A Captured Viral Interleukin 10 gene with Cellular Exon Structure

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Summary

We have characterised a novel, captured and fully-functional viral IL-10 homologue ($_{OvHV}$IL-10) from the gammaherpesvirus Ovine herpesvirus 2. Unlike IL-10 homologues from other gammaherpesviruses, the $_{OvHV}$IL-10 peptide sequence was highly divergent from that of the host species. The $_{OvHV}$IL-10 gene is unique amongst virus captured genes in that it has precisely retained the original cellular exon structure having five exons of similar sizes to the cellular counterparts. However, the sizes of the introns are dramatically reduced. The $_{OvHV}$IL-10 protein was shown to be a non-glycosylated, secreted protein of Mr 21,000 with a signal peptidase cleavage site between amino-acids 26 and 27 of the nascent peptide. Functional assays showed that $_{OvHV}$IL-10, in a similar way to ovine IL-10, stimulated mast cell proliferation and inhibited macrophage inflammatory chemokine production. This is the first example of a captured herpesvirus gene retaining the full cellular gene structure.

Introduction

The host-pathogen relationship is seen as a dynamic co-evolutionary process. On one side, host defence mechanisms have required ongoing adaptation to combat infection. Whereas, under survival pressure from sophisticated defence mechanisms, viruses have evolved immune evasion strategies. Some genes of large DNA viruses, such as herpesviruses, that encode immunomodulatory functions have extensive similarities in their encoded amino acid sequences to genes from the host. Counterparts of these genes are not universally found within herpesvirus subfamilies. This is in contrast to the majority of herpesvirus genes that are ancestrally related within either the herpesvirus family or sub-family (α, β or γ) and have either weak or no obvious sequence similarity with host genes (Holzerlandt *et al.*, 2002; McGeoch & Davison, 1999). This has led to the hypothesis that these immunomodulatory genes may have been recently captured from the host in the evolution of immune evasion (Chaston & Lidbury, 2001; Shackelton & Holmes, 2004). However, while much has been written on the function and phylogeny of captured genes, the mechanism of their capture remains cryptic. One possibility is direct recombination. This is feasible for viruses that replicate in the nucleus, such as herpesviruses. Even in herpesviruses most captured genes are single exons, unlike their cellular counterparts which contain introns (Bugert & Darai, 2000; Kotenko *et al.*, 2000; Moore *et al.*, 1990). Thus, it has been argued that insertion of an intron-less cDNA copy of spliced cellular RNA is more likely. The necessary reverse transcriptase for this process could be derived from co-infection with retroviruses and/or insertion of retroviruses into the viral genome (Brunovskis & Kung, 1995).

Interleukin-10 (IL-10) is a pleiotropic cytokine that has both immuno-stimulatory and immuno-suppressive activities. IL-10 inhibits the production of a number of pro-inflammatory cytokines in a variety of cell-types but is a co-stimulator for lymphocyte and

mast cell proliferation (Mosmann, 1994). IL-10 homologues have been acquired by a number of viruses. These may enhance viral pathogenesis by protecting infected cells from host anti-viral defence mechanisms and thereby promote virus survival. Homologues of IL-10 have been described in herpesviruses (Kotenko *et al.*, 2000; Moore *et al.*, 1990; Rode *et al.*, 1993) as well as the poxvirus orf (ORFV) (Haig *et al.*, 2002). Comparative studies on cellular and viral IL-10s are providing important insights into the structural and functional relationships of this cytokine (Hughes, 2002; Jones *et al.*, 2002; Zdanov *et al.*, 1997). These have been facilitated by the availability of the crystal structure of cellular and viral IL-10s and the cellular receptor complex that has allowed the identification of key residues involved in IL-10- IL-10 receptor interaction (Walter & Nagabhushan, 1995).

Malignant catarrhal fever (MCF) is a severe, usually fatal lymphoproliferative and inflammatory disease of cattle, pigs, deer and certain other susceptible ruminants such as bison (Reid & Buxton, 1989). The disease is mainly caused by either of two closely-related gammaherpesviruses that persist sub-clinically in their natural hosts. Alcelaphine herpesvirus 1 (AlHV-1) naturally infects wildebeest and is the cause of MCF in Africa. Domestic sheep are the reservoir of infection for Ovine herpesvirus 2 (OvHV-2), which causes MCF in other parts of the world (Baxter *et al.*, 1993). While AlHV-1 has been isolated and completely sequenced (Ensser *et al.*, 1997), the study of OvHV-2 has lagged behind due a lack of suitable culture systems. Recently, however, the complete sequence of OvHV-2 has been determined based on cosmid clones from an infected cell line (Hart *et al.*, 2007) and was confirmed by sequencing of DNA prepared from sheep-derived OvHV-2 virions (Taus *et al.*, 2007).

