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Genetic Diversity within Alphaherpesviruses: Characterization of a Novel Variant of Herpes Simplex Virus 2

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ABSTRACT

Very low levels of variability have been reported for the herpes simplex virus 2 (HSV-2) genome. We recently described a new genetic variant of HSV-2 (HSV-2v) characterized by a much higher degree of variability for the UL30 gene (DNA polymerase) than observed for the HG52 reference strain. Retrospective screening of 505 clinical isolates of HSV-2 by a specific real-time PCR assay targeting the UL30 gene led to the identification of 13 additional HSV-2v isolates, resulting in an overall prevalence of 2.8%. Phylogenetic analyses on the basis of microsatellite markers and gene sequences showed clear differences between HSV-2v and classical HSV-2. Thirteen of the 14 patients infected with HSV-2v originated from West or Central Africa, and 9 of these patients were coinfected with HIV. These results raise questions about the origin of this new virus. Preliminary results suggest that HSV-2v may have acquired genomic segments from chimpanzee alphaherpesvirus (ChHV) by recombination.

IMPORTANCE

This article deals with the highly topical question of the origin of this new HSV-2 variant identified in patients with HIV coinfection originating mostly from West or Central Africa. HSV-2v clearly differed from classical HSV-2 isolates in phylogenetic analyses and may be linked to simian ChHV. This new HSV-2 variant highlights the possible occurrence of recombination between human and simian herpesviruses under natural conditions, potentially presenting greater challenges for the future.

The *Herpesviridae* are a vast family of viruses, with >120 members affecting a diverse range of species, from invertebrates to primates. Sequence-based comparisons have shown that the three subfamilies of the *Herpesviridae*, the *Alpha*-, *Beta*-, and *Gammaherpesvirinae*, are descended from a common ancestor that existed at least 100 million years ago, with synchronous development of the virus and host lineages (1). Nonhuman primates (NHPs) harbor herpesviruses that are genetically more similar to human herpesviruses than to other mammalian herpesviruses. Moreover, simian herpesviruses cause diseases in their natural hosts that closely resemble those observed in humans infected with human herpesviruses (2). Herpes simplex virus 2 (HSV-2) establishes a latent infection in the sensory nerve ganglia and causes recurrent infections leading mostly to recurrent genital ulceration in humans. The prevalence of HSV-2 infection varies with geographic region, demographic feature (e.g., age and sex), study population, and HIV serostatus. The seroprevalence of HSV-2 in sub-Saharan Africa is among the highest in the world, exceeding 80% in some areas. In contrast, HSV-2 seroprevalence in Europe is only ~20% (3–5). The frequency of HSV-2 antibody detection is higher in HIV-1-infected individuals than in individuals not infected with HIV-1, with coinfection rates of ~85% being reported in sub-Saharan Africa (4). Recurrent HSV-2-related anogenital lesions, which are associated with high morbidity rates, are thought to facilitate the acquisition and transmission of HIV-1 and to favor disease progression by increasing plasma, genital, and rectal HIV-1 RNA levels (6).

HSV-2 has a linear double-stranded DNA genome of ~154 kbp, consisting of two extended regions of unique sequences (unique long [UL]/unique short [US]) containing >74 open reading frames and being flanked by a pair of terminal and internal inverted repeat elements (7, 8). The overall interstrain variability of HSV-2 is <0.5%, although the existence of distinct HSV-2 genogroups has been reported (9–12). The rate of evolution of HSV can be accounted for by diverse aspects of viral biology, such as polymerase fidelity and genomic architecture. Indeed, there is a cluster of highly conserved proteins (UL27 to UL33) surrounding the essential virus-encoded DNA polymerase (UL30) in a region of the genome with a low local mutation rate. Highly divergent proteins seemed to be preferentially encoded by genes located in the US segment, promoting genome diversity in response to environmental variation (13). Genetic recombination frequently occurs during HSV replication and constitutes a mechanism for generating genetic diversity complementary to nucleotide substitution (12, 14, 15).

In 2009, we used genotypic methods to investigate the drug

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TABLE 1 Primers and probes of the duplex real-time PCR assay for screening of HSV-2 isolates

Oligonucleotide	Target[a]	Positions[b]	Sequence (5′→3′) (direction)[c]
Primers			
H2_UL30-F	UL30 gene	2834–2854	GTACATCGGCGTCATCTGCG (F)
H2_UL30-R	UL30 gene	3142–3162	CAGGCGCTTGTTGGTGTACG (R)
Probes			
H2C-P	HSV-2c	2948–2974	HEX-TTACGACGATACCGTATCCGGAGCGG-TAMRA
H2V-P	HSV-2v	2948–2973	FAM-CTACAACGATTCCGTGTCCACCGCC-TAMRA

[a] UL, unique long; HSV-2c, classical HSV-2; HSV-2v, HSV-2 variant.
[b] Nucleotide position in the UL30 (DNA polymerase) gene according to the reference UL30 HSV-2 sequence (strain HG52) (GenBank accession number Z86099).
[c] F, forward; R, reverse; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine; HEX, hexachlorofluorescein.

susceptibility of a HSV-2 isolate recovered from a genital sample from a 46-year-old HIV-1-infected man (9, 16). Sequence analysis revealed an unexpectedly high rate of divergence of 2.4% for the UL30 DNA polymerase gene, contrasting with a low rate of divergence of 0.3% for the UL23 thymidine kinase (TK) gene. A variant-specific real-time PCR assay targeting the hypervariable region of the UL30 gene, which was considered to be a specific molecular signature, led to the identification of three other HSV-2 variants, designated HSV-2v, with respect to classical HSV-2 (HSV-2c). These four HSV-2v isolates were all obtained from patients from West Africa with HIV-1 coinfection (16).

The objective of this study was to identify additional isolates of HSV-2v for a more detailed characterization of the prevalence and epidemiological, genotypic, and phenotypic features of this new genetic variant of HSV-2.

MATERIALS AND METHODS

Viruses and DNA extraction. We retrospectively analyzed 505 clinical isolates of HSV-2 from a collection of frozen samples available in our laboratory. These isolates were recovered from patients with HSV infection hospitalized at La Pitié Salpêtrière-Charles Foix University Hospital during a period extending from January 2006 to December 2013. Viral DNA was extracted from viral stocks with a MagNA Pure Compact instrument (Roche Diagnostics, Meylan, France) and stored at −20°C for further analysis.

HSV-2v-specific real-time PCR assay. The relative frequency of HSV-2v with respect to HSV-2c was determined by a duplex real-time PCR assay with primers for the UL30 gene to facilitate discrimination between the two viruses (Table 1). The primers bound to a region of the UL30 gene that was highly conserved in HSV-2v and HSV-2c. They amplified a 328-bp fragment surrounding the HSV-2v-specific molecular signature (Fig. 1). The TaqMan specific probes for the identification of HSV-2v (H2V-P) and HSV-2c (H2C-P) were labeled at the 5′ end with 6-carboxyfluorescein (FAM) and hexachlorofluorescein (HEX), respectively. The amplification mixture (total volume, 25 μl) contained 10 μl of DNA, 1× LightCycler480 Probes Master (Roche Diagnostics), 200 nM forward and reverse primers, each probe at a concentration of 100 nM, and 0.38 U uracil-*N*-glycosylase (Invitrogen, Cergy-Pontoise, France). The reaction was performed on a LightCycler480 instrument (Roche Diagnostics), with the following cycle parameters: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 40 s at 60°C and a final cooling step for 1 min at 40°C.

