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Characterization of the Glycoprotein B Gene from Ruminant Alphaherpesviruses*

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Abstract. The complete open reading frame and promoter region of the glycoprotein B (*gB*) gene has been identified and sequenced from five poorly characterized alphaherpesviruses of ruminants, bovine herpesvirus 5 (BHV-5), buffalo herpesvirus 1 (BuHV-1), cervine herpesvirus 1 (CerHV-1), rangiferine herpesvirus 1 (RanHV-1), and caprine herpesvirus 1 (CapHV-1). One of the two regions identified with considerable sequence and length variation is also target of the immune system, as two B cell epitopes have been identified in this location [33]. Features shared with bovine herpesvirus 1 (BHV-1) gB include two broad hydrophobic regions, six *N*-glycosylation sites and ten conserved cysteine residues in the gB extracellular domain. Phylogenetic analysis showed that the studied ruminant alphaherpesviruses form, together with BHV-1, a consistent group within the α2 subgroup of the herpesviruses. BHV-5 and BuHV-1 are most closely related to BHV-1, followed by CerHV-1, RanHV-1 and more distantly by CapHV-1. A remarkable high degree of sequence similarity was observed between BuHV-1 and the neuropathogenic BHV-5.

Key words: BHV-1, glycoprotein B, phylogeny, ruminant alphaherpesvirus

Introduction

Bovine herpesvirus 1 (BHV-1) is a major pathogen of cattle distributed worldwide causing various symptoms, e.g., infectious bovine rhinotracheitis (IBR). Five ruminant herpesviruses are related to BHV-1; bovine herpesvirus 5 (BHV-5), buffalo herpesvirus 1 (BuHV-1), cervine herpesvirus 1 (CerHV-1), rangiferine herpesvirus 1 (RanHV-1), and caprine herpesvirus 1 (CapHV-1). BHV-5 causes fatal meningo-encephalitis in calves [1–4]. BuHV-1 was first isolated from buffaloes in Australia and was typed by serum neutralization as BHV-1 [5]. CerHV-1 was first isolated in 1982 from an outbreak of ocular disease in a red deer farm in Scotland [6]. The virus is widespread in free-living and farmed red deer [7]. RanHV-1 was isolated from caribou in Canada [8] and from reindeer in Finland [9,10], and USA [11]. CapHV-1 is distributed worldwide [12] causing generalized disease in young kids and unapparent infection in adult goats [13,14]. The significance of these herpesviruses is mainly based on their close relationship with BHV-1, which may have implications for diagnosis, as well as BHV-1 vaccination and eradication programs.

Herpesviruses are among the best characterized viruses regarding their genome structure. Complete genomes have been sequenced, as it is the case for BHV-1. However, sequence data from certain herpesviruses are still very limited or not available. The glycoprotein B (*gB*) gene is an essential component of BHV-1 [15,16], which plays an important role in virus entry [17–19]. It also represents a dominant viral

*The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers, AF359759 (BHV-5); AF359760 (BuHV-1); AF359761 (CerHV-1); AF359762 (RanHV-1) and AF359763 (CapHV-1).

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antigen that induces protective immunity in the natural host [20–23]. The gB antibodies are extensively targeted in diagnostic serology assays, mainly due to their high titers, early appearance and persistence after infection [24]. However, a general problem is the potential cross-reaction with other related herpesviruses. The *gB* gene has also been used to study the genetic relationship among herpesviruses [25–27] and sequence data of this gene are available from a large number of herpesviruses. Therefore, the comparison of gB sequences can be readily used to estimate the genetic relationship of newly characterized herpesviruses. In our preliminary study the genetic relationship among ruminant alphaherpesviruses was analysed based on gB and gD sequences [27]. In this preliminary study, only short genomic stretches were compared and BuHV-1 was not included.

In the present study the complete *gB* gene regions were sequenced and analysed from the five ruminant alphaherpesviruses. The sequences were compared to those of BHV-1 and other herpesviruses with two main purposes, (i) study the genetic relationship of this group of herpesviruses and (ii) provide information valuable for the development of specific serodiagnostic tests, with special regard to BHV-1. In addition, a comparative analysis of the different protein motifs involved in the biological function of the *gB* gene is provided.

