuller, A.O., and Spear, P.G. (1987). Anti-glycoprotein D antibodies that permit

adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. *Proc Natl Acadm Sci (USA)* 84, 5454-5458.

Geder, L., Lee, K.J., Dawson, M.S., Engle, R., Maliniak, R.M., and Lang, C.M. (1981). Induction of persistent infection in mice and oncogenic transformation of mouse macrophages with infectious bovine rhinotracheitis virus. *Am J. Vet. Res.* 42, 300-307.

Gibbs, E.P.J., and Rweyemamu, M.M. (1977). Bovine herpesvirus. I. Bovine herpesvirus-1. *Vet. Bull. (London)* 47, 317-343.

Gillespie, J.H., Mcentee, K., Kendrick, J.W., and Wagner, W.C. (1959). Comparison of infectious pustular vulvovaginitis virus with infectious bovine rhinotracheitis virus. *Cornell Vet.* 49, 288-297.

Glorioso, J., Schroder, C.H., Kumel, G., Szczesiul, M., and Levine, M. (1984). Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. *J. Virol.* 50, 805-812.

Gregersen, J.P., Pauli, G., and Ludwig, H. (1985). Bovine herpesvirus 1: Differentiation of IBR- and IPV-viruses and identification and functional role of their major immunogenic components. *Arch. Virol.* 84, 91-103.

Hayes, M.K., and Rock, D.L. (1990). Identification of a novel bovine herpesvirus type 1 immediate-early infected cell protein. *Arch. Virol.* 112, 291-300.

Herold, B.C., Wudunn, D., Soltys, N., and Spear, P.G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and infectivity. *J. Virol.* 65, 1090-1098.

Highlander, S.L, Sutherland, S.I., Gage, P.J., Johnson, D.C., Levine, M., and Glorioso, J.C. (1987). Neutralizing monoclonal antibodies for herpes simplex virus glycoprotein D inhibit virus penetration. *J. Virol.* 61, 3356-3364.

Holland, T.C., Homa, F.L., Marlin, S.D., Levine, M., and Glorioso, J. (1984). Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. *J. Virol* 52, 566-574.

Homan, E.J., and Easterday, B.S. (1982). Interactions of bovine herpesvirus type 1 and neurons in vitro. *J. Infect. Dis.* 146, 97.

Hughes, G., Babiuk, L.A., and van Drunen Little-van den Hurk, S. (1988). Functional and topographical analysis of epitopes on bovine herpesvirus type 1

glycoprotein IV. *Arch. Virol.* 103, 47-60.

Hutchings, D.L., van Drunen Little-van den Hurk, S., and Babiuk, L.A. (1990). Lymphocyte proliferative responses to separate bovine herpesvirus-1 proteins in immune cattle. *J. Virol.* 64, 5114-5122.

Izumi, K.M., and Stevens, J.G. (1990). Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. *J. Exp. Med.* 172, 487-496.

Johnson, D.C., Burke, R.L., and Gregory, T. (1990). Soluble forms of bovine herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. *J. Virol.* 64, 2569-2576.

Johnson, D.C., and Ligas, M.W. (1988). Herpes simplex virus lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. *J. Virol.* 62, 4605-4712.

Jones C., Delhon, G., Bratanich, A., Kutish, G., and Rock, D. (1990). Analysis of the transcriptional promoter which regulates latency-related transcript of bovine herpesvirus 1. *J. Virol.* 64, 1164-1170.

Kahrs, R.F. (1977). Infectious bovine rhinotracheitis: A review and update. *J. Am. Vet. Med. Assoc.* 171, 1055-1064.

Keller, P.M., Davison, A.J., Lowe, R.S., Bennet, C.D., and Ellis, R.W. (1986). Identification of the structure of the gene encoding gpII, a major glycoprotein of varicella zoster virus. *Virol.* 152, 181-191.

Kelley, R.B., Cozzarelli, N.R., Deutscher, M.P., Lehman, I.R., and Kornberg, A. (1970). Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. *J. Biol. Chem.* 245, 39.