Materials and Methods

Cloning and expression of the *Ov2.5* gene. The cell line BJ1035 (Buxton *et al.*, 1985), a bovine lymphoblastoid cell line which was derived from diseased cattle was used as the source of DNA and RNA for cloning. High molecular weight DNA was purified from this line using a standard SDS-proteinase K extraction protocol (Sambrook *et al.*, 1989). Total RNA was extracted using the RNeasy mini system (Qiagen) and oligo-dT primed cDNA generated using a standard protocol (Stewart *et al.*, 1996). The complete *Ov2.5* gene and cDNA were amplified separately, incorporating coding sequences for triple haemagglutinin (3xHA) and FLAG epitope tags at the 3'ends, at an annealing temperature of 55^{o}C using cloned *Pfu* DNA polymerase (Stratagene) and primers as follows: HA tagged sequence 5'-ATG CGG CCG CCG CCG CCA CCA TGG CAT TGG CCC ACC AAC TAC-3' (forward primer) and 5'-GCA GAT CTT TAA GCG TAG TCT GGA ACG TCG TAT GGG TAA GCG TAG TCT GGA ACG TCG TAT GGG TAA GCG TAG TCT GGA ACG TCG TAT GGG TAC TTG ACC CCA AAG TAG CTT TCC-3' (reverse primer); FLAG tagged sequence, above forward primer and 5'-GCA GAT CTT TAC TTG TCA TCG TCG TCC TTG TAG TCC TTG ACC CCA AAG TAG CTT TCC-3' (reverse primer), where the *Ov2.5*-specific sequences are underlined. PCR products were purified with a PCR purification kit (Qiagen), digested with *Not*I and *Bgl*II, inserted into *Not*I and *Bam*HI site of pKS (Stratagene) and the sequence verified. Sequencing was performed by MWG Biotech, Ebersberg, Germany. Verified sequences were then sub-cloned into the mammalian expression vector pVR1255, a kind gift of Vical Inc. (Norman *et al.*, 1997). These expression constructs (pVR1255-Ov2.5HA, pVR1255-Ov2.5cDNAHA and pVR1255-Ov2.5FLAG) were transfected into HEK 293T cells using a standard calcium phosphate protocol. Cell pellet and cell-free supernatant fractions were collected 48 hours post-infection.

The ovIL-10-FLAG was prepared with FLAG at the C-terminus of the polypeptide in the pAPEX-3 vector, and expressed ovIL-10-FLAG purified by M2-anti-FLAG (Sigma) gel affinity chromatography as described previously (Haig *et al.*, 2002).

Where indicated, N-linked glycosylation was inhibited by the inclusion of tunicamycin B2 (Sigma) at a final concentration of 5 µg/ml in the culture medium.

N-terminal sequence analysis. To prepare $_{OvHV}$IL-10 for N-terminal sequencing, cells were transiently transfected as above with pVR1255-Ov2.5HA. HA-tagged recombinant $_{OvHV}$IL-10 was then isolated from cell-free supernatant (CFS) from by affinity chromatography over monoclonal anti-HA (clone HA-7) conjugated to agarose (Sigma). Briefly, 5ml of CFS was repeatedly passed over a 2ml column of anti-HA-agarose previously equilibrated with TBS. The column was extensively washed and bound protein eluted with 0.1M glycine pH 2.8. Collected fractions were immediately made pH 7.5 by addition of 1M Tris pH 9.0. Analysis of the eluted protein fraction, concentrated 5-fold in Microcon 10K filtration cells (Millipore), was carried out by western blotting (see below). A single protein band of approximately 19kD was identified.

For N-terminal sequencing, the same fraction was run on a 4-12% Nupage Novex Bis-Tris gel (Invitrogen) under reducing conditions using a MES buffer system as per manufacturer's instructions. Separated protein was then transferred to Invitrolon PVDF membrane using the Novex wet transfer system as outlined by the relevant technical guide (Invitrogen). The blots were stained with 0.2% amido black in 40% methanol for 1 min and after destaining in distilled water, the 19kD band was used for sequencing by the Proteomics Unit at the Moredun Research Institute (Edinburgh, Scotland). N-terminal amino acid analysis was performed by Edman degradation chemistry using a PE Procise cLC494 protein sequencer (Applied Biosystems) in accordance with the manufacturer's recommendations.

Western blot analysis. Cell supernatant and cells were collected separately to detect expressed protein by the HA-tagged sequence. Cells (10^4 per well) or supernatant were admixed with 10 µl of SDS-PAGE sample buffer and boiled. Samples were loaded onto 12.5% SDS-PAGE gels, electrophoresed and blotted onto immobilon-P transfer membrane (Millipore). The membrane was then probed with 0.1 µg/ml of anti-HA-Biotin, High Affinity (3F10) (Roche) followed by 2 µg/ml of Horseradish peroxidase streptavidin (Vector Laboratories, USA). The blot was then developed using the ECL western blotting analysis system (Amersham Biosciences) and the light emission was detected by 15 seconds exposure to Hyperfilm ECL (Amersham Pharmacia Biotech).

