Isolation and characterization of a chimpanzee alphaherpesvirus

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Although both beta- and gammaherpesviruses indigenous to great-ape species have been isolated, to date all alphaherpesviruses isolated from apes have proven to be human viruses [herpes simplex virus types 1 (HSV1) and 2 (HSV2) or varicella-zoster virus]. If the alphaherpesviruses have co-evolved with their host species, some if not all ape species should harbour their own alphaherpesviruses. Here, the isolation and characterization of an alphaherpesvirus from a chimpanzee (ChHV) are described. Sequencing of a number of genes throughout the ChHV genome indicates that it is collinear with that of HSV. Phylogenetic analyses place ChHV in a clade with HSV1 and HSV2, the alphaherpesviruses of Old World monkeys comprising a separate clade. Analysis of reactivity patterns of HSV2-immune human sera and ChHV-immune chimpanzee sera by competition ELISA support this relationship. Phylogenetic analyses also place ChHV rather than HSV1 as the closest relative of HSV2.

INTRODUCTION

The family Herpesviridae is one of several virus families that maintain a very close relationship with their hosts over the lifetime of the host. In fact, phylogenetic analyses of viral genes indicate that herpesviruses have largely co-evolved with their host species (McGeoch & Cook, 1994; McGeoch et al., 1995, 2000). The mammalian alphaherpesviruses typically establish latent infections in sensory ganglia following the primary infection, thus providing a means for the virus to remain in its host for its lifetime despite the existence of an active immune response directed against the virus. Periodically, alphaherpesviruses reactivate from the latent state, during which times infectious virus is shed, thereby allowing transmission of the virus to new hosts.

Phylogenetic analyses have subdivided the alphaherpesviruses into two major groups: the simplexviruses and the varicellaviruses (McGeoch et al., 2000). With the exception of the human and simian varicellaviruses, all known primate alphaherpesviruses fall into the simplexvirus group. These include alphaherpesviruses isolated from humans [herpes simplex virus types 1 and 2 (HSV1 and HSV2)], Old World monkeys [macaques: Cercopithecine herpesvirus 1 or monkey B virus (BV); baboons: Cercopithecine herpesvirus 16 or herpesvirus papio 2 (HVP2); vervets: Cercopithecine herpesvirus 2 or simian agent 8 (SA8)] and South American monkeys [squirrel monkeys: Saimiriine herpesvirus 1 or herpesvirus saimiri 1 (HVS1); spider monkeys: Ateline herpesvirus 1 or herpesvirus ateles 1 (HVA1)].

Although both beta- and gammaherpesviruses have been isolated from apes, all alphaherpesvirus simplexvirus isolates obtained from apes have proven to be HSV1 or HSV2 (Smith et al., 1969; McClure et al., 1980; Heldstab et al., 1981; Eberle & Hilliard, 1989). Assuming co-speciation of the primate simplexviruses and their hosts, it is reasonable to expect that unique simplexviruses indigenous to ape species should exist, and that these viruses should be related very closely to the human HSVs. Serological testing of various ape species housed in US zoos has shown that many of these animals appear to be infected with HSV1 or HSV2. However, a few gorillas and chimpanzees were identified that, based on the reactivity pattern of their sera in competition ELISAs (cELISAs), appeared to be infected with viruses that were very similar but not identical to HSV2 (Eberle & Hilliard, 1989). Similarly, seropositive wild mountain gorillas with very limited exposure to humans exhibited a similar equivocal reactivity in cELISAs, again...
suggesting the existence of a gorilla virus related closely to HSV2 but distinct from it (Eberle, 1992). Thus, despite the absence of any characterized ape simplexviruses, there is reason to believe that such viruses exist. This report describes the identification of such a virus that appears to represent a chimpanzee simplexvirus.

METHODS

Cells and viruses. Vero cells were cultured in Dulbecco’s modified Eagle medium and used for preparation of virus stock and experimental procedures as described previously (Eberle & Hilliard, 1989). Viruses used included HSV1 strain F, HSV2 strain 186 and HVP2 strain OU1-76. The origins and propagation of these viruses have been described previously (Hilliard et al., 1989; Black & Eberle, 1997).

