

Short  
CommunicationGenetic characterization of the unique short segment of *Phocid herpesvirus type 1* reveals close relationships among alphaherpesviruses of hosts of the order CarnivoraB. E. E. Martina,<sup>1</sup> T. C. Harder<sup>2</sup> and A. D. M. E. Osterhaus<sup>1,3</sup>Correspondence  
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To further characterize phocid herpesvirus type 1 (PhHV-1) at the molecular level, a cluster of genes comprising the complete unique short (Us) region of PhHV-1 has been cloned and sequenced. Within this region, ORFs were detected that code for the equivalent of the Us 2 – protein of herpes simplex virus (HSV), a putative protein kinase, and for the glycoprotein equivalents gG, gD, gI and gE. In addition, two small ORFs downstream of gE, homologous to the Us 8.5 and Us 9 proteins of HSV were identified. Comparative analysis of the ORF encoding the gD equivalent of PhHV-1 identified the corresponding proteins of the alphaherpesviruses canine herpesvirus and, to lesser degree, feline herpesvirus as the closest relatives.

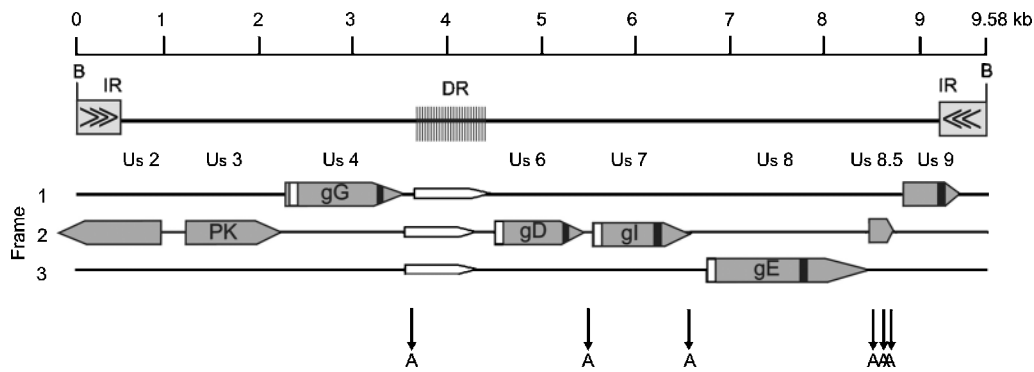
Two distinct species of phocine herpesviruses, an alphaherpesvirus and a gammaherpesvirus, have been described in pinniped species (Osterhaus *et al.*, 1985; Harder *et al.*, 1996). Natural infections with the alphaherpesvirus known as phocid herpesvirus type 1 (PhHV-1) are a major cause of morbidity in seal rehabilitation centres and are held responsible for a wide spectrum of clinical signs. These range from mild respiratory signs in adults to severe generalized and often fatal disease in seal pups and neonates (Osterhaus *et al.*, 1985; Borst *et al.*, 1986; Gulland *et al.*, 1997; Harder *et al.*, 1997). Reminiscent of alphaherpesvirus-induced disease in other mammals, host-mediated factors such as age and immune status also influence significantly the severity of PhHV-1-related disease in seals (Osterhaus, 1988; Have *et al.*, 1991; Harder *et al.*, 1997; Martina *et al.*, 2002). Serological surveys indicated a widespread occurrence of infections with PhHV-1 or other closely related alphaherpesviruses in free-ranging seal populations of the North Sea, as well as of Antarctic and Northern Pacific waters (Have *et al.*, 1991; Harder *et al.*, 1991; Zarnke *et al.*, 1997). Phylogenetic investigations of PhHV-1 have focussed on the conserved gB, the DNA polymerase equivalent, and fragments of the less conserved gD (Harder *et al.*, 1996; Harder & Osterhaus, 1997; King *et al.*, 1998). These studies identified the canine and feline herpesviruses (CHV and FHV, respectively) as the closest relatives of PhHV-1. To

further characterize PhHV-1 at the molecular level, we have cloned and sequenced a cluster of genes comprising the complete unique short (Us) region of PhHV-1.

