Comparison of ovine herpesvirus 2 genomes isolated from domestic sheep (*Ovis aries*) and a clinically affected cow (*Bos bovis*)

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The rhadinovirus *Ovine herpesvirus 2* (OvHV-2) is the causative agent of sheep-associated malignant catarrhal fever. OvHV-2 primarily affects ruminants and has a worldwide distribution. In this study, a composite sequence of OvHV-2 genomic DNA isolated from nasal secretions of sheep experiencing virus-shedding episodes was determined and compared with the sequence of OvHV-2 DNA isolated from a lymphoblastoid cell line derived from a clinically affected cow. The study confirmed the OvHV-2 sequence information determined for the cell line-isolated DNA and showed no apparently significant changes in the OvHV-2 genome during passage through a clinically susceptible species with subsequent maintenance *in vitro*. Amino acid identity between the predicted open reading frames (ORFs) of the two genomes was 94–100 %, except for ORF73, which had an identity of 83 %. Polymorphism in ORF73 was due primarily to variability in the G/E-rich repetitive central region of the ORF.

Malignant catarrhal fever (MCF) is a frequently fatal, lymphoproliferative disease syndrome of susceptible ruminants, including cattle, deer, bison and swine, caused by infection with one of a group of pathogenic gammaherpesviruses, particularly *Alcelaphine herpesvirus 1* (AlHV-1) and *Ovine herpesvirus 2* (OvHV-2) (Crawford et al., 1999; Loken et al., 1998; Plowright, 1990). Loss of livestock due to MCF can be significant, particularly in farmed deer and bison, and is most often the result of infection with OvHV-2, which is carried asymptomatically by virtually all domestic sheep (*Ovis aries*) (Baxter et al., 1993; Li et al., 1995, 2006; Reid, 1992). Considerable information concerning the epidemiology of OvHV-2, including the natural routes of transmission, is now available (Hüsey et al., 2002; Li et al., 2004); however, detailed molecular information about this virus is still lacking.

OvHV-2 has not yet been propagated *in vitro*, which has made it difficult to obtain sufficient virus DNA to sequence the genome. As a result, only a very limited amount of sequence information has been available (Coulter & Reid, 2002; Coulter et al., 2001; Dunowska et al., 2001). However, lymphoblastoid cell lines infected latently with OvHV-2 have been derived from clinically affected deer, cattle and rabbits (Buxton et al., 1984; Reid et al., 1983, 1989). Recently, Stewart and co-workers sequenced the complete OvHV-2 genome (GenBank accession no. AY839756) (Hart et al., 2007) by using viral DNA isolated from a lymphoblastoid cell line (BJ1035) derived from a cow with MCF (Schock et al., 1998). Analysis of this sequence showed that the OvHV-2 genome consists of a 130 kbp unique region containing 73 open reading frames (ORFs) flanked by multiple copies of a terminal repeat.

AlHV-1 is carried by wildebeest (*Connochaetes taurinus*) and can be propagated *in vitro* (Plowright, 1990). The complete genome sequence of one strain, C500, is available (Ensser et al., 1997). During prolonged culture, AlHV-1 loses the ability to induce MCF in cattle and rabbits (Handley et al., 1995; Wright et al., 2003). Examination of attenuated AlHV-1...
stocks revealed the presence of various genome rearrangements and it was suggested that the genes contained within the rearranged fragments were responsible for inducing clinical disease (Handley et al., 1995; Wright et al., 2003). Genome rearrangements and gene deletions, some of which are associated with changes in virus replication and pathogenicity, have also been reported for herpesvirus saimiri (HVS), mouse herpesvirus 68 (MHV-68) and Epstein–Barr virus (EBV) (Kieff, 1996; Kooomey et al., 1984; Macrae et al., 2001). Because of the variability found in other gammaherpesviruses, we felt that it was important to have OvHV-2 sequence information from more than a single source of virus DNA. Therefore, we sought to extend the work of Stewart and co-workers by sequencing the OvHV-2 genome isolated directly from domestic sheep, the natural carriers of the virus.