Cloning the sheep IL-10 gene. The ovine IL-10 gene was amplified from high molecular weight genomic DNA prepared from sheep peripheral blood mononuclear cells using the QIAamp DNA system (Qiagen). A 4kbp fragment carrying the IL-10 gene was amplified from several sheep DNA samples using the Expand Long Template PCR system (Roche) with primers OvIL10F1 (5'-CTC TTG CAA AAC CAA ACC ACA AGT C -3') and OvIL10R1 (5'- TGT GGG AGC TGA GGT ATC AGA GGT A-3') designed to anneal in the 5'- and 3'-untranslated regions of the gene. The PCR product was cloned into the pGEM-T-Easy vector and five clones containing the IL-10 gene were sequenced. The DNA sequences of the clones were compared to check for polymorphisms and PCR errors.

Analysis of *Ov2.5* polymorphism in OvHV-2 case samples. The complete *Ov2.5* gene was amplified by PCR from seven MCF case samples from five different areas of the UK. The *Ov2.5* gene was amplified from each DNA samples using primers Ov2.5start (5'-ATGGCATTGGCCCACCAACTAC-3') and Ov2.5stop (5'-CTTGACCCCAAAGTAGCTTTCC-3') with the Expand Long Template proofreading PCR system (Roche). PCR products from each sample were cloned into pGEM-T-Easy

(Promega) and five clones from each sample were sequenced. Individual sequences were obtained from each clone before deriving a consensus sequence for each sample.

IL-10 assays. The biological activity of $_{OvHV}$IL-10 protein and $_{ov}$IL-10 was assayed by measuring the proliferation of the murine mast cell line D36 (Schlaak *et al.*, 1994) in the presence of FLAG-tagged IL-10 protein and murine IL-4 as a non-proliferation-inducing co-stimulus (Haig *et al.*, 2002). D36 cells were maintained with 5U/ml murine IL-3 and 1U/ml murine IL-4 (BD Pharmingen, Oxford, UK). FLAG-tagged proteins and murine IL-10 (control) were added to 2 x 10^4 D-36 cells in 100µl IMDM in triplicate wells of 96 well plates along with 2U/ml murine IL-4 as co-stimulus. Cells were cultured for 48 hours, the final 14 hours with 18.5 kBq ^{3}H-thymidine per well. ^{3}H-thymidine incorporation (counts per minute – CPM) was measured using a β-scintillation counter (Packard Tricarb, Packard, Downes Grove, USA). IL-10 specificity was tested with a murine IL-10 receptor-specific neutralizing antibody (clone 1B1.3a, BD-Pharmingen) at 5ng/ml in cultures of D36 cells prior to the addition of the IL-10 reagents.

The anti-inflammatory activity of IL-10 was assayed by measuring the inhibition of IL-8 production by ovine alveolar macrophages (Haig *et al.*, 2002). The macrophages were obtained by broncho-alveolar lavage and purified by Percoll density gradient centrifugation (>98% purity)(Entrican *et al.*, 1999). FLAG-tagged proteins were added to cultures of macrophages (in triplicate wells of 96 well plates, 10^5 cells/well in 200µl IMDM) 1-2 hours prior to stimulation of the macrophages with 10ng/ml LPS (from *S. minnesota*, Sigma). One day later cell-free supernates were assayed for ovine IL-8 by specific ELISA (Haig *et al.*, 1996; Haig *et al.*, 2002). IL-10-FLAG specificity was tested using 5µg/ml anti-FLAG (murine Mab M2, Sigma) to neutralise the IL-10s for 1 hour at room temperature prior to adding the IL-10-anti-IL-10 complexes to the assay.

Phylogenetic Analysis. Multiple alignments were carried out on protein sequences using 6 different programs (MAFFT (Katoh *et al.*, 2005), Muscle (Edgar, 2004), T-coffee (Notredame & Suhre, 2004), ClustalW (Thompson *et al.*, 1994), hmmalign (http://hmmer.janelia.org/) and MOE (http://www.chemcomp.com). Trees were made by neighbour joining plus bootstrap using PHYLIP (http://evolution.genetics.washington.edu/phylip.html) and by Bayes MCMC (MrBayes) (Ronquist & Huelsenbeck, 2003). PAML (Yang, 2007) was used to investigate the possibility of positive selection.

RESULTS AND DISCUSSION.

Identification of the OvHV-2 *IL-10* gene. During the DNA sequencing of the complete OvHV-2 genome (Hart *et al.*, 2007), analysis of the non-coding regions between open-reading frames using the BLASTX algorithm (Altschul *et al.*, 1990) identified a putative gene near the left terminus of the unique portion of the genome, termed *Ov2.5.* This had the potential to encode a homologue of other IL-10 molecules (Fig. 1). The 883 bp gene was predicted to have five coding exons, four introns of 82-92 bp in size and encode a protein of 183 amino acid residues and predicted Mr of 21,000. Analysis of the putative protein using the algorithm SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; (Emanuelsson *et al.*, 2007)) revealed a consensus N-terminal secretory signal sequence 22 aa residues in length giving a predicted M_r of approximately 18,000 for the mature polypeptide. Database searching using the BLASTP algorithm (Altschul *et al.*, 1990) identified significant similarity to cellular and viral IL-10 family proteins, with greater similarity to IL-10 than to other IL-10 family members (IL-19, IL-22 or IL-24). Alignment of *Ov2.5* with the host (sheep) IL-10 coding sequence showed 52% nucleotide and 41% amino acid sequence identity. This is relatively divergent in sequence from the host IL-10

compared with many viral IL-10 homologues (e.g.EBV BCRF1 91% aa identity with human IL-10, EHV-2 E7 84% aa identity with horse IL-10).