Immunoenzyme assays. For preparation of ELISA antigens, Vero cells were infected and incubated until the entire cell monolayer exhibited cytopathic effect (CPE). Infected cells were harvested by scraping into the medium and pelleted by centrifugation at 500 g for 5 min. Cells were resuspended at 10⁷ ml⁻¹ in PBS containing 0.5 % Triton X-100, incubated at 37 °C for 10 min and the cell lysates were clarified by centrifugation at 14,000 g for 15 s. Supernatants were collected and adjusted to 1 mg protein ml⁻¹. These antigen preparations were used to coat 96-well plates for ELISA as described previously (Ohsawa et al., 1999).

cELISAs were performed basically as described previously (Eberle, 1992; Thompson et al., 2000). Briefly, twofold serial dilutions of sera were tested against each antigen to determine the dilution at which absorbance values began to decrease. Sera at this final dilution were incubated with serial twofold dilutions of soluble competing antigen in a polyvinyl 96-well plate at 37 °C for 30 min before being transferred in duplicate to wells of an antigen-coated plate for 30 min. The assays then continued as for the standard ELISA protocol. Results of cELISAs were expressed as percentage competition as described previously (Thompson et al., 2000).

Genetic analyses. Viral DNA was purified from infected cells on NaCl gradients as described previously (Black & Eberle, 1997). All PCR was performed by using standard 50 μl reactions consisting of 1 × PCR buffer, 2.5 mM MgCl₂, 25 pmol each primer, 1.5 M betaine, 5 % DMSO and 250 U Taq polymerase (Hirano et al., 2002; Payton et al., 2004). All primers were purchased from Sigma-Genosys. PCR products were either cloned into pCR-TOP2 (Invitrogen) or sequenced directly after purification on a Wizard PCR column (Promega). A partial genomic library of ChHV was prepared by cloning of KpnI restriction fragments into pUC19 as described previously (Ohsawa et al., 2002). All clones were end-sequenced and their genetic content was determined by BLAST searches against sequences deposited in GenBank. Some clones were sequenced in their entirety by a combination of primer walking and subcloning. In several instances, sequence was extended beyond the clone by PCR amplification of adjacent sequences from genomic DNA to obtain the complete sequence of some open reading frames (ORFs). All sequencing was performed by the Oklahoma Medical Research Foundation sequencing facility (Oklahoma City, OK, USA). Sequences were assembled and analysed by using the Vector NTi software package (Informax).

Phylogenetic analyses were performed by using the MEGA version 3.1 program package (Kumar et al., 2004). All sequences except those from a cynomolgous monkey isolate of BV (BVcy) and HVP2 (both unpublished) were extracted from GenBank. All ChHV sequences generated as part of this study have been deposited in GenBank.

Distances between aligned amino acid sequences were calculated by using the Poisson correction. Positions having gap characters in the alignment were eliminated from analyses and phylogenetic trees were constructed by neighbour-joining. Bootstrap resampling (500 replications) was used to assess the reliability of branch points.

Biosafety issues. With the exception of BV, all herpesviruses, including primate alphaherpesviruses known to cause severe cross-species infections, are classified as biosafety-level 2 agents. Nothing is known regarding the pathogenicity of the chimpanzee virus reported here, other than that it seems to behave in chimpanzees much as HSV1 does in humans. There is, however, no reason to expect that this chimpanzee virus is not capable of infecting humans. Consistent with the classification of other primate alphaherpesviruses of unknown pathogenicity for humans, we regard this virus as a biosafety-level 2 agent. Even so, all work with infectious virus conducted in this study was performed under biosafety-level 3 containment.

