Isolation and expression of three open reading frames from ovine herpesvirus-2

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Ovine herpesvirus-2 (OvHV-2), a member of the gammaherpesviruses (genus Rhadinovirus), asymptomatically infects its natural host, the sheep, but causes malignant catarrhal fever (MCF) in susceptible hosts, such as cattle, deer and pigs. A permissive cell culture system for virus replication has not been identified but viral DNA is present within lymphoblastoid cell lines (LCLs) established from cases of MCF. During this study, a cDNA expression library generated from LCLs was screened with sheep sera and two cDNAs were isolated. One cDNA contained two open reading frames (ORFs) that show similarity to ORFs 58 and 59 of alcelaphine herpesvirus-1 (AlHV-1), a closely related gammaherpesvirus that also causes MCF. Both ORFs 58 and 59 are conserved throughout the gammaherpesviruses. ORF 58 is predicted to be a membrane protein, while ORF 59 has been shown to be an early lytic gene that functions as a DNA polymerase processivity factor. The second cDNA clone contained a partial ORF showing limited similarity to AlHV-1 ORF 73, a homologue of the latency-associated nuclear antigen of human herpesvirus-8, which is associated with latent infections. The full-length OvHV-2 ORF 73 was cloned subsequently by PCR. The ORFs isolated from the library were cloned into a bacterial expression vector and the recombinant proteins tested for their reactivity to sera from OvHV-2-infected animals. An ORF 59 fusion protein was recognized specifically by sera from OvHV-2-infected cattle and will be used to develop a sero-diagnostic test.

Introduction

Within the sub-family Gammaherpesvirinae are a number of viruses that can cause malignant catarrhal fever (MCF), a lymphoproliferative disease of cattle, deer and bison that is usually fatal, although recovery and persistent infections have also been described (Collery & Foley, 1996; Heuschele et al., 1985; Michel & Aspeling, 1994; Milne & Reid, 1990; O’Toole et al., 1997; Plowright, 1990). Members of this group of viruses include alcelaphine herpesvirus-1 (AlHV-1) (Plowright et al., 1960), ovine herpesvirus-2 (OvHV-2) (Bridgen & Reid, 1991; Reid et al., 1989a), AlHV-2 (Reid & Rowe, 1973), hippotragine herpesvirus-1 (Reid & Bridgen, 1991) and a gammaherpesvirus identified recently in white-tailed deer (Li et al., 2000). A PCR product representing a novel gammaherpesvirus of goats, designated caprine herpesvirus-2 (CpHV-2), shows sequence similarity to the viruses of this sub-family; however, CpHV-2 has not been associated with MCF yet (Li et al., 2001).

AlHV-1, the most extensively studied MCF virus, is prevalent in sub-Saharan Africa, where it causes MCF in cattle following contact with apparently healthy wildebeest (Connochaetes species), the reservoir hosts for this disease (Mettam, 1923). AlHV-1 may be propagated in vitro and the complete sequence of the genome has been determined (Ensser et al., 1997), confirming AlHV-1 as a member of the sub-family Gammaherpesvirinae, genus Rhadinovirus. The genome consists of 70 open reading frames (ORFs), including ten genes that are unique to AlHV-1. It is these virus-specific genes that are expected to contribute to the particular pathology of MCF (Coulter et al., 2001).

OvHV-2 occurs worldwide, where it infects domestic sheep asymptomatically and causes sheep-associated MCF (SA-MCF) in large ruminants, such as cattle (Bridgen & Reid, 1991), deer (Denholm & Westbury, 1982; Reid et al., 1979, 1987), bison (Collins et al., 2000; Schultheiss et al., 1998), water buffalo and Banteng cattle (Ramachandran et al., 1982). Interestingly, OvHV-2 has also been shown to cause MCF in

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pigs (Loken et al., 1998). SA-MCF normally occurs sporadically, although epizootics, representing substantial economic losses, have been described previously (Collery & Foley, 1996; Pierson et al., 1973). Attempts to develop a permissive culture system for OvHV-2 have failed; however, lymphoblastoid cell lines (LCLs) have been established from clinically affected animals (Reid et al., 1983, 1989a) and have been found to contain OvHV-2 DNA (Bridgen & Reid, 1991). It is assumed that the virus persists in these cell lines as a latent infection. A fragment of viral DNA isolated from OvHV-2-infected LCLs has been used to develop a PCR test specific for the detection of OvHV-2 (Baxter et al., 1993) and has been employed worldwide for the diagnosis of MCF (Baxter et al., 1997; Ellis & Masters, 1997; Li et al., 1995; Loken et al., 1998; Mirangi & Kangee, 1997; Muller-Dobbies et al., 1998; Wiyono et al., 1994).

