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Macropodid herpesviruses 1 and 2 occupy unexpected molecular phylogenic positions within the *Alphaherpesvirinae*

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The molecular phylogeny of macropodid herpesviruses 1 and 2 (MaHV-1 and -2) has been investigated by cloning and sequencing the genes encoding glycoprotein B from both viruses. Phylogenetic reconstructions based on the putative amino acid sequences of glycoprotein B indicate that MaHV-1 and -2 are most closely related to the subfamily *Alphaherpesvirinae*. Within the *Alphaherpesvirinae*, MaHV-1 and -2 are closely associated with those herpesviruses that infect primates. This phylogenetic relationship does not fit the constraints of the proposed co-evolution theory described for other members of the *Alphaherpesvirinae* which have mammalian hosts.

Macropodid herpesviruses (MaHVs) have been implicated in fatal disease outbreaks amongst the captive marsupial populations of Australia (Finnie *et al.*, 1976; Dickson *et al.*, 1980). These outbreaks have resulted in the isolation of nine MaHVs which have been classified into two species called macropodid herpesvirus 1 and 2 (MaHV-1 and MaHV-2) (Johnson & Whalley, 1990). Serological evidence indicates that these viruses are widespread among Australian kangaroos and wallabies (family *Macropodidae*) (Webber & Whalley, 1978; Wilks *et al.*, 1981). MaHV-1 and -2 are represented by isolates from the parma wallaby (*Macropus parma*) and the dorcopsis wallaby (*Dorcopsis meulleri luctuosa*), respectively (Finnie *et al.*, 1976; Wilks *et al.*, 1981).

The family *Herpesviridae* is a large group of viruses that have double-stranded DNA genomes. Biological characteristics such as host symptoms, site of replication and site of latency have been used to describe three major subfamilies, *Alpha-*, *Beta-* and *Gammaherpesvirinae*, within the family *Herpesviridae*. Biological characteristics have been used to place MaHV-1 and -2 within the subfamily *Alphaherpesvirinae*.

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The nucleotide sequence data for glycoprotein B from MaHV-1 and -2 reported in this paper have been deposited in GenBank and assigned the accession nos AF061754 and AF061755 respectively.

The *Herpesviridae* is the best characterized at the molecular level of all the large DNA virus families. As a result the molecular phylogeny of the *Herpesviridae* has been well studied (McGeoch *et al.*, 1995). Current models of mammalian *Alphaherpesvirinae* evolution indicate that members of this subfamily have co-evolved with their respective hosts (McGeoch *et al.*, 1995). Consequently, this hypothesis predicts that the molecular evolutionary pattern of the *Alphaherpesvirinae* reflects that of their hosts. Importantly, the molecular datum used to elucidate these relationships has been derived from *Alphaherpesvirinae* which infect eutherian mammals. Absent from this literature is the molecular phylogenetic position of alphaherpesviruses which infect metatherian mammals (marsupials). It has been estimated that eutherian mammals and marsupials diverged from a common ancestor approximately 130 million years before present (MYBP) (Janke *et al.*, 1997) whereas the divergence of the *Alphaherpesvirinae* which infect eutherian mammals from a common ancestor in to the currently recognized species has been estimated at approximately 80 MYBP (McGeoch & Cook, 1994; McGeoch *et al.*, 1995). As a result co-evolution theory predicts that MaHV-1 and -2 should form a phylogenetic cluster divergent from alphaherpesviruses of eutherian mammals.

In this paper, we report the complete nucleotide sequences for the glycoprotein B (gB) genes from MaHV-1 and -2. We also report that molecular phylogenetic reconstructions based on the deduced gB amino acid sequences indicate an unexpected phylogenetic, though informative, position for MaHV-1 and -2 within the *Alphaherpesvirinae*.

Macropodid herpesviruses-1 and -2 were propagated in Potoroo kidney cells. Cells were cultured in Dulbecco's modified Eagle's medium containing 5–10% foetal calf serum at 37 °C in a 5% CO_2 atmosphere.

All DNA sequencing was performed using dideoxy sequencing chemistry utilizing the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit, with AmpliTaq DNA

Table 1. Comparison of the MaHV-1 and -2 glycoprotein B genes at both the nucleotide and amino acid levels

	Gene length (bp)	G+C (%)	Nucleotide similarity (%)	Amino acids	Amino acid identity (%)
MaHV-1	2664	51·3		887	
MaHV-2	2685	50·4	76·7	894	82·3

polymerase FS according to the manufacturer's instructions (Applied Biosystems). After recovery, sequencing products were resolved on an ABI automated A373 sequencer according to the manufacturer's instructions. Database searches were performed using either blastN or blastX search routines. The resultant DNA sequence data were aligned using AssemblyLIGN version 1.0.7 (Kodak).