Phenotypic characterization. The multistep growth of HSV-2v isolates was assayed in Vero cells, as previously described (17). The 50% effective concentrations (EC_{50}s) of acyclovir (ACV) and foscarnet (FOS) were determined in a plaque reduction assay performed as previously described (9, 18). HSV-2v isolates were considered to be resistant if the EC_{50}s were ≥7 μM for ACV and ≥330 μM for FOS.

HSV-2 microsatellite characterization. Microsatellite markers are widely used in epidemiological studies and have already proven useful for the precise characterization of HSV-2 isolates by length polymorphism analysis (19). Haplotype, defined as the combination of the lengths of the nine PCR products obtained in three multiplex PCR assays amplifying 12 polymorphic microsatellites (corresponding to nine loci), was used to differentiate between HSV-2 isolates. The microsatellites were given names beginning with an M, with this letter being followed by position numbers within noncoding regions of the HSV-2 genome, as previously described (Fig. 2) (19). The genetic relationships between the HSV-2v and HSV-2c isolates, based on the frequencies of particular amplicon sizes at

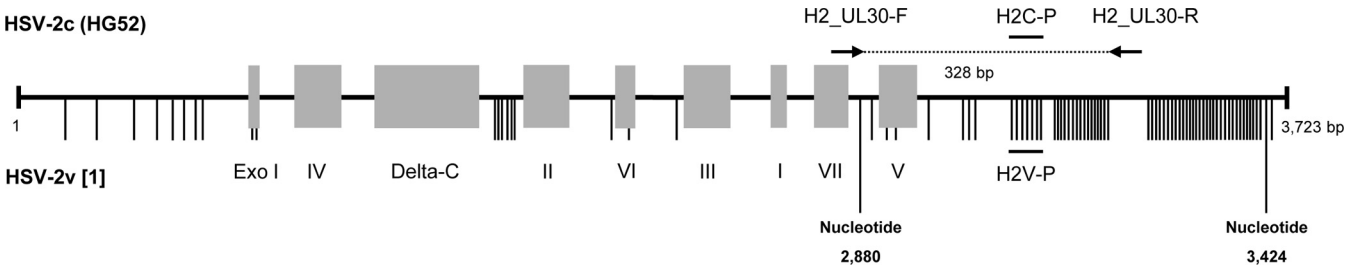

FIG 1 Variant-specific duplex real-time PCR assay targeting the HSV-2v molecular signature within the UL30 (DNA polymerase) gene. The conserved regions and functional domains of the DNA polymerase (3,723 bp) gene are indicated by gray boxes. They are named as follows: Exo I (nucleotides 1092 to 1122), domain IV (nucleotides 1314 to 1440), delta-C region (nucleotides 1596 to 1884), domain II (nucleotides 2097 to 2223), domain VI (nucleotides 2331 to 2388), domain III (nucleotides 2430 to 2550), domain I (nucleotides 2658 to 2703), domain VII (nucleotides 2829 to 2853), and domain V (nucleotides 2877 to 2907) (9). HSV-2c (top), embodied by reference strain HG52, and HSV-2v (bottom), embodied by HSV-2v isolate 1 (HSV-2v [1]), are represented separately. Nucleotide mutations identified in HSV-2v isolate 1 relative to the nucleotide sequence of the HG52 reference strain (GenBank accession number Z86099) are indicated by vertical bars. The duplex real-time PCR assay was designed to use primers generating a 328-bp amplicon (forward primer H2_UL30-F and reverse primer H2_UL30-R) and sequence-specific probes for discrimination between the two types of virus on the basis of the molecular signature located between nucleotides 2880 and 3424 (H2V-P and H2C-P, 5′-labeled hydrolysis probes with FAM and HEX for the detection of HSV-2v and HSV-2c, respectively).

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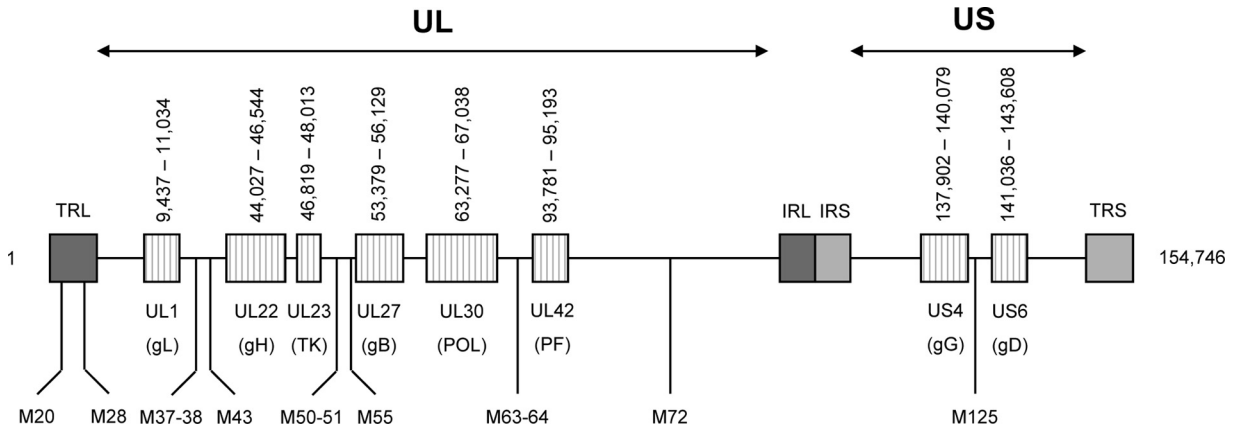

FIG 2 Linear map of the genomic distribution of the genes and microsatellites studied in the HG52 reference strain. The HSV-2 genome (HG52 reference strain [GenBank accession number Z86099]) is a linear double-stranded DNA molecule of 154,746 bp consisting of two covalently linked segments known as the unique long (UL) and unique short (US) sequences. Each segment is flanked by the terminal and internal inverted repeat sequences TRL-IRL (terminal internal repeat long) and IRS-TRS (terminal internal repeat short) (gray boxes). The hatched boxes represent the genes analyzed by conventional sequencing: DNA polymerase (POL) (UL30 gene), processivity factor of DNA polymerase (PF) (UL42 gene), thymidine kinase (TK) (UL23 gene), glycoprotein L (gL) (UL1 gene), gH (UL22 gene), gB (UL27 gene), gG (US4 gene), and gD (US6 gene). The labeled vertical bars represent microsatellite markers (M followed by position numbers, as previously identified [19]) analyzed by length polymorphism analysis: M20 (TRL), M28 (TRL), M37-38 (inter-UL10-UL11), M43 (inter-UL15-UL18), M50-51 (inter-UL25-UL26), M55 (inter-UL26-UL27), M63-64 (inter-UL37-UL38), M72 (inter-UL50-UL51), and M125 (inter-US4-US5).

the various microsatellite loci, were analyzed by generating a phylogenetic tree with the neighbor-joining (NJ) clustering method (20).

