A captured viral interleukin 10 gene with cellular exon structure

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We have characterized a novel, captured and fully functional viral interleukin (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 found universally within herpesvirus subfamilies. This is in contrast to the majority of herpesvirus genes, which are ancestrally related within either the herpesvirus family or subfamily (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 antiviral 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 studies have been facilitated by the availability of the crystal structures 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 subclinically in their natural hosts. Alcelaphine herpesvirus 1 (AHV-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 AHV-1 has been isolated and completely sequenced (Ensser et al., 1997), the study of OvHV-2 has lagged behind due to 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).

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 mass 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 was generated using a standard protocol (Stewart et al., 1996). The complete Ov2.5 gene and cDNA were amplified separately, incorporating coding sequences for triple HA-tagged sequence (5’-CTTGACCCCAAAGTAGCTTTCC-3’) and FLAG epitope tags at the 3’ ends, at an annealing temperature of 55 °C using cloned PitI DNA polymerase (Stratagene) and primers as follows: HA-tagged sequence 5’-ATGCCGCCGCGGCCACCATGCGATTTCGCCGACCAACTAGC-3’ (forward primer 1) and 5’-GCGATCTTTAAGCGATTTGCTGGGAACCGTGATGGCTATGGGTAAGCCGACTGTGGCAATTCGATGCTTTGCACCAATACTAG-3’ (reverse primer); FLAG-tagged sequence, forward primer 1 and 5’-GCGATCTTTAAGCGATTTGCTGGGAACCGTGATGGCTATGGGTAAGCCGACTGTGGCAATTCGATGCTTTGCACCAATACTAG-3’ (reverse primer); where the Ov2.5-specific sequences are underlined. PCR products were purified with a PCR purification kit (Qiagen), digested with NotI and BglII (Stratagene) and inserted into the NotI and BamHI sites of pKS (Stratagene). The sequence was verified by sequencing performed by MWG Biotech. Sequenced clones were then subcloned into the mammalian expression vector pVR1255 (a kind gift from 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 h post-infection.

The OvIL-10-FLAG was prepared with FLAG at the C terminus of the polypeptide in the pAPEX-3 vector. Expressed OvIL-10-FLAG was 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⁻¹ in the culture medium.

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

For N-terminal sequencing, the same fraction was run on a 4–12 % NuPage Novex Bistris gel (Invitrogen) under reducing conditions using a MES buffer system according to the manufacturer’s instructions. Separated protein was then transferred to Immobilon PVDF membrane using the Novex wet transfer system according to the manufacturer’s instructions (Invitrogen). The blots were stained with 0.2 % amido black in 40 % methanol for 1 min and, after destaining in distilled water, the 19 kDa band was sequenced by the Proteomics unit at the Moredun Research Institute. N-terminal amino acid analysis was performed by Edman degradation chemistry using a PE Prociase 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 (10⁶ 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 anti-HA-biotin, High Affinity (3F10) ml⁻¹ (Roche) followed by 2 µg horseradish peroxidase streptavidin ml⁻¹ (Vector Laboratories). The blot was then developed using the ECL Western blotting analysis system (Amersham Biosciences) and the light emission was detected by 15 s exposure to Hyperfilm ECL (Amersham Pharmacia Biotech).

Cloning the sheep IL-10 gene. The ovine IL-10 gene was amplified from high molecular mass genomic DNA prepared from sheep peripheral blood mononuclear cells using the QIAamp DNA system (Qiagen). A 4 kb fragment carrying the IL-10 gene was amplified from high molecular mass genomic DNA (Amersham Pharmacia Biotech).

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 sample using primers Ov2.5start
(5’-ATGGCATGGCCCAACCACTAC-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 and ovIL-10 proteins 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 5 U murine IL-3 ml⁻¹ and 1 U murine IL-4 ml⁻¹ (BD Pharmingen). FLAG-tagged proteins and murine IL-10 (control) were added to 2 × 10⁵ D-36 cells in 100 µl Iscove’s modified Dulbecco’s medium (IMDM) in triplicate wells of 96-well plates along with 2 U murine IL-4 ml⁻¹ as co-stimulus. Cells were cultured for 48 h, the final 14 h with 18.5 kBq [³H]thymidine per well. [³H]Thymidine incorporation (c.p.m.) was measured using a β-scintillation counter (Packard Tricarb, Packard). IL-10 specificity was tested prior to the addition of the IL-10 reagents, with a murine IL-10 receptor-specific neutralizing antibody (clone 1B1.3a; BD Pharmingen) at 5 ng ml⁻¹ in cultures of D36 cells.

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, 10⁵ cells per well in 200 µl IMDM) 1–2 h prior to stimulation of the macrophages with 10 ng LPS ml⁻¹ (from Salmonella enterica serotype minnesota; Sigma). One day later, cell-free supernates were assayed for ovine IL-8 by specific ELISA (Haig et al., 1996, 2002). IL-10-FLAG specificity was tested prior to adding the IL-10-anti-IL-10 complexes to the assay using 5 µg anti-FLAG ml⁻¹ (murine monoclonal antibody M2; Sigma) to neutralize the IL-10s for 1 h at room temperature.