RESULTS

Identification of a chimpanzee herpesvirus

A 5-year-old female chimpanzee (Pan troglodytes) group-housed with other male and female chimpanzees of varying ages developed acute oral and pharyngeal ulcers that were whitish with red borders. Four other chimpanzees in the group developed oral ulcers around the same time as the index animal. All continued to eat normally and showed no signs of discomfort. Oral ulcers resolved over 2–3 weeks in all of the animals and no further outbreaks were noted. While oral ulcers were still visible, the 5-year-old female was sedated for tissue biopsies and blood-chemistry profiles (all parameters were normal). A viral isolate (105640) was obtained from a biopsy specimen of a tongue lesion. When inoculated onto Vero cells, the virus exhibited CPE that was morphologically and temporally typical of HSV1: within 48 h, clumps of rounded-up cells appeared without any giant cells or syncytia commonly observed for HSV2, BV or HVP2 isolates (Fig. 1). Vero cell monolayers infected at low m.o.i. developed well-defined foci of infected cells that did not spread readily throughout the monolayer, indicating that the virus was highly cell-associated. Consistent with this, virus stocks having very high titres (>5 × 10⁷ p.f.u. ml⁻¹) were not readily obtainable.

PCR amplification of a divergent region in the extracellular domain of the glycoprotein gB (UL27) gene was performed to identify the isolate (Black & Eberle, 1997). Sequencing of the PCR product indicated that the isolate was very similar to HSV2. Over the 360 bp analysed, the chimpanzee isolate differed from HSV2 at 13 positions (3·6 %). Sequences of eight different strains of HSV2 varied at only four positions (1·1 %), suggesting that the chimpanzee herpesvirus isolate (hereafter designated ChHV) was not a variant strain of HSV2, but rather a distinct virus.

Although serum was not available from the animal that the virus was isolated from, sera from other chimpanzees housed at the same facility were made available and were tested by ELISA to obtain an estimate of the prevalence of infection. Of 21 sera tested, nine were positive (42·9 %) and
all of these sera reacted by ELISA with HSV1 and HSV2 antigen as well as HVP2 antigen.

Previous serological testing of captive apes residing in US zoos found that some animals exhibit virus-specificity profiles somewhat different from profiles of humans infected with HSV1 or HSV2 (Eberle & Hilliard, 1989), suggesting that these animals may be infected with a virus similar to but different from HSV1 or HSV2. Sera from two of the seropositive chimpanzees were therefore tested by cELISA to assess their reactivity profiles. Soluble HSV2 antigen only partially competed the reactivity of the chimpanzee sera with solid-phase ChHV antigen (Fig. 2d, f), but efficiently competed reactivity of human HSV2-positive sera with ChHV antigen (Fig. 2b). Conversely, soluble ChHV antigen only partially competed reactivity of HSV2-positive human sera with solid-phase HSV2 antigen (Fig. 2a), but completely inhibited reactivity of chimpanzee sera with solid-phase HSV2 antigen (Fig. 2c, e). Both HSV1 and HVP2 antigens were much less efficient at competing these antibody–antigen reactions, indicating that ChHV is antigenically related more closely to HSV2. Similar results were obtained with serum from a previously tested, zoo-housed chimpanzee (Fig. 2g, h), indicating that all three chimpanzees were probably infected with the same virus. These results demonstrate that whilst the chimpanzee viral isolate is antigenically very similar to HSV2, it is a distinct virus.

**Characterization of the chimpanzee herpesvirus**

To further characterize the ChHV isolate and confirm it as a new herpesvirus, a number of different regions of the genome were sequenced (Fig. 3). Contiguous sequences obtained included homologues of UL12–UL16, UL22–UL25, UL26–UL29, UL44–UL45, UL46–UL49 and US3–US8. Partial sequence data from several additional genes were also obtained, including UL20, UL21, UL30, UL52, RL2, RS1 and US1. Taken together, these sequences totalled about 35.5 kbp and represented genes encoding a variety of different classes of protein products with varying degrees of conservation among alphaherpesviruses. Genes sequenced in their entirety included homologues of HSV genes for several glycoproteins [UL27 (gB), UL44 (gC), US4 (gG), US5 (gJ), US6 (gD) and US7 (gI)], a capsid protein (UL28), tegument proteins [UL47 and UL48 (α-TIF)} and enzymes [UL13 (protein kinase) and UL23 (thymidine kinase)], as well as several other genes of less well-defined function (UL14, UL24 and UL49). Based on the genomic location and size of restriction fragments, the size and orientation of sequenced ORFs and the location of canonical polyadenylation and mRNA termination signal sequences, the genome of ChHV appears to be collinear with that of HSV2. Table 1 compares the size of ORFs and the percentage amino acid sequence identity for the predicted proteins of all genes sequenced in their entirety. In each case, the ChHV proteins were also related most closely to those of HSV2.