The PhHV-1 isolate PB84, obtained from a fatally diseased European harbour seal (Osterhaus *et al.*, 1985), was grown in Crandell Rees feline kidney cells (CrFK). Viral DNA was prepared from purified virions as described (Harder *et al.*, 1996).

A *Bam*HI restriction fragment library of PhHV-1 PB84 DNA was established in the phagemid pBluescript SK+ (Stratagene). A 290 bp PCR fragment, generated from the PhHV-1 gD equivalent gene as described (Harder *et al.*, 1996), was used as a probe in non-radioactive Southern blotting (ECL, Amersham) and was found to hybridize to a 9.6 kb fragment, which was sequenced to completion using nested sets of unidirectional deletion mutants and sequence-specific sequencing primers. Assembly and further analyses of the sequences, including phylogenetic studies, were achieved using the Husar clone of GCG (German Cancer Research Institute), including the PSORT II software (<http://psort.ims.u-tokyo.ac.jp>). The sequence of the 9.6 kb *Bam*HI fragment has been assigned GenBank accession no. AJ290955.

The complete sequence of the 9.6 kb *Bam*HI fragment consisted of 9578 nt. Using the FRAMES program of the GCG software, eight ORFs, each with homologues in the Us segment of other alphaherpesviruses of the genus *Varicellovirus*, were detected within this fragment. The



**Fig. 1.** Gene order within a 9.6 kb *Bam*HI fragment of PhHV-1 isolate PB84 comprising the complete Us region. B, *Bam*HI; IR, part of inverted repeat elements (449 bp each); DR, direct repeats ( $n=24$ ). ORFs are depicted in their approximate size and orientation. Shaded boxes represent ORFs with homologues in other varicelloviruses, whereas open boxes indicate lack of homology with other alphaherpesviruses. Vertical rectangles within ORFs indicate putative signal peptides (white) and membrane-spanning regions (black), respectively, whereas vertical arrows labelled 'A' mark consensus poly(A) sites.

order and orientation of each of the ORFs is depicted in Fig. 1. Numbering is according to the nomenclature used for herpes simplex virus type 1 (HSV-1). Inverted repeat (IR) regions of 449 bp were identified at the termini of the 9.6 kb fragment flanking the Us stretch of 8680 nt. The G+C content of the Us segment excluding the repeat elements amounts to 28%, while the IR and direct repeat (DR) regions hold 56.5 and 44% G+C, respectively. ORFs within the PhHV-1 Us extended into the IR elements at both sides. The central part of the Us segment is governed by 24 DRs of the sequence ATggTgTTTCATggggCgTTggg, interspersed between the ORFs encoding gG and gD.

Table 1 presents details of the ORFs and predicted properties of the deduced proteins. Most of the ORFs revealed

collinearity and a degree of sequence conservation when compared to their homologues in other alphaherpesviruses. Putative ORFs of 196–208 aa spanning the DR region in each reading frame apparently were unique to PhHV-1 (Fig. 1, open boxes). The deduced amino acid sequences of each of these ORFs consisted mainly of glycine-rich repeats to which no homologues were found in any of the databases screened. The repetitive sequence – MVFHGALGWCFMG RWDGVSWSVG – was conserved in all three reading frames and was perfectly repeated up to eight times. The vastly prevailing usage of rare codons in the DR ORFs (data not shown), however, suggests a low possibility for their actual expression.

To test this, CrFK cells ( $5 \times 10^6$ ) were infected with PhHV-1

**Table 1.** Properties of ORFs in the 9.6 kb *Bam*HI fragment of PhHV-1 comprising the Us region

ORF*	Location	Features of the putative product			
		Designation	Length (aa)	Properties†	N-linked glycosylation‡
Us 2	976–1	Us 2	> 325	Cytoplasmic protein?	–
Us 3	1220–2245	PK	341	Putative protein kinase	–
Us 4	2350–3588	gG	412	Type Ia membrane protein	10
DR1§	3578–4204	DR1	208	Glycine-rich repeats	–
DR2	3624–4220	DR2	198	Glycine-rich repeats	–
DR3	3640–4230	DR3	196	Glycine-rich repeats	–
Us 6	4352–5404	gD	350	Type Ia membrane protein	4
Us 7	5522–6631	gI	369	Type Ia membrane protein	11
Us 8	6798–8393	gE	531	Type Ia membrane protein	6
Us 8.5	8390–8662	Us 8.5	90	Cytoplasmic protein?	–
Us 9	8773–9186	Us 9	137	Type II membrane protein?	–