Materials and Methods

Viruses, Cells and Reagents

BHV-1 (strains Jura and K22), BHV-5 (N565), BuHV-1 (B6), CerHV-1 (Banffshire 82), RanHV-1 (Salla 82) and CapHV-1 (E/CH) were propagated in Madin–Darby bovine kidney (MDBK) cells grown in Eagle's minimum essential medium (MEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 10% foetal calf serum. Virus titers were determined by the tissue culture infective dose ($TCID_{50}$) method following standard procedures.

Sequencing Strategy

In order to sequence the *gB* gene regions, DNA was extracted from viral suspensions by the proteinase K/SDS/phenol-chloroform method [28]. Purified viral DNA was used to amplify overlapping regions of the *gB* gene. For the promoter region, a sense primer was selected upstream the translation initiation codon (−292 to −270) from a region of homology between BHV-1 and herpes simplex virus 1 (HSV-1), and the antisense primer was selected 47–70 bases downstream the starting codon of the gB [15,16]. For the gB open reading frame (ORF), a large panel of primers were selected considering conserved motifs present in the gB of BHV-1 and other related herpesviruses. The gB ORF of BHV-5 was the first fully determined by using this approach. After the gB of BHV-5 was completely sequenced, additional primers were selected from regions highly conserved between BHV-5 and BHV-1 in order to amplify overlapping gB sequences from the rest of ruminant alphaherpesviruses.

PCR and Sequencing

The amplification reactions were performed in 50 µl reaction mixtures containing 0.2 mM of each deoxynucleotide, 10 pmol of each primer, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 2 mM $MgCl_2$, 10% dimethyl sulphoxide (DMSO, Sigma, St. Louis, MI USA), 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer Cetus, Norwalk, CON. USA) and 25–50 ng of template DNA. The thermal profiles consisted of one initial cycle of 95°C for 10 min, to activate AmpliTaq Gold DNA polymerase and to properly denature the high GC-rich template DNA, and 35 cycles with the following profile, 96°C/min for denaturation, 50–68°C/min for annealing, depending on the melting temperature of the primers used and considering previous amplification results, and 72°C/0.5–2 min for extension, depending on the approximate expected size of the region to be amplified.

PCR products were purified with QIA quick PCR purification Kit (Qiagen). Both DNA strands were sequenced with an ABI PRISM device with dye terminators (Applied Biosystems Inc., Foster City, CA USA).

Sequence Analysis

The nucleotide and deduced amino acid sequences were aligned with the program package DNASTAR (DNASTAR Inc., Madison, WI USA), by using the clustal method [29]. The DNASTAR program was

used to analyse the promoter transcription factors, to construct phylogenetic trees, and to perform hydropathy analysis. Prediction of protein motifs was performed by the MOTIF program [30]. The TopPred II program [31] was used to predict the membrane region and cytoplasmic domains. The method described by Nielsen et al. [32] was used to predict putative signal peptide cleavage site.

Results

The length of the BHV-5 *gB* gene was 2,844 base pairs (bp) and it encoded a primary translation product of 947 amino acids, 15 more amino acids than the gB of BHV-1. The sizes of the *gB* gene of the other herpesviruses were; 2,739 bp (912 amino acids) for BuHV-1; 2,790 bp (929 amino acids) for CerHV-1; 2,796 bp (931 amino acids) for RanHV-1; 2,760 bp (919 amino acids) for CapHV-1. The nucleotide sequence identities in the *gB* gene among all the studied viruses are outlined in Table 1. The GC contents of the genes were similar in all studied viruses, spanning from 69.95% of BHV-1 to 72.97% of CerHV-1.

The alignment of the promoter region is shown in Fig. 1a. Potential promoter elements associated with mRNA transcription by the RNA polymerase II were conserved in all the studied viruses. The sequence TATAT, surrounded by GC-rich sequences, is located at position −149 to −145 and may function as a TATA box element. Upstream from the TATA box at position −184 to −174 is the decanucleotide GGGGCGTCAC, which matches the consensus binding sequence for the cellular transcription factor Sp1 (GC box) at 9 of 10 positions.

