Endotheliotropic elephant herpesvirus, the first betaherpesvirus with a thymidine kinase gene

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INTRODUCTION

A highly fatal haemorrhagic disease has been identified in Asian elephants at European and North American zoological gardens. In affected tissues, herpesvirus particles were detected by ultrastructural analysis and, by using PCR methods, a novel herpesvirus was identified. It was called endotheliotropic elephant herpesvirus (Ossent et al., 1990; Burkhardt et al., 1999; Richman et al., 1999). We previously reported the ultrastructural and genetic characterization of endotheliotropic elephant herpesvirus from a male Asian elephant that died from the disease at the Berlin zoological gardens in 1998. Two conserved genes, the glycoprotein B (gB) gene and the DNA polymerase (DPOL) gene, were sequenced completely and used for phylogenetic analysis. It appeared that the virus is a member of the subfamily Betaherpesvirinae with a very distant relationship to all other members of this subfamily. It was taxonomically named endotheliotropic herpesvirus 1 (EHV-1) (Ehlers et al., 2001). The novel genus Proboscivirus is currently proposed for classification, with EHV-1 as the type species and single member (McGeoch et al., 2006).

Here, we wanted to further extend knowledge of the EHV-1 genome, thereby possibly obtaining genetic hints regarding the fulminant pathogenicity of EHV-1 in Asian elephants. As no cell-culture system is available for EHV-1, this goal was difficult to achieve. However, with the technique of genome walking, which had already been used to investigate the EHV-1 gB and DPOL genes (Ehlers et al., 2001), we were able to extend the genomic characterization of EHV-1 to a locus of 60 kbp. Within this genomic region, 34 open reading frames (ORFs) were identified and analysed by computer-based analysis, including a prediction of three-dimensional protein structures. Most importantly, a thymidine kinase (TK) gene was identified and this feature represents a novelty in the subfamily Betaherpesvirinae.
METHODS

Sample collection, preparation of DNA and genome walking. Organ samples were collected post-mortem from the male elephant Kiba that died from endothelial disease at the Berlin zoological gardens in 1998. Total DNA was prepared from the heart by using a QIAamp DNA tissue kit (Qiagen). ElHV-1 sequence information was generated by genome walking as described previously for amplification of the DPOL locus of a novel porcine herpesvirus (Ulrich et al., 1999). In brief, an ElHV-1-positive heart sample from Kiba was used for DNA preparation, and genome walking was performed by Genexpress GmbH (Berlin, Germany) using a Universal GenomeWalker kit (Clontech) (Siebert et al., 1995). Six restriction enzymes were used to generate batches of digested genomic DNA, which was ligated to adaptors. ElHV-1 amplimers were generated by nested PCR using adaptor- and ElHV-1-specific primers.

Sequence determination. PCR fragments obtained by genome walking were sequenced by using BigDye Terminator chemistry and ABI 377 or ABI 3100 automated sequencers (Applied Biosystems). Additional overlapping PCRs were performed for sequence completion with 100 ng elephant DNA by using AmpliTaq Gold (Applied Biosystems) under the following conditions: 12 min activation at 95 °C, 45 cycles with 20 s at 95 °C, 30 s at a primer-dependent annealing temperature (usually 55–65 °C) and 2–3 min at 72 °C, followed by a final extension at 72 °C for 15 min. PCR products were purified of remaining primers and dNTPs by using Microspin S-300 HR or S-400 HR columns (Amersham Biosciences) and sequenced as described above. The double-stranded sequence was determined, with an eightfold redundancy on average. Each base pair was determined from at least two PCR amplimers generated with different primers.

Nucleotide and protein sequence analysis. The sequence files were assembled with the SeqMan module of the Lasergene software (version 6; GATC). ORF prediction and initial analysis was performed with MacVector (version 5.0; Oxford Molecular Group) and the Genequest module of Lasergene, using Borodowsky matrices. BLAST searches were performed in the NCBI database and the GAP program (with neither internal-gap penalty nor end-gap penalty) from the GCG package (Devereux et al., 1984) was used for calculating identity percentages.

ORF designation. Genome orientation and nomenclature of the ElHV-1 ORFs were adapted to the nomenclature of Human herpesvirus 6 (HHV-6A and HHV-6B) and other members of the genus Roseolovirus (Gompels et al., 1995; Dominguez et al., 1999). The ORFs unique to ElHV-1 that have no homologues in databases were named provisionally (ORFs A–F). Redesignation after detection of additional unique ORFs between the 5’ end of the sequence and the left terminus of the genome will be necessary.

