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Ovine herpesvirus 2 lytic cycle replication and capsid production

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Ovine herpesvirus 2 (OvHV-2) causes malignant catarrhal fever in cattle, pigs and deer. We have observed intact circular and linear OvHV-2 genomes in infected T cell lines derived from cows and rabbits. Bovine T cell lines were predominantly latently infected but rabbit T cell lines supported OvHV-2 productive cycle gene expression and virus capsids were demonstrated for the first time.

Malignant catarrhal fever (MCF) is a severe, usually fatal, lymphoproliferative and inflammatory syndrome of domestic cattle, pigs, deer and certain other susceptible ruminants such as bison. Cases of MCF in cattle usually occur sporadically. However, periodically limited epizootic outbreaks occur (Hamilton, 1990) where losses can be substantial. MCF is also the most important virus disease of farmed deer. The disease is mainly caused by either of two bovid gammaherpesviruses that persist subclinically in their natural host. These viruses are highly related to each other and to other gammaherpesviruses such as Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein–Barr virus (EBV) and murine gammaherpesvirus (MHV-68). Alcelaphine herpesvirus 1 (AlHV-1) naturally infects wildebeest and is the source of wildebeest-associated MCF in Africa (Plowright *et al.*, 1960). Domestic sheep are the reservoir of infection for the other main form of the disease, sheep-associated MCF (SA-MCF). The SA-MCF agent, ovine herpesvirus 2 (OvHV-2), is antigenically closely related to AlHV-1 (Herring *et al.*, 1989; Rossiter, 1981, 1983). Limited sequence analysis of the genome corresponding to part of ORF75 shows that OvHV-2 also shares homology at the DNA level with AlHV-1 and other gammaherpesviruses (Baxter *et al.*, 1993; Bridgen & Reid, 1991).

The detection of DNA in diseased animals and inapparently infected sheep has confirmed that OvHV-2 is the aetiological agent involved in SA-MCF (Baxter *et al.*, 1993; Li *et al.*, 1998; Muller-Doblies *et al.*, 1998; Wiyono *et al.*, 1994). OvHV-2 DNA can be found in epithelial tissues and B cells from sheep (Baxter *et al.*, 1997). However, in SA-MCF-affected ruminants, virus DNA has only been observed in hyperplastic T cells (Baxter *et al.*, 1993; Bridgen & Reid, 1991). These T cells can grow in culture into lines and will transmit disease back to cattle as well as to experimental animals such as rabbits and hamsters (Burrells & Reid, 1991; Buxton *et al.*, 1984, 1988). In turn, T cell lines can subsequently be isolated and passaged from these secondary hosts (Reid *et al.*, 1983).

AlHV-1 has been isolated, completely sequenced and will productively infect epithelial cell lines in culture (Ensser *et al.*, 1997; Plowright *et al.*, 1960). In contrast, research on OvHV-2 has been hampered by the lack of reagents and a consistent failure to isolate the virus and define a productive cell culture system. The aim of this work was to ascertain the nature of OvHV-2 genomes in T cell lines and to define a productive culture system for OvHV-2. Here, we show that infected rabbit T cell lines support aspects of the virus productive cycle.

To identify cells supporting productively replicating OvHV-2, we analysed infected T cell lines by Gardella (*in situ* lysis) gel electrophoresis and Southern blotting exactly as described previously (Gardella *et al.*, 1984; Usherwood *et al.*, 1996). This technique discriminates linear from covalently closed circular (CCC) viral genomes which are characteristic of cells supporting either productive or latent herpesvirus infection respectively. OvHV-2-infected T cell lines were derived from an affected cow (BJ/1035) or affected rabbits (BJ/2222, BJ/2223) as described previously (Buxton *et al.*, 1985). The control cell lines, S11 (Usherwood *et al.*, 1996) and BCP-1 (Boshoff *et al.*, 1998), are latently infected with the related MHV-68 and KSHV respectively. Equal numbers (2×10^6) of cells were loaded onto each lane of the gel. A Southern blot of the gel was probed simultaneously with ^{32}P-labelled DNA fragments derived from the OvHV-2 ORF75, the MHV-68 gp150 gene (Stewart *et al.*, 1996) and the KSHV K13 gene. The results are shown in Fig. 1. S11 (MHV-68-infected) and BCP-1 (KSHV-infected) cells contained both CCC and linear genomes but S11 cells contained a higher proportion of linear relative to circular genomes. Indeed, the amount of linear genomes in BCP-1 cells was on the borderline

