JOURNAL OF VIROLOGY, Mar. 1997, p. 2505–2511 Vol. 71, No. 3
0022-538X/97/$04.00+0

Strain Variability among Kaposi Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Genomes: Evidence that a Large Cohort of United States AIDS Patients May Have Been Infected by a Single Common Isolate

JIAN-CHAO ZONG,[1] CRAIG METROKA,[2] MARVIN S. REITZ,[3] JOHN NICHOLAS,[1] AND GARY S. HAYWARD[1]*

The Molecular Virology Laboratories, Department of Oncology, Johns Hopkins University School of Medicine,[1] and Institute of Human Virology, University of Maryland,[3] Baltimore, Maryland, and Memorial Hospital and Sloan-Kettering Institute, New York[2]

Received 6 August 1996/Accepted 22 November 1996

Previous analysis of the majority of Kaposi's sarcoma (KS) tumors, in both AIDS and non-AIDS populations, has revealed the consistent presence of two small subsegments (open reading frame 25/26 [ORF25/26] and ORF75) of a novel human gamma class herpesvirus genome referred to as KSHV or HHV-8. We have carried out DNA sequence comparisons with DNAs encompassing a total of 2,500 bp each over three separate PCR-amplified fragments from KS lesions and body cavity-based lymphoma (BCBL) samples from 12 distinct patients, including four African and two classical or endemic non-AIDS KS samples. The results revealed differences at 37 of 2,500 nucleotide positions (i.e., 1.5% overall variation). However, the 12 HHV-8 genomes examined fell into three distinct but very narrow subgroupings (A, B, and C strains). All A strain isolates differed from B strain isolates at 16 positions, but of the eight U.S. samples tested, six were A strains, and these differed at no more than two positions among them. Similarly, three of the four African samples were B strains, which differed from each other at only one position. The two C strain genomes also displayed only one nucleotide variation, but they differed from all A strains at 26 positions and from all B strains at 20 positions. One C strain genome was present in all six independent lesions from an AIDS KS patient with disseminated disease, and the other represented a mosaic A/C recombinant genome from the HBL6 cell line derived from a BCBL tumor. Evaluation of previous data suggests that B and C strains may predominate in Africa and that A strains predominate in classical Mediterranean samples. Although both B and C strains are represented in U.S. AIDS patients, the majority (70 to 80%) of samples from the mid-East Coast region at least appear to be virtually identical, supporting the concept that they may all derive from the spread during the AIDS epidemic of a single recently transmitted infectious agent.

Kaposi's sarcoma (KS) has been strongly implicated as having an infectious agent-like distribution (which is independent of human immunodeficiency virus [HIV]) in male homosexual AIDS patients, where it was also more aggressive and more common in the early 1980s than it is now (4, 5, 27). The classical form of KS skin lesion or nodules in elderly men of Mediterranean heritage described by Kaposi was claimed to be associated with a herpesvirus long before the AIDS epidemic developed. Boldogh et al. (6, 17) reported both herpesvirus-like particles, anti-cytomegalovirus (CMV) serology, and CMV antigens and nucleic acids in KS lesions. However, we and others could not confirm the claims for a human CMV (HCMV) association by Southern blotting or in situ hybridization with cloned HCMV DNA probes, with a set of matched tumor and uninvolved skin DNA samples of non-AIDS-associated African endemic KS from Zaire (2). Nevertheless, in late 1994, Chang et al. (12) reported the isolation and DNA sequence of two small fragments of a novel gamma herpesvirus-like genome (KSHV) within an AIDS KS DNA sample. These fragments were isolated by representational differential hybridization analysis, a technique developed by M. Wigler's group for detection of minor unique copy differences between two related mammalian genomes (25).