**Phylogenetic analysis.** Multiple alignments were carried out on protein sequences using six different programs (MAFFT (Katoh et al., 2005), MUSCLE (Edgar, 2004), T-coffee (Notredame & Suhre, 2004), CLUSTAL W (Thompson et al., 1994), hmmpalign (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 DNA sequencing of the complete OvHV-2 genome (Hart et al., 2007), analysis of the non-coding regions between open reading frames (ORFs) 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 and four introns of 82–92 bp and to encode a protein of 183 amino acid (aa) residues with a predicted Mᵣ of 21 000. Analysis of the putative protein using the SignalP 3.0 algorithm (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 Mᵣ 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. Epstein–Barr virus (EBV) BCRF1 91% aa identity with human IL-10, equine herpesvirus (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 genome (GenBank accession number AY839756). A diagrammatic representation of the gene structure is shown in Supplementary Fig. S1, available with JGV Online. Thus, the Ov2.5 product is a new member of the IL-10 family and we have named it OvHV-IL-10.

**Characterization of the OvHVIL-10 protein**

The OvHV-IL-10 was characterized by expressing recombinant protein that was tagged at the C terminus with three copies of an epitope from influenza virus haemagglutinin (3 × HA). 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...
Expression of OvHVIL-10 by transient transfection. (a) HA epitope-tagged genes in pVR1255 were transfected into HEK 293T cells. Extracts from pellets and supernatant were separated using 12.5% SDS-PAGE gels and analysed by Western blot. 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. *, Position of the OvHV-2 Ov2 protein. (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⁻¹) was added during transfection or infection as indicated. Molecular mass standards are indicated (kDa).

proteins with apparent Mᵣ of 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 3 × HA tag (3 kDa), with those predicted from sequence analysis for the nascent and mature forms of OvHVIL-10. To account 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 an Mᵣ of 40 000 on SDS-PAGE gels and a nuclear/cytosplastic distribution. An Ov2-HA product was contained only in the cell pellet. Thus the OvHVIL-10 seen in the supernatant fraction is secreted from cells and not derived from cell lysis.

Analysis of the OvHVIL-10 amino acid sequence using the algorithm NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) revealed one potential N-linked glycosylation site in OvHVIL-10. To determine whether OvHVIL-10 was N-glycosylated, cells were transfected with pVR1255-Ov2.5HA and then treated with tunicamycin B2 immediately after transfection. The results (Fig. 2a) showed that the Mᵣ of OvHVIL-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) was determined by Western blot analysis. Fig. 2(b) 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), OvHVIL-10 was not N-glycosylated.

Although bioinformatic analysis 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 OvHVIL-10 was purified from the supernatant of transfected cells and N-terminal sequence analysis was performed. This generated a good sequence for 15 residues, RVLPLRGNCCKLLQD, corresponding to residues 27–41 of OvHVIL-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 emphasizes the importance of experimental confirmation of signal sequences.

OvHVIL-10 retains IL-10 functions

To assess the biological activities of OvHVIL-10, FLAG epitope-tagged recombinant protein was produced in transfected CHO cells. Recombinant FLAG-tagged ovine IL-10 (OᵣIL-10) was produced in parallel and used as a positive control. FLAG-tagged OvHVIL-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 OvHVIL-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 OᵣIL-10. Mast cell proliferation was IL-10-specific, as it was almost completely inhibited by anti-IL-10 receptor treatment of the cells. Likewise, OvHVIL-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 neutralized the FLAG-IL-10. Thus, in spite of the divergence of its sequence, OvHVIL-10 is fully functional and exhibited typical IL-10 activity in both immunostimulatory and immunosuppressive assays.

OvHVIL-10 retains IL-10 functional motifs

The relative peptide sequence divergence of OvHVIL-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 OvHVIL-10 with that of other IL-10s. Amino acid residues involved in IL-10-binding to the human IL-10 (hIL-10) receptor are shown, as well as those thought to be important in stabilizing 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-10. All four putative IL-10 polypeptide-stabilizing residues are also conserved between OvHV-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-10. This would support the concept that Ile-87 alone is not sufficient to determine IL-10 immunostimulatory activity. Of the highly conserved residues Arg-27, Lys-34, Gln-38, Ser-141, Asp-144 and Glu-151 that form the IL-10R1-binding site (Josephson et al., 2002), only Ser-141 is not conserved in OvHV-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 stabilize 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-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 gammaherpesviruses (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 from the N terminus to position 38 in human protein and all gapped positions were removed. The resulting alignment was 138 aa 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 gammaherpesviruses (EHV-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 (Supplementary 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 a member of the Macavirus lineage of gammaherpesviruses (Ehlers et al., 2008; McGeoch et al., 2006). The 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 has 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 theoretically 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. 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 93 nucleotides (nt) for human/sheep compared with 184, 60, 153, 66 and 81 nt 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 align the highly truncated introns of Ov2.5 (80–92 bp) clearly 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 gammaherpesviruses are intronless (e.g. EBV BCRF1 and EHV-2 E7). To determine whether the presence of introns had an influence on expression of Ov2.5, pVIR1255 containing HA-tagged Ov2.5 cDNA was transfected into HEK 293T cells in parallel with the construct containing the genomic form of the coding sequence. The results showed that there was little difference in the intensity of the bands obtained after transfection of the intron-less Ov2.5 cDNA compared with the intron-containing gene (Fig. 2). 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 seven MCF case samples from five geographical locations in the UK. Six distinct Ov2.5 sequences were obtained, none of which was 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 four nucleotide positions. In all, 15 positions were polymorphic in the sequences analysed: there were 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 summarized in Supplementary Table S1.

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 minimize its genome size. Thus, Ov2.5 most likely represents a captured gene at the early stages of evolving into an intronless ORF (c.f. 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) which 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 to evolve novel genes rapidly 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 intronless ORF.

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