Conventional Sanger sequencing analysis. Overall genomic variability in HSV-2v was assessed and compared with that in HSV-2c by multilocus sequencing analysis, which confirmed the high degree of divergence of the UL30 gene in HSV-2v. We investigated sequence diversity for the following viral genes located in the US and UL regions: glycoprotein G (gG) (US4), gD (US6), gL (UL1), gH (UL22), TK (UL23), gB (UL27), and DNA polymerase processivity factor (PF) (UL42) (Fig. 2). Protocols for full-length gene sequencing were reported previously for the UL23 and UL30 (9); UL42 (21); and US4, US6, and UL27 (17) genes. We designed new primers for the amplification and sequencing of the UL1 and UL22 genes (Table 2). The gene amplification protocols were identical to those used for all other genes studied here, except that we used annealing temperatures of 53°C (UL1) and 61.5°C (UL22). We ensured that there were no PCR artifacts by carrying out sequencing twice, on both DNA strands, for all the genes studied. We also implemented sample handling precautions, to prevent PCR contamination before sequencing, in dedicated laboratory facilities. All nucleotide sequences were compared with that of the HG52 reference strain (GenBank accession number Z86099) by using Seqscape v2.5 software (8).

TABLE 2 Primers used for amplification and sequencing of the UL1 (gL) and UL22 (gH) genes

Target gene (size [bp])	Function	Primer	Sequence (5′→3′) (direction)
UL1-gL (675)	PCR	gL-F1	CGGGGGATATATAAAGCGGTA (F)
		gL-R1	CGTAGATGCGGAAAACATCA (R)
	Sequencing[a]	gL-1	CAACGCATCGCGTATCTCT (R)
		gL-2	CCAGAGGGCGTATCTGGTTA (F)
UL22-gH (2,517)	PCR	gH-F1	GGGAAAGGAAAGGAAACAGG (F)
		gH-R1	TCGGTCTGGTACTCCCTTTG (R)
	Sequencing[b]	gH-1	ATACAAGCTTACGGGCGTTG (R)
		gH-2	GTGGCGGTGAGATAGGTGAT (F)
		gH-3	CTGGCGTTCGTGTTGGAC (R)
		gH-4	CGAGAAACTCGGCGTACAG (F)
		gH-5	CTCCGGCCGAGATCATAGT (R)
		gH-6	CCAGGACAAAGACGCGATAC (F)
		gH-7	CAGCTGCTTTACATCCCAAA (R)
		gH-8	GTTGTGCGACAGTCCCTTG (F)

[a] Sequencing was performed by also using gL-F1 and gL-R1.
[b] Sequencing was performed by also using gH-F1 and gH-R1.

Phylogenetic trees, consensus sequences, and recombination analysis. Phylogenetic analyses were performed to assess the relationships between HSV-2v and other known primate simplex viruses. These analyses were carried out with the maximum likelihood method, implemented within MEGA6 software (22), in accordance with valid scientific recommendations (23, 24). We first carried out nucleotide substitution modeling on our data set with the Model Selection tool. The general time-reversible model with gamma distribution (GTR+G model) with five gamma categories was identified as the best-fit model with the lowest Bayesian information criterion (BIC) score. Phylogenetic trees were then constructed by comparing the full-length nucleotide sequences for each of the genes studied and the concatenated sequence alignment combining all genes according to their distribution across the UL and US regions (UL1, UL22, UL23, UL27, UL30, UL42, US4, and US6), to obtain a miniature HSV-2 genome. Sequences 1 to 14 corresponded to the 14 HSV-2v isolates, and sequences 15 to 18 and 19 to 22 corresponded to the HSV-2c isolates recovered from patients with and those without HIV coinfection, respectively. The phylogenetic trees also included other simplex viruses from humans (HSV-1 [reference strains 17, F, H129, and KOS] and HSV-2 [reference strains HG52 and SD90e]), Old World monkeys (cercopithecine herpesvirus 1, B virus [macaques], cercopithecine herpesvirus 16, HVP-2 [baboons], cercopithecine herpesvirus 2, SA-8 [vervets], and chimpanzee alpha-1 herpesvirus [ChHV] [chimpanzee]), and South American monkeys (saimiriine herpesvirus 1 [HVS-1]). The consensus nucleotide sequences for UL30 and US4 in HSV-2v (based on HSV-2v sequences 1 to 14) and HSV-2c (based on HSV-2c sequences 15 to 22) were generated with BioEdit (25). Similarity plots were generated and boot scanning was performed with SimPlot software (v3.5.1) to investigate potential recombination events within individually aligned sequences. The following parameters were used: Kimura two-parameter method, transition/transversion ratio of 2.0, and sliding window covering 200 nucleotides and shifted by 20 nucleotides at a time (26). A cutoff of 70% was used to determine whether the recombination signal was significant (27). We constructed phylogenetic trees (500 bootstrap replicates)

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TABLE 3 GenBank accession numbers of gene sequences from all HSV-2 isolates studied, obtained by classical Sanger sequence analysis[a]

Isolate	GenBank accession no. for gene							
	UL23 (TK)	UL30 (Pol)	UL42 (PF)	US4 (gG)	US6 (gD)	UL1 (gL)	UL22 (gH)	UL27 (gB)
HSV-2v_pat1	KC693017	JX905315	KF588478	KC693013	KF588418	KF588450	KF588430	KF588406
HSV-2v_pat2	KC693019	JX905318	KF588479	KC693016	KF588419	KF588451	KF588431	KF588407
HSV-2v_pat3	KC693018	JX905317	KF588480	KC693015	KF588420	KF588452	KF588432	KF588408
HSV-2v_pat4	KC693020	JX905316	KF588481	KC693014	KF588421	KF588453	KF588433	KF588409
HSV-2v_pat5	KF588498	KF588390	KF588482	KF588470	KF588422	KF588454	KF588434	KF588410
HSV-2v_pat6	KF588499	KF588391	KF588483	KF588471	KF588423	KF588455	KF588435	KF588411
HSV-2v_pat7	KF588500	KF588392	KF588484	KF588472	KF588424	KF588456	KF588436	KF588412
HSV-2v_pat8	KF588501	KF588393	KF588485	KF588473	KF588425	KF588457	KF588437	KF588413
HSV-2v_pat9	KF588502	KF588394	KF588486	KF588474	KF588426	KF588458	KF588438	KF588414
HSV-2v_pat10	KF588503	KF588395	KF588487	KF588475	KF588427	KF588459	KF588439	KF588415
HSV-2v_pat11	KF588504	KF588396	KF588488	KF588476	KF588428	KF588460	KF588440	KF588416
HSV-2v_pat12	KF588505	KF588397	KF588489	KF588477	KF588429	KF588461	KF588441	KF588417
HSV-2v_pat13	KM068898	KM068900	KM068902	KM068892	KM068890	KM068896	KM068894	KM068888
HSV-2v_pat14	KM068899	KM068901	KM068903	KM068893	KM068891	KM068897	KM068895	KM068889
HSV-2_pat15	KF588506	KF588398	KF588490	JQ956303	JQ956351	KF588462	KF588442	JQ956327
HSV-2_pat16	KF588507	KF588399	KF588491	JQ956304	JQ956352	KF588463	KF588443	JQ956328
HSV-2_pat17	KF588508	KF588400	KF588492	JQ956305	JQ956353	KF588464	KF588444	JQ956329
HSV-2_pat18	KF588509	KF588401	KF588493	JQ956306	JQ956354	KF588465	KF588445	JQ956330
HSV-2_pat19	KF588510	KF588402	KF588494	JQ956314	JQ956362	KF588466	KF588446	JQ956338
HSV-2_pat20	KF588511	KF588403	KF588495	JQ956315	JQ956363	KF588467	KF588447	JQ956339
HSV-2_pat21	KF588512	KF588404	KF588496	JQ956316	JQ956364	KF588468	KF588448	JQ956340
HSV-2_pat22	KF588513	KF588405	KF588497	JQ956317	JQ956365	KF588469	KF588449	JQ956341

[a] gG; glycoprotein G; gD; glycoprotein D; gL; glycoprotein L; gH; glycoprotein H; gB; glycoprotein B; PF, processivity factor of DNA polymerase; Pol, DNA polymerase; TK, thymidine kinase; UL; unique long; US, unique short.

to validate recombination signals, using nucleotide fragments flanking putative recombination breakpoints.