DNA sequences from KSHV (now more frequently referred to as HHV-8) have subsequently been identified by PCR in virtually every KS sample tested, whether from homosexual AIDS patients or from endemic African and classical Mediterranean HIV-negative KS sources (3, 7, 9, 13, 15, 19, 23, 31). They have also been described in a small fraction of AIDS non-Hodgkin lymphomas, especially body cavity-based lymphoma (BCBL) (11) and multicentric Castleman's disease (36), and, occasionally, in normal apparently uninvolved skin samples (although at much lower levels than in the adjacent KS tissue itself). KS is only rarely found in nonhomosexual AIDS patients in the United States, and there are occasional HHV-8 DNA-positive homosexual KS patients who are HIV negative. KS also is rare in HIV-positive hemophiliacs, but it may occur occasionally in HIV-negative renal and cardiac transplant patients. Some recent studies suggest that HHV-8 is detectable in peripheral mononuclear cells in up to 20% of homosexual AIDS patients (37), although reports of wider occurrence (34) are still largely controversial (1, 8). Recent serology studies (16, 20) found that antibodies to HHV-8 are present in the serum from most KS patients as well as in a moderate to high proportion of normal sera from those parts of the world associated with the classic and endemic forms of KS (70% in Uganda, 5 to 20% in Italy and Greece). However, seropositiv-

* Corresponding author. Mailing address: Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, 725 N. Wolfe St., WBSB 317, Baltimore, MD 21205. Phone: (410) 955-8684. Fax: (410) 955-8685.

ity in normal non-AIDS sera from elsewhere (the United States, United Kingdom, Japan, etc.) is extremely low. These results lead to major questions about whether HHV-8 infection shows the ubiquitous widely distributed pattern typical of all other human herpesviruses, or whether it occurs only in isolated pockets of the human population, where it may be spreading from a relatively recent exogenous nonhuman source. It is also not known at present whether the HHV-8 genomes found in KS tumors, etc., represent newly acquired infectious virus or reactivation from cryptic latent infections in response to immunosuppression associated with HIV or some other as yet unknown agent.

We have consistently found both the open reading frame 26 (ORF26) and ORF25 HHV-8 DNA fragments, together with an additional third HHV-8 locus encompassing a viral thymidylate synthetase gene, in 18 of 18 KS lesion and BCBL sources tested by direct nonnested PCR analysis (32), but have not found them in any abundance in saliva or enlarged lymph nodes from patients with HIV-associated lymphadenopathy or in numerous control DNA samples from other normal or tumor tissue. HHV-8 DNA has not been found to be retained in any of the spindle-shaped KS cell lines that have been developed over the years from KS lesions (26, 30), leading to as yet unanswered questions about whether the virus is present only in infiltrating inflammatory cells and whether it is present in a lytic or latent mode in KS tumors. However, HHV-8 DNA sequences have been reported to be 50 times more abundant in BCBL tumor cell lines than in KS lesion samples, although most of the former also carry Epstein-Barr virus (EBV) genomes, and are clonal but without c-*myc* rearrangements (11, 22). An HHV-8-positive BCBL line without EBV present has also been described recently and was shown to produce herpesvirus-like particles after tetradecanoyl phorbol acetate induction (35).

The three herpesviruses that are most homologous to HHV-8 are all gamma class herpesviruses. EBV and herpesvirus saimiri (HVS) have been classified as prototypes of the gamma-1 and gamma-2 subclasses, respectively, because they differ greatly in (G+C) content, genome structure, and gene arrangement and have a selective ability to transform and establish latent states in B cells or T cells. Isolates of both HVS and EBV fall into several distinct subtype categories. Three groups of HVS genomes, referred to as A, B, and C, differ primarily in gene organization at the left-hand end of the genome, a region which is associated with biological differences in the ability to transform T cells. In EBV, most isolates fall clearly into one or the other of the A and B subtypes, which have exactly the same gene content, but are characterized by having highly distinctive alleles for EBNA-1, EBNA-2, EBNA-3, and the EBERs, with the EBNA-2 protein in particular retaining only 54% identity (14), which is a significant fraction of the evolutionary distance (35% identity) between either subtype of human EBNA-2 and the baboon EBV (herpesvirus papio) version (24). EBV-A and -B subtypes differ in their efficiency of transforming primary B lymphocytes, and both subtypes coexist in some AIDS patients, although type A isolates predominate in posttransplant lymphoproliferative disease. Type A isolates predominate in the United States and type Bs predominate in Africa, but some isolates especially from AIDS patients appear to represent recombinants between the two.