The aim of this work was to investigate the expression of OvHV-2 antigens in LCLs derived from bovine cases of SA-MCF. A cDNA expression library was constructed using mRNA isolated from LCLs and the resulting plaques were screened with pooled sheep sera, as evidence suggests that all normal sheep mount a strong immune response to OvHV-2 proteins (Herring et al., 1989; Rossiter, 1981). It was anticipated that this approach would identify viral antigens that may be involved in the maintenance of a latent OvHV-2 infection within these cell lines and which may be used for the development of sero-diagnostic tests.

Methods

- **Lymphoblastoid cell lines.** LCLs BJ1044, BJ1004 and BJ1035 were derived from SA-MCF-affected cattle. BJ1001 is an LCL derived from a rabbit infected with AlHV-1, BJ1265 was derived from a rabbit reaching with SA-MCF and BJ1178 was derived from normal bovine peripheral blood lymphocytes cultured with 5 µg/ml Concanavalin A for 48 h (Reid et al., 1989a).

- **Western blotting of LCLs.** Approximately 1 × 10⁶ cells from each LCL were harvested, centrifuged at 2000 g for 10 min and the supernatants removed. For LCLs BJ1178, BJ1044 and BJ1001, the pellets were washed in 10 ml PBS (136 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.47 mM KH₂PO₄). Cell pellets from LCLs BJ1004 and BJ1035 were stored unwashed at −20 °C. All pellets were resuspended in 10 ml sample buffer (50 mM Tris–HCl, pH 6.8, 1% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and proteins were separated by SDS–PAGE and blotted onto a nitrocellulose membrane with 5 µg/ml Concanavalin A for 48 h (Reid et al., 1989a).

- **Cloning into pTrxFus expression vector.** Fragments 59U1 and 59U3 were amplified from clone H at an annealing temperature of 55 °C using the reverse primer 59L (5′-GAGGAAAGAAGTGTCCAGATCTT-TGIGCC 3′), with either forward primer 59U1 (5′-GCTTGGATAC-GTCTAGAGCCTGTGCG 3′) or 59U3 (5′-GGCAACACTA-TCTGACGAGGGAGTT 3′) (see Fig. 3a). Fragment 58U3 was amplified from clone I using forward primer 58U3 (5′-AAGCATATA-TCTAATGCTTGAGGAAAAGT 3′) and reverse primer 58L1 (5′-AATCTGGGATGCTTCAAGCCTGTGCTTATTTT 3′) at an annealing temperature of 50 °C (see Fig. 3b). Fragment 22U3 was amplified from clone II using primers 22U3 (5′-AAGGCCCCGCTCAGGGGCTC-TCCAGTA 3′) and 22L (5′-AGCATACCAGCCTGAAAATTTA-TGGTGGAAAG 3′) at an annealing temperature of 53 °C. All amplifications were carried out using BioTag DNA polymerase (Bio-line) with the recommended buffers. PCR fragments were digested with the appropriate restriction enzymes, the positions of which are indicated by underlining, and cloned into the pTrxFus expression vector. The construct used to provide the control fusion protein was supplied by Colin Melnes (Moredun Research Institute, Midlothian, UK) and contains the OvIFNR gene of orf virus cloned into the pTrxFus expression vector (Haig et al., 1998). All pTrxFus constructs were expressed in GL724 cells (Invitrogen) and were found to be insoluble. Fusion proteins 59U3, 58U3 and OvIFNR were solubilized in one-tenth of the original culture volume of 7 M urea in 50 mM Tris, pH 8.5, and 2 mM EDTA, and purified using Thiobond resin (Invitrogen), as described in the manufacturer's instructions. Thiobond resin purification was not successful for fusion proteins 59U1 and 22U3. Lysates of these fusion proteins were washed in one-tenth of the original culture volume of 3 M urea and 1% Triton-X-100 to remove non-specific contaminants.

- **Immunoscreening.** The cDNA expression library was immuno-screened, as described by Stratagene, using a 1/400 dilution of pooled sheep serum. Bound antibody was detected using a 1/10000 dilution of donkey anti-sheep IgG labelled with alkaline phosphatase, followed by colour development. Positive plaques were picked, purified by repeating the screening procedure and excised from the phage vector in the form of the pBK-CMV phagemid vector using the in vivo excision method described by Stratagene.