Computer-based prediction of three-dimensional protein structures. The location of the kinase domains within the ORFs of ElHV-1 TK (ORF E) and protein kinase (PK) (U69), respectively, was determined by using the Pfam database of hidden Markov models. Sequence and secondary-structure analysis was performed in the Structure Prediction Meta Server (http://cubic.bioc.columbia.edu/predictprotein/doc/meta_intro.html) and yielded the modelling template PDB 1vyw_C (human Cdk2; Pevarello et al., 2004) for ElHV-1 PK (U69) and PDB 2kub (herpes simplex virus 1 (HSV-1) TK; Bennett et al., 1999) for ElHV-1 TK (ORF E). A structural model of the ElHV-1 kinase domain was built in SwissModel by using default settings. Although Cdk2 was identified as a high-scoring template for U69, sequence identity was weak. Therefore, the conserved PK domain of U69, as defined by subdomains (SDs) I–XI, was identified by multiple and pairwise alignments of U69 to various PKs and subsequently aligned to Cdk2. Where necessary, the automated alignment between U69 and Cdk2 was corrected manually to ensure an optimized superimposition. On this basis, structural modelling of the U69 PK domain was achieved in SwissModel. Model evaluation was performed by ProQ with reference to secondary-structure predictions by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

RESULTS AND DISCUSSION

Determination of novel ElHV-1 sequences

Novel ElHV-1 sequences were obtained by PCR-based genome walking using DNA from the heart of Kiba. The genome-walking approach was chosen because neither a cell-culture system producing infectious virus nor a permanently infected cell line harbouring latent ElHV-1 was available. The PCR products were sequenced at both ends and verified by BLAST analysis. To confirm the organization of the PCR fragments, new overlapping PCRs were designed by using the initial sequence data and the confirmatory products were sequenced in both orientations. By using this approach, 59 467 bp of ElHV-1 genomic DNA sequence was obtained.

General ElHV-1 sequence features

The overall G+C content was 42 mol%, which was not distributed uniformly, but ranged from 30 to 60%. No general depression of the CpG dinucleotide frequency, like in many gammaherpesviruses and viral hosts, was found (ratio observed/expected, 1.08) (Honess et al., 1989). Direct and inverted repeats of >50 bp were only seen in the non-coding region between ORFs U41 and U42 (Table 1). This repeat cluster may represent the oriLyt of ElHV-1, as demonstrated experimentally for the homologous position in HHV-6B, murine cytomegalovirus (MCMV) and rat cytomegalovirus (Dominguez et al., 1999; Masse et al., 1997; Vink et al., 1997).

Coding capacity and overall gene arrangement of ElHV-1

The conserved genes of herpesviruses are arranged in a common block organization, I–VII (Gompels et al., 1995). The 60 kbp region of ElHV-1 includes the entire second, third, fourth, fifth and part of the sixth conserved gene block. The overall arrangement of the blocks resembles the genomes of betaherpesviruses, in particular those of the roseoloviruses HHV-6A, HHV-6B and Human herpesvirus 7 (HHV-7). BLAST analysis revealed that the ElHV-1 ORFs had the most closely related counterparts in the human herpesviruses HHV-6B, HHV-6A and HHV-7, as well as in human cytomegalovirus (HCMV). The HHV-6/HHV-7 nomenclature was used for all ElHV-1 ORFs with homologues in the human roseoloviruses (Fig. 1; Table 1). ORFs that exhibited a similarity of <20% upon comparison with other betaherpesvirus ORFs were preliminarily named A–F.
Table 1. Potential ORFs in ElHV-1 and homologous ORFs in HHV-6B and HCMV

<table>
<thead>
<tr>
<th>Polarity</th>
<th>ElHV-1</th>
<th>HHV-6B</th>
<th>HCMV</th>
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<tbody>
<tr>
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<td>Contig position</td>
<td>Size (bp)</td>
<td>Size (aa)</td>
</tr>
<tr>
<td>U38</td>
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</tr>
<tr>
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<td>7927–5849</td>
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<td>692</td>
</tr>
<tr>
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<tr>
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<td>23093–21348</td>
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<tr>
<td>U4</td>
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</table>

NSS, No significant similarity to GenBank sequences (<20% identity).

*Splicing predicted by analysis in silico.
Conserved gene blocks of ElHV-1

Conserved gene block II is identical in gene content to that of HHV-6 and HHV-7. ORFs U38–U41 encode DPOL (ORF U38), gB (ORF U39), a transport protein (ORF U40) and the major DNA-binding protein (ORF U41) (Fig. 1; Table 1). Between conserved gene blocks II and III, the HCMV genome has an insertion of approximately 8 kbp that was reported to contain the HCMV ORFs UL58–UL68 (Dunn et al., 2003; Murphy et al., 2003a, b; Yu et al., 2003). Others consider this region as non-coding (Davison et al., 2003; Dolan et al., 2004). This 8 kbp insertion is not present in the ElHV-1 genome, as is also the case in the HHV-6A, HHV-6B and HHV-7 genomes.