Sources of KS and BCBL DNA and gammaherpesvirus DNA samples. A variety of discarded KS-related autopsy and biopsy samples from both AIDS and non-AIDS patients were acquired from the Memorial Hospital and Sloan-Kettering Institute (New York, N.Y.) and from Zaire in 1983 and 1984 (2), and a variety of additional samples were collected from Johns Hopkins Hospital and the National Institutes of Health in 1995. These included AIDS KS autopsy samples labelled C282 and ASM70 to -80 (New York, N.Y., 1984); endemic African KS DNA biopsies 431KAP and 431NSC (Zaire, 1984); AIDS-associated female KS DNA biopsies TS1, TS2, and TS3 (Uganda, 1995); fresh frozen AIDS-associated AKS1 and non-AIDS-associated EKS1 lesion biopsies (Baltimore, Md., 1995); an AIDS KS lesion DNA sample referred to as AKS2 (National Institutes of Health, 1995); and a paraffin-embedded AIDS-associated KS lesion, AKS4 (Baltimore, Md., 1995). Other HHV8-positive samples included the BCBL tumor DNA (BCBL-R; National Institutes of Health, 1995) that was used to prepare our phage lambda HHV8 genome library (32) and DNA from the HHV-8- plus EBV-positive HBL6 cells, which are derived from the BCL1 cell line described by Cesarman et al. (10). DNA from all tumor, biopsy, and tissue or cell culture samples were extracted with pronase and phenol followed by ethanol precipitation, dialysis, and treatment with Chelex. Confirmation of the presence of HHV8 DNA sequences was initially carried out by direct PCR band amplification with the ORF26, ORF75, UPS75, and TS primer pairs as described in the accompanying report by Nicholas et al. (32).

DNA Source		500-bp ORF26 (895) 926	* 981	989	* 1032	1033	1055	* 1086	1094	* 1132	* 1139 (1395)	Subgroup
1.KSHV AIDS°	Col	A	T	C	C	(C)	G	C	G	A	A	A′
2.BC1 AIDS Lym°	Col	(T)	T	C	C	T	G	C	G	A	A	A
3.BC2 AIDS Lym°	Col	(T)	(C)	(G)	(A)	T	(T)	C	G	(G)	(C)	B′
4.ST1 AIDS KS	Uganda	A	(C)	C	C	T	G	(T)	G	A	(C)	C
5.ST2 AIDS KS	Uganda	A	(C)	C	(A)	T	G	C	G	(G)	(C)	B
6.ST3 AIDS KS	Uganda	A	(C)	C	(A)	T	G	C	G	(G)	(C)	B
7.C282 AIDS KS LN	NY	A	T	C	C	T	G	C	G	A	A	A
8.ASM70 Lung KS	NY	A	(C)	C	C	T	G	(T)	(A)	A	(C)	C
ASM72 Lung KS-2	NY	A	(C)	C	C	T	G	(T)	(A)	A	(C)	C
ASM74 Skin KS	NY	A	(C)	C	C	T	G	(T)	(A)	A	(C)	C
ASM75 Hylar LN KS	NY	A	(C)	C	C	T	G	(T)	(A)	A	(C)	C
ASM77 Lung KS-3	NY	A	(C)	C	C	T	G	(T)	(A)	A	(C)	C
ASM80 Lung KS-1	NY	A	(C)	C	C	T	G	(T)	(A)	A	(C)	C
9.431KAP Endemic KS	Zaire	A	(C)	C	C	T	G	C	G	(G)	(C)	B
431NSC Normal Skin	Zaire	A	(C)	C	C	T	G	C	G	(G)	(C)	B
10.AKS1 AIDS KS	Bal	A	T	C	C	T	G	C	G	A	A	A
11.AKS2 AIDS KS	NIH	A	T	C	C	T	G	C	G	A	A	A
12.AKS4 AIDS KS	Bal	A	T	C	C	T	G	C	G	A	A	A
13.BCBLR AIDS Lym	NIH	A	T	C	C	T	G	C	G	A	A	A
14.HBL6 AIDS Lym	Col	A	T	C	C	T	G	C	G	A	A	A
15.EKS1 Non-AIDS KS	Bal	A	T	C	C	T	G	C	G	A	A	A