- **Southern blot analysis.** AlHV-1 DNA was isolated from a virion preparation of the WC1 isolate of AlHV-1, as described previously (Bridgen et al., 1989). The AlHV-1 DNA, digested with Smal, and calf thymus DNA (Roche), cut with EcoRI, were loaded onto a 0.8% agarose gel and the restriction fragments were separated by electrophoresis. The gel was soaked in denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 30 min and neutralizing solution (1 M ammonium acetate and 0.02 M NaOH) for 30 min and was blotted overnight onto a nylon Hybond-N* membrane (Amersham). Membranes were prehybridized in standard buffer containing 50% formamide (DIG-system) (Roche) for 1–2 h at 37 °C. Probes were labelled with digoxigenin (DIG), as described by Roche, and incubated with the membranes overnight at 37 °C in the above hybridization buffer. Membranes were washed twice for 5 min in 2× SSC containing 0.1% SDS at room temperature and then in 0.1× SSC containing 0.1% SDS at 45 °C. DIG-labelled DNA was detected using the chemiluminescent substrate CSPD, as described by the manufacturer (Roche).

- **Sequencing.** Plasmids were prepared for sequencing using the Qiagen Plasmid Purification kit. Sequencing was carried out using an ABI automated sequencer at MRI and also by Ian Bennett [Royal (Dick) School of Veterinary Studies, Edinburgh, UK]. Sequence analysis and database searches were performed using the software packages of the University of Wisconsin Genetics Computer Group and DNAStar.

**Construction of the cDNA expression library.** Total RNA was prepared from three bovine OvHV-2-infected LCLs (BJ1044, BJ1004 and BJ1035). Equal amounts of RNA from each cell line were pooled and poly(A)+ RNA prepared using a Poly(A)+ Quick mRNA Isolation kit (Stratagene). The poly(A)+ RNA (5 µg) was used to synthesize double-stranded cDNA, which was then ligated into the λZAP Express vector (Stratagene), as described in the manufacturer's instructions.

**Blot was then placed in detection buffer (100 mM Tris–HCl, pH 9.5, and 100 mM NaCl) containing 225 µg/ml NBT (Roche) and 87.5 µg/ml BCIP (Roche).**
many of the bacterial proteins and were then solubilized in 7 M urea, providing preparations enriched for the fusion proteins.

**Western blotting of fusion proteins.** Samples from an enriched preparation of fusion protein 59U1 and from ThioBond resin purified fusion proteins 59U3 and OVFNR were mixed with sample buffer and equivalent amounts loaded onto 15% SDS–PAGE gels. The proteins were electrophoresed and blotted onto a nitrocellulose membrane. The membrane was then cut into nine sections (A–I), each containing one lane each of the fusion proteins. Each filter was incubated with a different primary antibody: filter A in pooled sheep serum (1/100 dilution), filter B in normal bovine serum (1/100 dilution) and filter C in anti-thioredoxin antibody (1/5000 dilution). Filters D–I were each incubated in a 1/100 dilution of sera from suspected bovine cases of SA-MCF as follows: filter D in BJ1196; E in BJ1865; F in BJ1868; G in B3881; H in B4153; and I in B4551. After washing in TBS-T, filters A, B and D–I were incubated in a 1/100,000 dilution of donkey anti-sheep IgG labelled with alkaline phosphatase (Sigma), while filter C was incubated in a 1/10,000 dilution of rabbit anti-mouse IgG labelled with alkaline phosphatase (Dako). The filters were washed in TBS-T and placed in detection buffer containing 225 µg/ml NBT (Roche) and 87.5 µg/ml BCIP (Roche).

**PCR of the complete OvHV-2 ORF 73.** Genomic DNA was isolated from an OvHV-2-infected rabbit LCL (BJ1265) using a Nucleon kit (Scotlab) and used as the template for amplification of ORF 73 using forward primers 75U1 (5′ ATGGGGTTAGTTGAAAGGAAATTACATT 3′) and 75U5 (5′ GCTACAATATGTCTTCTAGCAAGTTG 3′) and reverse primers 22L3 (5′ CCGCTAGAAAGGCTTCCTCATAAGGCTTT 3′), 22L4 (5′ GGGCTCTATTATGTTGGTCCCAATA 3′), 22L5 (5′ GTTGAAGGATGAGGCGAGTG 3′), 22L6 (5′ TATTCAGAAGCACACAGTATCTATTACCTCA 3′) and 75U4 (5′ CCAGCTGTAAACCTGAACCTGATTGA- NBT/BCIP. Sizes of markers (on the left) and antigens (on the right) are indicated in kDa. The resulting PCR products were cloned into either the pCR4 Blunt-TOPO vector or the pCR4-TOPO vector (Invitrogen).