Statistical analyses. Statistical analyses were performed with MedCalc software. We considered P values of <0.05 to be statistically significant.

Nucleotide sequence accession numbers. All sequences determined in this study have been deposited in the GenBank database under the accession numbers listed in Table 3.

RESULTS

Characteristics of patients infected with HSV-2v. The 505 HSV-2 clinical isolates obtained over the 8-year study period (2006 to 2013) were screened retrospectively in a specific duplex real-time PCR assay, and 14 HSV-2v isolates recovered from different patients were identified (HSV-2v isolates 1 to 14). This corresponds to an overall prevalence of 2.8%. The mean annual rate of positive tests for the variant form was reasonably stable at 3.1% (standard deviation, 1.9%). The 14 patients infected with HSV-2v comprised 7 men and 7 women (median age, 41 years; range, 5 to 63 years) (Table 4). All but one of the patients originated from Africa (10 patients from West Africa and 3 from Central Africa), and 9 were coinfected with HIV (HIV-1 in 7 patients and HIV-2 in the other 2). HSV-2v infection was significantly associated with an African origin ($P = 0.0001$) and HIV coinfection ($P = 0.0223$) of the patients (Table 5). Overall, the detection rate for HSV-2v was significantly higher than that for HSV-2c in HIV-infected patients originating from Africa (odds ratio = 4.6 [confidence interval, 1.6 to 13.6]; $P = 0.0068$). HSV-2v infection did not appear to be associated with attenuated or enhanced virulence as a function of

TABLE 4 Characteristics of patients infected with HSV-2v

Patient	Sex[a]	Age (yr)	Origin	HIV infection	Site of infection
1	M	48	Guinea-Conakry	HIV-1	Genitals
2	F	36	Niger	HIV-1	Buttock
3	F	38	Nigeria	HIV-1	Anus
4	F	30	Ivory Coast	HIV-1	Genitals
5	M	5	Unspecified (African)	No	Buttock
6	M	52	Equatorial Guinea	HIV-1	Buttock
7	F	22	Mali	No	Genitals
8	M	44	Mali	No	Genitals
9	M	46	Ivory Coast	HIV-2	Genitals
10	M	55	Democratic Republic of Congo	No	Genitals
11	F	29	Ivory Coast	HIV-2	Buttock
12	F	61	Ivory Coast	HIV-1	Genitals
13	F	32	Democratic Republic of Congo	No	Genitals
14	M	63	Unspecified (Caucasian)	HIV-1	Buttock

[a] M, male; F, female.

TABLE 5 Epidemiological features of HSV-2v

Feature	No. (%) of patients with feature		P[a]
	HSV-2c ($n = 491$)	HSV-2v ($n = 14$)	
Coinfection with HIV	160 (32.6)	9 (63.4)	0.0223
HIV-1	152 (31.1)	7 (50)	
HIV-2	8 (1.6)	2 (14.3)	
Originating from West or Central Africa	185 (37.7)	13 (92.9)	0.0001
Coinfection with HIV and originating from West/Central Africa	110 (22.4)	8 (57.1)	0.0068

[a] P values of <0.05 were considered statistically significant.

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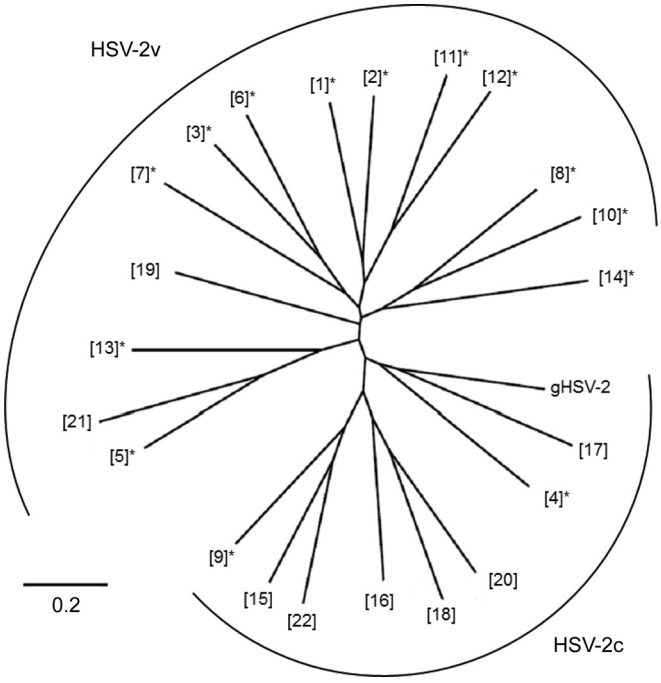

FIG 3 Phylogenetic tree based on microsatellite markers, depicting the genetic relationships between variant and classical HSV-2 clinical isolates. The neighbor-joining unrooted tree was constructed from the distance matrix derived from microsatellite allele frequencies reported by Nei et al.(20). The HSV-2 isolates are identified beside the corresponding branches: HSV-2v isolates, marked with an asterisk (isolates 1 to 14), and classical HSV-2c isolates (isolates 15 to 22). The solid bar beside the phylogenetic tree indicates length based on similarity. The haplotype of the gHSV-2 laboratory strain is included.

the HIV serostatus of the patients. Interestingly, an analysis of sequential isolates obtained during recurrent genital herpes episodes in patient 1 indicated that HSV-2v and HSV-2c could coexist in the same individual (data not shown).

Phenotypic characterization of HSV-2v. The cytopathic effect and replication profile of HSV-2v observed in Vero cells were similar to those of the gHSV-2 laboratory strain (data not shown). All HSV-2v isolates were susceptible to both ACV and FOS in the

TABLE 6 Overall genetic variability among the 14 variant and 8 classical HSV-2 isolates as assessed by conventional Sanger sequence analysis

Gene	Size (bp)	Function	Mean % divergence[a]	
			HSV-2v	HSV-2c
UL30	3,723	DNA polymerase	**2.1**	0.1
US4	2,100	Glycoprotein G	**1.7**	0.4
UL22	2,518	Glycoprotein H	0.9	0.3
UL42	1,413	Processivity factor	0.9	0.3
UL27	2,751	Glycoprotein B	0.6	0.3
UL1	1,598	Glycoprotein L	0.6	0.0
US6	1,182	Glycoprotein D	0.4	0.1
UL23	1,195	Thymidine kinase	0.3	0.2

[a] For each of the genes studied, the mean percent divergence was significantly higher ($P < 0.05$) for the 14 HSV-2v isolates than for the 8 HSV-2c isolates, with the corresponding nucleotide sequences from the HG52 strain being used as a reference (GenBank accession number Z86099). High percentages of divergence (>1.5%) are shown in boldface type.