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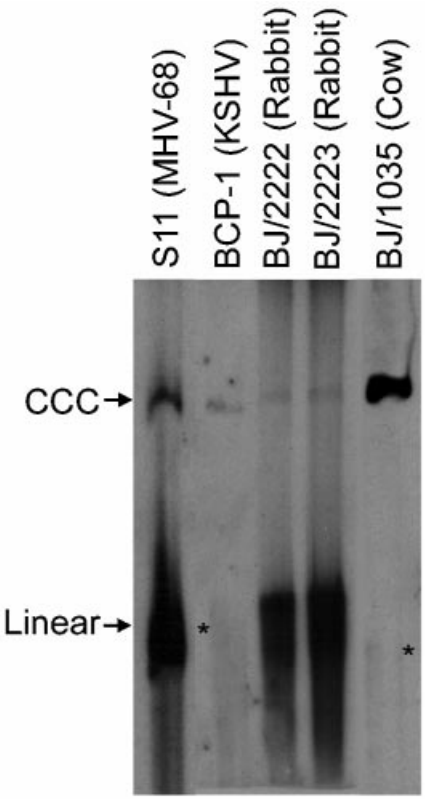

Fig. 1. Gardella gel analysis of lymphoid cell lines. Cells were lysed *in situ* in the agarose gel and then electrophoresed and analysed by Southern blotting. Cell lines used were as indicated above the tracks. The blot was probed simultaneously with ^{32}P-labelled probes specific for MHV-68, KSHV and OvHV-2 followed by autoradiography. The positions of covalently closed circular (CCC) and linear viral DNA are indicated at the left. Asterisks indicate the positions of linear DNA in two cases that were visible on the autoradiograph but did not reproduce photographically.

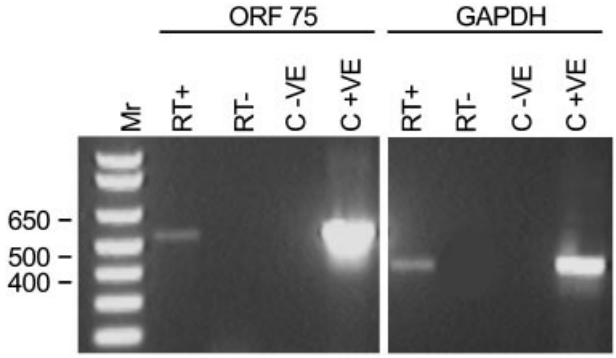

Fig. 2. RT–PCR analysis of OvHV-2-infected rabbit T cell line. RNA was extracted from cells and analysed by RT–PCR using primers specific for GAPDH and ORF75 as indicated above the gel. Products were electrophoresed through 2% agarose gels containing ethidium bromide and visualized using a UV transilluminator. Reactions were performed in the presence (RT+) or absence (RT−) of reverse transcriptase as well as in the absence of template (C-VE). Positive controls (C+VE) of infected cell DNA and mouse cell cDNA were included for the ORF75 and GAPDH primers respectively. Molecular mass determinations were made using a DNA ladder (1 kb plus, Life Technologies) as indicated (Mr). The positions of relevant markers are shown at the left.

of detection (shown by asterisk). This corresponds to a relatively high level of spontaneous reactivation (up to 20% of cells) in S11 (Usherwood *et al.*, 1996), compared with BCP-1 where the level of spontaneous reactivation is only 1–2% of cells (Talbot *et al.*, 1999). An OvHV-2-infected bovine T cell line (BJ/1035) contained predominantly circular genomes and a barely detectable level of linear genomes (shown by asterisk). In contrast, two OvHV-2-infected rabbit T cell lines (BJ/2222 and BJ/2223) contained comparatively large amounts of linear genomes with only a relatively small amount of circular genome apparent. These results strongly suggested that the OvHV-2-infected bovine line that we studied contained predominantly latently infected cells, whereas the two rabbit lines contained latently infected cells but also a relatively large proportion of cells that might be undergoing productive replication.