Kit, S., Kit, M., and McConnel, S. (1988). Intramuscular and intra vaginal vaccination of pregnant cows with thymidine kinase-negative, temperature-resistant infectious bovine rhinotracheitis virus (bovine herpesvirus 1). *Vaccine* 4, 55-61.

Kit, S., Kit, M., and McConnell, S. (1986). Intramuscular and intravaginal vaccination of pregnant cows with thymidine kinase-negative, temperature-resistant infectious bovine rhinotracheitis virus (bovine herpesvirus 1). *Vaccine* 4, 55-61.

Kit, M., Kit, S., Little, S.P., Richard, D., Marchi Di., and Gale, C. (1991). Bovine

herpesvirus-1 (Infectious bovine rhinotracheitis virus)-based viral vector which expresses foot-and-mouth disease epitopes. *Vaccine* 9, 564-572.

Kit, S., and Kit, M. (1991). Genetically engineered herpesvirus vaccines. In: Progress in medical virology. (Melnick J.L. ed.). Basel, Karger 38, 128-166.

Kit, S., and Qavi, H. (1983). Thymidine kinase (TK) induction after infection of TK-deficient rabbit cell mutants with herpesvirus type 1 (BHV-1): Isolation of TK$^-$ BHV-1 mutants. *Virol.* 130, 381-389.

Koerner, T.J., Hill, J.E., Myers, A.M., and Tzagoloff, A. (1991). High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. *Methods in Enzymology*. 194, 477-490.

Kornfeld, R., and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.* 54, 631-664.

Kumel, G., Kaerner, H.C., Levine, M., Schroder, C.H., and Glorioso, J.C. (1985).Passive immune protection by herpes simplex virus specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenecity. *J. Virol.* 56, 930-937.

Kutish, G., Mainprize, T., and Rock, D. (1990). Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. *J. Virol.* 64, 5730-5737.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of protein. *J. Mol. Biol.* 157, 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680-685.

Langeland, N., Oyan, A.M., Marsden, H.S., Cross, A., Glorioso, J.C., Moore, L.J., and Haarr, L. (1990). Localization on the herpes simplex type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. *J. Virol.* 64, 1271-1277.

Lawrence, W.C., D'urso, R.C., Kundel, C.A., Whitbeck, J.C., and Bello, L.J. (1986). Map location of the gene for a 130,000-dalton glycoprotein of bovine herpesvirus 1. *J. Virol.* 60, 405-414.

Liang, X., Babiuk, L.A., and Zamb, T.J. (1991). Pseudorabies virus gIII and bovine herpesvirus 1 gIII share complementary functions. *J. Virol.* 65,5553-5557.

Liang, X., Babiuk, L.A., Van Drunen Little-van den Hurk, S., Fitzpatrick, D.R., and Zamb, T.J. (1991). Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII and gIV. *J. Virol.* 65, 1124-1132.

Liang, X., Babiuk, L.A., and Zamb, T.J. (1992). An in vivo glycoprotein gIII-negative bovine herpesvirus 1 (BHV-1) mutant expressing B-galactosidase: Evaluation of the role of gIII in virus infectivity and its use as a vector. *Virol.* 189, 629-639.

Liang, X., Babiuk, L.A., Zamb, T.J. (1993). Mapping of heparin-binding structures on bovine herpesvirus 1 and pseudorabies virus gIII glycoproteins. *Virol.* 194, 233-43

Lin, H., Parmacek, M.S., Morle, G., Bolling, S., and Leiden, M.J. (1990). Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 82, 2217-2221.

Little, S.P., Jofre, J.T., Courtney, R.J., and Schaffer, P.A. (1981). A virion-associated glycoprotein essential for infectivity of herpes simplex type 1. *Virol.* 115, 149-160.

Ludwig, G.V., and Letchworth, G.J. (1987). Temporal control of bovine herpesvirus 1 glycoprotein synthesis. *J. Virol.* 61, 3292-3294.

Ludwig H. (1983). Bovine herpesviruses. In: The herpesviruses. vol II. Roizman B (ed). p. 135-214. Plenum Press. N.Y.