**Results**

**Western blotting of LCLs**

Prior to the construction of the cDNA expression library, OvHV-2-infected LCLs were examined for expression of viral antigens by Western blotting (Fig. 1). Proteins prepared from OvHV-2-infected bovine LCLs BJ1044, BJ1004 and BJ1035, an AlHV-1-infected rabbit LCL BJ1001 and uninfected bovine cells BJ1178 were separated by SDS–PAGE, blotted and probed with sera pooled from several domestic sheep. The sheep sera recognized a major antigen of approximately 83 kDa in all the infected bovine cell lines. Two additional antigens, with molecular masses of 75 and 60 kDa, were also detected in the BJ1004 and BJ1035 bovine LCL preparations. These lower molecular mass proteins may represent distinct antigens or may be breakdown products of the 83 kDa antigen, resulting from a freeze-thaw cycle that these cells had undergone prior to the addition of sample buffer. The absence of these antigens in the AlHV-1-infected control cells and in the control bovine lymphocytes indicates that the OvHV-2-infected bovine LCLs express virus-specific antigens that can be detected by pooled sheep serum.

**Isolation of viral antigens from the cDNA expression library**

A cDNA expression library was constructed using mRNA isolated from OvHV-2-infected LCLs of bovine origin (BJ1044, BJ1004 and BJ1035). Over 6 × 10⁸ plaques from the library were screened with pooled sheep serum and approximately 100 immuno-positive plaques were isolated. The inserts from these positive clones were excised in the form of the pBK-CMV phagemid vector, labelled with DIG and hybridized against each other on Southern blots (results not shown). This analysis revealed that although many of the clones were identical to each other, 21 clones were distinct. The DIG-labelled inserts of the 21 clones were used to probe AlHV-1 and bovine DNA on Southern blots (Fig. 2). One clone,
Fig. 3. Alignment of the amino acid sequences of (a) ORF 59 and (b) ORF 58 from OvHV-2 and AlHV-1. Sequences were aligned using the CLUSTAL method (MEGALIGN; DNASTAR). Identical residues are shaded in dark grey and similar residues are shaded in light grey. Lines represent gaps in the sequence. The boxed residues in (a) indicate the location of a putative nuclear localization signal. The locations of PCR primers are indicated with arrows above the amino acid sequence.

designated clone H, hybridized to an AlHV-1 DNA fragment of approximately 8 kb under high stringency conditions, with no hybridization to calf thymus DNA, indicating that it is of viral origin. A second clone, clone I, also hybridized to AlHV-1 DNA under high stringency conditions, particularly to fragments of approximately 6.5 and 3.8 kb, although the probe also hybridized to DNA throughout the track and resulted in a smeared signal. A smeared signal was also observed throughout the calf thymus track with this probe under the same conditions. Thus, the origin of this clone was ambiguous. The remaining 19 clone inserts either hybridized only to bovine DNA or did not bind at all.

Phage representing the 21 distinct clones were examined for their reactivity to sera from clinical cases of SA-MCF and to bovine convalescent sera. Only plaques formed by clones H and I were recognized by these sera (results not shown). Thus, clones H and I were considered to be putative viral antigens and investigated further.

**Sequence analysis of isolated cDNA clones**

Sequence analysis of the two immuno-positive cDNA clones, H and I, revealed significant similarity to the AlHV-1 genome. Clone H is 2311 bp in length and contains two putative ORFs that show strong similarity to ORFs 58 and 59 of AlHV-1. The presence of two ORFs within this single cDNA clone, with only one poly(A)⁺ tail (situated just downstream of ORF 58), suggests that these genes are transcribed as a bicistronic message. ORF 59 is situated at the 5' end of the cDNA clone, in-frame with the β-galactosidase
protein of the vector. Thus, it is assumed that it is the product of this ORF that was recognized by sheep sera during the immunoscreening of the library. OvHV-2 ORF 59 is 1171 bp long and is predicted to encode a protein of 389 amino acids with a calculated molecular mass of 42.6 kDa. OvHV-2 ORF 59 has counterparts in all other gammaherpesviruses sequenced and exhibits the greatest similarity to AlHV-1 ORF 59 (63% amino acid identity). Most of the residues conserved between OvHV-2 and AlHV-1 ORF 59 lie within the first 290 amino acids of the proteins, although there is also a short stretch of conserved residues at the C terminus (Fig. 3a). These conserved residues form a consensus bipartite nuclear localization signal (Dingwall & Laskey, 1991), suggesting that this is a nuclear protein.