TABLE 7 Variations of HSV DNA polymerase, DNA processivity factor, thymidine kinase, and glycoprotein (gB, gD, gH, gL, and gG) sequences, at both the nucleotide and amino acid levels, among the 14 HSV-2v isolates investigated

Gene	Mean % nucleotide identity (range)[a]	No. of nucleotide mutations	Mean no. of mutations per strain (range)	% silent mutations	Mean % amino acid identity (range)[a]	No. of amino acid changes	Mean no. of changes per strain (range)	% variation of total codons
UL30 (Pol)	97.9 (97.6–99.2)	124	78.1 (31–92)	59.7	97.4 (97.0–99.0)	50	31.8 (12–37)	4
US4 (gG)	98.3 (97.4–99.7)	81	36.6 (6–55)	37	96.9 (95.3–99.3)	51	21.4 (5–33)	7.3
UL22 (gH)	99.1 (98.8–99.8)	39	21.9 (4–20)	41	98.9 (98.3–100)	23	9.5 (0–14)	2.7
UL42 (PF)	99.1 (98.7–99.7)	27	12.9 (4–18)	51.9	98.9 (98.3–99.6)	13	5.3 (2–8)	2.8
UL27 (gB)	99.4 (98.9–99.8)	55	15.1 (5–29)	41.8	99.2 (98.6–99.8)	32	7.5 (2–13)	3.5
UL1 (gL)	99.4 (99.1–100)	8	4.1 (0–6)	50	98.7 (98.2–100)	4	2.9 (0–4)	1.8
US6 (gD)	99.6 (99.3–100)	15	4.3 (0–8)	80	99.6 (99.2–100)	3	1.4 (0–3)	0.8
UL23 (TK)	99.7 (99.5–99.9)	13	3.4 (1–6)	38.5	99.3 (98.9–99.7)	8	2.5 (1–4)	2.1

[a] The HG52 strain sequence was used as the reference sequence (GenBank accession number Z86099).

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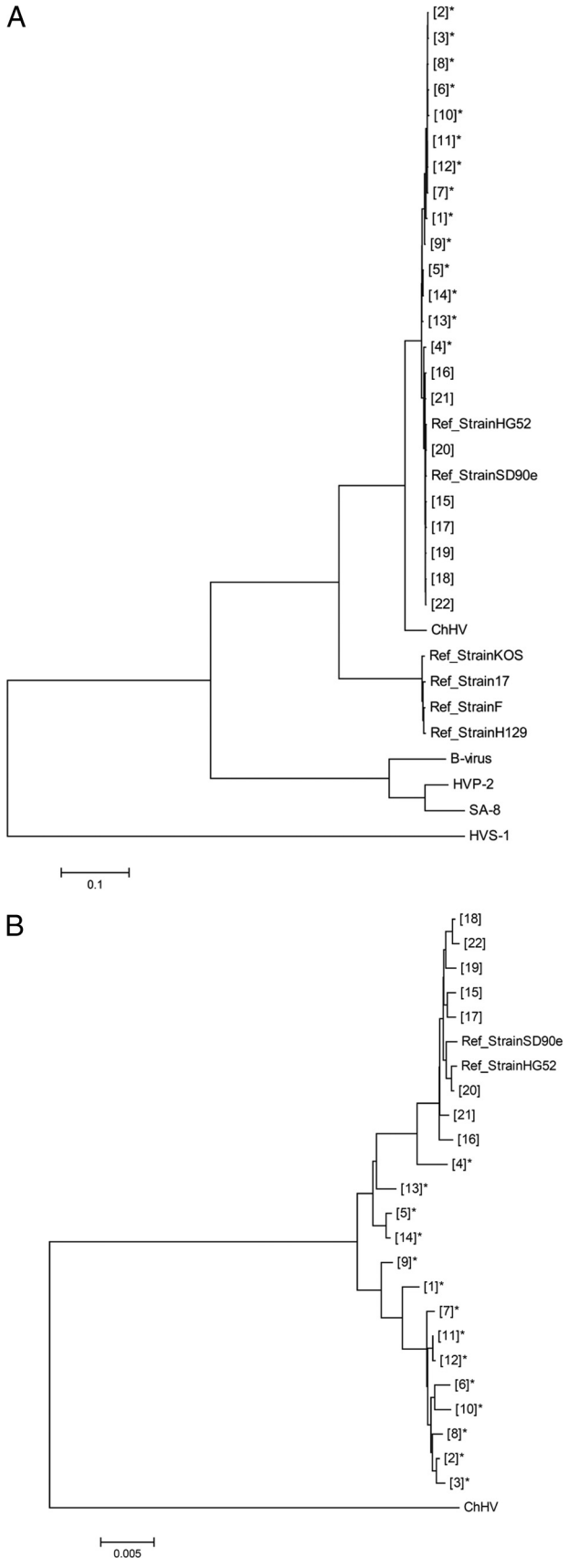

antiviral drug susceptibility assay, with EC_{50}s of <7 μM for ACV and <330 μM for FOS being obtained.

Genotypic characterization of HSV-2v. Each HSV-2 isolate was characterized by determining its microsatellite haplotype—the combination of the PCR product lengths obtained in the three multiplex assays—for rapid evaluation of the genetic profiles of the 14 HSV-2v and 8 HSV-2c isolates. As reported in a previous study (19), haplotypes were obtained for all isolates, making it possible to generate an unrooted phylogenetic tree with the NJ algorithm from microsatellite allele frequencies. Moreover, all HSV-2v haplotypes were correctly discriminated from each other, and all HSV-2v isolates other than HSV-2v isolates 4 and 9 (12/14) clustered together (Fig. 3). We then performed additional studies with conventional sequencing analysis approaches to assess the multilocus variability of this new HSV-2v. The results are presented in detail in Tables 6 and 7. The mean percent divergence for each of the genes studied was significantly higher for the 14 HSV-2v than for the 8 HSV-2c isolates ($P < 0.05$), taking the corresponding nucleotide sequences from HG52 as a reference. In contrast to the high degree of nucleotide divergence observed for the UL30 and US4 genes in the 14 HSV-2v isolates (mean divergences of 2.1% and 1.7%, respectively), the other genes studied (UL1, UL22, UL23, UL27, UL42, and US6) had much lower levels of variability, below 1% (Table 6). The interisolate identities of the nucleotide sequences obtained for the genes from the 14 HSV-2v isolates studied ranged from 97.9% (UL30) to 100% (UL1 and US6). With respect to the reference sequences from HG52, 124 nucleotide mutations (mean, 78.1 per strain) and 81 nucleotide mutations (mean, 36.6 per strain) were identified within the UL30 and US4 coding sequences, respectively. The interisolate amino acid sequence identities were 96.9% for gG and 97.4% for the DNA polymerase. There were 51 amino acid substitutions in gG and 50 in the DNA polymerase, corresponding to 7.3% and 4.0% of the codons encoding these proteins, respectively (Table 7).