°Data from Chang et al. (1994) and Cesarman et al. (1995)
*Key subgroup indicator loci.

FIG. 1. Sequence variability and similarity among HHV-8 samples from different sources (block I, the ORF26 gene). A summary of the results of PCR DNA sequence analysis over a 500-bp region of ORF26 for 18 HHV-8-positive DNA samples from patients 4 to 15 and comparison with published data for three samples (patients 1 to 3) obtained by groups at the Columbia (Col) College of Medicine, New York, are given (11, 12). Only data for the variable positions are included, with all differences from our prototype strain A sample pattern (AKS1) being designated by circled nucleotides. The positional nomenclature used for ORF26 follows that of Chang et al. (12). The ORF26 primers used to give a 330-bp product were LGH 1701 5′-GGATGGATCCCTCTGACAACC-3′ and LGH 1702 5′-ACGTGGATCCGTGTTGTCTACG-3′, based on the KS330 Bam fragment of Chang et al. (12). Lym, lymphoma; LN, lymph node; Bal, Johns Hopkins University School of Medicine, Baltimore, Md.; NIH, National Institutes of Health; NY, Memorial Hospital and Sloan-Kettering Institute, New York.

DNA sequence variability among HHV-8 samples within the ORF26 region. Nucleotide sequence data from PCR-amplified products of a 500-bp DNA segment encompassing the original ORF26 fragment have been compiled from 16 HHV-8-positive KS lesion samples and two BCBL tumors (Fig. 1). The comparison includes the original published data from the Chang et al. (12) KSHV sample (Fig. 1, row 1) and two BCBL samples (BCL1 and BCL2) (10) from Columbia University (Fig. 1, rows 2 and 3). Deviations from our prototype KS sequence (AKS1)

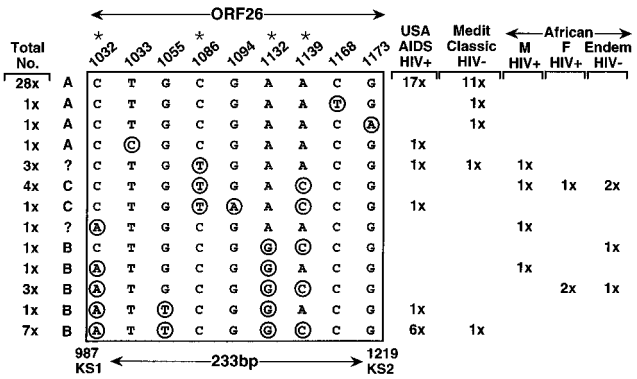

FIG. 2. Sequence variability among all KS samples reported. The data are compiled from the 12 patients in our studies, together with the results presented by Cesarman et al. (11), Collandre et al. (13), Huang et al. (19), Kemeny et al. (21), and Moore and Chang (31) over the 233-bp region of ORF26 between the KS1 and KS2 primers originally recommended by Chang et al. (12). Variations from our AKS1 strain A pattern are indicated by circles, and the number of distinct examples of each genotype are listed by total on the left and under geographic epidemic, classical, and endemic categories on the right. Asterisks denote positions that are considered key strain designators (see Fig. 5). Medit, Mediterranean; M, male; F, female; Endem, endemic.