The putative start codon of a second ORF, 1056 bp in length, lies only 6 bp after the termination codon of ORF 59 and in the same reading frame. This second ORF is predicted to encode a protein of 351 amino acids with an estimated molecular mass of 40.6 kDa. This predicted protein is identical in length to AlHV-1 ORF 58 and exhibits 60.4% amino acid identity to it (Fig. 3b). OvHV-2 ORF 58 is predicted to encode a protein that is very hydrophobic and is a potential plasma membrane protein (PSORT; Nakai & Kanehisa, 1992).

The second immuno-positive cDNA clone isolated from the cDNA library, clone I, is 1043 nucleotides in length and was found to contain an ORF of 930 bp, open at the 5' end (due to the absence of an initiation codon) with a poly(A)+ tail 98 bp downstream from the predicted termination codon. Database searches revealed that this partial ORF is similar to ORF 73 of AlHV-1 and may therefore represent an OvHV-2 ORF 73 homologue. The greatest similarity occurs in the C-terminal region, with 43.8% sequence identity over 121 amino acids. The N-terminal region of the predicted OvHV-2 protein consists of repeats rich in glycine, glutamic acid and proline residues, followed by a short stretch of glutamic acid residues. There is little sequence identity between the OvHV-2 and AlHV-1 homologues in these regions. A consensus bipartite nuclear localization signal lies at the start of the conserved C-terminal region and the protein is therefore predicted to be a nuclear protein (PSORT; Nakai & Kanehisa, 1992).

Expression and immunoreactivity of OvHV-2 fusion proteins

The ORF 59 fusion proteins, 59U1 and 59U3, were expressed as a 66 and a 59 kDa protein, respectively. Fusion protein 58U3 consists of the 3' half of ORF 58 and was expressed as a 33.5 kDa protein. The ORF 73 fusion protein, 22U3, and OVIFRN, the orf virus fusion control protein, were expressed as a 22 and a 36 kDa protein, respectively. Fusion proteins 59U3, 58U3 and OVIFRN were purified using ThioBond resin and enriched preparations of fusion proteins 59U1 and 22U3 were prepared.

The OvHV-2 fusion proteins were tested for their ability to react with sera from suspected clinical cases of SA-MCF.

Fig. 4. An enriched preparation of fusion protein 59U1 (lanes 1, 4, 7, 10, 13, 16, 19, 22 and 25) and ThioBond resin-purified preparations of fusion proteins 59U3 (lanes 2, 5, 8, 11, 14, 17, 20, 23 and 26) and OVIFRN (lanes 3, 6, 9, 12, 15, 18, 21, 24 and 27) were separated on 15% SDS–PAGE gels and blotted onto a nitrocellulose membrane. Blot A was incubated in a 1/100 dilution of pooled sheep sera; blot B was incubated in a 1/100 dilution of normal bovine serum; blot C was incubated in a 1/5000 dilution of mouse anti-thioredoxin antibody and blots D–I were each incubated in a 1/100 dilution of serum from a suspected bovine case of SA-MCF as follows: blot D was incubated in serum BJ1196; E in BJ1865; F in BJ1868; G in B3881; H in B4153; and I in B4551. Blots A, B and D–I were each incubated in a 1/10000 dilution of donkey anti-sheep IgG labelled with alkaline phosphatase, while filter C was incubated in a 1/10000 dilution of rabbit anti-mouse–alkaline phosphatase conjugate. The filters were washed and placed in detection buffer containing NBT/BCIP.