Lupton, H.W., and Reed, D.E. (1980). Evaluation of experimental subunit vaccines for infectious bovine rhinotracheitis. *Am. J. Vet. Res.* 41, 383-390.

Marshal, R.L., Israel, B.A., and Letchworh III, G.J. (1988). Monoclonal antibody analysis of bovine herpesvirus-1 glycoprotein antigenic areas relevant to natural infection. *Virol.* 165, 338-347.

Marshal, R.L., Rodriguez, L.L., and Letchworth III, G.J. (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus 1) by biochemical and immunological methods. *J. Virol.* 57, 745-753.

Maxam, A.M., and Gilbert, W. (1977). Chemical sequencing of DNA. *Proc. Natl. Acad. Sci. (USA)* 74, 560-564.

Maxam, A.M., and Gilbert, W. (1980). Sequencing end-labeled DNA with base specific chemical cleavages. *Methods Enzymol.* 65, 499.

Mayfield, J.E, Good, P.J., Van Oort, H.J., Campbell, A.R., and Reed, D.E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesviruses 1 (Cooper strain. *J. Virol.* 47:259-264.

Mettenleitner, T.C., Laszlo, Z., Zuckermann, F., Sugg, N., Kern, H., and Ben-Porat, T. (1990).. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. *J. Virol.* 64, 278-286.

Mettenleitner, T.C., Zsak, L., Zuckermann, F., Sugg, N., Kern, H., and Ben-Porat, T. (1990). Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. *J. Virol.* 64, 278-286.

Metzler, A.E., Matile, H., Gassman, U., Engels, M., and Wyler, R. (1985). European isolates of bovine herpesvirus 1: A comparison of restriction endonuclease sites, polypeptides, and reactivity with monoclonal antibodies. *Arch. Virol.* 85, 57-69.

Metzler, A.E., Schudel, A.A., and Engles, M. (1986). Bovine herpesvirus 1: Molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. *Arch. Virol.* 87, 205-217.

Miller, J.M., Whetstone, C.A., Bello, L.J., and Lawrence, W.C. (1991). Determination of the ability of thymidine kinase-negative deletion mutantof bovine herpesvirus 1 to cause abortion in cattle. *Am. J. Vet. Res.* 52, 1038-1042.

Misra, V., Blumenthal, R.M., and Babiuk, L.A. (1981). Proteins specified by bovine herpesvirus 1 (infectious bovine rhinotracheitis). *J. Virol.* 40, 367-378.

Misra, V., Nelson, R., Smith, M. (1988). Sequence of a bovine herpesvirus type-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. *Virol.* 166, 542-9

Mittal, S.K., and Field, H.J. (1989). Analysis of the bovine herpesvirus type 1 thymidine kinase (TK) gene from wild-type virus and TK-deficient mutants. *J. Gen. Virol.* 70, 901-918.

Moretti, B., Orfei, Z., Mondino, G., and Persechino, A. (1964). Infectious bovine rhinotracheitis, clinical observations and isolation of virus. *Vet. Ital.* 15, 676.

Narita, M., Inui, S., Nanba, K., and Shimizu, Y. (1981). Recrudescence of infectious bovine rhinotracheitis virus and associated neural changes in calves treated with dexamethasone. *Amer. J. Vet. Res.* 42, 1192-1197.

Nettleton, P.F., and Sharp, J.M. (1980). Infectious bovine rhinotracheitis virus

excretion after vaccination. *Vet. Rec.* 107, 379.

Okazaki, K., Matsuzaki, T., Sugahara, Y., Okada, J., Hasebe, M., Iwamura, Y., Ohnishi, M., Kanno, T., Shimizu, M., Honda, E., and Kono, Y. (1991). BHV-1 adsorption is mediated by the interaction of glycoprotein gIII with heparine like moeity on the cell surface. *Virol.* 181, 666-670.