Phylogenetic analyses were performed to assess the relationship of the chimpanzee virus to other known primate simplexviruses. Predicted amino acid sequences were aligned, distances were calculated (excluding positions in the sequence alignments having a gap character) and trees were constructed by neighbour-joining (Kumar et al., 2004). Fig. 4(a) shows a phylogenetic tree generated by using UL27 (gB glycoprotein) sequences. As in previous analyses, the two New World monkey viruses (HVS1 and HVA1) formed a clade distinct from viruses of catarrhine primates (Eberle & Black, 1993; McGeoch et al., 1995, 2000). Consistent with accepted evolutionary relationships of the host species and co-speciation of simplexviruses and their hosts, ChHV
Fig. 2. Antigenic relatedness of ChHV and HSV2. cELISAs were performed as described in Methods. The serum–solid-phase antigen reactions that were competed were as follows: human HSV2-immune serum (a, b), sera from two chimpanzees residing at the facility where ChHV was isolated (c–f) and serum from a chimpanzee housed in a US zoo (g, h) were reacted with HSV2 antigen (a, c, e, g) or ChHV antigen (b, d, f, h). Soluble antigens used to compete these antibody–antigen reactions were uninfected cell (●), HSV1 (■), HSV2 (□), ChHV (▲) and HVP2 (△).
together with HSV1 and HSV2 formed a hominoid-virusclade that was separate from the Old World monkey viruses(BV, SA8 and HVP2), which constituted a third clade. Thebranch length separating ChHV and HSV2 relative tobranch lengths separating various strains of HSV2 from eachother again indicates that ChHV is distinct from HSV2 andis not simply a variant strain of HSV2.

Similar analyses were performed by using amino acidsequences for 12 genes (UL13, UL14, UL23, UL24, UL27,UL28, UL44, UL47, UL48, UL49, US6 and US7) that wereassembled into a single, concatenated sequence totalling5430 residues (Fig. 4b). The topology of the resulting treewas consistent with that obtained for gB sequences: ChHVand HSV2 were related more closely to each other thaneither virus was to HSV1 and these three hominoid virusesformed one clade, whilst the cercopithecine monkey viruses(BV, HVP2 and SA8) formed a separate clade. Furthermore,bootstrap-resampling tests gave bootstrap-confidence levels(BCLs) of 99–100 % for all interior branches (Kumar et al.,2004). Use of other distance-estimation and tree-buildingmethods, including maximum parsimony, produced treeswith identical topology and BCL values of 99–100 % for allinterior branches, indicating that the phylogenetic relationshipsdervied for these five primate simplexviruses are veryrobust. When sequences for individual genes were used, ineach case, the overall tree topology was the same, althoughthere was variation in branch lengths (reflecting varyingdegrees of conservation of different genes).

The finding that HSV2 is related more closely to ChHV than toHSV1 raises questions regarding the relationship ofthesee three viruses relative to the accepted co-speciation ofmammalian simplexviruses and their hosts. It was thereforeof interest to obtain an estimate of the time of divergence ofthese viruses relative to the divergence of their host lineages.As the gB protein is strongly conserved and widely used forcomparative purposes, we used the aligned amino acidsequences of the gB protein to estimate the time of diver-gence of these viruses as described by McGeoch & Cook(1994). Pairwise-distance values were determined for eachpair of gB sequences and these distance values were plottedagainst the estimated time of divergence of their host species.As primate-speciation events were used to calibrate themolecular clock and as (i) not all published estimates ofprimate speciation events were obtained by using the same

Table 1. Relatedness of ChHV proteins to homologous proteins of other primate simplexviruses