We previously demonstrated the presence of infectious OvHV-2 in nasal secretions of sheep experiencing shedding episodes, defined as $\geq 100000$ OvHV-2 genome copies per $2 \mu g$ nasal secretion sample DNA (Li et al., 2004; Taus et al., 2005). Therefore, we used nasal secretions from sheep experiencing shedding episodes as the source of OvHV-2 DNA for this study. Fifteen OvHV-2-infected sheep (6–9 months old) were obtained from the US Sheep Experiment Station, Dubois, ID, USA, and nasal secretions were collected daily as described previously (Kim et al., 2003; Li et al., 2004). Four sheep experiencing shedding episodes were identified and samples from these sheep were clarified by centrifugation and treated with DNase I (0.5 mg ml$^{-1}$; Roche Applied Science) to remove unprotected virus and cellular DNA. Two of the samples were also treated with RNase A (2 mg ml$^{-1}$; Puregene RNase solution; Gentra Systems). Virion DNA was released by the addition of proteinase K (0.5 mg ml$^{-1}$; Sigma-Aldrich) and SDS (0.3 %), samples were extracted twice with phenol/chloroform/isooamyl alcohol (24:24:1) and the DNA was precipitated. The four samples were combined to give a final yield of 700 ng DNA. This DNA was sheared by sonication (Sonifier Cell disruptor 350, 12 s, power setting #1; VWR International) and a library was constructed by using a TOPO Shotgun Subcloning kit (Invitrogen Life Technologies) according to the manufacturer’s directions. Eight hundred and seventy-seven clones were picked, arrayed on nylon filters and screened for clones containing sheep DNA by using hybridization to a random digoxigenin (DIG)-labelled probe (DIG DNA Labelling and Detection kit; Roche Applied Science), generated from genomic sheep DNA purified from peripheral blood buffy-coat cells (Li et al., 2004). The 784 clones that did not hybridize to the sheep probe were sequenced by using a BigDye Terminator v3.1 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (both from Applied Biosystems). Sequence assembly was done by using Vector NTI Advance 9.0 (InforMax Software; Invitrogen) and PHRAP, using the complete sequence of OvHV-2 strain BJ1035 as a scaffold, which simplified orientation and positioning of contigs. One hundred and eighty-five clones from the initial library yielded 80 % coverage of the genome in 23 contigs.

A new source of virus DNA was required to obtain sequence coverage of the 22 gap regions. Material pelleted from nasal secretions containing high levels of OvHV-2 also contains virus DNA (H. Li, N. S. Taus & D. L. Traul, unpublished data), which is presumably cell-associated. Pelleted material from nasal secretions collected from nine sheep was used to isolate viral DNA. Briefly, samples were frozen, thawed and cleared of debris by using centrifugation at 2600 $g$ for 30 min. Viral particles were concentrated by centrifugation at 15 000 $g$ for 4 h and DNA was isolated by using the procedure of Yu et al. (1999). The presence of OvHV-2 DNA was confirmed by using a previously described PCR assay for a tegument gene (Li et al., 2004). This DNA was used as template for a PCR designed to amplify regions of the OvHV-2 genome missing from the original library. PCR products were cloned into pCR2.1-TOPO or pCR4Blunt (Invitrogen) and two to four clones from each gap region were sequenced.

The final composite genome sequence was obtained from the nasal secretions of 13 sheep and contained 131 621 nt. Because the OvHV-2 DNA used to determine the genome sequence was obtained from different sheep, the final sequence does not represent a single isolate and may reflect a mixture of isolates. The 5’ end of this sequence corresponded to nt 164 of the BJ1035 sequence (GenBank accession no. AY839756), located in the 40 bp repeat element adjacent to the terminal repeat, and the 3’ end of the composite sequence extended into the reported terminal repeat. Given the limited amount of virus DNA available to us and the difficulties associated with sequencing DNA with a high G+C content, such as that found in herpesvirus terminal repeats, we did not attempt to sequence the ends of the OvHV-2 genome completely. Comparison of the secretion-derived OvHV-2 sequence with that of the BJ1035-derived sequence revealed that the two viruses are very similar, with ORF amino acid identity ranging from 94 to 100 %, except for ORF73, which has an identity of 83 % (Fig. 1).