Okazaki, K., Honda, E., Minetoma, T., and Kumagai, T. (1987). Bovine herpesvirus type 1 gp87 mediates both attachment of virion to susceptible cells and haemagglutination. *Arch. virol.* 97, 297-307.

Osorio, F.A., Reed, D.E., Van der maaten, M.J., and Metz, C.A. (1985). Comparison of the herpesviruses of cattle by DNA restriction endonuclease analysis and serologic analysis. *Am. J. Vet. Res.* 46, 2104-2109.

Owen, L.J., and Field, H.J. (1988). Genomic localization and sequence analysis of the putative bovine herpesvirus-1 DNA polymerase gene. *Arch. Virol.* 98, 27-38.

Pastoret, P.P., Babiuk, L.A., Misra, V., and Griebel, P. (1980a). Reactivation of temperature-sensitive and non-temperature sensitive infectious bovine rhinotracheitis vaccine virus with dexamethasone. *Infect. Immun.* 29, 483-488.

Pastoret, P.P., Burtonboy, G., AGuilar-Setien, A., Godart, M., Lamy, M.E., and Schoenars, F. (1980b). Comparison between strains of infectious bovine rhinotracheitis virus (bovid herpesvirus 1), from respiratory and genital origins, using polyacrylamide gel electrophoresis of structural proteins. *Vet. Microbiol.* 5, 187-194.

Pellett, P.E., Biggin, M.D., Barrell, B., and Roizman, B. (1985a). Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. *J. Virol.* 56, 807-813.

Pellett, P.E., Kousoulas, K.G., Pereira, L., and Roizman, B. (1985b). Anatomy of herpes simplex virus 1 strain F glycoprotein B gene: Primary sequence and predicted protein structure of the wildtype and of monoclonal antibody resistant mutants. *J. Virol.* 53, 243-253.

Petrovskis, E.A., Timmins, J.G., Armentrout, M.A., Marchioli, C.C., Yancey, Jr., R.J., and Post, L.A.. (1986). DNA sequence of the gene for pseudorabies virus gp50, a glycoprotein without N-linked glycosylation. *J. Virol.* 59, 216-223.

Puga, A., and Notkins, A.L. (1987). Continued expression of a poly(A)$^+$ transcript

of herpes simplex virus type 1 in trigeminal ganglia of latently infected mice. *J. Virol.* 61, 1700-1703.

Rauh, I., Weiland, F., Fehler, F., Keil, G., and Mettenleitner, T.C. (1991). Pseudorabies virus mutants lacking the essential glycoprotein II can be complemented by glycoprotein gI of bovine herpesvirus-1. *J. Virol.* 65, 621-631.

Riggio, M.P., Cullinane, A.A., and Onions, D.E. (1989). Identification and nucleotide sequence of the glycoprotein gB gene of equine herpesvirus 4. *J. Virol.* 63, 1123-1133.

Robbins, A.K., Dorney, D., Wathen, M.W., Whealy, M.E., Gold, C., Watson, R.J., Holland, L.E., Weed, S.D., Levine, M., Glorioso, J., and Enquist, L.W. (1987). The pseudorabies virus gII gene is closely related to the gB glycoprotein genes of herpes simplex virus. *J. virol.* 61, 2691-2701.

Robbins, A.K., Watson, R.J., Whealy, M.E., Hays, W.W., and Enquist, L.W. (1986a). Characterization of a pseudorabies virus glycoprotein gene with homology to herpes simplex virus type 1 and type 2 glycoprotein C. *J. Virol.* 58, 339-347.

Rock, D.L, Hagemoser, W.A., Osorio, F.A., and Reed, D.E. (1986). Detection of bovine herpesvirus type 1 RNA in trigeminal ganglia of latently infected rabbits by in situ hybridization. *J. Gen. Virol.* 67,2515-2520.

Rock, D.L, Nesburn, A.B., Ghiasi, H., Ong, J., Lewis, T.L., Lokensgard, J.R., and Wechsler, S.L. (1987). Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *J. Virol.* 61(12), 3820-3826.

