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# Complete sequence and analysis of the Ovine herpesvirus 2 genome.

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## SUMMARY

Ovine herpesvirus 2 (OvHV-2) is endemic in sheep populations worldwide and causes malignant catarrhal fever, a lymphoproliferative disease, in cattle, bison and deer. OvHV-2 has been placed in the gammaherpesvirus subfamily and is highly related to alcelaphine herpesvirus 1. Here, we report the cloning, sequencing and analysis of the complete OvHV-2 genome derived from a lymphoblastoid cell line from an affected cow (BJ1035). The unique portion of the genome consists of 130,930 bp with a mean GC content of 52 %. The unique DNA is flanked by multiple copies of terminal repeat elements 4,205 bp in length with a mean GC content of 72 %. Analysis revealed 73 open-reading frames (ORFs), the majority (62) of which showed homology with other gammaherpesvirus genes. A further subset of 9 ORFs is shared with only the related alcelaphine herpesvirus 1. Three ORFs are entirely unique to OvHV-2, including a spliced homologue of cellular interleukin 10 that retains the exon structure of the cellular gene. The sequence of OvHV-2 is a critical first step in the study of the pathogenesis and treatment of malignant catarrhal fever.


## INTRODUCTION

Malignant catarrhal fever (MCF) is a severe, usually fatal lymphoproliferative and inflammatory syndrome of domestic cattle, pigs, deer and certain other susceptible ruminants such as bison. Cases of MCF in cattle usually occur sporadically. However, periodically limited epizootic outbreaks occur (Hamilton, 1990) where losses can be substantial. The disease is mainly caused by either of two closely-related bovid gammaherpesviruses (γHVs), alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2), that persist sub-clinically in their natural host. These viruses are highly related in biological properties and sequence to each other and to a recently-identified virus

porcine lymphotropic herpesvirus 1 (PLHV-1) that causes post-transplant lymphoproliferative disease in pigs (Goltz et al., 2002). OvHV-2 and AlHV-1 are more distantly related to other γHVs such as herpesvirus saimiri (HVS), Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV) and the murid herpesvirus 4 (MuHV-4 or MHV-68). AlHV-1 naturally infects wildebeest (*Connochaetes spp*) and is the source of wildebeest-associated MCF in Africa (Plowright et al., 1960). OvHV-2 is endemic in domestic sheep (*Ovis aries*), which act as a reservoir of infection for the other main form of the disease, sheep-associated MCF (SA-MCF) (Baxter et al., 1993, Li et al., 1998, Muller-Doblies et al., 1998, Wiyono et al., 1994). Aside from sporadic outbreaks in domestic cattle, SA-MCF is the most important virus disease of farmed deer and has recently been reported in pigs (Albini et al., 2003, Loken et al., 1998). SA-MCF is also currently a disease of great concern in Indonesia, affecting Bali cattle and in the U.S.A. where bison are particularly susceptible (Li et al., 2006, O'Toole et al., 2002, Schultheiss et al., 2000).

In the reservoir species, sheep, OvHV-2 DNA has been found by PCR in B cells in the bloodstream, lymph nodes and the respiratory, alimentary, and urogenital tracts (Baxter et al., 1997, Hussy et al., 2002). OvHV-2 DNA has also been detected in nasal and ejaculatory secretions suggesting possible respiratory and sexual transmission mechanisms (Hussy et al., 2002, Li et al., 2004). Moreover, cattle have been experimentally infected with nasal secretions from infected sheep showing that respiratory transmission is likely (Taus et al., 2006). In contrast, in SA-MCF-affected ruminants, virus DNA is usually detected by PCR in lymph nodes and spleens (Muller-Doblies et al., 2001) and has been observed by *in situ* hybridization in hyperplastic T cells in brain lesions (Simon et al., 2003). Thus, to enable the study of the interaction of OvHV-2 with host cells, T-lymphoblastoid cell lines with the morphology of large granular lymphocytes (LGLs)

have be established in culture from the tissues of MCF-affected animals (Reid et al., 1989, Reid et al., 1983, Schock & Reid, 1996, Swa et al., 2001). These T cell lines contain OvHV-2 DNA and antigen (Baxter et al., 1993, Bridgen & Reid, 1991, Swa et al., 2001) and can transmit MCF experimentally to rabbits and hamsters (Buxton et al., 1988, Buxton et al., 1984) which are used as animal models. OvHV-2-positive LGLs generally have a T cell phenotype, are constitutively and indiscriminately (non-MHC-restricted) cytotoxic and produce a range of cytokines, but not IL-2 (Schock et al., 1998, Schock & Reid, 1996, Swa et al., 2001). Our current hypothesis is that MCF is due to indiscriminate tissue damage caused by dysregulated cytotoxic T cells generated as a consequence of infection. The LGL T cells in culture represent the virus-infected cells *in vivo* and are invaluable for virus-cell interaction studies in MCF.

AlHV-1 has been isolated, will productively infect epithelial cell lines in culture and has been completely sequenced (Ensser et al., 1997, Plowright et al., 1960). In contrast, research on OvHV-2 has lagged behind due to the lack of a productive tissue culture system and reagents. This work describes the complete sequence of the OvHV-2 genome as a first step in the detailed molecular analysis of SA-MCF. An accompanying manuscript by Taus et al. describes a comparison of our sequence with that of OvHV-2 derived from the nasal secretions of sheep.

## MATERIALS AND METHODS

**Cells and virus.** The OvHV-2-infected bovine T cell line BJ1035 was derived from a cow with SA-MCF (Schock et al., 1998). This line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM glutamine (Invitrogen, Paisley, UK), 100 IU of Penicillin per ml, 100 μg of streptomycin per ml, 10 % heat- inactivated foetal

bovine serum and 350 IU of IL-2 per ml (PROLEUKIN, Chiron Therapeutics, Emeryville, CA).

**Construction of a cosmid library.** A cosmid library containing OvHV-2 DNA was generated from BJ1035 cells using Supercos1 (Stratagene), modified as described previously (Cunningham & Davison, 1993) (a kind gift of Dr A. Davison). High molecular weight DNA extracted from BJ1035 cells was partially digested with MboI such that the products were on average 40 kb in size. A cosmid library was then generated in Supercos1 from the MboI-digested cut BJ1035 DNA as described in the Supercos1 manual (Stratagene). Briefly, cosmid arms generated from vector by restriction enzyme digestion and dephosphorylation were ligated to MboI cut BJ1035 DNA. Ligated DNA was packaged into recombinant λ phage using a Gigapack III XL packaging kit (Stratagene) according to the manufacturer's instructions. The library was then amplified once using *E. coli* XL1-Blue MR and stored at -80 °C.