Phylogenetic relationships between HSV-2v and other simplex viruses. The phylogenetic analysis of concatenated HSV-2 gene sequences (UL1, UL22, UL23, UL27, UL30, UL42, US4, and US6) clearly revealed two distinct genetic groups supported by high bootstrap values (Fig. 4A and B). All HSV-2v isolates other than HSV-2v isolate 4 clustered together and were more distantly related to HSV-2c (isolates 15 to 22 and HSV-2 reference strains SD90e and HG52) and HSV-1 reference strains. Despite the lower

FIG 4 Genetic analysis of the concatenated sequence alignments of the UL1, UL22, UL23, UL27, UL30, UL42, US4, and US6 genes from human and NHP simplex viruses. Phylogenetic trees were constructed by the maximum likelihood method, implemented in MEGA6 software (22). The bar indicates the number of substitutions per site. Sequences of HSV-2v isolates 1 to 14 correspond to identified HSV-2 variant isolates, marked with an asterisk, and sequences of HSV-2 isolates 15 to 18 and 19 to 22 correspond to HSV-2c isolates recovered from HIV-infected and HIV-negative patients, respectively. The corresponding concatenated nucleotide sequences from HSV-2 reference strains HG52 and SD90e (GenBank accession numbers Z86099 and KF781518, respectively); HSV-1 reference strains 17, F, H129, and KOS (GenBank accession numbers X14112, GU734771, GU734772, and JQ780693, respectively); Old World monkeys (cercopithecine herpesvirus 1, B virus [macaques], cercopithecine herpesvirus 16, HVP2 [baboons], cercopithecine herpesvirus 2, SA-8 [vervets], and chimpanzee alpha-1 herpesvirus [ChHV] [chimpanzee] [GenBank accession numbers AF533768, AY714813, DQ149153, and JQ360576, respectively]); and South American monkeys (saimiriine herpesvirus 1 [HVS-1] [GenBank accession number HM625781]) were also included. Panel B focuses on the HSV-2 and ChHV sequences.

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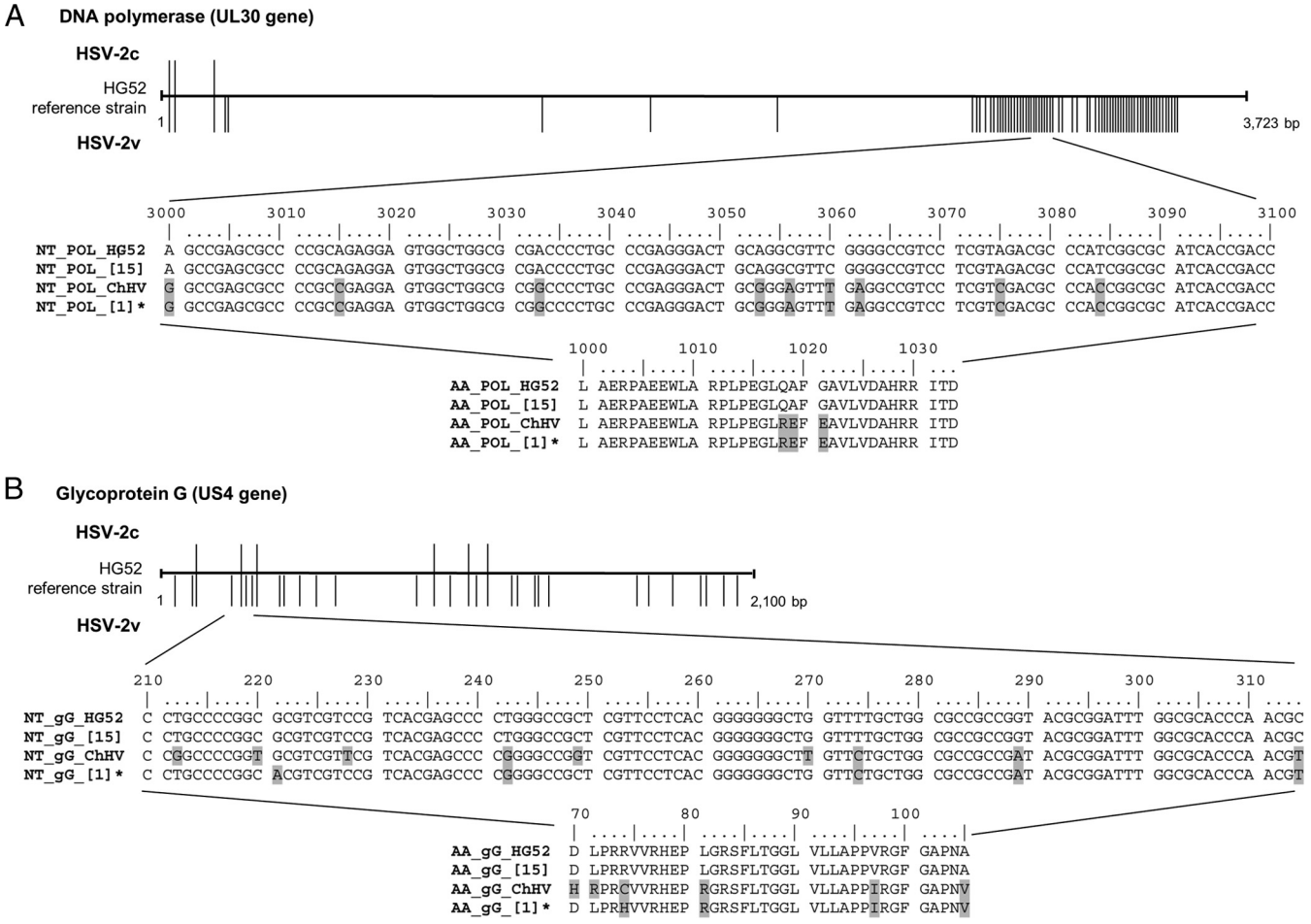

FIG 5 Nucleotide sequence differences for UL30 (DNA polymerase [POL]) (A) and US4 (gG) (B) between classical HSV-2 and the HSV-2 variant in comparison with ChHV UL30 DNA polymerase (3,723 bp) and US4 glycoprotein G (2,100 bp). Nucleotide consensus sequences for HSV-2c (isolates 15 to 22) and HSV-2v (isolates 1 to 14) are compared with the respective gene sequences from ChHV indexed in the GenBank database under accession number JQ360576. For each gene, nucleotide sequence differences observed in HSV-2c (top) or HSV-2v (bottom) and ChHV with respect to the nucleotide sequence of the HG52 reference strain (GenBank accession number Z86099) are indicated by vertical bars. For each panel, a representative set of nucleotide (NT) and amino acid (AA) alignments for HSV-2c and HSV-2v (embodied by HSV-2 isolate 15 and HSV-2v isolate 1, respectively) and sequences from across the region with high similarity to ChHV were included.