The alignment of the amino acid sequences of the predicted gB primary translation product from the studied herpesviruses is presented in Fig. 1b. Two regions of significant length and sequence variation were identified. One is located near the amino terminal and the other in the central part of the protein flanking the conserved protease motif RRARR. The amino acid sequence similarities are presented in Table 1. The hydropathic analysis showed two main hydrophobic regions in all the studied viruses (Fig. 2). One hydrophobic peak is near the amino terminus and the other near the carboxyl terminus. The gB amino-terminal leader is predominantly hydrophilic in all the studied viruses. According to the sequences of suid herpesvirus 1 (SHV-1) and BHV-1, but not of HSV-1, this hydrophilic region is due to the overlapping of a part of the ICP18.5 assembly protein ORF, upstream the gB coding region, and the leader sequence of the *gB* gene. This overlapping region consists of 47 amino acids in BHV-1 [16]. According to the sequences obtained from the five studied herpesviruses, the ICP 18.5 assembly protein ORF also overlaps the leader sequence of the gB by 47 amino acids in CerHV-1 and CapHV-1; 48 amino acids in BHV-5 and BuHV-1; and 52 amino acids in RanHV-1. Analysis of the amino acid sequence at the amino terminus confirms the presence of a signal peptide cleavage site in all the studied viruses [32] (Fig. 1b). The final length of the polypeptide after cleavage of the putative signal sequence would be 865 amino acids for BHV-1, 879 amino acids for BHV-5, 844 amino acids for BuHV-1, 862 amino acids for CerHV-1, 859 amino acids for RanHV-1 and 852 amino acids for CapHV-1.

Computer analysis of the gB proteins revealed a remarkable conservation of the different motifs. The cleavage site for the protease was conserved,

Table 1. Percent of sequence identity between *gB* genes of ruminant alphaherpesviruses

	BHV-1	BHV-5	BuHV-1	CerHV-1	RanHV-1	CapHV-1
BHV-1	—	91.9	91.9	88.7	82.3	77.3
BHV-5	87.4	—	97.6	88.2	82.1	80.4
BuHV-1	87.7	97.9	—	89.4	83.9	80.8
CerHV-1	85.8	91.5	92.3	—	84.3	81.8
RanHV-1	84.0	74.0	77.1	77.9	—	79.7
CapHV-1	78.5	83.1	83.7	84.6	82.5	—

In the upper triangle the percentage of identity of amino acid sequences, while in the lower triangle the percentage of identity of nucleotide sequences is shown.