To obtain empirical confirmation of *Ov2.5* gene and mRNA structure, RNA extracted from the OvHV-2 positive bovine T cell line BJ1035 was amplified by RT-PCR using primers specific for *Ov2.5*. Products were then sequenced. This confirmed that, as predicted, there were five exons corresponding to co-ordinates 3576 - 3761, 3844 - 3903, 3985 - 4137, 4229 – 4294 and 4375 - 4458 of the OvHV-2 BJ1035 genome (Genbank accession number AY839756) A diagrammatic representation of the gene structure is shown in supplemental Figure S1. Thus, the *Ov2.5* product is a new member of the IL-10 family and we have named it $_{OvHV}$IL-10.

Characterisation of the $_{ovHV}$IL-10 protein. The $_{OvHV}$IL-10 was characterised by expressing recombinant protein that was tagged at the carboxy terminus with three copies of an epitope from influenza virus haemagglutinin (3xHA). Tagged versions of the gene were cloned into the mammalian expression vector pVR1255 and transfected into HEK 293T cells. Western blot analyses of both cell pellet and cell-free supernatant (Fig. 2A) showed that proteins of apparent M_r 24,000 and 21,000 were produced in cells transfected with pVR1255 containing the genomic form of *Ov2.5* and that the smaller form only was found secreted in the cell supernatant. These Mr correspond well, after accounting for the 3xHA tag (3,000), with those predicted from sequence analysis for the nascent and mature forms of $_{OvHV}$IL-10. To control for the presence in the supernatant of proteins derived from lysed cells, HEK 293T cells were transfected with pVR1255 containing an HA-tagged cDNA of the OvHV-2 *Ov2* gene (Hart *et al.*, 2007), the product of which is known to have a M_r of 40,000 on SDS-PAGE gels and a nuclear/cytoplasmic distribution. An *Ov2-HA* product was contained only in the cell pellet. Thus the $_{OvHV}$IL-10 seen in the supernatant fraction is secreted from cells and not derived from cell lysis.

Analysis of the $_{OvHV}$IL-10 aa sequence using the algorithm NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) revealed one potential N-linked glycosylation site in $_{OvHV}$IL-10. To determine if $_{OvHV}$IL-10 was N-glycosylated, cells were transfected with pVR1255-Ov2.5HA, then treated with tunicamycin B2 immediately after transfection. The results (Fig. 2A) showed that the M_r of $_{OvHV}$IL-10 was unchanged after tunicamycin B2 treatment. As a control for tunicamycin treatment, MHV-68 infected cells were processed as for transfected cells and the Mr of MHV-68 glycoprotein B (gB) determined by Western blot analysis. Figure 2B shows that the Mr of MHV-68 gB was reduced from approximately 105,000 to 95,000 after treatment showing that tunicamycin was effective in blocking N-linked carbohydrate addition under these conditions. Thus, unlike some other IL-10 molecules (Moore *et al.*, 1990), $_{OvHV}$IL-10 was not N-glycosylated.

Although bioinformatic analysis had indicated the presence of an N-terminal signal sequence, we wished to determine the precise signal peptidase cleavage site and hence the N-terminus of the secreted mature protein. Thus, HA-tagged recombinant $_{OvHV}$IL-10 was purified from the supernatant of transfected cells and N-terminal sequence analysis performed. This generated a good sequence for 15 residues of RVLPLRGNCKLLLQD corresponding to residues 27 – 41 of $_{OvHV}$IL-10. This confirmed the presence of a signal sequence and identified a signal peptidase cleavage site between residues Glu-26 and Arg-27, giving a mature peptide of 157 aa residues. This empirically derived N-terminal sequence of the mature peptide differed from that predicted by SignalP and emphasises the importance of experimental confirmation of signal sequences.

$_{OvHV}$IL-10 retains IL-10 functions. To assess the biological activities of $_{OvHV}$IL-10, FLAG epitope-tagged recombinant protein was produced in transfected CHO cells. Recombinant FLAG-tagged ovine IL-10 ($_{Ov}$IL-10) was produced in parallel and used as a positive control. FLAG-tagged $_{OvHV}$IL-10 was used in these experiments rather than HA-tag as the relevant

controls and assays for IL-10 were already established using the FLAG-tag system (Haig *et al.*, 2002). Recombinant IL-10s were tested in standard biological assays for IL-10, namely, mast cell proliferation and inhibition of macrophage inflammatory chemokine (IL-8) production assays.