level of divergence of the UL30 gene (0.8%) in HSV-2v isolate 4, this isolate was significantly different from HSV-2c, lying in an intermediate position between HSV-2v and HSV-2c. We investigated possible links between HSV-2v and NHP simplex viruses by subjecting sequences from B virus, HVP-2, SA-8, ChHV, and HVS-1 to a phylogenetic analysis. We found that the simian simplex virus sequences could be used as reliable outgroups, with the exception of ChHV (28), which was closely related to HSV-2 isolates (Fig. 4A). We generated phylogenic trees for each of the genes studied to confirm the findings for our concatenated gene analysis. Similar tree topologies were obtained, confirming that most of the HSV-2v sequences clustered in a distinct, separate genetic group, for all genes other than UL23 (data not shown). We investigated the highly divergent UL30 and US4 genes of HSV-2v further, comparing them with their counterparts from HSV-2c and ChHV. Interestingly, multiple-sequence nucleotide alignments revealed similarity between HSV-2v and ChHV sequences, particularly in the HSV-2v UL30-specific molecular signature. We investigated the relationship between HSV-2v and ChHV in more detail by aligning the sequences of the UL30 and US4 genes of HSV-2v isolates 1 to 14 and HSV-2c isolates 15 to 22 separately to generate consensus sequences for these two groups. These consensus sequences were compared with the corresponding sequences from ChHV and the reference strain HG52. For each gene, the nucleotide mutations present in HSV-2v, HSV-2c, and ChHV were highlighted (Fig. 5). The alignments showed that the mutations identified in HSV-2v and HSV-2c occurred at nucleotide positions also mutated in ChHV: 71 mutations for HSV-2v (located mostly in the specific molecular signature between nucleotide positions 2926 and 3130) and 3 for HSV-2c among the 3,723 nucleotides of the UL30 gene (Fig. 5A) and 31 for HSV-2v and 6 for HSV-2c among the 2,100 nucleotides of the US4 gene (Fig. 5B). The phylogenetic profile of UL30 was also analyzed with SimPlot. Using the HSV-2v isolate 1 sequence as a query, we detected recombination breakpoints around nucleotide positions 1940 and 3370 of the UL30 sequence, with HSV-2c and ChHV as the two putative parental strains (Fig. 6A). In the phylogenetic tree generated with the UL30 sequences between these potential recombi-

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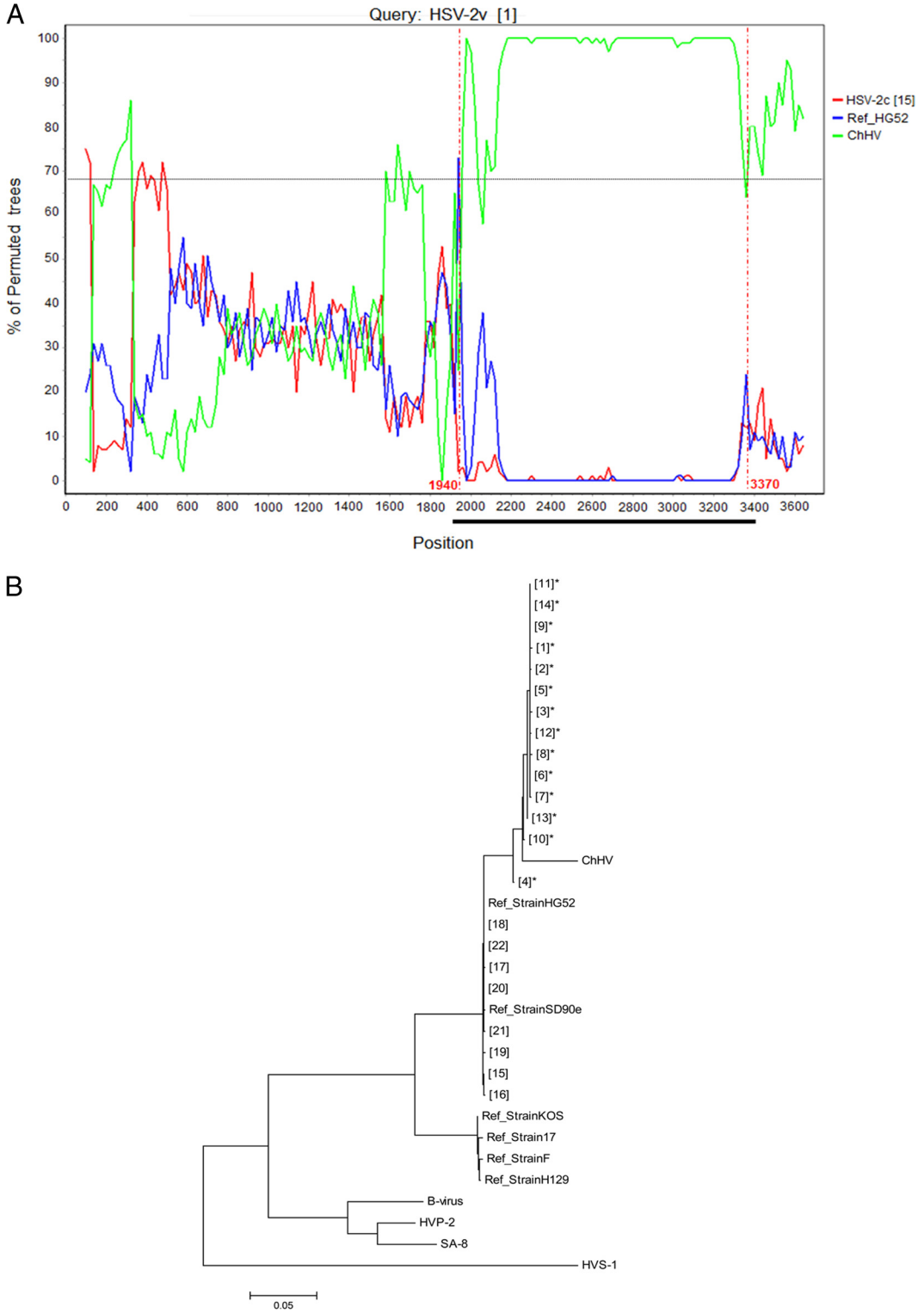

FIG 6 Boot-scanning (A) and phylogenetic (B) analyses of evidence for recombination in UL30 gene sequences. (A) The query sequence from HSV-2v (embodied by HSV-2v isolate 1) is shown at the top. The sequences (classical HSV-2, embodied by HSV-2 isolate 15, the HG52 reference strain, and ChHV) compared with the query sequence are indicated on the right. Sequence similarity was calculated by the Kimura two-parameter method with a transition/transversion ratio of 2.0. SimPlot software generates a graph of the percentage of trees (on the *y* axis) permuted with respect to the nucleotide position in the UL30 sequence (*x* axis), obtained with a sliding window covering 200 nucleotides, shifted 20 nucleotides at a time. The recombination cutoff value of 70% is indicated by the dashed line. The positions of the putative recombination breakpoints are indicated in red at the bottom. The black line below the boot-scanning plot indicates the region of alignment used to substantiate the recombination signal in the phylogenetic analysis shown in panel B. (B) Phylogenetic tree (500 bootstrap replicates) based on the UL30 segment shown in panel A, encompassing the putative recombination breakpoints (nucleotide positions 1940 to 3370). This tree was constructed to validate the recombination signals obtained by the maximum-likelihood method, implemented in MEGA6 software (22). The bar

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nation breakpoints, HSV-2v and ChHV clustered together, except for HSV-2v isolate 4, which was again separate from HSV-2v but was also separate from HSV-2c (Fig. 6B). These data are consistent with the hypothesis of a new HSV-2 variant that presumably arose through recombination. Boot-scanning analysis of the US4 gene suggested that scattered recombination events concerning small regions had occurred between HSV-2v and ChHV, whereas no significant recombination signals were detected in the other genes analyzed in this study (data not shown).