Gene block III (ORFs U42–U46) is not collinear in gene content with those of HHV-6 and HHV-7. ORF U42 encodes a transactivator, ORF U43 a helicase–primase complex protein, ORF U44 a protein of unknown function and ORF U46 glycoprotein N. A homologue to the HHV-6 ORF U45 is missing in ElHV-1. Instead, two genes without any significant similarity are present (ORFs A and C), the latter possessing a high content of serine, threonine and proline residues. Interspersed is a U4 homologue and ORF B, which exhibits a very weak similarity to HCMV UL27. As HHV-6 U45 encodes a dUTPase, present in all other herpesviruses (Davison & Stow, 2005), ORFs A or C might encode a distantly related dUTPase. Functional experiments are needed to clarify this issue. A protein of obvious similarity to HHV-6 ORF U47 (HHV-6 glycoprotein O; 738 aa), positioned between blocks III and IV, is also missing. Instead, the much shorter ORF D (213 aa) is present. In this regard, it should be mentioned that the gO glycoproteins of HCMV, MCMV, HHV-6A, HHV-6B and HHV-7 do not share regions of significant sequence conservation, but have similar numbers of cysteine residues (five or six) and N-glycosylation sites (six to 26), as well as comparable pl values (9–10) (Huber & Compton, 1998). With seven cysteine residues, five of them in the N-terminal half, seven potential N-glycosylation sites and a pl value of 9.5, ElHV-1 ORF D shows clear parallels. Therefore, ORF D may encode a specific gO version of ElHV-1.

Block IV of ElHV-1, ORFs U48–U53, contains all block IV counterparts of HHV-6 and HHV-7, including glycoprotein H (ORF U48), a fusion protein (ORF U49), a virion protein (ORF U50), a G protein-coupled receptor (ORF U51), a protein of unknown function (ORF U52) and a protease (ORF U53). Interspersed between ORFs U48 and U49, an unconserved gene (ORF E) is located that reveals significant similarity to the thymidine kinase genes of alpha- and gammaherpesviruses. Thus, ORF E was identified as a novel TK gene contained in a betaherpesvirus genome.

Genes with significant similarity to ORFs U54 and U55, positioned between blocks IV and V in HHV-6 and HHV-7, are not present in ElHV-1. Instead, ORF F was found, which displays only very low similarity to these two HHV-6 genes (24 and 20%, respectively). As U54 and U55, as well as their counterparts in HCMV (UL82–UL84), represent a family of dUTPase-related genes (Davison & Stow, 2005), ORF F may also be a member of this group. The small block V (ORFs U56–U57) is complete and encodes a capsid and the major capsid protein. Homologues to ORFs U58 and U59, positioned between blocks V and VI in HHV-6 and HHV-7, are present in ElHV-1, but their function is not known.

Fig. 1. Map of the ORFs of the ElHV-1 60 kbp locus. ORFs are shown as arrows. Description of ORFs and abbreviations are given in Table 1. Black arrows indicate ORFs found in two or all three subfamilies of the family Herpesviridae. Open arrows symbolize ORFs specific for ElHV-1. The grey arrow symbolizes the TK ORF. A scale in kbp is given above the map. Base 1 is the first base of the genome stretch determined so far by genome walking. The gene blocks conserved among herpesvirus genomes are indicated with dotted-line bars and numbered I–VI.
In the determined part of block VI (HHV-6B U60–U77), ORFs U62–U65 and U67–U68 encode proteins with unknown function. ORF U66 encodes the viral cleavage and packaging protein (terminase). The terminase gene occurs in all sequenced herpesvirus genomes as two exons, separated by a large intron that contains other ORFs on the opposite strand. In our ORF analysis, this situation appeared initially to be similar for EIHV-1, i.e. two exons (245 and 375 aa, respectively) separated by a large intron. However, the deduced protein (620 aa) exhibited a gap of 40 aa in a highly conserved region as seen in a multiple terminase alignment, and was therefore too short to suggest functionality. Directly downstream of exon 1, however, an additional short exon could be predicted through strong splice-acceptor and -donor sites, thereby providing the corresponding coding region of 40 aa. As depicted in Fig. 1 and listed in Table 1, we propose that the EIHV-1 terminase possesses three exons, namely ORF U66Ex1–3, with exon 3 located between ORFs U59 and U63 (in HHV-6A and HHV-6B, this exon is named U60 and U66Ex2, respectively).