are indicated at the circled positions. Note that the ORF26 HHV-8 block represents a well-conserved virion protein-coding region displaying 60% amino acid identity among the HHV-8 and other gamma herpesviruses versions. The analyses of all three female African AIDS patient samples (ST1, ST2, ST3 [Fig. 1, rows 4, 5, and 6]) and the AKS1 KS sample (row 10) were carried out both directly on the PCR products and after TA tail cloning of a separate batch of PCR products into the pBluescript plasmid, without any discrepancies between the two procedures. Similarly, a KS lesion sample and a positive adjacent (supposedly uninvolved) skin sample from an endemic HIV-negative Zaire patient 9 (431KAP and 431NSC [Fig. 1, row 9]) also showed identical and unique sequence patterns, as did two of the three independent lesion samples from African HIV-positive females (ST2 and ST3 [Fig. 1, rows 5 and 6]). All six skin and visceral KS lesions sequenced from a single New York AIDS patient (samples ASM70 to -80; row 7) proved to be identical to one another, but differed significantly from all other isolates described. Overall, the nucleotide variability seen among the eight distinct genome types was 2.0% over 500 bp for ORF26, although only a few of these variations lead to amino acid changes.

Division into three subgroups. Comparison of combined data from five published studies (11, 13, 19, 21, 31) plus our own results for a total of 53 distinct HHV-8-positive KS isolates over the somewhat limited distance of the 230-bp standard ORF26 PCR fragment (12) revealed interesting patterns (Fig. 2). First, although there were nine polymorphic nucleotide positions overall, 23 of the 27 U.S. AIDS patient samples reported fell into just two subgroups—those shown as the A and B prototypes on the top and bottom lines in Fig. 2. The major exceptions were the samples from our multiple-lesion ASM70 patient referred to as subgroup C; otherwise, the three other samples showed only 1-bp changes from the A or B prototypes, including the original sample described by Chang et al. (12). Second, the majority of the classical Mediterranean samples reported (11 of 15) also fell into the prototype A subgroup. Third, none of 12 African samples matched precisely either of the U.S. AIDS or classic KS A and B prototype genotypes, but rather represented scattered slightly diverged variants (six in all) of primarily B prototypes and included some putative C strains resembling ASM70. Overall, the samples fell into 13 distinct genotypes, but most notably fully half (28 of 53) were identical over this range forming the prototype A subgroup. Seven more samples have the prototype B subgroup pattern, which differs from the A group pattern at four of the nine variable positions. Importantly, none of the 12 African samples fell into either of these two extremes, but rather all showed more individual variability. Obviously, this represents fairly limited data from just a short region of the genome, and some of the samples examined might be epidemiologically or geographically related (19). However, even within our more extended studies, the same narrow but polarized A, B, and C patterns persisted over the larger 500-bp segment of ORF26 (Fig. 1) and also at the second and third genomic locations as described below (ORF75 and UPS75).

Confirmation of subgrouping within and upstream from the ORF75 gene region. To extend the analysis over an additional larger region, we also carried out direct PCR sequencing of the same sets of KS and BCBL samples over two adjacent segments of the HHV-8 ORF75 gene. The second segment (block II) involved a 515-bp block of HHV-8 ORF75 described by Chang et al. (12), which represents a relatively poorly conserved N-terminal portion of that protein with less than 30% amino acid identity among HHV-8 and the three other sequenced gamma herpesvirus versions. The third segment (block III) involved an adjacent 1,520-bp region encompassing the remainder of the N terminus of ORF75 and 860 bp of sequence upstream from the ORF75 initiator codon (positions 1341 to 1343), which was first sequenced as the right-hand end of a phage λ clone (λ B5-1) insert derived from our BCBL-R genomic library (32) (GenBank accession no. U85269). Each DNA sample was cycle sequenced on both strands from multiple PCR-amplified products derived from a series of 15 distinct primers covering the region. Among the 12 different HHV-8 patient samples, all 7 of the A subgroup genomes were identical within the 515-bp ORF75 block (Fig. 3), although the original representational differential hybridization analysis