**Screening cosmid library.** Bacterial colonies from the cosmid library were screened by colony hybridisation at high stringency (Sambrook et al., 1989) using probes of known OvHV-2 sequence. DNA for use as probes of 300 - 500 bp in length were generated by PCR amplification using either DNA extracted from OvHV-2 infected cell lines or cosmid DNA as a template. DNA probes were labelled with $\alpha$-$^{32}$P dCTP using a random-primed DNA labelling kit (Roche). Positive colonies underwent a second round of screening. Cosmid DNA was prepared from colonies positive on the second screen using QIA-spin mini prep kits (QIAGEN). The ends of the inserted DNA were then sequenced using the vector-specific primers as follows: 5'-AAGGAAACGACAGGTGCTG and 5'-CGAAAATGTCCACCTGACGTC which lie either side of the insert sites in the modified Supercos 1.

**DNA sequencing.** DNA sequencing was performed using the di-deoxy chain termination sequencing method. Sequencing of cosmid ends, splinkerette products and plasmids containing the terminal repeat elements was performed using either the in-house sequencing service at the Dept of Veterinary Pathology, University of Edinburgh or via Lark Technologies.

Sequencing of the four overlapping cosmid clones was performed by a shotgun library approach using pCR4bluntTopo (Invitrogen). Plasmid subclones were cycle-sequenced with Big-Dye terminator version 1.0 reagents (Applied Biosystems) and analyzed on a MegaBace 1000 sequencer (Amerham Biotech) or a ABI 377 sequencer (Applied Biosystems). Computer-assisted assembly was done with Lasergene SeqMan (DNASTAR Inc.) with a 5 to 7 fold redundancy.

**Polymerase chain reaction amplification.** Polymerase chain reaction (PCR) reactions of 50 µl total volume contained 1 x PCR reaction buffer (20 mM Tris pH 8.4, 50 mM KCl, Invitrogen), 1.5 mM $MgCl_2$, 250 µM of each of dATP, dGTP, dCTP, dTTP (Ultrapure dNTP set, Amersham Biosciences) 200 pmol of each primer, 100-500 ng DNA template and 1U *Taq* DNA polymerase (Invitrogen). PCR primers were obtained from MWG-Biotech. PCR programs generally consisted of 30-40 cycles of 30 s denaturing at 94 ºC, 1 min annealing at 55 – 60 $^{o}$C and extension at 72 ºC for 1 min/Kb of product. To generate PCR products across the genome termini, the high GC PCR kit (Roche) was used in combination with primers homologous to the ends of the known sequence. The genome co-ordinates of the primers were as follows: sense, 128,577 – 128598 and anti-sense, 467- 487.

**Analysis of OvHV-2 gene splicing by RT-PCR.** Total RNA was isolated from BJ0135 cells by extraction using RNeasy kits (Qiagen) according to the manufacturer's guidelines, digested with RQ1 DNase (Promega) (0.1 U/µl) for 30 min at 37 °C, extracted sequentially in phenol:chloform and chloroform and then precipitated in ethanol. cDNA was

synthesized from 2µg of total RNA with Superscript II reverse transcriptase primed with an oligo(dT)-adapter primer (Gibco-BRL) in a 20 µl reaction according to the manufacturer's recommendations. One-microliter aliquots of cDNA were then amplified by PCR using primers specific for OvHV-2 ORFs. Amplified cDNAs were then analysed by gel electrophoresis, inserted into the cloning vector pCR2.1TOPO and multiple clones for each cDNA were sequenced. The genome co-ordinates for primers used for RT-PCR are as follows. Ov2, sense 2813-2792, antisense 2162-2183; Ov2.5, sense 3576-3597, antisense 4455-4434; Ov6, sense 79,355-79375, antisense 80,327-80307; ORF57, sense 89,060-89,072, antisense 90,482-90,460; Ov8, sense 81,538-81560, antisense 83,906-83886; Ov8.5, sense 117,777-117796, antisense 118,950-118930.

**Genome walking by splinkerette PCR.** Splinkerette PCR is a method of extending from known to unknown sequence by amplification of DNA sequences which lie between a single known primer and a nearby restriction site (Devon et al., 1995). Splinkerette PCR was performed on BJ1035 DNA exactly as described previously (Devon et al., 1995). Oligonucleotide adaptors specific for the enzymes BamHI, HindIII, SalI and EagI were utilised. PCR products generated were cloned using a pCR 2.1-TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Multiple clones (5-7) from each walk were sequenced.

**Nucleotide and protein sequence analysis.** The EMBOSS (Rice et al., 2000) package of programs was used for final assembly of sequences and analysis of sequences, including gene finding. The definition of open-reading frames was performed by using the GeneMarkS program with the eukaryotic virus option (Besemer et al., 2001) as well as by comparison with other herpesviruses and mammalian genes using the NCBI BLAST programs (Altschul et al., 1990, Gish & States, 1993). Annotation was performed using the Artemis program (Rutherford et al., 2000).

## RESULTS AND DISCUSSION

**Molecular cloning and sequencing of the OvHV-2 genome**. There is currently no productive culture system for OvHV-2. DNA for sequencing the OvHV-2 genome was therefore obtained from the OvHV-2-positive bovine T cell line BJ1035. This cell line was derived from an MCF-affected cow. We were unable to clone the BJ0135 line prior to sequencing. Indeed, it has never been possible to clone T cell lines carrying OvHV-2 (H.W. Reid unpublished observations). However, previous studies had shown that BJ1035 contain a mean of between 40 and 400 copies of episomal OvHV-2 genome per cell (J. Hart, J.P. Stewart, unpublished observations).

The cloning and sequencing strategy is shown in Fig. 1 and the co-ordinates of relevant clones are detailed in Table 1. A cosmid library was made using total high molecular weight DNA from BJ1035 cells. An initial OvHV-2 cosmid (c75) was isolated using a probe consisting of part of ORF75 (Bridgen & Reid, 1991). Cosmid clones corresponding to a large part of the unique portion of the genome were then successively isolated using probes derived from the ends of the cosmid inserts. The DNA sequence of these cosmids was determined after shotgun cloning into pCR4bluntTopo and sequencing. In spite of repeated attempts we were unable to isolate further cosmid clones that spanned or contained genome termini. To complete the genome sequence, successive splinkerette PCRs were performed, walking away from the known sequence. However, upon reaching high GC content and repetitive sequence, corresponding to terminal repeat elements (TRs) we were unable to proceed any further with this technique.