ORF U69 encodes a serine/threonine PK (a kinase potentially possessing additional ganciclovir-phosphorylating activity) and ORF U70 for an alkaline exonuclease (Fig. 1; Table 1).

**EIHV-1 is a very distantly related member of the subfamily Betaherpesvirinae**

At the nucleotide and amino acid levels, almost all EIHV-1 genes with orthologues in other herpesviruses are related more closely to those of the subfamily Betaherpesvirinae than to those of the subfamilies Gammaherpesvirinae and Alphaherpesvirinae (with the exception of the TK gene). However, the percentages of identity are consistently low. The highest value (44 %) was obtained with DPOL. Five ORFs showed no significant similarity (Table 1). This confirms the result of the phylogenetic analysis published previously (Ehlers et al., 2001) that EIHV-1 is a very distant betaherpesvirus.

**EIHV-1 encodes a TK and a serine/threonine PK**

Sequence alignments of EIHV-1 genes with a series of viral and cellular genes provided initial evidence that EIHV-1 ORF E is a herpesvirus TK with homology to gammaherpesvirus TKs and alphaherpesvirus TKs (Fig. 2a), whilst EIHV-1 U69 is a herpesvirus PK with homology to HHV6 U69 and HCMV UL97 (Fig. 3a). This is indicated mainly by the presence of highly conserved SDs I–V (for TK) or I–XI (for PK), respectively. Distinct functional properties were assigned to some of these SDs. For example, SD I of TKs, consisting of the motif ‘-GXXGXGKT-’, forms a so-called glycin loop (or phosphate-binding P loop) and is considered to be characteristic for ATP-binding sites (Saraste et al., 1990; Matte et al., 1998). EIHV-1 TK shows sequential and structural similarity to the HSV-1 TK (Fig. 2a, b). The sequence alignment for EIHV-1 TK includes the five conserved SDs. Particularly, SD I (including glycine residues of the P loop) conforms completely to the consensus sequence motif. In addition, the structural model contains all five SDs as indicated (Fig. 2b).

As far as the SDs of typical eukaryotic cellular PKs are concerned, SDs I–V form the so-called ATP-binding site, whilst SDs VI–XI are involved in the generation of the catalytic centre (Hanks et al., 1988; Hanks & Hunter, 1995). A similar conservation of sequence motifs in SDs I–XI was identified for the herpesvirus PKs (Fig. 3a). By analogy, it is likely that herpesvirus PKs are very similar in their structural organization. Despite low overall sequence identity, a homology-based structure prediction for EIHV-1 PK on the cellular PK Cdk2 was successful and indicated the position of several of the conserved SDs (Fig. 3b). Note distinct three-dimensional locations of the putative ATP-binding site in SDs I–III and a part of the putative catalytic centre in SDs VI–IX. Thus, EIHV-1 TK and PK contain typical sequential (Figs 2a, 3a) and structural (Figs 2b, 3b) features of herpesvirus kinases and it is highly suggestive that they represent a TK and PK, respectively.