The results showed that $_{OvHV}$IL-10 induced dose-dependent proliferation of D36 murine mast cells in conjunction with IL-4 (Fig. 3A). The magnitude of dose-dependent induction of proliferation was similar to that observed for $_{ov}$IL-10. Mast cell proliferation was IL-10-specific as it was almost completely inhibited by anti-IL-10 receptor treatment of the cells. Likewise, $_{OvHV}$IL-10 inhibited IL-8 production by LPS-stimulated ovine macrophages in a dose-dependent fashion (Fig. 3B). IL-10 specificity was confirmed in this case by the addition of anti-FLAG that neutralised the FLAG-IL-10. Thus, in spite of the divergence of its sequence, $_{OvHV}$IL-10 is fully functional and exhibited typical IL-10 activity in both immunostimulatory and immunosuppressive assays.

$_{OvHV}$IL-10 retains IL-10 functional motifs. The relative peptide sequence divergence of $_{OvHV}$IL-10 from both viral and cellular homologues enabled analysis of functionally relevant residues. Fig. 4 shows an alignment of the predicted amino acid sequence of $_{OvHV}$IL-10 with that of other IL-10s. Amino-acid residues involved in IL-10-binding to the hIL-10 receptor are shown, as well as those thought to be important in stabilising the structural core of the IL-10 protein. Seventeen of 27 residues that are involved in the binding of hIL-10 with its receptor are conserved in $_{OvHV}$IL-10. All four putative IL-10 polypeptide-stabilising residues are also conserved between $_{OvHV}$IL-10 and hIL-10. An isoleucine at position 87 of the hIL-10, thought to be important for cell-stimulatory activity of hIL-10 (Ding *et al.*, 2000; Haig *et al.*, 2002) is not conserved in $_{OvHV}$IL-10. This would support the concept that Ile-87 alone is not sufficient in determining IL-10 immuno-stimulatory activity. Of the highly conserved residues Arg-27, Lys-34, Gln-38, Ser-141, Asp-144 and Glu-151

that form the IL10R1-binding site (Josephson *et al.*, 2002), only Ser-141 is not conserved in $_{OvHV}$IL-10. In addition, a range of structural features are conserved in the *Ov2.5* sequence, including the cysteine residues involved in disulphide bonds and two salt bridges that stabilise the receptor binding site structure (Lys-34 to Asp-144 and Arg-27 to Glu-151). Thus, in spite of the relatively high divergence of $_{OvHV}$IL-10 from its cellular counterparts, there was relatively high conservation of structurally-relevant residues that enabled binding to receptor and both immunostimulatory and immunosuppressive function.

The origins of the Ov2.5 gene. To study the origin of the *Ov2.5* gene, phylogenetic relationships of IL-10s from representative mammalian species (n = 11) and γ-herpesviruses (n = 5) were analysed extensively using neighbour joining plus bootstrap and by Bayes MCMC (Ronquist & Huelsenbeck, 2003). Comparison of the protein sequence alignments using different alignment programs (see Methods) revealed that the N-terminal region cannot be aligned with confidence. Therefore all residues in the alignment N-terminal to position 38 in human protein, were removed, as well as all gapped positions. The resulting alignment was 138 characters long. It was noted that the host IL-10 sequences are highly similar, while the OvHV2 sequence is highly diverged from all others . Both of these features limit the potential for resolution of phylogenetic trees and it was impossible to produce a reliable phylogenetic tree incorporating *Ov2.5* with high statistical confidence.

PAML analysis using only the cDNA sequences corresponding to the 138 residues of the trimmed protein alignment revealed no evidence for positive selection. However, as with the construction of trees, this was also confounded by the degree of divergence of *Ov2.5*.

It has been suggested that there have been multiple acquisitions of IL-10 in the γ-herpesviruses (Equine herpesvirus 2 and the Epstein-Barr-related viruses) (Hughes, 2002;

McGeoch, 2001). The case for multiple acquisitions is supported by the differing relative genomic locations of the IL-10 homologues found in the individual gammaherpesviruses (Supplemental Fig. S2). Multiple viral acquisitions of IL-10 suggest a strong selective advantage in specific hosts. This is most likely due to the immunosuppressive actions of IL-10 (Kurilla *et al.*, 1993). OvHV-2 has been classified as member of the *Macavirus* lineage of gammherpesviruses (Ehlers *et al.*, 2008; McGeoch *et al.*, 2006). Sequence of the *Ov2.5*-equivalent region is available for only AlHV-1 and porcine lymphotropic herpesvirus 1 (PLHV-1) of the *Macavirus* lineage and neither of these viruses have an *Ov2.5* homologue, suggesting that acquisition of *Ov2.5* is a relatively recent occurrence. Indeed, OvHV-2 and AlHV-1 have homologous genes flanking the site of *Ov2.5* insertion and it will be of great interest to study the same genomic region in other closely related Macaviruses.

Ov2.5* intron-exon boundaries correspond with those of ovine *IL-10. All sequenced cellular *IL-10* genes including human and mouse had, like *Ov2.5*, five exons and four introns that varied from approximately 300 to 1000 bp in length. The sequence of the sheep *IL-10* gene had not been determined and it was formally possible that the sheep IL-10 had a different gene structure or smaller introns than other mammalian counterparts. Thus, to compare directly *Ov2.5* with host, the ovine *IL-10* was cloned and sequenced from sheep genomic DNA (accession AJ748588). The positions of the intron-exon boundaries in *Ov2.5* corresponded exactly with those of the ovine (and human) *IL-10* (coding exons of 165/168, 60, 153, 66 and 93nt for human/sheep compared with 184, 60, 153, 66 and 81nt for *Ov2.5*). Nucleotide sequence identity between sheep *IL-10* and *Ov2.5* was 45-55% in the exons. However, it was not possible to clearly align the highly truncated introns of *Ov2.5* (80-92bp) with those of the sheep gene (297-1092 bp).