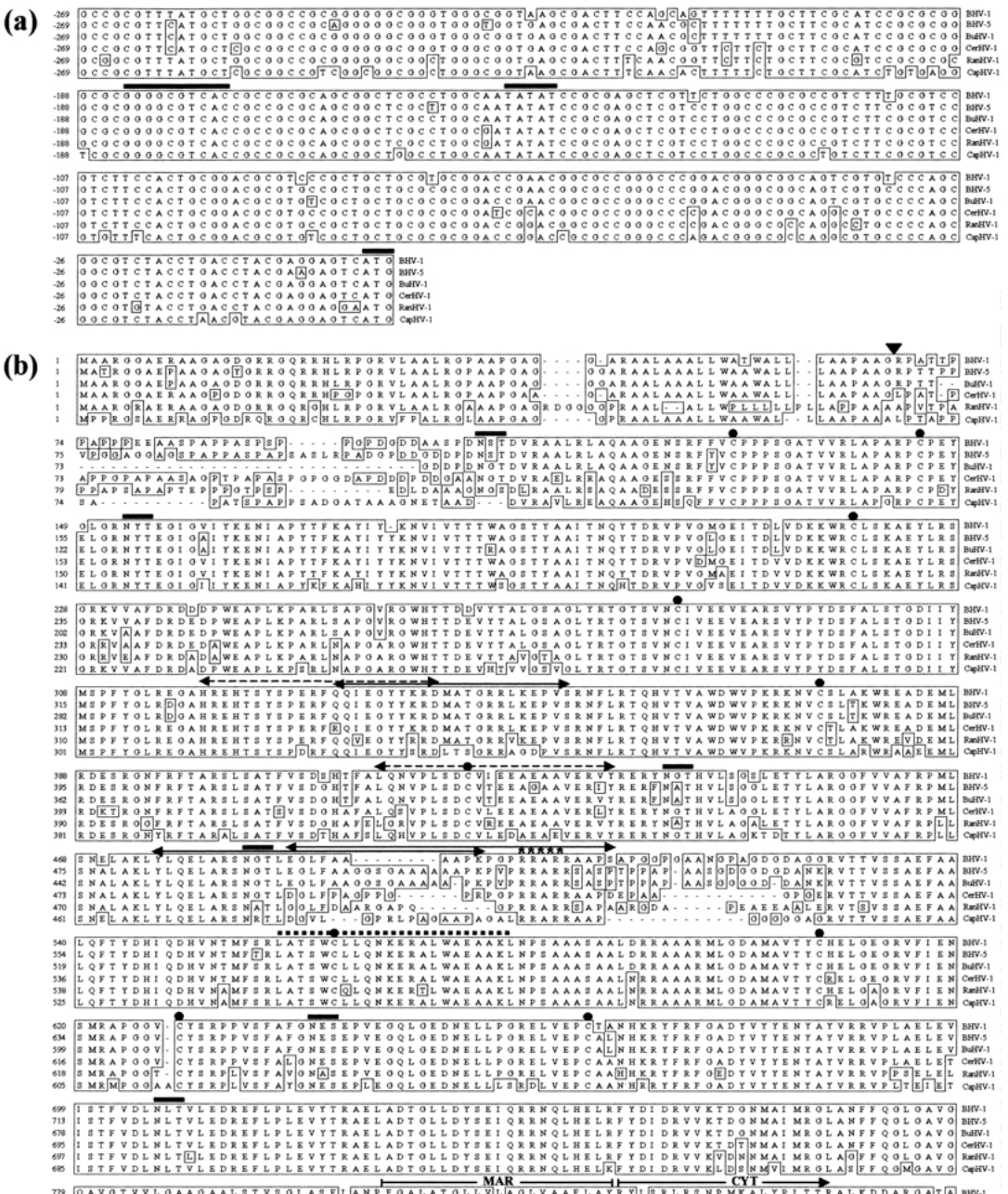

Fig. 1. Alignment of the promoter region (a) and deduced amino acid sequence (b) of the *gB* gene from six ruminant alphaherpesviruses. Dashes indicate gaps introduced to achieve maximal amino acid matching. Putative GC and TATA boxes in the promoter and gB starting codon are indicated by a black line over the sequences. Potential *N*-glycosylation sites are shown in the gB polypeptide by a black line over the sequences (Note that for CapHV-1, the first *N*-glycosylation site is between amino acids 94–96). A triangle points at the peptide cleavage site. Dots show conserved cysteine residues. A dotted line indicates the leucine zipper motif. A line of asterisks indicates the conserved protease motif. Arrows demarcate the B cell epitopes (continues line) and T cell epitopes (discontinues line). The membrane anchor region and cytoplasmic domains are indicated.

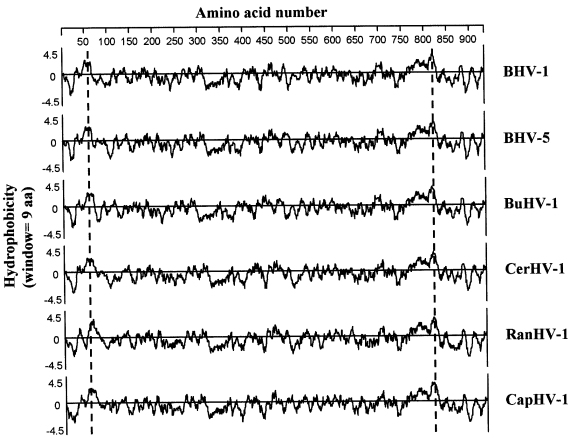

Fig. 2. Hydropathy analysis of gB from six ruminant alphaherpesviruses. The two prominent hydrophobic domains are indicated by discontinue lines. Kyte–Doolittle hydrophobic values are given on the left margin. Above the midline (0) indicates increasing hydrophobicity, while below indicates increasing hydrophilicity.