Since most of the OvHV-2 genomes in BJ1035 are circular (Rosbottom et al., 2002, Thonur et al., 2006), PCR amplification using primers homologous to the ends of the known sequence and a high GC PCR kit (Roche) was used to generate independent

plasmid clones that completed a circular sequence. Analysis of several independent clones (represented by p3.5) revealed that they were identical in sequence and that there was a GC-rich region of approximately 600 bp within each clone. Since the TR elements of γHVs are GC-rich we surmised that this could correspond to one copy or part of the terminal repeat (TR) element. However, it was impossible to determine the boundaries of the unique region and TR using this clone. Analysis revealed a unique HindIII site in the centre of the 600 bp GC-rich region in p3.5. To determine the exact nature of the terminal repeat unit, BJ1035 DNA was therefore cut with HindIII and analysed by Southern blotting using p3.5 as a probe (not shown). This revealed a hyper-molar fragment of approximately 4.2 kbp in length corresponding to one TR unit. The 4.2 kbp HindIII fragment was subsequently cloned into the vector pBluescript KS+ (Stratagene). A number of clones with the same sequence containing one unit of the TR element (pH4.2) were isolated. Comparison of the sequences of p3.5 and pH4.2 allowed the definition of the boundaries of the unique and TR DNA and additionally showed that the PCR fragment in p3.5 was generated from a circular defective OvHV-2 genome containing only a single, deleted TR unit. The boundaries of the TR and unique DNA were further confirmed by PCR analysis and sequencing.

In line with the convention for other γHVs, the OvHV-2 sequence was assembled in the same orientation as that of herpesvirus saimiri (HVS) (Albrecht et al., 1992) with the sequence of one copy of the TR element placed after the end of the unique sequence. The sequence of the unique region was 130,930 bp in length with a mean GC content of 52% and the sequence of one TR unit was 4,205 bp in length with a mean GC content of 72%.

**Repeat regions in the unique portion of the OvHV-2 genome.** In addition to the terminal repeats, analysis of the unique portion of the genome using the EMBOSS

programmes ‘equicktandem’ and ‘einverted’ revealed six tandem and two inverted repeat structures. These are shown in Fig. 2 and detailed in the GenBank entry. Five of the tandem repeat elements are contained within coding regions, two within the unique ORF Ov8.5 and three within ORF73. Repeats within ORF73 homologues are also seen in related viruses such as AlHV-1 and HHV-8. Repeats at the same position as those within Ov8.5 are seen in a number of γHVs (e.g. bovine herpesvirus 4 (BoHV-4), MHV-68 and KSHV) and are known to form part of the lytic origins of replication in these viruses (AuCoin et al., 2002, Deng et al., 2004, Lin et al., 2003, Zimmermann et al., 2001). Thus, this region of the genome may also act as a lytic origin in OvHV-2. The two inverted repeats are located in a long region of apparently non-coding DNA between ORFs 11 and 17.5. Although not conserved in sequence, these repeats are positionally analogous to two inverted repeats in the AlHV-1 genome (Ensser et al., 1997). They may therefore perform a conserved function such as origin of DNA replication.

**Coding potential of the OvHV-2 genome.** Computer assisted analysis predicted 73 ORFs in the OvHV-2 genome (Fig. 2 and Table 2). Potential protein-coding ORFs were identified by the following criteria: ORF size larger than 60 amino acids, presence of potential transcriptional start and stop sites, a high GeneMark score and homology to other known herpesvirus or cellular ORFs. In line with the nomenclature of other γHVs, where applicable, ORFs were assigned the number of the homologue in HVS. When possible, ORFs with homologues shared only with AlHV-1 were assigned the same number as in AlHV-1 but with the Ov prefix for Ovine. ORFs with no homologues in HVS and AlHV-1 were assigned an Ov prefix with numbers between the adjacent Ov ORFs.

The arrangement of ORFs within the genome was highly similar to other γHVs. The genome structure, ORF content and the similarity of amino-acid content was closest to AlHV-1 with many AlHV-1 ‘unique’ ORFs being found in OvHV-2 also. A comparison of the

AlHV-1 and OvHV-2 genomes is shown in Fig. 3. Conserved OvHV-2 ORFs were arranged in four blocks co-linear with other γHVs as indicated in Table 2 and Fig. 3.

**Non-conserved and unique ORFs.** Outside the four conserved blocks are ORFs that are unique to OvHV-2 or only conserved in the highly-related AlHV-1 (Albrecht et al., 1992) and PLHV-1 (Goltz et al., 2002). The validity of all candidate OvHV-2 unique ORFs (those with an Ov prefix) has been confirmed as mRNA corresponding to all these ORFs were expressed in OvHV-2 infected cells (Thonur et al., 2006)

The left end of the unique region contains four ORFs. ORFs Ov2 and Ov3 are homologous to AlHV-1 A2 and A3 respectively. Ov2 contains two exons as confirmed by RT-PCR and sequencing. It encodes a protein containing a basic leucine zipper (bZIP) motif, is homologous to ATF-like, CREB and Jun dimerisation proteins and is therefore likely to be a transcription factor. The product of Ov3, contains a putative signal peptide to residue 22 and, like AlHV-1 A3, is homologous to proteins of the semaphorin family. However, Ov3 is shorter by 200 residues at the carboxyl terminus as compared with A3, the truncation occurring just after the consensus Sema domain (InterPro IPR001627). There is a short stretch of co-linear homology between A3 and the region downstream of the stop codon for the Ov3 ORF. In addition, the Ov3 ORF has a long predicted 3' untranslated region making the gene a similar length (predicted TATA to polyA signal) to the A3 gene. It seems likely, therefore that the Ov3 ORF is the product of truncation of a longer ORF. The Ov3 protein shows the greatest similarity in amino-acid sequence to semaphorin 7A (CDw108) and poxvirus semaphorins e.g. vaccinia virus A39R. Sempahorin 7A is expressed by lymphoid and myeloid cells and, like A39R, is a potent monocyte activator (Comeau et al., 1998, Holmes et al., 2002). Thus, Ov3 may be involved in the modulation of the host response to OvHV-2.

There are no homologues of the AlHV-1 A1 and A4 ORFs in OvHV-2. Instead there are two unique ORFs Ov2.5 and Ov3.5. Ov2.5 contains 5 exons (as confirmed by RT-PCR and sequencing) and encodes a homologue of cellular interleukin 10 (IL10). This ORF is interesting in that it retains precisely the exon structure of the cellular gene, unlike other herpesvirus IL10 homologues. Ov2.5 has also been shown to encode a functional IL10 molecule that can block cytokine secretion by macrophages and stimulate proliferation of mast cells (J.P Stewart and D.Haig, Unpublished observations) and may thus be involved in the modulation of the host response to OvHV-2. In contrast, Ov3.5 has no significant homology to any known protein, encodes a peptide of 163 amino-acid residues and contains a putative signal peptide. It is likely therefore to be a secreted from the infected cell.