To determine whether infected rabbit T cells supported OvHV-2 gene expression normally associated with the productive cycle, we performed RT–PCR analysis. Total RNA was extracted from a rabbit T cell line (BJ/2319) or uninfected rabbit lymphoblasts using an RNeasy kit (Qiagen) and then 1 μg was reverse-transcribed using a combination of random hexamer primers (Roche) and Superscript II RT (Life Technologies) as described previously (Roy *et al.*, 2000). RT products were amplified by PCR as described previously (Usherwood *et al.*, 2000), using 40 cycles and primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense 5′ CCTTCATTGACCTCAACTACAT, antisense 5′ CCAAAGTTGTCATGGATGACC) and OvHV-2 ORF75 (sense 5′ GCTTCAGCTTACTCCCTTTAC, antisense 5′ TCAATCAGGTTCAGGTTTACAG). ORF75 in the related EBV and MHV-68 is a late productive cycle gene (Hudson *et al.*, 1985; B. Ebrahimi, personal communication) and is therefore an indicator of productive cycle gene expression. As shown in Fig. 2, a product of the correct size (400 bp) was amplified using GAPDH primers from positive control mouse cDNA and the OvHV-2-infected cell cDNA. No products were seen in control reactions where either RT (RT−) or template (C−) was omitted. Likewise, products 540 bp in length were amplified using ORF75 primers from positive control OvHV-2-infected cell DNA and OvHV-2-infected cell cDNA, but not from negative control samples or uninfected rabbit cells (not shown). Thus, ORF75 was expressed in the OvHV-2-infected rabbit T cell line. This shows that gene expression normally associated with the productive cycle was occurring in the rabbit T cell line. This concurs with the detection of linear genomes in infected rabbit T cell lines.

We surmised that if rabbit T cells contained linear virus DNA and expressed a gene associated with the productive cycle in other gammaherpesviruses then they might produce virus particles. In an initial screen, infected bovine and rabbit T cell lines were examined by transmission electron microscopy. However, after inspection of several hundred cells from

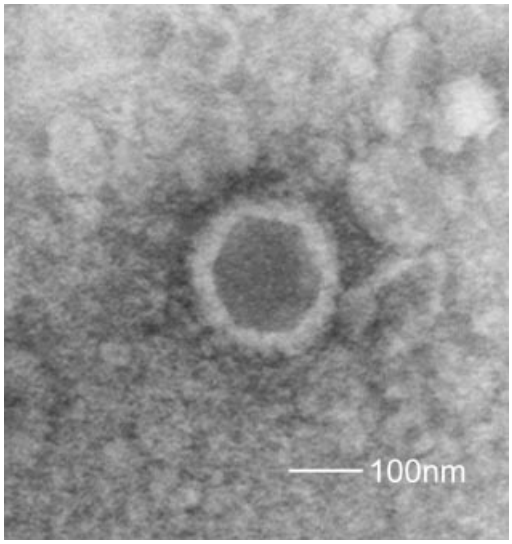

Fig. 3. Transmission electron micrograph of an OvHV-2 particle. Infected rabbit T cells were lysed, the virus pelleted and then visualized by negative staining using transmission electron microscopy.

different preparations of each type of cell line we were unable to detect viral particles. We therefore adopted an alternative approach based upon the concentration of viral particles by pelleting that had proved successful for certain KSHV-infected cell lines. Infected cells were lysed by freeze–thawing and viral pellets were prepared and examined by transmission electron microscopy exactly as described by Arvanitakis *et al*. (1996). Fig. 3 shows that characteristic herpesvirus capsids were observed in extracts from infected rabbit T cells. These appeared to be reminiscent of A-type capsids that do not contain viral DNA and are non-infectious. Thus, infected rabbit T cells not only display markers of the productive cycle but also produce visible capsids. However, the lack of mature capsids and particles argues that the majority of cells are undergoing an abortive productive cycle.

Previous results had implied that OvHV-2-infected T cell lines were capable of producing infectious viral particles because they were capable of causing MCF after experimental transfer into rabbits and hamsters (Burrells & Reid, 1991; Buxton *et al*., 1984, 1988). While the results presented here represent a significant advance in OvHV-2 research in delineating cell lines that support gene expression that is normally associated with the productive cycle, they still fall short of defining an amenable, fully productive culture system. The presence of linear genomes in cells infected by other gammaherpesviruses is directly correlated with the presence of cells undergoing full productive replication. There are clearly significant numbers of linear viral genomes and evidence of productive cycle gene expression in infected rabbit T cells. However, neither gives an indication of the proportion of cells involved and RT–PCR gives no idea of the levels of gene expression. The inability to detect viral particles in intact cells argues for one of two conclusions. Firstly, that only a very small percentage of cells is undergoing productive replication resulting in particles or, alternatively, that there are a larger percentage of cells undergoing a productive cycle that is largely abortive with respect to particle production. These limitations aside, the delineation of cell types maintaining either latency or aspects of the productive cycle will enable the study of these phases of the virus life-cycle in more detail, in particular the delineation of antigens associated with each phase. However, the key to the analysis of this virus is the determination of the complete DNA sequence of the genome. The definition of an *in vitro* culture system supporting productive infection provides the substrate for such a sequencing project.

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