**Presence of a TK and a PK gene in EIHV-1 might contribute to the fulminant pathogenicity in Asian elephants**

PKs regulate many cellular functions (Edelman et al., 1987) and herpesviruses utilize their PKs to regulate their own replicative process and modify the cellular machinery (Purves et al., 1993). It is a well-established fact that TK and PK genes in herpesviruses are generally not absolutely essential for viral growth in vitro, but their deletion reduces the viral pathogenic potential substantially in vivo. TK-negative HSV mutants did not reactivate from latency (Coen et al., 1989) and a murine gammaherpesvirus 68 mutant with a TK deficiency showed a severe attenuation in the lung (Coleman et al., 2003). Attenuation in vivo was also observed in TK-negative mutants of channel catfish virus (Zhang & Hanson, 1995). TK-negative mutants were discussed as a basis for the development of vaccines for the protection of cattle, horses, cats and chickens against the alphaherpesviruses Equid herpesvirus 1 (Kaashoek et al., 1996), Equid herpesvirus 1 (Slater et al., 1993), Felid herpesvirus 1 (Yokoyama et al., 1996) and infectious laryngotracheitis virus (Han et al., 2002), respectively. The PK encoded by HCMV UL97 is important for efficient replication of HCMV (Prichard et al., 1999; Michel & Mertens, 2004; Marschall et al., 2005). Therefore, the UL97 protein is considered as a determinant of virus replication and spread in vivo, contributing to major aspects of HCMV pathogenicity. The homologous PK in varicella-zoster virus (VZV) (ORF47) phosphorylates the VZV major immediate-early transactivator, the IE62 protein (Ng et al., 1994). The VZV PK is important for replication in dendritic cells and spread to other cells (Hu & Cohen, 2005), as well as for VZV infection and cell-to-cell spread in human skin (Besser et al., 2003). Obviously, both types of viral kinases contribute to herpesvirus pathogenicity in vivo.
**Fig. 2.** Conserved sequence elements and structural model of ElHV-1 TK. (a) Sequence alignment of ElHV-1 TK to related herpesvirus TKs. Partial amino acid sequences (positions as indicated) of HSV-1 TK (Wagner et al., 1981; Prichard et al., 2006), VZV TK (Grose et al., 2004) and EBV TK (Wu et al., 2005) were aligned. Five conserved regions (SDs I–V) and a conserved glutamine position (Q) are indicated by black boxes. (b) Predicted three-dimensional structure of the ElHV-1 TK kinase domain (Pro51–Val328). ElHV-1 TK was modelled on the homologous template HSV-1 TK. SDs I–V are marked in black.
Generally, betaherpesviruses encode a PK (HCMV UL97 homologue), but – in contrast to alpha- and gammaherpesviruses – they do not encode a TK homologue. With ElHV-1, the first betaherpesvirus encoding a TK in addition to a PK has now been identified. This reflects the unique evolutionary position of the elephant herpesvirus among the herpesviruses of vertebrates, as observed previously in phylogenetic analyses of two conserved ElHV-1 proteins (Ehlers et al., 2001). As (i) elephants, the hosts of ElHV-1, are members of the mammalian superorder Afrotheria and (ii) the completely sequenced betaherpesviruses infecting hosts of the superorders Laurasiatheria (Artiodactyla) and Euarchontoglires (Primates, Rodentia, Scandentia) do not contain a TK gene, it can be speculated that the ancestor of currently existing primates, rodent, artiodactyl and scadent betaherpesviruses lost the TK gene after the separation of the afrotherian superorder from other superorders in mammalian evolution.

ElHV-1 exhibits fulminant pathogenicity in Asian elephants, but is apathogenic in African elephants. It has been argued that the virus has the opportunity to cross the species barrier from African to Asian elephants in zoological gardens where both species are housed (Richman et al., 1999). Crossing of the species barrier, associated with high mortality in the new host, is known for several herpesvirus species. For example, the HSV-related Cercopithecine herpesvirus 1, also known as B virus, is apathogenic in rhesus macaques, but causes fatal encephalitis in humans (Whitley & Hilliard, 2001). Vice versa, HSV-1 can also infect monkeys with often fatal consequences (Mätz-Rensing et al., 2003). Pseudorabies virus, an alphaherpesvirus of pigs, can infect dogs and cats with rabies-like symptoms and death within a short time (Mettenleiter, 2000). Crossing of the species barrier was also documented within the subfamily Gammaherpesvirinae. Ovine herpesvirus 2, a clinically inapparent virus in sheep, can infect cattle and pigs. As a consequence, the so-called malignant catarrhal fever develops, a lymphoproliferative disease with high mortality (Reid et al., 1984; Albini et al., 2003). ElHV-1 is thought to exhibit cross-species infective properties by developing endotheliotropic elephant disease in the foreign host, the Asian elephant. Although the potential to enter Asian elephant cells is obviously a prerequisite, the unique genetic make-up with respect to
Novel treatment options for endotheliotropic herpesvirus disease in Asian elephants?

Famciclovir (FAM) has been used for treatment in two juvenile Asian elephants that presented with symptoms suggestive of endotheliotropic herpesvirus disease and tested PCR-positive for ElHV-1. After a treatment of 3–4 weeks, they recovered (Schmitt et al., 2000). FAM is administered in human alphaherpesvirus infections (HSV-1, HSV-2). With the finding of a TK gene in ElHV-1, the use of FAM in ElHV-1-infected, clinically ill elephants makes sense retrospectively. However, ElHV-1 contains also a U69/-UL97-homologous PK gene. This opens up the possibility of therapeutic alternatives, i.e. the use of ganciclovir, aciclovir or penciclovir, as these nucleoside analogues are phosphorylated and thereby activated by pUL97 or related kinases (Zimmermann et al., 1997; Talarico et al., 1999; Marschall et al., 2002). Especially, derivatives of these drugs with increased oral bioavailability, such as valganciclovir, might be promising. A first step could be the initial testing of phosphorylation of these drugs by the ElHV-1-encoded kinases in vitro. As soon as a viral isolate is available, the determination of the drugs' efficacy in inhibiting ElHV-1 replication in cell-culture experiments will be possible.

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