Between conserved ORFs 03 and 06 lies the Ov4.5 ORF. This encodes a protein with homology to the EBV BALF1, EHV-2 E6 and cellular Bcl-2 proteins. Comparative sequence analysis of the AlHV-1 genome showed that a homologous ORF, now termed A4.5, was not reported in the published description of the sequence (Ensser et al., 1997). A second OvHV-2 ORF, Ov9, also encodes a Bcl-2 homologue. This ORF is situated at the right hand end of the unique region and is homologous to AlHV-1 A9. Thus, like EBV, it appears that OvHV-2 (and AlHV-1) encodes two Bcl-2 family homologues. In EBV, BHRF1 is antiapoptotic whereas the role of BALF1 is controversial, being reported as both pro- and antiapoptotic in transfected cell lines (Bellows et al., 2002, Marshall et al., 1999). Recent deletion analysis has, however, assigned an essential role in EBV transformation of B cells for both proteins by protecting newly-infected cells from apoptosis prior to the establishment of latency. Thus, it is possible that in OvHV-2-infected cells the Ov4.5 and Ov9 products promote survival of infected lymphocytes and the establishment of latency.

The Ov5 ORF is located downstream of ORF9/DNA polymerase and overlaps with ORF10. This ORF is predicted to encode a G-protein coupled receptor (GPCR) that is homologous to the AlHV-1 A5 except it is predicted to have a longer C-terminal (intracellular) tail than A5. Iteration of the PSI-BLAST programme revealed that Ov5 is also related to EBV BILF1 and more weakly to cellular interleukin 8 (IL8) receptors. BILF1 functions as a constitutively signalling (ligand-independent) GPCR that alters intracellular signalling (Beisser et al., 2005, Paulsen et al., 2005) and so Ov5 protein may fulfil a similar role in OvHV-2 infected cells.

OvHV-2, like AlHV-1 and PLHV-1 is unusual amongst γHVs in that it has no ORF28 homologue. ORF28 encodes a non-essential virion glycoprotein in other γHVs (Bortz et al., 2003, May et al., 2005). Thus, the function of ORF28 is either redundant or is performed by a separate glycoprotein in this sub-group of γHVs. However, unlike AlHV-1, OvHV-2 does encode an ORF49 homologue. The ORF49 homologue of EBV (BRRF1) has been shown to act as a transcriptional transactivator that co-operates with the viral BRLF1 transactivator (ORF50) to induce lytic replication (Hong et al., 2004). PLHV-1 also contains an ORF49 homologue so AlHV-1 is highly unusual in not encoding a homologous ORF and its absence is not a consistent feature of this group of γHVs.

In between ORF50 and ORF52 lie three ORFs Ov6, Ov7 and Ov8. These all have homologues in AlHV-1 (A6, A7 and A8) and in PLHV-1. Like the PLHV-1 homologue, Ov6 was shown to consist of three exons as determined by RT-PCR analysis and sequencing. The product of Ov6 contains a leucine zipper motif in its carboxyl-terminal region and there are consensus DNA binding motifs towards the N-terminus. It also has significant sequence similarity to the CCAAT/enhancer binding protein family consistent with it having a putative role in transcriptional transactivation. In addition, although there is no direct sequence relationship, Ov6 is positionally-analogous to EBV BZLF1 and KSHV/HHV-8 K8

that are both transactivators of the viral lytic cycle. It seems likely, therefore that Ov6 may fulfil a similar function during OvHV-2 infection. The product of Ov7 contains a predicted signal peptide and N-linked glycosylation motifs and thus is likely to be a viral glycoprotein. Although there is no sequence similarity, Ov7 is positionally analogous to EBV BZLF2 whose product is involved in entry of EBV into B cells via binding to HLA-DR (Spriggs et al., 1996). Ov7 protein may also therefore be involved in receptor binding. Ov8 was shown to consist of two exons by RT-PCR and sequencing. These splice sites are conserved in the homologous ORFs in AlHV-1 and PLHV-1 and correspond to the regions of homology between these proteins. Thus, the AlHV-1 A8 and PLHV-1 A8 may be spliced in a similar fashion. The product of Ov8 was predicted to contain a transmembrane anchor near the carboxyl-terminus and N-linked glycosylation sites and so, like Ov7 is likely to be a virus glycoprotein. Also like Ov7, although there is no sequence similarity, Ov8 is positionally analogous to EBV BLLF1 (gp350/220), KSHV/HHV-8 K8.1 and MHV-68 ORF51 all of which encode glycoproteins involved in binding to cellular receptors (Birkmann et al., 2001, Stewart et al., 2004, Tanner et al., 1987). Thus, Ov8 protein may also be involved in binding to cellular receptors.

Downstream of ORF73 lies Ov8.5, which is entirely unique to OvHV-2 and shows no obvious similarity with any viral or cellular genes. Ov8.5 is predicted to encode a proline-rich (24%) peptide of Mr 42 K that contains no consensus motifs as defined by the PROSITE database. However, RNA from Ov8.5 is found in OvHV-2 infected cells (Thonur et al., 2006) and so this is a bone fide ORF. The N-terminal region of Ov8.5 protein is encoded by two direct DNA repeat elements. Direct repeat elements that form part of the origins of viral DNA replication are present in the analogous genomic location in other γHVs e.g. BoHV-4, KSHV and MHV-68 (AuCoin et al., 2002, Deng et al., 2004, Lin et al., 2003, Zimmermann et al., 2001). In BoHV-4 and KSHV unique ORFs (Bo11, Bo12 and

K12) are also found surrounding the repeat. Thus, the presence of a direct repeat and unique ORF at an analogous genomic location to Ov8.5 appears to be a common feature of γHVs. Further studies are required to show whether this region acts as an origin of DNA replication and to assign a function to Ov8.5.

Directly upstream of Ov8.5 is ORF73. This shows significant homology with ORF73s of other γHVs including KSHV/HHV-8. However, the homology is largely restricted to the carboxyl terminal region. Like other homologues, OvHV-2 ORF73 incorporates a large acidic repeat domain. Variability in the length of the acidic repeat domain is seen between isolates of KSHV and herpesvirus saimiri ORF73 proteins (Ensser et al., 2003, Gao et al., 1999, Zhang et al., 2000). In an accompanying study Taus *et al*. show that ORF73 derived from the nasal secretions of sheep varied from the sequence reported here in the length of the acidic domain. It is not clear what functional difference this variability makes. However, differences in ORF73 sequence may be useful for epidemiological studies. The KSHV/HHV-8 ORF73 was described as latency-associated nuclear protein (LANA) that functions to trans-activate the viral latent origin of replication (Hu et al., 2002). It seems likely, therefore that the OvHV-2 homologue will have a similar function.

The final unique ORF found was Ov10. This lies at the right hand end of the unique region between Ov9 and the terminal repeats. This ORF shows limited similarity with AlHV-1 A10. The predicted Ov10 protein has a potential transmembrane anchor at the carboxyl-terminus and four consensus nuclear localisation signals. Thus, Ov10 protein may localise to the nucleus of infected cells.