<table>
<thead>
<tr>
<th>ChHV</th>
<th>Protein function</th>
<th>Amino acid identity (%) to homologous proteins of*</th>
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<tr>
<td>ORF</td>
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<tr>
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<td>US7</td>
<td>376</td>
<td>92</td>
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</tbody>
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*Percentage identity of predicted amino acid sequences for each gene is indicated, with the number of amino acid residues in the open reading frame of each virus indicated in parentheses.
methodology and (ii) there is wide variation in the literature of the estimated time for some speciation events using different estimation approaches (palaeontological, molecular data analysis, etc.), we used a range of published values for the host-speciation events (McGeoch & Cook, 1994; Hayasaka et al., 1996; Kumar & Hedges, 1998; Yoder & Yang, 2000; Glazko & Nei, 2003; Raaum et al., 2005). A best-fit line was drawn through the resulting lines (representing pairwise gB distance values vs speciation-time values for the corresponding host pair). The resulting plot (Fig. 5) was overall very similar to that of McGeoch & Cook (1994). Based on this, divergence of HSV2 and ChHV is estimated to have occurred approximately 5 million years ago (MYA). This is within the accepted range for the time of divergence of the human and chimpanzee lineages (4–6 MYA). In contrast, divergence of HSV1 from both ChHV and HSV2 is estimated to have occurred some 13–14 MYA, well before the split of the human and chimpanzee lineages.

**DISCUSSION**

HSV1 has previously been isolated from captive gorillas, and sera of many captive chimpanzees and gorillas display serological reactivity profiles consistent with these animals being infected with either HSV1 or HSV2 (Heldstab et al., 1981; Eberle & Hilliard, 1989). However, a few captive chimpanzees and gorillas were found to exhibit reactivity profiles suggestive of infection with a virus very similar but not identical to HSV1 or HSV2. This same similar-but-not-identical reactivity was observed for all positive sera from wild mountain gorillas that had minimal close contact with humans (Eberle, 1992). Although the evidence is indirect, these studies imply the existence of ape simplexviruses that are antigenically very similar but not identical to the human HSVs. Sera from two chimpanzees co-housed with the chimpanzee from which ChHV was isolated displayed the same type of cELISA reactivity profile seen in some zoo-housed chimpanzees, suggesting that these animals were infected with a virus that is similar to but distinct from HSV2. Taken together with the genetic data, the results presented here indicate that the ChHV isolate is not a variant of human HSV transmitted to the chimpanzee, but rather represents an actual chimpanzee simplexvirus.

Previous phylogenetic analyses have provided convincing support for the hypothesis that the primate simplexviruses and their host species have co-speciated over time.
(McGeoch & Cook, 1994; McGeoch et al., 2000). Accepting this, a simplexvirus indigenous to chimpanzees would not only be predicted to exist, but should also be related more closely to HSV1 and HSV2 than to viruses indigenous to cercopithecine monkeys (BV, SA8 and HVP2). Phylogenetic trees generated by using different distance algorithms and based on sequences for a number of different viral genes consistently placed ChHV in a clade with HSV1 and HSV2. cELISA analyses of seropositive chimpanzee sera similarly indicated that ChHV is antigenically related most closely to HSV2. These results are consistent with what is known regarding the relatedness of the human and simian alphaherpesviruses, with evolutionary co-speciation of the mammalian alphaherpesviruses and their hosts and with the predicted properties of an ape simplexvirus.

Phylogenetic and antigenic analyses both indicate that HSV2 is related more closely to the chimpanzee virus than to HSV1. Excluding HSV1, the interrelatedness of all of the other primate simplexviruses is consistent with accepted phylogenetic relationships of their host species, including consistency in the estimated times for divergence of the respective virus and host lineages. The splitting of the two human viruses (HSV1 and HSV2) prior to the split of the human- and chimpanzee-virus lineages is the one aspect of the primate simplexvirus phylogeny that, on the surface, may appear inconsistent with strict host–virus co-speciation. However, HSV1 and HSV2 may represent two sympatric virus lineages that diverged prior to human–ape speciation, with HSV2 and ChHV representing two extant viruses of the ‘HSV2 lineage’. Several characteristics of HSV1 that are not found in either HSV2 or ChHV (or any of the simian simplexviruses) could be taken as supportive evidence that HSV1 may represent a distinct hominoid (or even hominid) simplexvirus lineage. These include a large deletion within the US4 (gG) gene (McGeoch et al., 1985, 1987) and the existence of a small ORF between UL20 and UL21 (UL20.5) that appears to be both transcribed and translated in HSV1 (Ward et al., 2000). Whether or not these differences are truly characteristic of an ‘HSV1 lineage’ of hominoid simplexviruses will require isolation and characterization of additional simplexviruses from apes.