Viral DNA was purified as previously described (Dorman *et al.*, 1985). An ordered *Eco*RI genomic library of the MaHV-2 genome was constructed. The 5′ and 3′ termini of the resultant clones were sequenced and open reading frames putatively identified using the blastX search routine (Altschul *et al.*, 1990). Due to the collinear arrangement of the herpesviral genomes, MaHV-2 clones could be arranged in a putative genomic order. Because of the location of an *Eco*RI site within UL27, a larger *Kpn*I genomic fragment was then cloned. The genomic *Kpn*I fragment was subcloned following digestion with *Sac*I and a 3·5 kbp fragment identified which encoded UL26 at the 5′ end and UL28 at the 3′ end and hence was deemed to contain the entire UL27 open reading frame. The complete nucleotide sequence of this fragment was then determined as described below.

The UL27 gene from the MaHV-2 was used to probe a Southern blot of restriction enzyme-digested DNA from MaHV-1 (data not shown). A 13 kb *Bam*HI restriction fragment was putatively identified as containing the UL27 homologue of MaHV-1. After cloning, blastX search analysis indicated that this fragment encoded the herpesviral gene homologues UL22 at the 3′ end and UL29 at the 5′ end and thus should contain the homologue of UL27.

A 3·6 kbp *Sac*I subclone contained a genomic segment from UL26 to UL28. Nested deletions of two clones with the *Sac*I insert in opposite orientations were constructed from the 5′ end using the Erase-a-Base system (Promega). Where the nested deletion clones failed to give unambiguous sequence overlapping sequence oligonucleotides were synthesized to complete the sequencing of the fragments. The nucleotide sequence of all fragments was determined in both directions. Using these methods the nucleotide sequences of both strands the MaHV-1 and -2 gB genes were determined.

The gB gene of MaHV-1 is 2661 bp in length encoding 887 amino acid residues, while the MaHV-2 gB gene is 2682 bp in length encoding 894 amino acid residues. The properties of the two gB genes, including nucleotide similarity and amino acid identity, are summarized in Table 1.