DNA Source		515-bp ORF75 (115) 159 167 192 237 417 442 462 496 528 563 618 (630)	Subgroup
1.KSHV AIDS°	Col	A C G G C T A C G A T	A′
4.ST1 AIDS KS	Uganda	A T G G C C A T G A T	A
5.ST2 AIDS KS	Uganda	G T G G T C G T G G C	B
6.ST3 AIDS KS	Uganda	G T G T C G T G G C	B
7.C282 AIDS KS LN	NY	A T G G C C A T G A T	A
8.ASM70 Lung KS	NY	G T G A C C G T T G C	C
ASM72 Lung KS-2	NY	G T G A C C G T T G C	C
9.431KAP Endemic KS	Zaire	G T G G T C G T G G C	B
10.AKS1 AIDS KS	Bal	A T G G C C A T G A T	A
11.AKS2 AIDS KS	NIH	A T G G C C A T G A T	A
12.AKS4 AIDS KS	Bal	A T G G C C A T G A T	A
13.BCBLR AIDS Lym	NIH	A T G G C C A T G A T	A
14.HBL6 AIDS Lym	Col	G T A A C C G T T C C	C
15.EKS1 Non-AIDS KS	Bal	A T G G C C A T G A T	A

°Data from Chang et al. (1994).
*Key subgroup indicator loci.

FIG. 3. Sequence variability and similarity among HHV-8 samples from different sources (block II, the ORF75 gene). A summary of the results of PCR DNA sequence analysis for 13 HHV-8-positive DNA samples from patients 4 to 15 over a 515-bp segment of ORF75 is given. Only those nucleotides present at variable positions are given, with positions that differ from those of our prototype AKS1 strain A sample enclosed in circles. The ORF75 primers used to give a 530-bp product were LGH 1984 5′-CTAGAGATCTGTTTAGTCCGGAG-3′ (positions 115 to 131) and LGH 1704 5′-GTACGGATCCACGGAGCATAC-3′ (positions 605 to 631) based on the KS631 Bam DNA sequence of Chang et al. (12). Col, Columbia College of Medicine, New York; NY, Memorial Hospital and Sloan-Kettering Institute, New York; Bal, Johns Hopkins University School of Medicine, Baltimore, Md.; NIH, National Institutes of Health; Lym, lymphoma; LN, lymph node.