Rock, D.L, Beam, S.L., and Mayfield, J.E. (1987). Mapping of bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. *J. Virol.* 61(12), 3827-3831.

Rock, D.L., Hagemoser, W.A., Osorio, F.A., and Mcallister, H.A. (1988). Transcription from the pseudorabies virus genome during latent infection. *Arch. Virol.* 98, 99-106.

Roizman, B., Desrosiers, R.C., Fleckenstein, B., Lopez, C., Minson, A.C., and Studdert, M.J. (1992). The family herpesviridae: an update. *Arch. Virol.* 123, 425-449.

Roizman, B. (1990a). Herpesviridae: A brief introduction. *In* "Fields' Virology." (B.N. Fields, D.M., Knipe, R.M. Chanock, J.L. Melnick, B. Roizman, and R.E.

Shope, Eds.), 2nd ed., pp. 1787-1793. Raven Press Ltd., New York.

Roizman, B., and Sears, A.E. (1990). Herpes simplex viruses and their replication. *In* "Fields' Virology". (B.N. Fields, D.M. Knipe, R.M. Chanock, J.L. Melnick, B. Roizman, and R.E. Shope, Eds.), pp. 1795-1841. Raven Press Ltd., New York.

Roizman, B., Carmichael, L.E., Deinhardt, F., De the, G., Nahmias, A.J., Plowright, W., Rapp, F., Sheldrick, P., Takahashi, M., and Wolf, K. (1981). Herpesviridae. Definition, provisional nomenclature, and taxonomy. *Intervirology* 16, 201-217..

Roizman B., and Sears, A.E. (1990). Herpes simplex viruses and their replication. Fields B.N, and Knipe D.M (eds). vol 2. p.1795-1841. Raven Press N.Y.

Rossi, C.E., and Kiesel, G.K. (1982). Effect of infectious bovine rhinotracheitis virus immunization on viral shedding in challenge-exposed calves treated with dexamethasone. *Amer. J. Vet. Res.* 47, 1139-1141.

Rouse, B.T., and Babiuk, L.A. (1978). Mechanisms of recovery from herpesvirus infections-A review. *Canad. J. Comp. Med.* 42, 415-427.

Rouse, B.T., and Horohov, D.W. (1984). Cytotoxic T lymphocytes in herpesvirus infections. *Vet. Immunol. Immunopathol.* 6, 35-66.

Rziha, H.J., Mettenleiter, T.C., Ohlinger, V., and Wittmann, G. (1986). *Virol.* 155, 600-613.

Sambrook J., Fritsch, E., and Maniatis, T. (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor laboratories, Cold Spring Harbor, N.Y.

Sanger, F., Nicklen, S.,and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. (USA)* 74, 5463-5467.

Schudel, A.A., Carillo, B.J., Wyler, R., and Metzler, A.E. (1986). Infections of calves with antigenic variants of bovine herpesvirus-1 (BHV-1) and neurological disease. *Zbl. Vet. Med. B* 33, 303-310.

Scott, H.A., Whalley, J.N., Mattick, J.S., Underwood, P.A., Aboud, L., Williams, K.L., and Kirckland, P. (1988). Identification of major antigenic proteins of bovine herpesvirus 1 and their correlation with virus neutralizing activity. *Vet. Microbiol.* 16, 109-121.

Seal, B.S., St. Joer, S.C., and Lee Taylor, R.E. (1985). Restriction endonuclease analysis of bovine herpesvirus-1 DNA and nucleic acid homology between isolates. *J. Gen. Virol.* 66, 2787-2792.

Seal, B.S., Whetstone, C.A., Zamb, T.J., Bello, L.J., Lawrence, W.C. (1992). Relationship of bovine herpesvirus 1 immediate-early, early, and late gene expression to host cellular gene transcription. *Virol.* 188, 152-9

Seal, B.S., Irving, J.I., and Whetstone, C.A. (1991). Transcriptional analysis of the bovine herpesvirus 1 Cooper isolate: Temporal analysis and characterization of immediate-early, early and late RNA. *Arch. Virol.* 121, 55-73.