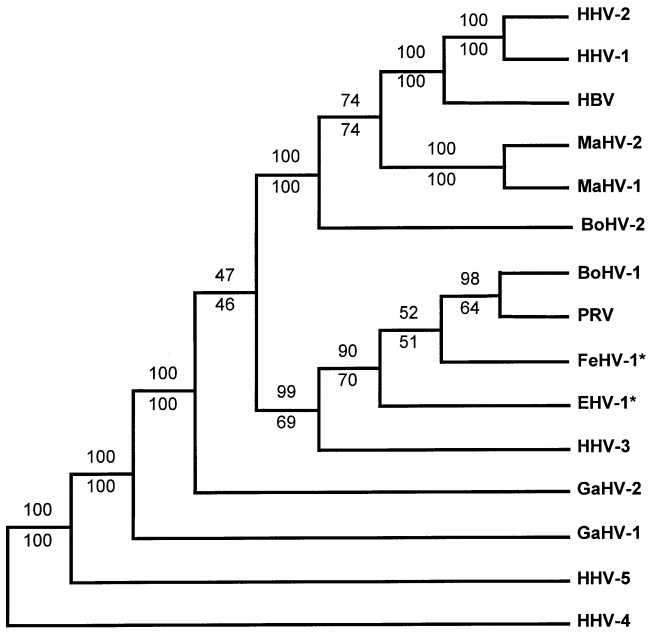

Fig. 1. Phylogenetic analysis of the glycoprotein B amino acid sequences of MHV-1 and -2 compared to selected herpesviruses. This is an unrooted tree. Numbers indicate the percentage of trees in which that branching pattern occurs when using the neighbour-joining (above the branches) and maximum parsimony (below the branches) methods following bootstrap analysis. An asterisk (*) indicates that FeHV-1 and EHV-1 branching positions were reversed in the maximum parsimony construction compared to the neighbour-joining analysis. Virus abbreviations are listed below with full names, common virus names and abbreviations. GenBank sequence accession numbers are also provided. MaHV-1, macropodid herpesvirus 1 (parma wallaby herpesvirus), AF061754; MaHV-2, macropodid herpesvirus 2 (dorcopsis wallaby herpesvirus), AF061755; HHV-2, human herpesvirus 2 (herpes simplex virus 2, HSV2), P08666; HHV-1, human herpesvirus 1 (herpes simplex virus 1, HSV1), P10211; HBV, cercopithecine herpesvirus 1 (herpesvirus B), U14664; BoHV-2, bovine herpesvirus 2, P12641; BoHV-1, bovine herpesvirus 1, M23257; PRV, pseudorabies virus (Aujeszky's disease), P08355; FeHV-1, felid herpesvirus 1 (feline herpesvirus 1), A56602; EHV-1, equid herpesvirus 1 (equine herpesvirus 1), P28922; HHV-3, human herpesvirus 3 (varicella-zoster virus, VZV), P09257; GaHV-2, gallid herpesvirus 2 (Marek's disease herpesvirus 1, MDV), X91985; GaHV-1, gallid herpesvirus 1 (infectious laryngotracheitis virus, ITLV), P24904; HHV-5, human herpesvirus 5 (human cytomegalovirus, HCMV), M60931; HHV-4, human herpesvirus 4 (Epstein-Barr virus, EBV), P03188.

It has previously been reported that amino acid sequence data give more robust and informative phylogenetic relationships (McGeoch & Cook, 1994). As a result the phylogenetic inferences presented here were determined using putative amino acid sequence data. The amino acid sequences of gB from MaHV-1 and -2 were aligned to the homologous gene products from selected alphaherpesviruses using ClustalW version 1.9 (Thompson *et al.*, 1994). Human herpesvirus 5 and human herpesvirus 4 were included as representatives of the gammaherpesvirus and betaherpesvirus subfamilies respect-

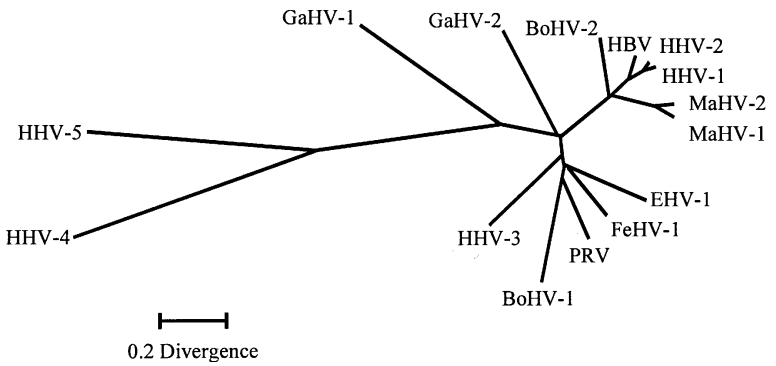

Fig. 2. Neighbour-joining distance tree for glycoprotein B from selected herpesviruses. Divergences between pairs of aligned amino acid sequences were computed and distance trees derived by the neighbour-joining method. The tree is shown in unrooted form with a divergence scale. Virus abbreviations are as in the legend to Fig. 1.

ively. For phylogenetic reconstructions, regions of the alignment that could not be aligned were deleted, as were those areas where gaps were introduced to facilitate alignment; thus only amino acid replacements were evaluated (data not shown). The final alignment was 774 amino acids in length and is available on request. This alignment was then used to calculate putative molecular phylogenetic relationships for MaHV-1 and -2 compared to other herpesviruses.

Phylogenetic trees were computed using the neighbour-joining distance method and maximum parsimony routines, with 1000 bootstrap replicated analyses being performed on both algorithms. The phylogenetic inference programs utilized were from PHYLIP version 3.57c (Felsenstein, 1993). Distance analyses were carried out using the 250 PAM substitution probability matrix of Schwartz & Dayhoff (1978) as previously recommended (McGeoch & Cook, 1994).

The resultant phylogenetic trees from each method were consistent, except for the position of FeHV-1 and EHV-1. A combined phylogenetic tree indicating bootstrap scores and branching patterns of maximum parsimony and distance matrix is illustrated in Fig. 1. In both cases the three major subdivisions of the *Herpesviridae* were distinguished. MaHV-1 and -2 cluster most parsimoniously within the *Alphaherpesviridae*, which is consistent with their biological classifications. The relationships between the major subfamilies of the *Herpesviridae* have been described in detail elsewhere and therefore not all UL27 sequences were included in this study. A complete description of these relationships for eutherian mammals can be found else where (McGeoch *et al*., 1995).