**Conserved spliced ORFs.** The region encoding ORF40/41 in OvHV-2 consisted of one continuous ORF with regions that were homologous to ORFs 40 and 41 of other herpesviruses. These ORFs are conserved amongst all herpesviruses and code for a

protein which is complexed with helicase and primase. In many γHVs such as EBV (Fixman et al., 1995) and KSHV (AuCoin & Pari, 2002, Wu et al., 2001), the coding sequence for this protein is formed by splicing of two separate ORFs 40 and 41. In other γHVs, such as MuHV-4, there is a continuous ORF but this is still spliced at conserved splice sites (J.P. Stewart, unpublished). The sequence of ORF40/41 of OvHV-2 contained conserved splice sites which corresponded to regions of homology with other γHV ORF40 and 41s. It seems likely therefore that OvHV-2 ORF40/41 is spliced in a similar fashion to that of MuHV-4. Interestingly, the OvHV-2 sequence derived from the nasal secretions of sheep described by Taus et al. in a complementary study contains an additional two nucleotides in the predicted intron in ORF40/41 resulting in two separate ORFs. To confirm the sequence of the BJ1035 virus, PCR products across the region were generated from BJ1035 cellular DNA, sequenced and found to be identical to the original sequence. Thus, within the OvHV-2 species there are variants with either one single or separate ORF40/41s. However, this difference is likely to be silent as the predicted final transcripts are identical.

The transcripts for viral terminase (ORF29), transcriptional transactivator (Rta/ORF50) and ORF57 are known to be formed from the splicing of two exons in other γHVs. Consensus potential splice donor and acceptor sites for these OvHV-2 homologues (Table 2) were present and determined by comparative sequence analysis.

**Similarity to other MCF-associated viruses.** It has been proposed that the γHVs associated with MCF be placed in their own genus, Macavirus (McGeoch et al., 2006), based on evolutionary relatedness of conserved ORF sequences. The sequence presented here confirms this new grouping showing that OvHV-2 is highly similar to AlHV-1 and PLHV-1, not only in the nucleotide similarity of conserved ORFs, but also in terms of ORFs that are only present in this group of viruses. These Macavirus-specific ORFs are

likely to be involved in host-specific pathogenesis and the development of MCF. Thus, the comparative genetic analysis of OvHV-2 and related viruses enabled by the completion of this sequence will be core to the understanding of the mechanisms underlying MCF.

The sequence of OvHV-2 derived from the nasal secretions of sheep published in a complementary study by Taus et al. shows that while the two genomes are extremely similar, there are differences. Since the outbreaks of MCF in European cattle are sporadic in nature, it has been hypothesised that they could be due to the generation of more pathogenic OvHV-2 variants. Analysis of the sequence differences between the nasal secretion virus (derived from the reservoir species) and the sequence derived from a clinically affected cow (BJ1035) will be important to determine whether these are relevant to pathogenicity in cattle.

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**FIGURES**

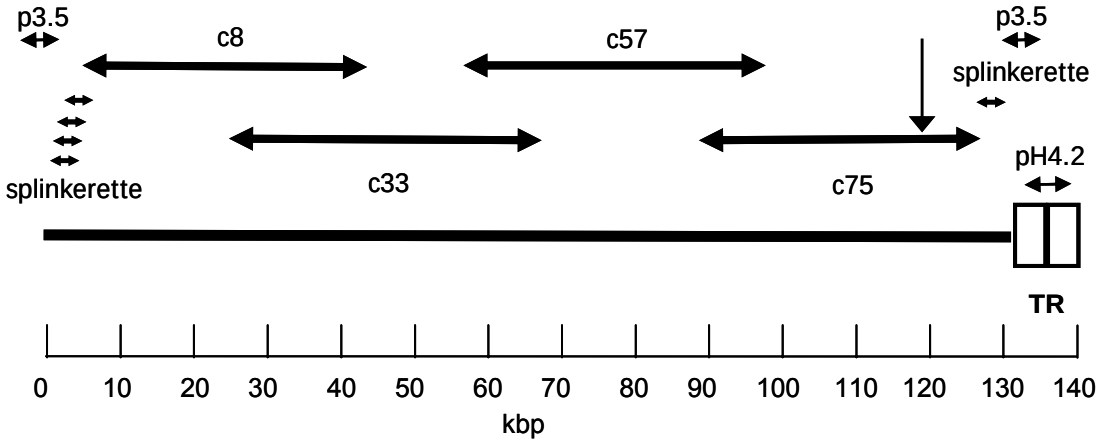
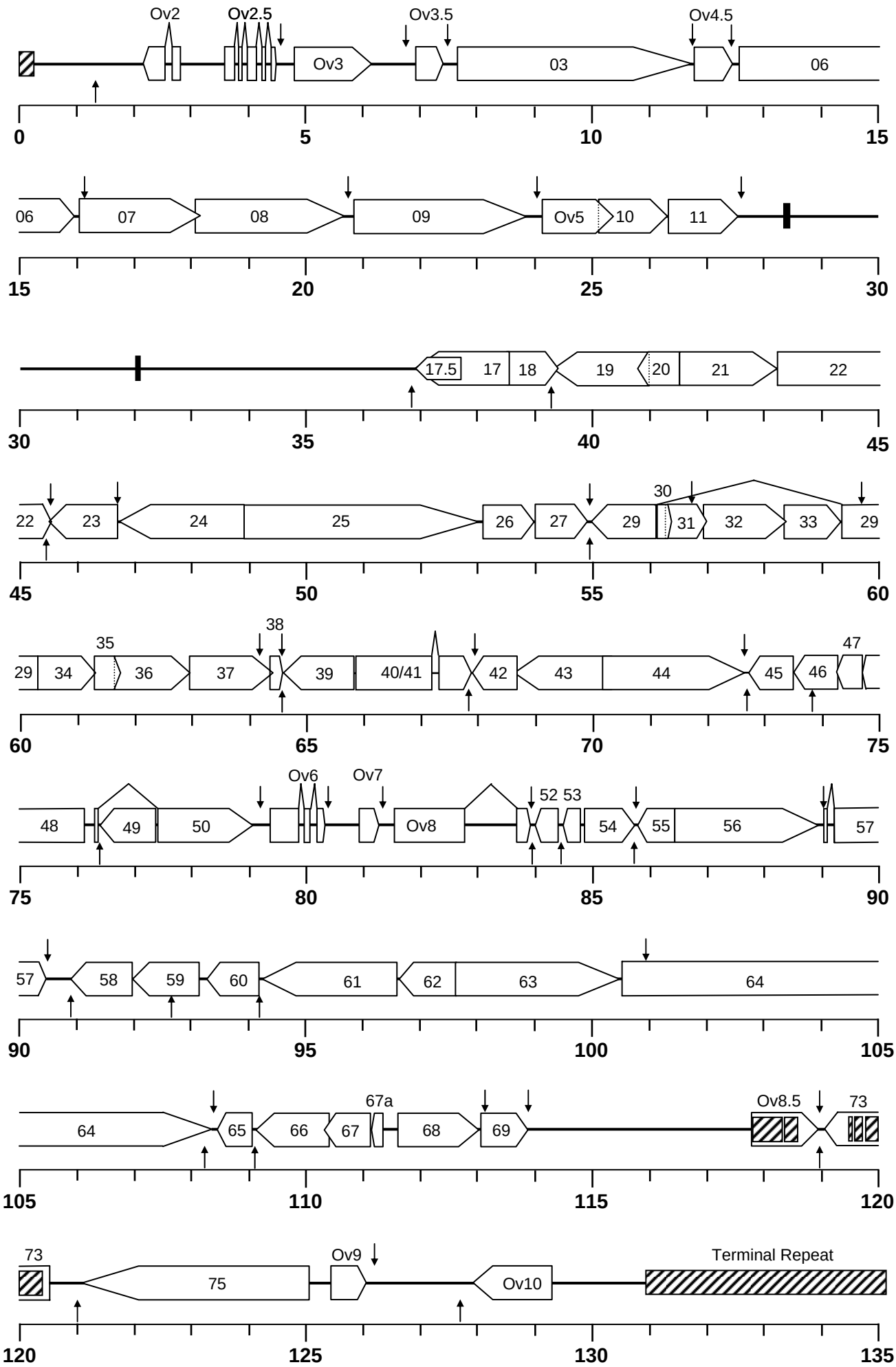