Attempted alignments with the extremities of the host introns showed some conservation of the 10-20 bases closest to the exons (>50% identity).

Absence of introns does not influence $_{OvHV}$IL-10 expression *in vitro*. The gene sequences of IL-10 homologues in other gammaherpesvirus are intron-less (e.g. Epstein-Barr virus BCRF1, Equine herpesvirus 2 E7). To determine if the presence of introns had an influence on expression of *Ov2.5 per se*, pVR1255 containing an HA-tagged *Ov2.5* cDNA was transfected in parallel with the construct containing the genomic form of the coding sequence into HEK 293T cells. The results (Fig. 2) showed that there was little difference in the intensity of the bands obtained after transfection of the intron-less *Ov2.5* cDNA as compared with the intron-containing gene. This was repeated on two separate occasions with similar results. This suggests that there was no advantage or disadvantage of the gene containing introns for the level of protein expression, at least in tissue culture systems.

Sequence divergence of *Ov2.5* in UK isolates. To examine the functional importance of the *Ov2.5* gene in OvHV-2, conservation of the *Ov2.5* sequence was analysed in 7 MCF case samples from 5 geographical locations in the UK. Six distinct *Ov2.5* sequences were obtained, none of which were identical to the BJ1035-derived sequence. Two sequences (5, 6) that were identical came from the same geographical location, while a further two samples from a second location (11, 12) differed at only 4 nucleotide positions. In all, 15 positions were polymorphic in the sequences analysed: 5 in the exons and 10 in introns, but only one polymorphism gave rise to a coding change in $_{OvHV}$IL-10. This change (Pro8-Gln) is within the predicted leader peptide and is unlikely to affect the activity of the mature

protein, suggesting that selection is acting on the *Ov2.5* gene to maintain function. These changes are summarised in supplemental data Table 1.

Potential origin and evolution of *Ov2.5*. This is the first example of a cellular gene captured by a virus that has retained the exact, intact cellular gene structure in terms of the number and position of its introns and exons. It is thought that the most likely way in which virus genes are captured from the host is in the form of cDNA copies (Brunovskis & Kung, 1995; Shackelton & Holmes, 2004). While it is still possible that OvHV-2 captured IL-10 as the cDNA copy of an unspliced nuclear RNA, the fact that *Ov2.5* has retained all cellular intron-exon boundaries suggests that it could also have arisen by direct recombination with cellular DNA in the nucleus. Thus, at least in herpesviruses which replicate in the nucleus, acquisition of host genes could occur via the capture of host genomic DNA. The integration of the EBV genome into multiple sites in human cell lines (Gulley *et al.*, 1992; Henderson *et al.*, 1983) demonstrates that recombination between virus and host genomes can occur and may be responsible for the acquisition of *Ov2.5* by OvHV-2.

The small size of the introns in the *Ov2.5* gene compared to cellular counterparts suggests that there has been successive deletion, probably in response to selection pressure on the virus to minimise its genome size. Thus, *Ov2.5* most likely represents a captured gene at the early stages of evolving into an intron-less open-reading frame (q.v. EBV and EHV-2 IL-10). This concept is supported by the *IL-10* genes of human cytomegaloviruses (CMV) and simian CMVs (e.g. rhesus CMV) that are also spliced, with human and simian CMV *IL-10* retaining two and three exons respectively (Kotenko *et al.*, 2000; Lockridge *et al.*, 2000).

There appeared to be no obvious advantage to retaining introns in terms of levels of $_{OvHV}$IL-10 protein expression (Fig. 2). It is possible, however, that introns contribute to mRNA stability in a specific cellular environment or stage of viral infection. In this regard, it is significant that we have recently identified that the $_{OvHV}$IL-10 gene is expressed during OvHV-2 latency (Thonur *et al.*, 2006) and that it has recently been shown that a version of the human CMV IL-10 was expressed during viral latency (Jenkins *et al.*, 2004). This contrasts with the un-spliced Epstein-Barr virus IL-10 that is only expressed during the productive phase of the viral life cycle (Stewart & Rooney, 1992). Thus, retention of introns may be important for efficient expression during latency.

The *Ov2.5* gene indicates that direct capture of genes as genomic DNA from the host cell could occur in herpesviruses as part of viral evolution. This is an efficient way for DNA viruses that have relatively stable genomes rapidly to evolve novel genes that are of a selective advantage. However, the unique nature of the structure of this gene and the divergence of the coding sequence suggest that rapid change and selection can occur after acquisition resulting in an intron-less open-reading frame.