Fusion proteins 59U1, 59U3 and OVIFRN were electrophoresed, blotted onto a nitrocellulose membrane and incubated with three control sera: pooled sheep serum, bovine serum from an uninfected animal and an anti-thioredoxin antibody (Fig. 4, blots A–C, respectively). As expected, the anti-thioredoxin antibody detected the 66 kDa 59U1 fusion protein, the 59 kDa 59U3 fusion protein and the 36 kDa OVIFRN protein (Fig. 4, blot C). This antibody also detected an additional protein of approximately 55 kDa in the 59U3 sample (Fig. 4, lane 8), presumably the result of proteolytic digestion of the 59 kDa protein. Several other minor pro-
Fig. 5. (a) The genomic arrangement of OvHV-2 ORFs 73 and 75. ORFs are shown as open boxes with an arrow indicating the direction of transcription. Primer locations are shown by short arrows. (b) A representation of the predicted OvHV-2 ORF 73 protein. Amino acid positions are marked above the protein. G, glycine; E, glutamic acid; P, proline; V, valine.

teolytic digestion products were also detected with this antibody in both the 59U1 and 59U3 samples. The pooled sheep serum recognized both of the ORF 59 fusion proteins (Fig. 4, lanes 1 and 2) and also reacted slightly with the control OvIFNR fusion protein, which was unexpected as OvIFNR is not known to be an antigen (Fig. 4, lane 3). Normal bovine serum did not react with 59U3, OvIFNR or the full-length 59U1 fusion protein. However, a protein of approximately 34 kDa present in the 59U1 preparation was detected by normal bovine serum (Fig. 4, lane 4). This protein may represent a contaminating bacterial protein present within the unpurified 59U1 preparation. These results confirm that the OvHV-2 fusion proteins react with sera from OvHV-2-infected sheep and also show that they are not recognized by serum from a normal bovine. To determine if these proteins are recognized by OvHV-2-infected bovine sera, blots D–I were incubated with six different bovine sera from both infected and uninfected animals. Three of the bovine sera (BJ1196, B3881 and B4153; Fig. 4, blots D, G and H, respectively) reacted with the 59U1 and 59U3 fusion proteins but not with the OvIFNR control. Reaction with serum BJ1196 was weak but reproducible (Fig. 4, blot D). Serum BJ1868 recognized only several low molecular mass proteins present in the 59U1 sample, presumed to be contaminating bacterial proteins (Fig. 4, lane 16). Sera BJ1865 and B4551 (Fig. 4, blots E and I, respectively) did not recognize any of the fusion proteins. These bovine sera had been tested previously using the fluorescence antibody test (FAT) (Reid et al., 1989b), which is currently used to diagnose cases of OvHV-2 infection. When the results of the Western blots were compared with those of the FAT, it was found that sera positive by FAT (BJ1196, B3881 and B4153) also reacted with the ORF 59 fusion proteins on blots, while sera BJ1865, BJ1868 and B4551, which were FAT-negative, did not react with the fusion proteins. The absence of any reaction to the OvIFNR protein with the bovine sera indicates that the observed reactions are not due to the presence of the thioredoxin fusion partner. Thus, these results indicate that the ORF 59 fusion proteins are recognized specifically by sera from OvHV-2-infected cattle and may therefore be suitable for development as a sero-diagnostic test such as an ELISA.

The Western blotting experiment was repeated using the OvHV-2 ORF 58 (58U3) and ORF 73 (22U3) fusion proteins. However, these fusion proteins were recognized by all of the bovine sera used, irrespective of whether they were positive or negative in the FAT test (results not shown). Thus, these antigens are not considered to be suitable for use in the diagnosis of OvHV-2 infection.

Cloning of the complete ORF 73

Attempts to isolate a cDNA clone containing the full-length OvHV-2 ORF 73 by repeat immunoscreening of the cDNA library were not successful. Baxter et al. (1993) had isolated previously a 549 bp genomic clone (Bp4a1) that contains the 3’ end of ORF75. Thus, the complete OvHV-2 ORF 73 was cloned by PCR amplification of the region between the 3’ end of ORF 75 (Bp4a1) and the 3’ end of ORF 73 (clone I) (Fig. 5). The template used for all of the amplifications was genomic DNA isolated from an OvHV-2-infected rabbit cell line (BJ1265). Only one source of OvHV-2 was used as a PCR template, as it has been found that the number and content of repeat sequences within ORF 73 of human herpesvirus-8 (HHV-8) vary from one isolate to another (Gao et al., 1999; Zhang et al., 2000).