**Figure 1.** Molecular cloning of the OvHV-2 genome. High molecular weight DNA was extracted from the OvHV-2 infected cow LGL line BJ1035 and initially used to construct a cosmid library. The positions of individual clones are shown as horizontal arrows above the representation of the viral genome as a solid line (unique DNA) or open squares (terminal repeats). The nucleotide base numbers are shown below in kbp. The first cosmid clone (c75) was isolated using a probe to a known sequence (vertical arrow). Subsequent cosmids were isolated using probes derived from the cosmid ends. Sequences to the end of the unique portion of the genome and the terminal repeats were derived by a series of splinkerette walks and finally cloning of the terminal repeats into plasmid as indicated in the text.


Ov2
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90
95
100
105
67a
Ov8.5
73
64
65
66
67
68
69
105
110
115
120
73
Ov9
Terminal Repeat
75
Ov10
120
125
130
135

**Figure 2.**

Organisation of the OvHV-2 BJ1035 genome. ORFs are shown by open arrows with the arrow showing the direction of transcription/translation and non-coding DNA as a solid line. The ORFs are drawn to scale with the relative co-ordinates shown below in kbp. Splice sites are shown as lines above connecting exons. Potential polyadenylation signals (AATAAA or ATTAAA) are shown for the respective forward (↓) or reverse (↑) DNA strands. Major repetitive elements are shown as shaded squares, direct repeats filled with hashes and inverted repeats as solid bars.

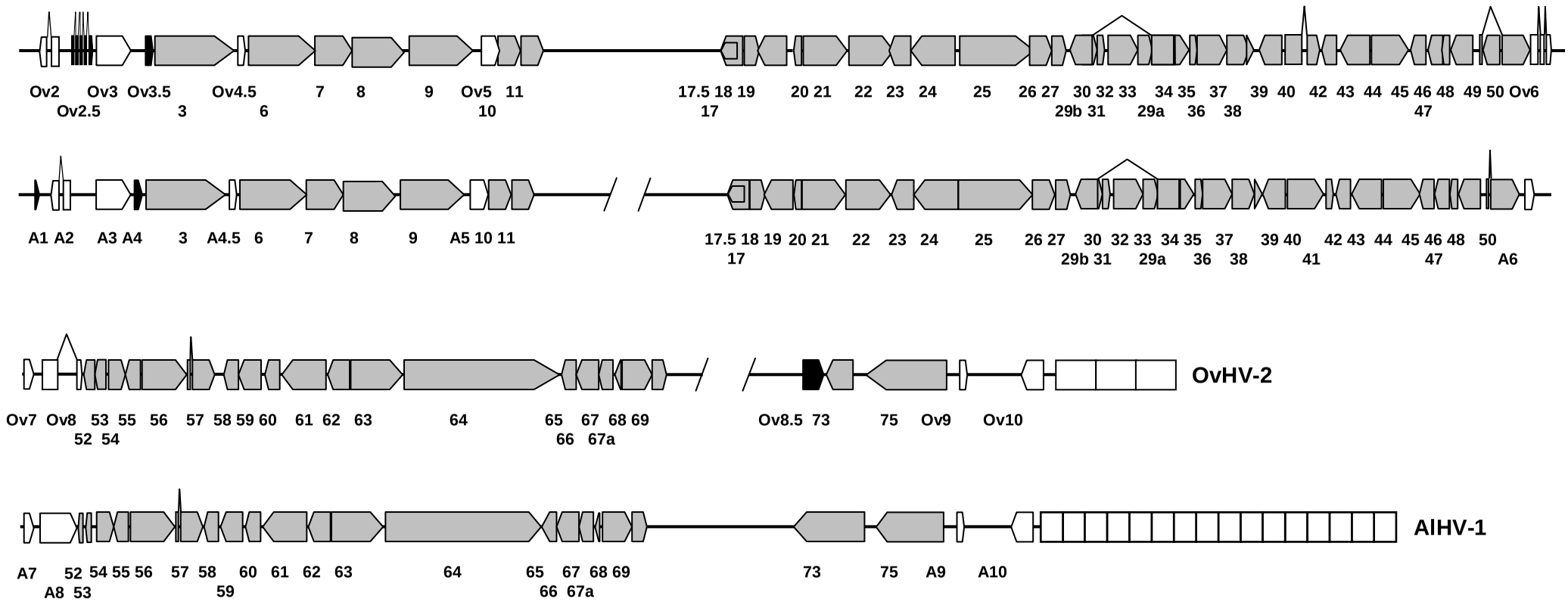


**Figure 3.**

Comparison of the genome organizations of OvHV-2 and AlHV-1. The organization of OvHV-2 is shown relative to that of AlHV-1 below. ORFs are shown as block arrows with shading representing the conservation of genes as follows. Light shading, ORFs common to γHVs; no shading, ORFs only found in OvHV-2 and AlHV-1; solid shading, ORFs unique to OvHV-2 or AlHV-1.