DISCUSSION

HSV-2 is a DNA virus with low rates of mutation during replication, for which recombination constitutes an important mechanism of genomic evolution (15). The HSV DNA polymerase, which is required for the duplication of genomic DNA and the maintenance of genome integrity, possesses exonuclease activities to correct nucleotide misincorporation events (29). Low levels of intratypic variability (<0.5%) have been reported for the HSV-2 genes encoding the DNA polymerase and glycoproteins (9, 11, 12, 17). The high degree of genetic divergence of the UL30 (mean, 2.1%) and US4 (mean, 1.7%) genes reported here for 14 independent isolates is therefore highly suggestive of a new HSV-2 variant, which we have called HSV-2v (Tables 6 and 7). Phylogenetic results based on microsatellite markers and gene sequences showed that HSV-2v clearly differs from HSV-2c (Fig. 3 and 4).

HSV-2v was detected almost exclusively in patients originating from West or Central Africa, raising questions about the geographic origin of this new virus. Preliminary surveys performed in our laboratory revealed the presence of HSV-2v in Burkina Faso, a landlocked country in West Africa, and in Martinique, an island under French jurisdiction to which enslaved West Africans were transported in the past (data not shown). Obviously, further studies are required to explore the precise worldwide distribution of this new HSV-2v. Two clades of HSV-2 were recently described, based on the DNA sequences of the gG, gE, and gI genes and corresponding to isolates collected at different geographic sites: Tanzania and Scandinavia (12). The geographic distribution of HSV-2 strains may be similar to those of other human alphaherpesviruses, such as HSV-1 and varicella-zoster virus (VZV), for which several major clades closely related to geographic regions have been described (14, 30). According to specific clade definition criteria, HSV-2v could be referred to as a new African HSV-2 clade. However, despite the African origin of HSV-2v (West and Central Africa), the recently described unique amino acid signature in gG from an East African subset of HSV-2 isolates was not detected in the isolates studied here (31). Furthermore, all the HSV-2v isolates studied were obtained from different patients, nine of whom were also infected with HIV, probably reflecting the high prevalence of HIV infection in sub-Saharan African countries (4). Nevertheless, epidemiological and molecular investigations are required to study the specific impact of HSV-2v/HIV coinfection on pathogenesis. Whatever the HIV serostatus of the patient, HSV-2v infection did not seem to be associated with particularly severe or atypical clinical forms.

New viral variants may generate new challenges in terms of diagnosis, antiviral treatments, and vaccine development. HSV-2v displayed typical behavior in cell culture and in specific molecular tests targeting surface glycoproteins, suggesting that HSV-2v may be circulating undetected elsewhere. Only gG, which was highly divergent in HSV-2v, elicited a predominantly type-specific serological response (32). Viral genetic diversity, such as that observed for HSV-2v, may theoretically affect the performance of molecular detection tests. For example, molecular assays may fail to detect HSV-2v due to primer/probe mismatches in the conserved region of UL30 sequences targeted in these tests (33, 34). There is no easy way to identify HSV-2v with current serological tools. Only the molecular test described here can differentiate HSV-2v infections from HSV-2c infections. This work highlights the need to update public databases continually with the gene sequences of putative new viral variants to facilitate the development of reliable diagnostic assays. All 14 HSV-2v isolates identified were susceptible to ACV and FOS. However, the high degree of nucleotide diversity observed for the HSV-2v DNA polymerase gene might alter the fidelity and/or the proofreading activity of this enzyme, resulting in error-prone DNA synthesis. This would lead to an increase in the spontaneous mutation rate for UL23 TK, potentially favoring the generation of drug-resistant viral subpopulations. Next-generation sequencing (NGS) may facilitate investigation of this potential phenomenon during HSV-2v infection. The development of vaccines for preventing HSV-2 infection would also have significant medical implications. All promising HSV-2 vaccines to date, including the GlaxoSmithKline gD2 vaccine, have failed in clinical trials (35). Concomitant high levels of genetic diversity for the viral glycoproteins involved in viral entry (gD, gH/gL, and gB) might affect the antigenic properties of HSV-2v and the development of a new candidate vaccine in the future.

The differential clustering of HSV-2v isolates on phylogenetic trees (Fig. 4A and 6B) was suggestive of potential recombination with ChHV. No outliers are evident in Fig. 6. Homologous recombination requires closely related genomes. Intraspecific recombination is frequent in alphaherpesviruses, whereas interspecific recombination is possible but remains rare, particularly between animal and human viruses displaying low levels of sequence similarity (15). Our results indicate that all HSV-2v isolates harbor a specific UL30 molecular signature that perfectly matches a nucleotide segment in the ChHV UL30 gene. As previously reported, ChHV is a true chimpanzee simplex virus rather than a variant of human HSV transmitted to chimpanzees (28). Moreover, ChHV is closely related to, but different from, HSV-2, from which it is separated by a mean distance of ~8% (23). Thus, HSV-2v might be a true human HSV-2 that has acquired segments of the ChHV genome by recombination (Fig. 5 and 6). No other significant recombination signals were found in any of these genes, whereas

indicates the number of substitutions per site. Sequences of HSV-2v isolates 1 to 14 correspond to the identified HSV-2 variant isolates, marked with an asterisk, and sequences of HSV-2 isolates 15 to 18 and 19 to 22 correspond to HSV-2c isolates recovered from HIV-infected and HIV-negative patients, respectively. The corresponding nucleotide sequences for HSV-2 reference strains HG52 and SD90e (GenBank accession numbers Z86099 and KF781518, respectively); HSV-1 reference strains 17, F, H129, and KOS (GenBank accession numbers X14112, GU734771, GU734772, and JQ780693, respectively); Old World monkeys (cercopithecine herpesvirus 1, B virus [macaques], cercopithecine herpesvirus 16, HVP2 [baboons], cercopithecine herpesvirus 2, SA-8 [vervets], and ChHV [chimpanzees] [GenBank accession numbers AF533768, AY714813, DQ149153, and JQ360576, respectively]); and South American monkeys (HVS-1 [GenBank accession number HM625781]) are included.

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recombination has been shown to occur on many occasions in the HSV genome (36). A reasonable alternative to the recombination hypothesis is that the HSV-2v UL30 sequence is simply basal for HSV-2. Further studies are required to assess the precise degree of relatedness of HSV-2v and ChHV. Thanks to NGS, it should be possible to obtain a whole-genome sequence for HSV-2v, facilitating the identification of potential recombination events involving ChHV.

In conclusion, the novel variant of HSV-2 described here, HSV-2v, may constitute a new African HSV-2 clade. This virus does not seem to display significantly altered biological properties, a specific drug resistance pattern, or obvious enhanced virulence. However, this particular human simplex virus should be taken into account during diagnosis and for vaccination. Furthermore, this new HSV-2v highlights the feasibility of recombination between human and simian herpesviruses under natural conditions, and this may pose greater challenges in the future.

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