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Figure Legends

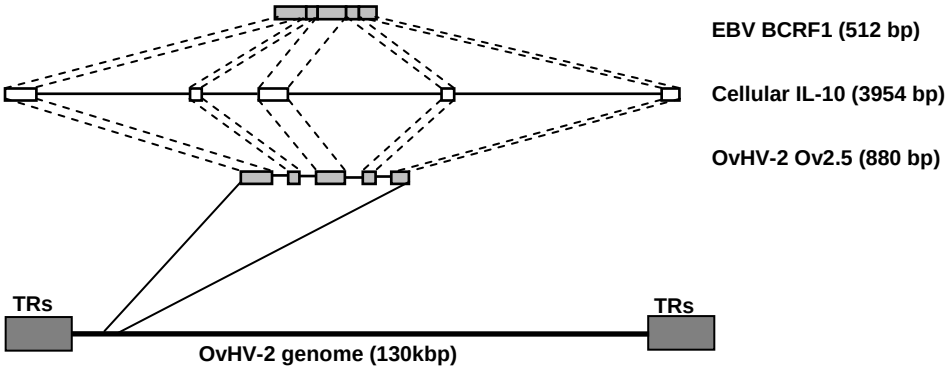
Figure 1. Structure of the OvHV-2 *Ov2.5* gene. Upper section shows the structure of *Ov2.5* and the related cellular and Epstein-Barr virus (EBV) IL-10 genes. Exons are shown as shaded boxes and introns as lines. Dotted lines show the relationship of homologous exons. The lower section shows the position of the *Ov2.5* within the virus genome. The unique portion of the genome is shown as a solid line bounded by terminal repeats shown as shaded boxes.

Figure 2. Expression of $_{OvHV}$IL-10 by transient transfection. Panel A. HA epitope-tagged genes in pVR1255 were transfected into HEK 293T cells. Extracts from pellets and supernatant were analysed by Western blotting using 12.5% SDS-PAGE gels. Proteins on the blots were detected using rat monoclonal anti-HA. Both the intact genomic form of *Ov2.5* and the cDNA were studied along with the cDNA for the *Ov2* gene which encodes a nuclear transcription factor. Panel B. As a control for tunicamycin treatment, BHK-21 cells were infected with MHV-68 and Western blotted as above using rabbit polyclonal anti-MHV-68 gB or pre-immune rabbit serum as primary antibody. Tunicamycin (Tc, 5 μg/ml) was added during transfection or infection as indicated. The sizes of molecular weight standards ($x10^{-3}$) are indicated at the side

Figure 3. Functional analysis of $_{OvHV}$IL-10. Panel A. $_{OvHV}$IL-10-FLAG stimulates mast cell proliferation in conjunction with IL-4. $_{OvHV}$IL-10-FLAG was used at 20ng/ml, 8ng/ml and 2ng/ml (20, 8, 2 in parentheses) along with 4 units/ml murine IL-4 (IL-4) in the D36 murine mast cell proliferation assay. $_{ov}$IL-10-FLAG was used as a control at 20 ng/ml, 10 ng/ml and 1 ng/ml. Anti-IL-10 receptor antibody (anti-IL10R) was used at 100ng/ml as specificity control.

Panel B. $_{OvHV}$IL-10 suppresses IL-8 production from LPS-stimulated macrophages. $_{OvHV}$IL-10-FLAG and $_{ov}$IL-10 were used at the concentrations shown along with LPS at 100ng/ml. IL-8 titres were measured by ELISA. * represents P<0.01 compared to LPS control. Anti-FLAG was used as a specificity control. Error bars represent ± s.e.m.

Figure 4. Conservation of structural motifs. CLUSTAL-W Alignment of the mature polypeptide sequences of IL-10 homologues from man, sheep, horse, mouse, Epstein-Barr virus (EBV), Equine herpesvirus 2 (EHV-2), and Ovine herpesvirus 2 (OvHV-2). Residues identical in all sequences are highlighted in black with white text. The positions of structurally relevant cysteines are marked C and the positions of the main helices (A-F) in the human IL-10 structure are shown above the alignment (Josephson *et al.*, 2001). Residues predicted to contact the IL-10R1 are marked * (Jones *et al.*, 2002; Josephson *et al.*, 2001). Residues involved in stabilising the structural core of the protein are marked $ and IL-10 dimerisation contacts from the human IL-10 structure are marked as +. The boxed regions (helix A, loop AB and helix F) form the receptor binding region. The isoleucine at position 87, potentially involved in immunostimulatory activity of IL-10, is marked # (Haig *et al.*, 2002).