While there is at present no evidence for the existence of an ‘HSV1 lineage’ virus in apes or monkeys, such viruses may well exist. If divergence of HSV1 and HSV2 occurred prior to the divergence of the human–ape lineages, as results presented here suggest, apes might be expected to similarly harbour two different simplexvirus lineages, analogous to HSV1 and HSV2 of humans. While many captive gorillas and chimpanzees do show serological evidence of infection with a virus related very closely to HSV1, what little evidence there is to date indicates that these viruses are actually HSV1 that was probably obtained from humans during captivity.

**Fig. 5.** Estimation of the time of divergence of HSV1, HSV2 and ChHV. Distance values between aligned gB amino acid sequence pairs are plotted against estimated divergence times (millions of years ago; MYA) for various primate-lineage specification events (black bars). Speciation events for priamtes and their alphaherpesviruses used and their respective divergence time ranges were: (a) rhesus/cynomolgous macaques – BVrh/BVcy, 1·4–2·2 MYA; (b) macaques/baboons – BV/HVP2, 8·6–10·9 MYA; (c) baboons/cercopithecine monkeys – HVP2/SA8, 10·3–12·9 MYA; (d) platyrhine speciation [spider/squirrel monkey] – HVS1/HVA1, 18 MYA; (e) humans/cercopithecines – HSV1 + HSV2/BV + HVP2 + SA8, 21–25 MYA; (f) humans/platyrhines – HSV1 + HSV2/HVS1 + HVA1, 32–56 MYA. The estimated divergence time for humans and chimpanzees (4–6 MYA) is indicated by the stippled area. Arrows above the best-fit line represent the point where pairwise-distance values for HSV2/ChHV (grey), HSV1/ChHV (white) and HSV1/HSV2 (black) intersect the line, giving estimated divergence times of 5, 13 and 14 MYA, respectively.
In addition, testing of captive apes has not revealed any serological profiles suggestive of infection with two different HSV-like viruses. Similar testing did not suggest the presence of a virus related closely to HSV1 in wild mountain gorillas (Eberle, 1992). Thus, although indirect, evidence to date does not suggest the existence of multiple ape simplexviruses analogous to HSV1 and HSV2. It is possible that viruses of the ‘HSV1 lineage’ were lost from all but the human lineage as hominoid speciation progressed.

If apes do harbour only a single simplexvirus, as appears to be the case for monkeys, then humans are the only primate species having an HSV1-like virus. Given the apparent divergence of the HSV1 and HSV2 lineages before divergence of the human–chimpanzee/gorilla lineages (and in the temporal vicinity of the human–African ape/orangutan lineages), it is possible that the ape lineages subsequently lost the HSV1-like virus lineage. Alternatively, HSV1 may have originated in humans as a zoonotic infection. A greater rate of evolutionary change might be expected in a virus following cross-species transmission as it adapts to the new host species and, consequently, the ‘molecular clock’ would not ‘tick’ at the same rate as for viruses being maintained in their natural host species. Thus, if the HSV1 lineage did originally enter humans as a zoonotic infection, the HSV1 lineage may have evolved more rapidly than the other primate virus lineages, resulting in an overestimation of the divergence time between HSV1 and HSV2 (and ChHV). However, lacking any potential source for such an event in the form of a closely related virus, there is no direct evidence to support this possibility. Obviously, isolation and characterization of additional simplexviruses from other ape species are needed to clarify these issues.

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REFERENCES


outbreak of B virus infection in a colony of DeBrazza’s monkeys 

Identification of a novel expressed open reading frame situated 
between genes \textit{U}1\textsubscript{20} and \textit{U}1\textsubscript{21} of the herpes simplex virus 1 genome. 