Table 1. Genome co-ordinates of clones used in sequence determination

| Clone Name | Description | Enzyme used to Generate Clone | Genome co-ordinates | |
|---|---|---|---|---|
| | | | From | to |
| c8 | Cosmid from library | MboI | 2,628 | 44,562 |
| c33 | Cosmid from library | MboI | 25,375 | 68,382 |
| c57 | Cosmid from library | MboI | 58,319 | 98,615 |
| c75 | Cosmid from library | MboI | 89,612 | 128,522 |
| pSPL1 | Plasmid generated from splinkerette | HindIII | 1,623 | 2,679 |
| pSPL2 | Plasmid generated from splinkerette | BamHI | 824 | 1,668 |
| pSPL3 | Plasmid generated from splinkerette | SalI | 410 | 887 |
| pSPL4 | Plasmid generated from splinkerette | EagI | 135 | 495 |
| pSPLR1 | Plasmid generated from splinkerette | BamHI | 128,486 | 128,666 |
| p3.5 | Plasmid generated from PCR across genome termini | | 128,577<br>1 | 131,582<br>487 |
| pH4.2 | Plasmid generated from genomic DNA | HindIII | 130,931<br>131,167 | 131,172<br>135,135 |

Table 2

| Gene Block[a] | OvHV-2 ORF | | Strand | Expression[b] | Start Codon[c] | Stop Codon[c] | TATA[d] | Poly(A)[d] | Length (aa) | AlHV-1 % id[e] | HVS % id[e] | Description[f] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ov2 | exon 2 | - | + | SA:2569 | 2159 | | 1326 | 186 | 56 | N/A | Basic leucine zipper motif. Homologue of AlHV-1 A2 |
| | | exon 1 | - | | 2813 | SD:2664 | 2840 | | | | | |
| | Ov2.5 | exon 1 | + | | 3576 | SD:3761 | 3520 | | | | | |
| | | exon 2 | + | | SA:3844 | SD:3903 | | | | | | |
| | | exon 3 | + | | SA:3985 | SD:4137 | | | | | | |
| | | exon 4 | + | | SA:4229 | SD:4294 | | | | | | |
| | | exon 5 | + | + | SA:4375 | 4458 | | 4503 | 182 | N/A | N/A | vIL-10 |
| | Ov3 | | + | + | 4801 | 6177 | 4641 | 6755 | 458 | 50 | N/A | Semaphorin. Homologue of AlHV-1 A3 |
| | Ov3.5 | | + | + | 6911 | 7402 | 6843 | 7432 | 163 | N/A | N/A | Signal peptide |
| | ORF3 | | + | | 7654 | 11739 | 7609 | 11759 | 1361 | 52 | 28 | FGARAT; tegument protein |
| | Ov4.5 | | + | + | 11795 | 12433 | 11758 | 12429 | 212 | 50 | N/A | vBcl-2. Homologue of EBV BALF1 |
| | ORF06 | | + | | 12575 | 15964 | 12501 | 16156 | 1129 | 79 | 50 | Single-stranded DNA binding protein |
| I | ORF07 | | + | | 16039 | 18087 | 15627 | | 682 | 65 | 41 | Subunit of terminase |
| | ORF08 | | + | | 18071 | 20662 | 17905 | 20763 | 863 | 76 | 44 | Glycoprotein B |
| | ORF09 | | + | | 20857 | 23853 | 20718 | 24031 | 998 | 75 | 56 | DNA polymerase, catalytic subunit |
| | Ov5 | | + | + | 24135 | 25388 | 23830 | | 417 | 49 | N/A | GPCR. Homologue of AlHV-1 A5 and EBV BILF1 |
| | ORF10 | | + | | 25108 | 26328 | 24887 | | 406 | 56 | 21 | Non-essential for replication |
| | ORF11 | | + | | 26330 | 27562 | 26103 | 27600 | 410 | 56 | 30 | Virion component. Non-essential for replication |
| | ORF17 | | - | | 38557 | 36899 | 38646 | 36852 | 552 | 50 | 34 | Maturational protease; capsid protein |
| | ORF17.5 | | - | | 37726 | 36899 | 37873 | 36852 | 275 | 42 | 29 | Major scaffold protein |
| | ORF18 | | + | | 38514 | 39344 | 38350 | | 276 | 68 | 45 | |
| | ORF19 | | - | | 40997 | 39318 | 41111 | 39299 | 559 | 74 | 41 | Tegument protein; putative portal capping protein |
| | ORF20 | | - | | 41538 | 40786 | 41641 | | 250 | 61 | 41 | Nuclear protein. |
| II | ORF21 | | + | | 41537 | 43246 | 41233 | | 569 | 47 | 29 | Thymidine kinase |
| | ORF22 | | + | | 43267 | 45519 | 43165 | 45522 | 750 | 63 | 27 | Glycoprotein H |
| | ORF23 | | - | | 46714 | 45512 | 46920 | 45463 | 400 | 58 | 34 | Tegument protein |
| | ORF24 | | - | | 48909 | 46720 | 49012 | 46702 | 729 | 70 | 41 | |
| | ORF25 | | + | | 48908 | 53011 | 48789 | | 1367 | 81 | 57 | Major capsid protein forming pentons and hexons |
| | ORF26 | | + | | 53077 | 53991 | 52980 | | 304 | 83 | 48 | Capsid triplex protein |
| | ORF27 | | + | | 53997 | 54878 | 53945 | 54955 | 293 | 51 | 28 | Glycoprotein. Involved in cell-cell spread |
| | ORF29 | exon 2 | - | | SA:56110 | 54965 | | 54969 | 687 | 82 | 58 | ATPase subunit of terminase |
| | ORF30 | | + | | 56128 | 56379 | 56021 | 56738 | 83 | 57 | 33 | |
| | ORF31 | | + | | 56301 | 56975 | | | 224 | 67 | 41 | Essential for replication |
| | ORF32 | | + | | 56924 | 58354 | 56868 | | 476 | 48 | 25 | Nuclear capsid localisation |
| | ORF33 | | + | | 58344 | 59363 | 58288 | 59679 | 339 | 52 | 37 | Tegument protein |
| | ORF29 | exon 1 | - | | 60270 | SD:59353 | 60410 | | | | | |
| | ORF34 | | + | | 60269 | 61282 | | | 337 | 63 | 37 | |
| | ORF35 | | + | | 61260 | 61721 | 60970 | | 153 | 59 | 29 | Tegument protein |
| | ORF36 | | + | | 61615 | 62937 | | 64175 | 440 | 55 | 33 | Protein kinase |
| III | ORF37 | | + | | 62921 | 64378 | 62729 | | 485 | 79 | 42 | Alkaline exonuclease |
| | ORF38 | | + | | 64333 | 64518 | 64224 | 64525 | 61 | 49 | 47 | Myristylated tegument protein; role in egress |