The complete OvHV-2 ORF 73 consists of 1488 bp and is predicted to encode a protein of 495 amino acids with an estimated molecular mass of 49 kDa. The ORF 73 protein can be divided into five regions: a short N-terminal region (32 aa), which contains a putative nuclear localization signal; a 244 aa region consisting entirely of glycine, glutamic acid, proline and valine residues; 48 contiguous glutamic acid residues; a 38 aa region of glycine, glutamic acid and proline residues; and a 133 aa C-terminal region that contains a second putative nuclear
localization signal (Fig. 5b). OvHV-2 ORF 73 exhibits most similarity to its AlHV-1 homologue (47.3% nucleotide identity and 38.1% amino acid identity). The two homologues share many features, including the repeat regions and similar C-terminal regions; however, OvHV-2 ORF 73 (495 amino acids) is considerably smaller than its AlHV-1 equivalent (1300 amino acids). Sequence identity to the other gammaherpesvirus homologues is much lower, although there is some similarity among the C-terminal regions of all ORF 73 homologues.

Discussion

During this study, a cDNA expression library constructed from OvHV-2-infected LCLs was immunoscreened with sheep sera and the immunopositive clones then screened for hybridization to AlHV-1 DNA on Southern blots. This approach, while unlikely to identify all viral gene expression within the LCLs, ensured that (a) viral clones were selected for against the high background of bovine gene expression and (b) the viral clones isolated were immunogenic and would therefore be of use in the sero-diagnosis of OvHV-2 infection. Two viral cDNA clones, designated clones H and I, were isolated from OvHV-2-infected LCLs. Clone H contains two ORFs showing similarity to ORFs 58 and 59 of AlHV-1, while clone I contains an incomplete ORF exhibiting limited similarity to AlHV-1 ORF 73. The complete OvHV-2 ORF 73 was cloned subsequently from OvHV-2 genomic DNA using PCR. The relationship between the OvHV-2 antigens isolated from the library and the antigens observed when LCL preparations were immunoblotted with pooled sheep serum (Fig. 1) has still to be determined, as specific antisera are not available yet.

The presence of both ORFs 58 and 59 in cDNA clone H indicates that these genes are transcribed as a bicistronic message. cDNAs containing both ORFs 58 and 59 have also been isolated from cells infected with HHV-8 (Chan et al., 1998; Katano et al., 1999a); however, Chan et al. (1998) found that these cDNAs were only one of seven RNA species transcribed from this locus. Similarly, in Epstein–Barr virus (EBV), human cytomegalovirus and HHV-6, three, four and six differently sized RNA species, respectively, have been detected at this locus (Leach & Mocarski, 1989; Pari et al., 1993; Pitzner et al., 1987; Zhou et al., 1997). Such complex transcription may be important for regulating the expression of these genes.

Little is known about the expression and function of ORF 58, which is conserved throughout the gammaherpesviruses. The EBV ORF 58 homologue, BMRF2, has been shown recently to be highly expressed in the epithelium of hairy leukoplakia lesions (Peñaranda et al., 1997). The protein possesses multiple hydrophobic domains (Modrow et al., 1992; Peñaranda et al., 1997) and contains an arginine–glycine–aspartic acid (RGD) motif, which also occurs in viral proteins involved in cellular attachment and penetration (Cuzange et al., 1994; Liebermann et al., 1991; Roivainen et al., 1991). The RGD motif is not present in the other gammaherpesvirus homologues, including OvHV-2.

OvHV-2 ORF 59 also has homologues in all gammaherpesviruses sequenced. The EBV homologue, BMRF1, and the HHV-8 ORF 59 encode early phosphoproteins that localize to the nucleus and, in EBV, form part of the early antigen diffuse component (Chan et al., 1998; Chan & Chandran, 2000; Cho et al., 1985; Li et al., 1987; Pearson et al., 1983). Both proteins bind dsDNA, function as a processivity factor for the viral DNA polymerase and are absolutely required for lytic virus replication (Chan & Chandran, 2000; Chen et al., 1995; Chio et al., 1985; Fixman et al., 1992, 1995; Kiehl & Dorsky, 1995; Lin et al., 1998; Tsurumi, 1993). BMRF1 also transactivates the oriLyt BHLF1 promoter (Zhang et al., 1996, 1997). The presence of ORF 59, the homologue of an early lytic gene, in the OvHV-2-infected LCLs suggests that a proportion of the cells within the LCLs had entered a lytic cycle of infection. However, as virus particles have never been observed in these cell lines (Hussy et al., 2001; Reid et al., 1989a), it can be concluded that either only a very small population of cells was lytically infected or the lytic infection was aborted prior to virion assembly.