\*Nomenclature according to HSV, except for DRs 1–3.

†Properties were predicted using PSORT II software.

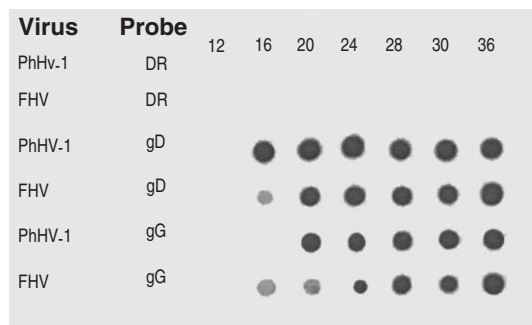
‡Number of putative N-linked glycosylation sites in extracytoplasmic domains.

§Glycine-rich repeat sequences were identical in all three DR ORFs, although encoded in different reading frames.

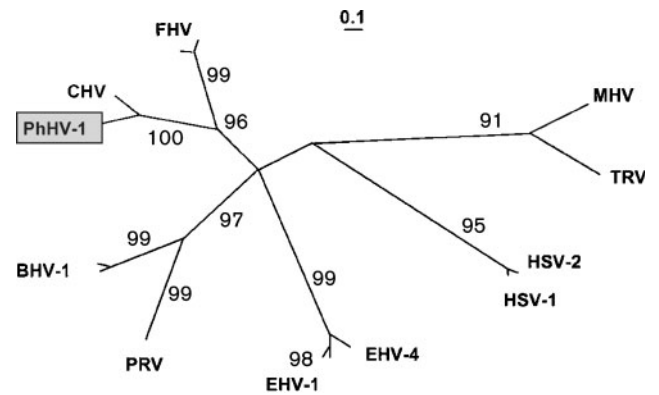
or FHV at an m.o.i. of 5. At intervals of 0, 2, 4, 6, 8, 12, 16, 20, 24, 28, 30 and 36 h post-infection (p.i.), cells were collected for RNA isolation and were frozen at  $-80^{\circ}\text{C}$  until all samples were collected. Total cellular RNA was prepared from  $5 \times 10^6$  virus- (PhHV-1 or FHV) or mock-infected cells using the High Pure RNA Isolation kit (Roche), according to the manufacturer's instructions. An extra treatment of RNA samples with 1 U RNase-free DNase was employed to ensure total degradation of contaminant DNA. Absence of viral DNA was confirmed by PCR/hybridization procedures using gD-specific primers and probe (see below). RNA (25  $\mu\text{g}$ ) was transcribed into cDNA. Approximately 100 ng DNA was then spotted onto a hybond  $\text{N}^+$  membrane (Amersham) and hybridized at  $42^{\circ}\text{C}$  using biotin-labelled probes specific for gG, DR and gD (gG, 5'-<sup>2410</sup>TCA GAACTATGTTATGCAGATCCC<sup>2433</sup>-3'; DR, 5'-<sup>3617</sup>ATGG TGTTTCATGGGGCGTTGGG<sup>3629</sup>-3'; gD, 5'-<sup>4516</sup>ATCTAC AGATCCATGTGGTATG<sup>4536</sup>-3'). Hybridization procedures were conducted as described (Fouchier *et al.*, 2000).