We identified 74 ORFs in the unique region of secretion-derived OvHV-2, compared with 73 ORFs identified in the BJ1035-derived genome. The presence of two additional nucleotides in the secretion-derived sequence, nt 67130–67131, resulted in a frameshift such that two ORFs, 40 and 41, were present in this region of the genome, accounting for the additional ORF in secretion-derived OvHV-2. A number of other gammaherpesviruses, including EBV, Kaposi’s sarcoma–associated herpesvirus (KSHV), HVS, *Bovine herpesvirus 4* (BoHV-4), rhesus rhadinovirus (RRV) and AlHV-1, are also predicted to encode homologues of ORFs 40 and 41 (Albrecht et al., 1992; Alexander et al., 2000; Ensser et al., 1997, 2003; Russo et al., 1996; Zimmermann et al., 2001). In EBV, the BBLF 2 and 3 (ORF40 and 41 homologues) transcripts are spliced to form a single transcript that is translated into the helicase–primase accessory protein (Fixman et al., 1995), and in KSHV, the ORF40 and 41 transcripts are also spliced and
predicted to give rise to a single helicase–primase-associated protein (AuCoin & Pari, 2002). The precedent of a single ORF for 40 and 41, as seen in the BJ1035-derived sequence, was established in MHV-68, which has a single ORF predicted to encode the helicase–primase complex component (Virgin et al., 1997); however, this continuous ORF is also spliced at conserved sites (J. P. Stewart, unpublished data). Whether the OvHV-2 ORF40/41 transcript results from splicing of two independent transcripts or from splicing of a single ORF, the predicted spliced coding transcript is the same in both the nasal secretion- and BJ1035-derived virus. Further analysis of the genes encoding the OvHV-2 helicase–primase complex awaits the development of an in vitro propagation system.

Differences in predicted amino acids resulting from nucleotide insertions or deletions were identified in ORFs 17, Ov3 and Ov10 (Table 1). ORF17, the homologue of the herpes simplex virus maturational protease in secretion-derived OvHV-2, is predicted to encode a protein of 550 aa, compared with 552 aa predicted for BJ1305. Ov3, a homologue of AlHV-1 A3 and Ov10, unique to OvHV-2, are genes of unknown function and, in the secretion-derived virus, are predicted to encode proteins of 459 and 468 aa, respectively. It is unknown whether the differences in these three ORFs have an effect on the functions of the affected proteins.

ORF73 of KSHV encodes the latency-associated nuclear antigen (LANA) (Kedes et al., 1997; Rainbow et al., 1997). LANA is a multifunctional protein involved in stable maintenance of viral genomes during latency and regulation of viral and cellular genes (Verma & Robertson, 2003). Positional homologues of ORF73 have been identified in a number of gammaherpesviruses, although there is considerable variability in the predicted length and amino acid identity of the homologues (Albrecht et al., 1992; Alexander et al., 2000; Ensser et al., 1997, 2003; Virgin et al., 1997; Zimmermann et al., 2001). ORF73 homologues range in length from 253 aa (BoHV-4) to 1300 aa (AlHV-1) and the repetitive/acidic region of KSHV ORF73 is absent in the MHV-68, RRV and BoHV-4 homologues; however, the proteins are postulated to have similar functions during virus latency (Albrecht et al., 1992; Alexander et al., 2000; Ensser et al., 1997, 2003; Virgin et al., 1997; Zimmermann et al., 2001).

As we were unable to determine a complete ORF73 from our initial pooled viral DNA sequences, we amplified, cloned and sequenced ORF73 by using virus DNA isolated from nasal secretions of three individual sheep. Two of the sheep, 802 and 809, were obtained in the year 2003, and the third, 1056, was obtained in 2004. The primers (22L6 and 75U5) used to amplify the entire ORF73 were described by Coulter & Reid (2002). Cycling conditions were 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min 30 s, 72°C for 2 min, followed by a final extension at 72°C for 7 min. PCR was performed by using the Expand Long Template PCR system (Roche Applied Science). Examination of the PCR products using gel electrophoresis and ethidium bromide staining showed that only a single product was amplified from each sheep (data not shown).
Table 1. Predicted amino acid polymorphisms in three ORFs of nasal secretion- and BJ1035-derived OvHV-2