The most striking feature of both analyses is the grouping of MaHV-1 and -2 with the primate herpesviruses. Very high bootstrap scores (100%) were associated with the branching patterns of both MaHV-1 and -2 in the methods utilized. The scores support the inferred tree topology with MaHV-1 and -2 being most closely related to the primate alphaherpesviruses. The molecular phylogeny of the *Herpesviridae* has been examined using both DNA and protein sequence data for gB. As highlighted by McGeoch *et al*. (1995) it is important that the sequence datum used for phylogenetic reconstructions is from either a core gene or sets of core genes. As gB is one of the most commonly sequenced genes of the *Herpesviridae* it was chosen as the core gene in order to evaluate the molecular phylogenetic position of MaHV-1 and -2. Further, herpesvirus phylogenetic reconstructions based on the amino acid sequence of gB have previously been shown to be robust and informative as these phylogenetic reconstructions were representative of reconstructions based on core genes or combined data sets (McGeoch & Cook, 1994; McGeoch *et al*., 1995).

A distance tree drawn with branch lengths proportional to computed divergences between the gB amino acid sequences (Fig. 2) also separates the major families as previously reported (McGeoch *et al*., 1995). The distance tree supports the previous conclusion that MaHV-1 and -2 are most closely related to the primate herpesviruses.

Molecular phylogenetic reconstructions allow the evaluation of how virus evolution correlates with host evolution. This approach has been particularly informative for the mammalian alphaherpesviruses, with currently available data indicating that the mammalian alphaherpesviruses have co-evolved with the respective hosts (McGeoch & Cook, 1994; McGeoch *et al*., 1995).

As metatherian and eutherian mammals are thought to have diverged from a common ancestor at least 80 MYBP, under the constraint of co-evolution it could be expected that MaHV-1 and -2 would form a group divergent from other alphaherpesviruses. The molecular phylogenies presented here indicate a more complex relationship than has previously been proposed. It has been suggested that the avian alphaherpesviruses are likely to have originated from an event of horizontal transfer from mammals to birds, thus preserving the co-evolution theory (McGeoch *et al*., 1995). The introduction of the marsupial herpesviruses into the data set further complicates co-evolution. For co-evolution to be the major route of speciation with in the alphaherpesviruses then MaHV-1 and -2 should have formed a branch of the phylogenetic tree which was ancestral to known mammalian alphaherpesviruses, perhaps even the avian members of this subfamily. The co-speciation model for the alphaherpesviruses does not allow for the inferred position of marsupial herpesviruses.

The phylogenetic clusters of the alphaherpesviruses indicate that complex mechanisms are involved in virus speciation. The recognized genera of the alphaherpesviruses,

Simplexvirus and *Varicellovirus*, contain more than one herpesvirus for some host species. The grouping of BoHV-2 in the lineage with the primate and marsupial viruses also appears to contrast with the co-evolution theory. Alphaherpesviruses with mice and rats as a primary host have not been reported despite the close association of these groups with other eutherian mammals, providing ample opportunity for horizontal transfer, such has been proposed for the avian alphaherpesviruses.

The constructed phylogenies for the two alphaherpesvirus genera indicate that the two groups have possibly radiated separately from a common ancestor. Following divergence from a common ancestor the progenitor for each genus could have co-evolved with the respective mammalian hosts.

There are many putative species within the subfamily alphaherpesviruses for which no molecular data are currently available; obtaining and including these sequences in the data from a wider variety of species will be essential in establishing the evolutionary trends within alphaherpesviruses. A monotreme-specific alphaherpesvirus would also provide an interesting insight into alphaherpesvirus evolution, though none has been recorded.

Here we have reported the first attempt to construct the molecular phylogenies of marsupial alphaherpesviruses. These reconstructions indicate that the proposed co-evolution theory for placental mammal alphaherpesviruses is inconsistent with the contemporary position of marsupial alphaherpesviruses. On currently available data this proposed theory is incongruent with calculated phylogenies. The reconstructed phylogeny presented here, which includes MaHV-1 and -2, supports the previously reported lineages within the alphaherpesviruses (McGeoch *et al.*, 1995). These lineages correspond to the two currently recognized genera of the alphaherpesviruses. More molecular data are required from members representing both genera to establish if the virus-host co-evolution theory can be applied individually to these taxonomic groupings rather than the alphaherpesviruses as a whole. Clearly further studies are required to resolve these issues and should include viral genes with cellular homologues, such as the uracil glycosylase gene, for which rooted molecular phylogenies can be determined. This would allow for a direct comparison of viral phylogeny to mammalian phylogeny to be done.

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