ORF 59 has been found to be a major antigen in both EBV and HHV-8 infections. In EBV, an ELISA using BMRF1 as the antigen has been shown to be specific for patients with nasopharyngeal carcinoma (Nadala et al., 1996). HHV-8 ORF 59 has been isolated from several cDNA libraries following immunoscreening (Chan et al., 1998; Chandran et al., 1998; Katano et al., 1999a) and an ELISA using recombinant HHV-8 ORF 59 has revealed that 32% of patients with AIDS-related Kaposi’s sarcoma are positive for anti-ORF 59 antibodies (Katano et al., 1999a). The results of the immunoblotting experiments carried out in this study show that OvHV-2 ORF 59 recombinant proteins are recognized specifically by sera from OvHV-2-infected cattle. These ORF 59 recombinant proteins will now be used in the development of an OvHV-2-specific ELISA.

ORF 73 has homologues in most, but not all, gammaherpesviruses sequenced and is associated with latent infections. Transcripts containing ORF 73 have been identified in cells latently infected with HHV-8 (Chandran et al., 1998; Dittmer et al., 1998; Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997; Sarid et al., 1999; Talbot et al., 1999), herpesvirus saimiri (Hall et al., 2000) and murine herpesvirus-68 (Virgin et al., 1999). Thus, the expression of ORF 73 in OvHV-2-infected LCLs is consistent with the assumption that OvHV-2 persists within these cell lines as a latent infection. However, it is interesting to note that ORF 73 may not be latency specific, as motifs characteristic of herpesvirus immediate-early genes have been identified within the promoter of HHV-8 ORF 73 (Talbot et al., 1999).

The protein encoded by HHV-8 ORF 73, designated latency associated nuclear antigen (LANA, LNA or LNA-1) (Gao et al., 1996a; Kedes et al., 1997; Kellam et al., 1997;
Rainbow et al., 1997), accumulates in discrete spots in the nuclei of cells latently infected with HHV-8 and also in tumour cells, resulting in a characteristic punctate staining pattern (Dupin et al., 1999; Katano et al., 1999b; Kellato et al., 1997; Parravicini et al., 2000; Rainbow et al., 1997). HHV-8 LANA is associated with several functions. For example, it acts as a transcriptional regulator (Krithivas et al., 2000; Schwam et al., 2000) and can repress the transcriptional activity of p53, the tumour suppressor protein, resulting in a reduction in the apoptosis of HHV-8-infected cells (Friberg et al., 1999). LANA interacts also with the retinoblastoma protein and transforms primary rat fibroblast cells in cooperation with HRAS, indicating that it may be involved in HHV-8-induced onco genesis (Radkov et al., 2000). It has also been proposed that LANA ensures the maintenance of the virus episome during mitosis by tethering the HHV-8 genome to the host chromosome through interactions with host histone H1 and a cis-acting element located at the left end of the HHV-8 genome (Ballestas et al., 1999; Cotter & Robertson, 1999).

The immunogenicity of LANA forms the basis of several successful serological assays, including immunofluorescence assays, Western blotting and ELISAs (Gao et al., 1996a, b; Katano et al., 1999b, 2000; Kedes et al., 1996; Lenette et al., 1996; Olsen et al., 2000; Rainbow et al., 1997; Simpson et al., 1996; Zhu et al., 1999). Results of immunoblotting experiments with the OvHV-2 ORF 73 C-terminal fusion protein were disappointing, as sera from infected animals did not appear to recognize the fusion protein specifically. However, it is possible that a fusion protein of the entire ORF may be of use in immunoassays.

The cloning of OvHV-2 ORFs 58, 59 and 73 is significant, as they represent the first complete OvHV-2 genes to be isolated. ORFs 59 and 73 were expressed in the cDNA library established from OvHV-2-infected LCLs and were recognized by sera from both OvHV-2-infected sheep and cattle. The function of these ORFs is not known; however, they represent important antigens of OvHV-2 infection and may play a role in the pathogenesis of the disease in vivo. It is anticipated that vaccination against these antigens or interference with their mode of action may disrupt latent OvHV-2 infection and may prevent the onset of disease symptoms. In addition, it has been shown that an ORF 59 recombinant protein is recognized specifically by sera from OvHV-2-infected cattle and will be used in the development of a sero-diagnostic test.

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