<table>
<thead>
<tr>
<th>Virus*</th>
<th>ORF</th>
<th>Length (aa)</th>
<th>Amino acid identity (%)†</th>
<th>Alignment† (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>17</td>
<td>550</td>
<td>98.9</td>
<td>STSTSTST-- (aa 268–275)</td>
</tr>
<tr>
<td>BJ1035</td>
<td>552</td>
<td></td>
<td></td>
<td>STSTSTSTST (aa 268–277)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Ov3</td>
<td>459</td>
<td>98.9</td>
<td>VFTANSTSV (aa 412–420)</td>
</tr>
<tr>
<td>BJ1035</td>
<td>458</td>
<td></td>
<td></td>
<td>VFT-NSTSV (aa 412–419)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Ov10</td>
<td>468</td>
<td>94.2</td>
<td>VKKKK-T (aa 234–239); EENGAT (aa 297–302)</td>
</tr>
<tr>
<td>BJ1035</td>
<td>468</td>
<td></td>
<td></td>
<td>VKKKKKKT (aa 234–240); EE-GAT (aa 298–303)</td>
</tr>
</tbody>
</table>

*Sheep, OvHV-2 DNA isolated from nasal secretions of sheep, GenBank accession no. DQ198083; BJ1035, OvHV-2 DNA isolated from a bovine lymphoblastoid cell line, GenBank accession no. AY839756.
†Identity and alignment determined by using the CLUSTAL_W algorithm as implemented by AlignX (Vector NTI Advance 9.0; Invitrogen). The scoring matrix was BLOSUM62mt2. The gap-introduction penalty was 10 and the gap-extension penalty was 0.1.

The PCR products were gel-purified, cloned into pCR-Blunt-II or pCR2.1-TOPO and sequenced. Four clones each from sheep 802 and 1056 and seven clones from sheep 809 were sequenced. Nucleotide and amino acid sequences were compared by using Vector NTI Advance 9.0. The sequences of ORF73 from each individual sheep were identical and consisted of 1551 bp (Ov1056), 1635 bp (Ov809) and 1656 bp (Ov802), predicted to encode proteins of 516, 544 and 551 aa, respectively (Fig. 1). The longest sequence for ORF73, from Ov802, was included in the final composite OvHV-2 genome sequence deposited in GenBank.

Alignment of the three secretion-derived ORF73 sequences, Ov802, Ov809 and Ov1056, showed an overall amino acid identity of 94–98%. The identity was 100% for the 32 N-terminal residues and also for the C-terminal 136 residues (Fig. 1). The middle region of the molecule contained variable numbers of repeated P, G, E and V residues, comprising a G/E-rich region with an identity of 91–98% among the three isolates (Fig. 1). The sequence of ORF73 determined for the BJ1035-derived virus is 1488 nt and is predicted to encode a protein of 495 aa (Fig. 1). Alignment of BJ1035-derived ORF73 with Ov802, Ov809 and Ov1056 showed that the N-terminal 32 aa of BJ1035 ORF73 had 88% identity with the three sheep secretion-derived sequences and the C-terminal 136 aa had 99% identity, whilst the G/E-rich repetitive region had an identity of 79–88% between the four isolates (Fig. 1). Polymorphisms in the length and number of repeated residues in the central region of ORF73 have been identified in KSHV isolates and in the HVS ORF73 homologue (Ensser et al., 2003; Gao et al., 1999; Zhang et al., 2000). Polymorphism of KSHV ORF73 has revealed that some individuals are infected with two genotypes of the virus (Gao et al., 1999), although it is not known whether dual infections have an effect on the development of disease in such individuals. Examination of the OvHV-2 ORF73 homologue from a greater number of samples will be needed to determine whether dual infection with different viral genotypes is present in sheep and clinically affected animals.

In this study, we isolated OvHV-2 DNA from the nasal secretions of 13 sheep experiencing intense virus-shedding events and determined a composite genome sequence. Comparison of this sequence with the BJ1035-derived OvHV-2 sequence revealed that the two viruses are highly similar and no genome rearrangements, such as those that occur in AIHV-1, were detected. This indicates that the pathology of MCF observed in clinically susceptible species is not due to changes in the genome structure, but to other factors, such as virus gene regulation or differences in virus cell tropisms between clinically susceptible species and sheep. We detected polymorphisms in several genes between secretion- and BJ1035-derived OvHV-2, with the greatest density found in ORF73, the LANA homologue. As a function has not been defined for the repeat region of ORF73 homologues, it is unclear what effect the variability of this region might have on the ORF73 protein.

This study, in combination with the work of Stewart and colleagues (Hart et al., 2007), provides important information about the structure of the OvHV-2 genome and forms the basis for future studies of viral pathogenesis and immunological control of MCF.

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References