Simard, C., Nadon, F., Seguin, C., Laboissiere, S., and Trudel, M. (1990). Gene mapping of infectious bovine rhinotracheitis viral DNA genome. *Arch. Virol.* 110, 63-75.

Simard, C., Laboissiere, S., Seguin, C., and Trudel, M. (1991). Genomic heterogeneities in bovine herpesvirus type 1 viral isolates: A major variant selected from a field isolate. *Intervirology* 32, 117-126.

Smith, P.C., Nusbaum, K.E., Kwapien, R.P., Stringfellow, D.A., and Driggers, K. (1990). Necrotic oophoritis in heifers vaccinated intravenously with infectious bovine rhinotracheitis virus vaccine during estrus. *Am. J. Vet. Res.* 51, 969-972.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separared by gel electrophoresis. *J. Mol. Biol.* 98, 503.

Stevens, J.G., Wagner, E.K., Devi-rao, G.B., Cook, M.L., and Feldman, L.T. (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. *Science* 235, 1056-1059.

Straub, O.C. (1990). Infectious bovine rhinotracheitis virus. *In* "Virus Infections of Ruminants." (Z. Dinter and B. Morein, Eds.), pp. 71-108. Elsevier Science Publishers, New York.

Studdert, M.J. (1989). Bovine encephalitis herpesvirus. *Vet. Rec.* 125, 584.

Szewczyk, B., and Summers, D.F. (1988). Preparative elution of proteins blotted to immobilon membranes. *Anal. Biochem.* 168, 48-53.

Thiry, E., Saliki, J., Pastoret, P.P., Lambert, A.F., and Ligot, J. (1984). *Vet. Rec.* 115, 248-249.

Thiry, E., Dubuisson, J., and Pastoret, P.P. (1986). Pathogenesis, latency and

reactivation of infections by herpesviruses. *Rev. Sci. Tech. Off. Int. Epiz.* 5, 809-819.

Thiry, E., Saliki, J., Schwers, A., and Pastoret, P.P.(1985). *Vet. Rec.* 116, 599-600.

Tikoo, S.K., Zamb, T.J., and Babiuk, L.A. (1993). Analysis of bovine herpesvirus 1 glycoprotein gIV truncation and deletions expressed by recombinant vaccinia viruses. *J. Virol.* 67, 2103-2109.

Tikoo, S.K., Parker, M.D., van-den-Hurk, J.V., Kowalski, J., Zamb, T.J., Babiuk, L.A. (1993). Role of N-linked glycans in antigenicity, processing, and cell surface expression of bovine herpesvirus 1 glycoprotein gIV. *J. Virol.* 67, 726-33

Tikoo, S.K., Fitzpatrick, D.R., Babiuk, L.A., and Zamb, T.J. (1990). Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. *J. Virol.* 64, 5132-5142.

Todd, J.D., Volenec, F.J., and Paton, I.M. (1972). Interferon in nasal secretions and sera of calves after intranasal administration of avirulent infectious bovine rhinotracheitis virus: Association of interferon in nasal secretions with early resistance to challenge with virulent virus. *Infect. Immun.* 5, 699-706.

Todd, J.D., Volenec, F.J., and Paton, I.M. (1971). Intranasal vaccination against infectious bovine rhinotracheitis: Studies on early onset of protection and use of the vaccine in pregnant cows. *J.A.V.M.A.* 159, 1370-1373.

Van Drunen Little-van den Hurk, S., Parker, M.D., Massie, B., van den Hurk, J.V., Harland, R., Babiuk, L.A., and Zamb, T.J. (1993). Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. *Vaccine* 11, 25-35

Van Drunen Little-van den Hurk, S., and Babiuk, L.A. (1986). Synthesis and processing of bovine herpesvirus type 1 glycoproteins. *J. Virol.* 59, 401-410.

Van Drunen Little-van den Hurk, S., Hughes, G., and Babiuk, L.A. (1990a). The role of carbohydrate in the antigenic and immunogenic structure of bovine herpesvirus type-1 glycoproteins gI and gIV. *J. Gen. Virol.* 71, 2053-2063.