A negative PCR and hybridization using gD primers and probe, respectively, confirmed that residual contaminant viral DNA was not present in the RNA samples (data not shown). No DR transcripts were detected in infected cell cultures (Fig. 2). The DR probe was shown to recognize PhHV-1 DNA at the conditions described (data not shown). The ORFs encoding gG and gD were used as positive control for detection of transcription. In both PhHV-1- and FHV-infected cultures, gG and gD transcripts were detected around 16–20 h p.i. (Fig. 2).

The deduced sequence of the PhHV-1 gD equivalent (350 aa) was used for extensive homology searches. Homologous sequences, as retrieved from the GenBank and SWISS-PROT databases, were aligned using PILEUP (GCG) and their phylogenetic relationships were examined by maximum-likelihood analysis (PUZZLE, Husar clone of GCG; Strimmer & von Haeseler, 1997), using the JTT substitution model and assuming a uniform rate heterogeneity. The gD proteins encoded by CHV and, to a lesser



**Fig. 2.** Detection of RNA transcripts in CrFK cells infected with PhHV-1 and FHV. RNA transcripts for gD and gG were detected at 16–20 h p.i. using biotin-labelled probes. No DR transcripts were detected.



**Fig. 3.** Unrooted phylogenetic tree of gD equivalent proteins of alphaherpesviruses. Branch lengths are drawn in proportion to genetic distances. Numbers represent percentages of quartet puzzling analysis (1000 puzzling steps). Sequence accession numbers from GenBank (GB), SWISS-PROT (SW) and Protein Information Resource (PIR) databases are as follows: phocid herpesvirus type 1 (PhHV-1, isolate PB84); canid herpesvirus (CHV), GB:U84223; felid herpesvirus (FHV), GB:S72415 and GB:D42113; Marek's disease herpesvirus (MHV), SW:Q05100; turkey rhinotracheitis virus (TRV), PIR:JQ2351; HSV-1, SW:p03171; HSV-2, SW:E43674; equine herpesvirus type 1 (EHV-1), SW:P24727 and GB:M87497; EHV-4, GB:S65633; pseudorabies virus (PRV), SW:P07645; bovine herpesvirus type 1 (BHV-1), SW:Q08100 and SW:P24906.

extent, FHV turned out to be the closest relatives of PhHV-1 gD (Fig. 3). Between the gD equivalent proteins of CHV and PhHV-1, 68.1% amino acid identity (76.0% homology) was detected. This included all of six cysteine residues and four potential *N*-linked glycosylation sites in PhHV-1 gD. A fifth site present in the extracytoplasmic domain of CHV gD was missing from the PhHV-1 gD sequence. There was significant less homology to FHV gD (62.2% homology and 52.0% identity, respectively).

We have identified, cloned and sequenced the gene cluster comprising the complete Us region of the pinniped alphaherpesvirus PhHV-1. Within this region, ORFs were detected that code for the equivalent of the Us 2– protein of HSV, a putative protein kinase, and for the glycoprotein equivalents gG, gD, gI and gE. In addition, two small ORFs downstream of gE, homologous to Us 8.5 and Us 9 of HSV, were identified.

Alphaherpesviruses of terrestrial carnivores and PhHV-1 share significant homology at the antigenic and immunogenic levels (Osterhaus *et al.*, 1985; Lebich *et al.*, 1994; Harder *et al.*, 1996, 1998). Using the deduced amino acid sequences of the gB equivalents in phylogenetic analysis, CHV and FHV were identified previously as the closest relatives of PhHV-1 (Harder & Osterhaus, 1997). Similar phylogenetic relationships were now obtained using gD. The particularly close relationship between PhHV-1, CHV and FHV is emphasized also by the genetic organization of the

Us segment. This bears a remarkable resemblance between these viruses, with the exception, however, of the central DR elements detected between the ORFs of gG and gD in the PhHV-1 sequence and at the same location in the Us region of FHV, but not of CHV (Willemse *et al.*, 1995; Haanes & Tomlinson, 1998). The repeated sequence stretches of FHV and PhHV-1 show some homology above background (about 46 % nucleotide identity in 150 bp).

No DR transcripts were detected using a biotin-labelled probe. However, it cannot be excluded that low level transcripts were not detected by the non-radioactive Southern blot system used in our study.

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