EBV BCRF1 (512 bp)
Cellular IL-10 (3954 bp)
OvHV-2 Ov2.5 (880 bp)
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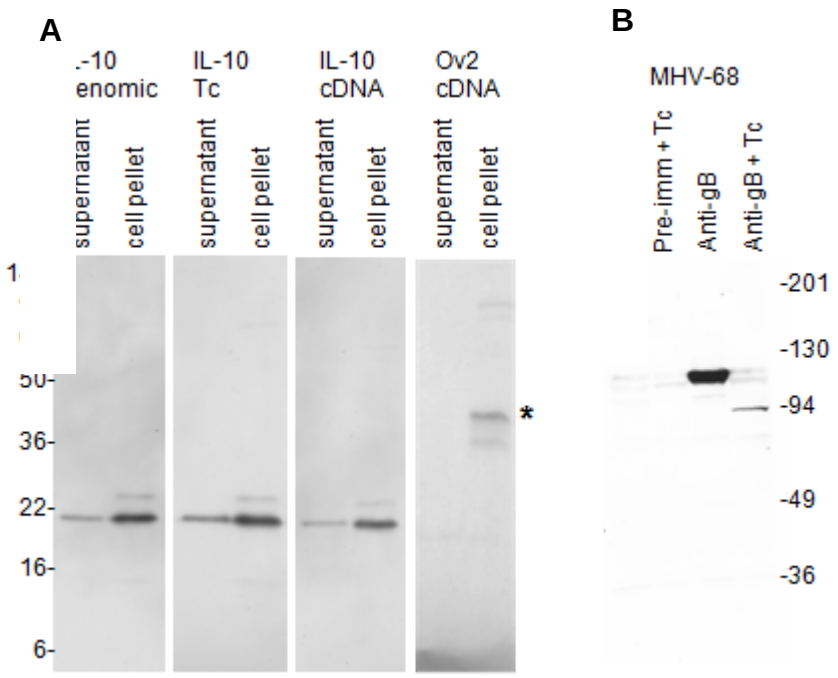
TRs
OvHV-2 genome (130kbp)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Pre-imm + Tc
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Anti-gB + Tc
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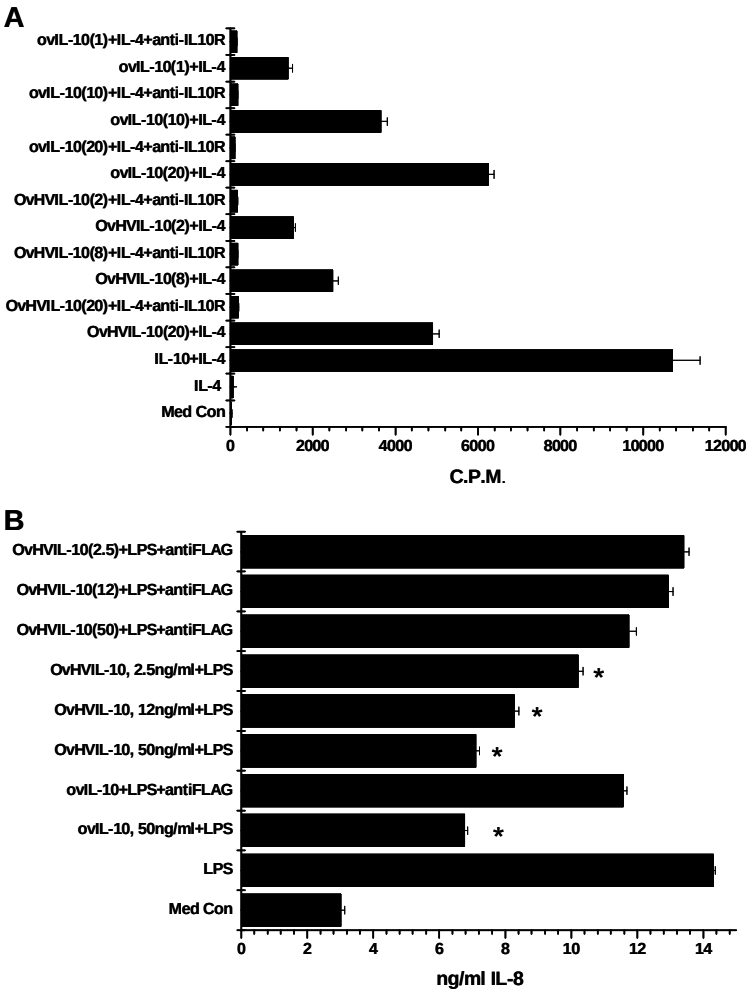
A
ovIL-10(1)+IL-4+anti-IL10R
ovIL-10(1)+IL-4
ovIL-10(10)+IL-4+anti-IL10R
ovIL-10(10)+IL-4
ovIL-10(20)+IL-4+anti-IL10R
ovIL-10(20)+IL-4
OvHVIL-10(2)+IL-4+anti-IL10R
OvHVIL-10(2)+IL-4
OvHVIL-10(8)+IL-4+anti-IL10R
OvHVIL-10(8)+IL-4
OvHVIL-10(20)+IL-4+anti-IL10R
OvHVIL-10(20)+IL-4
IL-10+IL-4
IL-4
Med Con
0
2000
4000
6000
8000
10000
12000
C.P.M.
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OvHVIL-10(2.5)+LPS+antiFLAG
OvHVIL-10(12)+LPS+antiFLAG
OvHVIL-10(50)+LPS+antiFLAG
OvHVIL-10, 2.5ng/ml+LPS
OvHVIL-10, 12ng/ml+LPS
OvHVIL-10, 50ng/ml+LPS
ovIL-10+LPS+antiFLAG
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2
4
6
8
10
12
14
ng/ml IL-8

Figure 4