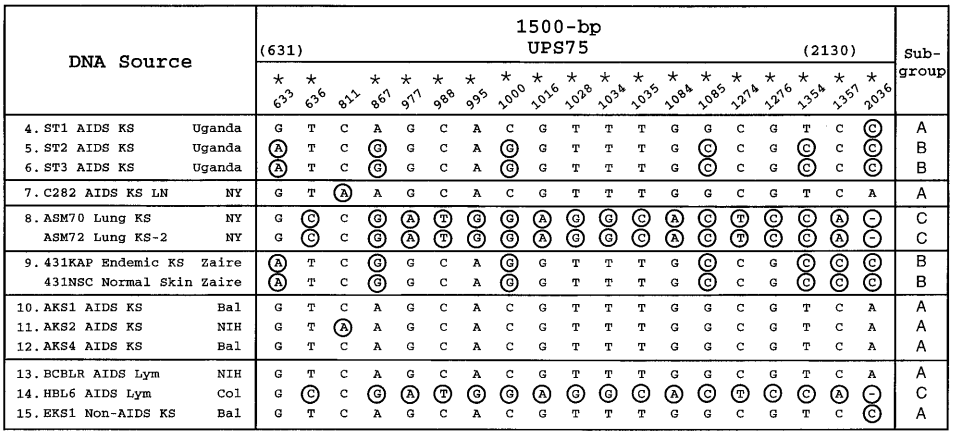

FIG. 4. Sequence variability and similarity among HHV-8 samples from different sources (block III, the UPS75 gene region). A summary of the results of PCR DNA sequence analysis for 14 HHV-8-positive DNA samples from patients 4 to 15 over a 1,500-bp segment immediately to the right of ORF75 encompassing the N terminus and upstream noncoding region of ORF75 is given. Only those nucleotides present at variable positions are given, with positions that differ from those of our prototype AKS1 strain A samples enclosed in circles. No data for this region of the original KSHV DNA (patient 1 [12]) are available. The complete 2,151-bp sequence of the combined ORF75/UPS75 blocks for BCBL-R is available from GenBank (accession no. U85269). Multiple overlapping PCR primer pairs were used. The most useful minimal set to cover the 15 variable positions between 618 and 1,278 were LGH 2000 5′-GGAAACAGGGTGCTGTG-3′ (positions 541 to 557) and LGH 2051 5′-CTCAGTTTATCAGGGCA-3′ (positions 882 to 898), giving a 350-bp product, and LGH 2070 5′-GGTTCTAGCAGGGTCTGT-3′ (positions 907 to 924) and LGH 2034 5′-CATGGCCTACGACGTCAC-3′ (positions 1327 to 1344), giving a 415-bp product. NY, Memorial Hospital and Sloan-Kettering Institute, New York; LN, lymph node; Bal, Johns Hopkins University School of Medicine, Baltimore, Md.; NIH, National Institutes of Health; Lym, lymphoma; Col, Columbia College of Medicine, New York.

KSHV sample of Chang and Moore (referred to as subtype A′) differs at three positions. The five B or C strain samples showed between 5- and 6-bp differences each from the prototype A strain pattern over a total of 11 variable positions. The nucleotide position nomenclature used throughout these studies conforms to that introduced by Chang et al. (12), despite the fact that ORF75 is oriented in the opposite direction compared to ORF26 in the viral genome.

Extension of this type of analysis even further into the adjacent 1,520-bp region upstream from the original ORF75 PCR fragment (UPS75 in Fig. 4) revealed 18 more variable positions. All samples that were considered B or C type within the ORF75 block again fell into the B or C subclass within the upstream UPS75 block. Furthermore, AKS1, AKS4, and BCBL-R all proved to be identical over the entire 1,500 bp evaluated, whereas C282, AKS2, and EKS1 showed only 1-bp changes (at position 811 or 2036 in UPS75). On the other hand, the variant C strain ASM70 differed from C282 at 18 positions over this stretch (1.1%). Among these specimens, the AIDS KS lesions (C282 and AKS1) were biopsied 12 years apart in New York and Baltimore, yet they showed only 1-bp differences over the entire 2,500-bp sequenced.

Diagnostic positions and recombinant genomes. Overall, among a total of 38 variable positions out of 2,500 that we have now identified (1.5% overall variation), 10 of the nucleotide changes at these loci occur only in the A strain pattern, 6 occur in the B strain pattern, and 15 occur in the C strain pattern. Therefore, these are considered key diagnostic positions and are summarized in reverse type in Fig. 5. Interestingly, the A and B subgroups differed by only 1 bp (at position 2036) be-