| | ORF39 | | - | | 65790 | 64564 | 65868 | 64568 | 408 | 80 | 44 | Glycoprotein M |
| | ORF40 | exon 1 | + | | 65834 | SD:67180 | 65773 | | | | | |
| | ORF40 | exon 2 | + | | SA:67304 | 67861 | | 67956 | 634 | 48 | 26 | Complexed with helicase-primase |
| | ORF42 | | - | | 68631 | 67858 | 68713 | 67845 | 257 | 64 | 3 | Tegument protein |
| | ORF43 | | - | | 70291 | 68603 | 70642 | | 562 | 83 | 54 | Capsid portal protein |
| | ORF44 | | + | | 70146 | 72638 | 70084 | 72637 | 830 | 78 | 55 | Helicase |
| | ORF45 | | - | | 73479 | 72694 | | 72684 | 261 | 37 | 22 | Nuclear phosphoprotein. Essential for replication |
| | ORF46 | | - | | 74267 | 73512 | 74361 | | 251 | 71 | 56 | Uracil DNA glycosylase |
| | ORF47 | | - | | 74706 | 74251 | 74752 | 73839 | 151 | 45 | 28 | Glycoprotein L |
| | ORF48 | | - | | 76101 | 74761 | 76148 | | 446 | 41 | 25 | |
| | ORF50 | exon 1 | + | | 76272 | SD:76328 | 75986 | | | | | |
| | ORF49 | | - | | 77341 | 76367 | 77394 | 76365 | 324 | N/A | 23 | Transcriptional control, EBV BRRF1 homolog |
| | ORF50 | exon 2 | + | + | SA:77379 | 79043 | | 79196 | 573 | 36 | 22 | Transcriptional control, Rta |
| | Ov6 | exon 1 | + | | 79355 | SD:79879 | 79287 | | | | | |
| | Ov6 | exon 2 | + | | SA:79989 | SD:80093 | | | | | | |
| | Ov6 | exon 3 | + | + | SA:80189 | 80329 | | 80384 | 256 | 28 | N/A | Leucine zipper motif. Homologue of AlHV-1 A6 |
| | Ov7 | | + | + | 80901 | 81266 | 80846 | 81338 | 121 | 60 | N/A | Putative glycoprotein. Homologue of AlHV-1 A7 |
| | Ov8 | exon 1 | + | | 81538 | SD:82756 | | | | | | |
| | Ov8 | exon 2 | + | + | SA:83704 | 83906 | | 83906 | 473 | 41 | N/A | Putative glycoprotein. Homologue of AlHV-1 A8 |
| | ORF52 | | - | | 84375 | 83965 | 84433 | 83953 | 136 | 45 | 26 | |
| | ORF53 | | - | | 84765 | 84457 | 84821 | 84461 | 102 | 51 | 35 | Glycoprotein N |
| | ORF54 | | + | | 84834 | 85715 | 84766 | 85750 | 293 | 57 | 36 | dUTPase |
| | ORF55 | | - | | 86415 | 85759 | 86479 | 85723 | 218 | 74 | 43 | |
| | ORF56 | | + | | 86414 | 88927 | | 89052 | 837 | 67 | 43 | Primase |
| | ORF57 | exon 1 | + | | 89060 | SD:89111 | 88953 | | 433 | 51 | 23 | |
| | ORF57 | exon 2 | + | | SA:89214 | 90463 | | 90485 | | | | Post-transcriptional regulator, Mta |
| | ORF58 | | - | + | 91970 | 90915 | | 90929 | 351 | 60 | 26 | Membrane spanning. Complexed with ORF27 |
| | ORF59 | | - | + | 93146 | 91977 | 93241 | | 389 | 63 | 33 | Processivity factor of DNA polymerase |
| | ORF60 | | - | | 94195 | 93278 | 94304 | 93660 | 305 | 80 | 57 | Ribonucleotide reductase (small subunit) |
| IV | ORF61 | | - | | 96604 | 94247 | 96794 | 94202 | 785 | 71 | 51 | Ribonucleotide reductase (large subunit) |
| | ORF62 | | - | | 97642 | 96635 | 97797 | | 335 | 63 | 32 | Capsid triplex protein |
| | ORF63 | | + | | 97641 | 100484 | 97494 | 100947 | 947 | 61 | 27 | Tegument protein |
| | ORF64 | | + | | 100489 | 108363 | 100391 | 108392 | 2624 | 50 | 24 | Large tegument protein |
| | ORF65 | | - | | 109063 | 108428 | 109170 | 108234 | 211 | 41 | | Capsid protein on hexon tips |
| | ORF66 | | - | | 110427 | 109120 | 110574 | 109121 | 435 | 60 | 36 | |
| | ORF67 | | - | | 111122 | 110346 | 111277 | | 258 | 68 | 51 | Capsid docking protein on nuclear lamina |
| | ORF67a | | - | | 111384 | 111130 | 111482 | | 84 | 69 | 48 | Interacts with terminase |
| | ORF68 | | + | | 111604 | 113022 | 111534 | 113134 | 472 | 61 | 39 | Nuclear capsid localisation |
| | ORF69 | | + | | 113029 | 113883 | 112941 | 113844 | 284 | 74 | 44 | Capsid docking protein on nuclear lamina |
| | Ov8.5 | | + | + | 117777 | 118949 | 117677 | 118969 | 390 | N/A | N/A | Proline rich |
| | ORF73 | | - | + | 120533 | 119046 | 120686 | 118971 | 495 | 43 | | LANA homologue; episomal maintenance |
| | ORF75 | | - | | 125054 | 121104 | 125099 | 121023 | 1316 | 58 | 29 | FGARAT; virion tegument protein |
| | Ov9 | | + | + | 125436 | 126056 | 125375 | 126207 | 206 | 50 | | vBcl-2 |
| | Ov10 | | - | + | 129326 | 127920 | 129392 | 127708 | 468 | 22 | | Putative nuclear protein. Homologue of AlHV-1 A10 |

a Blocks of conserved core herpesvirus genes are separated by lines and indicated by Roman numerals.
b Indication of expression of mRNA specific for this ORF in OvHV-2 infected cells either in this study or others (Coulter & Reid, 2002, Thonur et al., 2006)
c The position of the respective ORF on the genome is given from the first nucleotide of the first methionine codon or splice acceptor (SA) to the last nucleotide of the stop codon or splice donor; (SD).
d The first nucleotides of putative TATA boxes and polyadenylation signals (AATAAA or ATTAAA) are given.
e %id, percent identity of herpesvirus ORFs to the respective OvHV-2 ORF (calculated with the BLAST algorithm with default parameters).
f Possible functions for the OvHV-2 proteins are deduced from the homologous ORFs described in other herpesviruses