Van Drunen Little-van den Hurk, S., and Babiuk., L.A. (1985). Effect of Tunicamycin and Monesin on biosynthesis, transport, and maturation of bovine herpesvirus type 1 glycoproteins. *Virol.* 143, 104-118.

Van Drunen Little-van den Hurk, S., van den Hurk, J.V., Gilchrist, J.E., Misra, V., and Babiuk, L.A. (1984). Interactions of monoclonal antibodies and bovine

herpesvirus type 1 (BHV-1) glycoproteins. *Virol.* 135, 466-479.

Van Drunen Little-van den Hurk, S., Gifford, G.A., and Babiuk, L.A. (1990b). Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. *Vaccine* 8, 358-368.

Van der Maaten, M.J., Miller, J.M., and Whetstone, C.A. (1985). Ovarian lesions induced in heifers by intravenous inoculation with modified-live infectious bovine rhinotracheitis virus on the day after breeding. *Am. J. Vet. Res.* 46, 1996-1999.

von Heinje, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14, 4683-4690.

Weijer, W.J. (1988). Antibodies against synthetic peptides of herpes simplex virus type 1 glycoprotein D and their capability to neutralize viral infectivity in vitro. *J. Virol.* 62, 501-510.

Watson, R.J. (1983). DNA sequence of the herpes simplex virus type 2 glycoprotein D gene. *Gene* 26, 307-312.

Watson, R.J., Weis, J.H., Salstrom, J.S., and Enquist, L.W. (1982). Herpes simplex virus type-1 glycoprotein D gene: nucleotide sequence and expression in Escherichia coli. *Science* 218, 381-384.

Weinmaster, G.A., Misra, V., McGuire, R., Babiuk, L.A., and Declercq E. (1982). Bovid herpesvirus type 1 (Infectious bovine rhinotracheitis)-induced thymidine kinase. *Virol.* 118, 191-201.

Whalley, J.M., Robertson, G., Scott, N., Hudson, G., Bell, C., and Woodworth, L. (1989). Identification and nucleotide sequence of a gene in equine herpesvirus 1 analogous to the herpes simplex virus gene encoding the major envelope glycoprotein gB. *J. Gen. Virol.* 70, 383-394.

Whitbeck, J.C., Bello, L.J., and Lawrence, W.C. (1988). Comparison of bovine herpesvirus gI and the herpes simplex virus type 1 gB gene. *J. Virol.* 62, 3319-3327.

Wirth, U.V., Vogt, B., and Schwyzer, M. (1991). The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. *J. Virol.* 65, 195-205.

Wirth, U.V., Vogt, B., and Schwyzer, M. (1991). The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced

transcription units. *J. Virol.* 65, 195-205.

Wirth, U.V., Gunkel, K., Engels, M., and Schwyzer, M. (1989). Spatial and temporal distribution of bovine herpesvirus type 1 transcripts. *J. Virol.* 63, 4882-4889.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P.L. (1990). Direct gene transfer into mouse muscles in vivo. *Science* 247, 1465-1468.

Wudunn, D., and Spear, P.G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* 665, 4317-4324.

Wyler, R., Engles, M., and Schwyzer, M. (1989). Infectious bovine rhinotracheitis / vulvovaginitis (BHV-1). In: Herpesvirus diseases of cattle, horses, and pigs. Developments in veterinary virology. G. Wittman (ed). Kluwer Academic Publisher, Boston, Mass.

Yates, W.D.G. (1982). A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can J. Comp. Med.* 46, 225-263.

Zhang, Y-F, and Wagner, E.K. (1987). The kinetics of expression of individual herpes simplex type 1 transcripts. *Virus Genes* 1, 49-60.

Zygraich, N., Lobmann, M., Vascoboinic, E., Berge, E., and Huygelen, C. (1974). *In vivo* and *in vitro* properties of a temperature sensitive mutant of infectious bovine rhinotracheitis virus. *Res. Vet. Sci.* 16, 328-335.