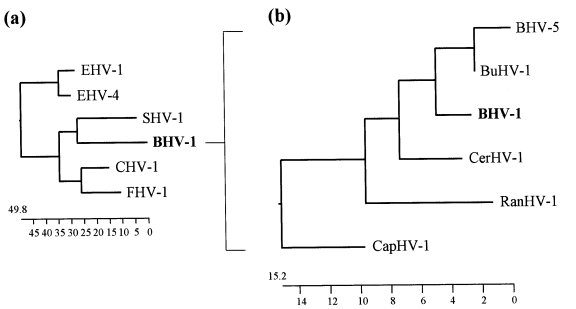

Fig. 3. Phylogenetic trees inferred by amino acid sequences from the gB regions. (a) Non-ruminant alphaherpesviruses related to BHV-1 (EHV, equine herpesvirus; SHV, suid herpesvirus; CHV, canine herpesvirus; FHV, feline herpesvirus); (b) Ruminant alphaherpesviruses. The length of the branches for each tree is indicated.

suggesting that the gB is proteolytically processed in all the studied viruses. Furthermore, six *N*-glycosylation sites and ten cysteine residues in the gB extracellular domain were conserved (Fig. 1b). A leucine zipper-like sequence, located downstream and near the protease domain, which role is to 'zip' the two gB subunits together, was also conserved.

The T and B cell epitopes recognized in the BHV-1 gB [33] and marked in Fig. 1b, had a variable degree of sequence conservation. The highest variability was observed in the B cell epitopes mapped between amino acids 475–496 and 487–508. The latter region includes the protease-cleavage site that renders the gB as two subunits linked by disulphide bonds.

The phylogenetic tree inferred by the complete gB amino acid sequences, shows that the studied viruses form a consistent group within the *alphaherpesvirinae* subfamily (Fig. 3), and particularly within the $\alpha 2$ subgroup of herpesviruses [34]. BHV-5 and BuHV-1 appear the most closely related viruses to BHV-1, followed in order by CerHV-1, RanHV-1 and CapHV-1. The most closely related virus to BuHV-1 is the neuropathogenic BHV-5. Interestingly, these two viruses differ considerably in pathogenicity, but show, however, a remarkable high degree of sequence similarity (Table 1, Fig. 3). The same topography was observed in a phylogenetic tree inferred by the gB nucleotide sequences (data not shown).

Discussion

Although of variable length, the gB homologues showed a remarkable conservation of all the protein motifs, suggesting that its biological function is the same in all the studied viruses. The analysis of the gB homologue sequences demonstrates that all of them contain structures characteristic of membrane-bound glycoproteins, including an amino-terminal signal sequence and carboxy-terminal hydrophobic transmembrane domain.

The estimation of the exact evolutionary relationship among viruses, in particular among highly stable DNA viruses, probably requires analysis of complete genomes. However, since this task is very complicated for viruses with large genomes, like herpesviruses, the phylogeny is usually inferred from individual genes. In this sense, the *gB* gene has emerged as the most frequently studied gene to examine the genetic distances among herpesviruses because of its large size and because by herpesvirus standards they are well conserved. The results obtained by analysing the viruses on the genetic level showed that all the studied viruses form a consistent group within the $\alpha 2$ subgroup of herpesviruses being SHV-1 the most closely related virus to them. Interestingly, the first time that BuHV-1 was isolated, it was serologically typed as BHV-1 [5]. However, the present phylogenetic analysis showed that instead of BHV-1, the most closely related virus to BuHV-1 is the neuropathogenic BHV-5. In fact, the two *gB* genes from BuHV-1 and BHV-5 are practically identical. In order to shed light on the origin of

the BHV-5 infections in calves, it would, therefore, be of interest to verify whether infection with BuHV-1, that cause no significant symptoms in buffaloes, is able to generate neurological disorders in calves.

Successful control of IBR depends on the use of efficient, sensitive and specific diagnostic tests. However, the diagnosis of the disease is hampered by the cross-reactivity between BHV-1 and the closely related ruminant alphaherpesviruses. The poor characterization of this group of viruses has complicated the development of a specific diagnostic assay for BHV-1 infections. From the diagnostic point of view, it is interesting that one of the two regions identified with the most significant sequence and length variation, the one flanking the protease domain (Fig. 1), it is also target of the immune system, as two B cell epitopes have been previously identified in this location [33]. Therefore, this region could eventually serve for the development of specific serodiagnostic assays that could ultimately distinguish BHV-1 infections from the rest of ruminant alphaherpesvirus infections, contributing to the control of IBR.

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