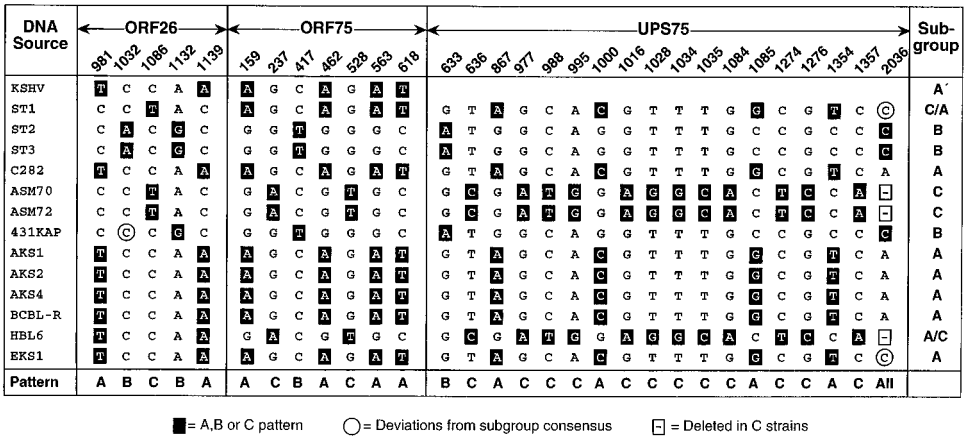

FIG. 5. HHV-8 A, B, and C strain or subgroup indicator loci. A summary of the sequence changes (white type in black boxes) at 28 variable positions in blocks I (ORF26), II (ORF75), and III (UPS75) that are considered diagnostic for the three HHV-8 genomic subgroups described here is shown. Rare variations that do not fit the A, B, or C consensus patterns are circled. The position numbers given match those denoted by asterisks in Fig. 1, 3, and 4. Note that the ASM70 and ASM72 samples represent two distinct KS lesions from the same patient.

tween UPS75 positions 1360 and 2130, but all three C strain DNA samples examined (ASM70, ASM72, and HBL6) appeared to be deleted over much of this region (Fig. 5), because they failed to yield PCR-amplified products with at least four sets of primers that were effective with all other HHV-8 samples (32).

Finally, the HBL6 cell line sample derived from BCBL1 cells (10) proved to be closely related to the ASM70 series C strain sample, except that in this case, the ORF26 locus (Fig. 1) and the IE1-B region (not shown) near ORF11 in the divergent locus B (DL-B) (32) both resembled the typical A strain pattern, whereas the ORF75 and UPS75 blocks (Fig. 3 and 4) were both matched closely to the C strain pattern of ASM70. ST1 showed a similar type of switch between the ORF26 and ORF75 regions. Therefore, both HBL6 and ST1 are considered mosaics or recombinants between the A/C and C/A strains, respectively (Fig. 5). The alternative possibility that these patients may instead have dual infections and that some primers demonstrated selectivity is ruled out in the ORF75/UPS75 blocks at least, where a large number of independent primers were used, and all variant positions were confirmed on both strands from multiple PCR products.

In summary, all 12 separate patient samples fell into one of three distinct subgroupings, namely A, B, and C, with the B strain prototype (represented by 431KAP) differing from the A strain (represented by AKS1) at 15 positions and from the C subgroup (represented by ASM70) at 20 positions, whereas the C prototype (ASM70) differed from the A prototype (AKS1) at 26 positions and from the B prototype (431KAP) at 20 positions. In contrast, the seven A subgroup samples differed among themselves at only one of two positions. Similarly, the three B subgroup samples (ST2, ST3, and 431KAP) varied at only one position, and the two C isolates (ASM70 and HBL6) also differed at only one position within the entire 2,000-bp ORF75/UPS75 region.

Variability in the EBV BNRF1 gene equivalent to that in ORF75 of HHV-8. For comparative purposes, we also examined strain variability in the N-terminal segment of the BNRF1 gene of EBV (which is the EBV homolog of HHV-8 ORF75) in the region that matches the 515-bp sequenced block II in HHV-8. This is a relatively poorly conserved lytic cycle protein, with only 28 to 32% amino acid identity within this segment among the EBV, EHV-2, HVS, and HHV-8 versions. Total cell DNA from cultured human Raji and Jijoye B lymphoblasts and from marmoset B95-8 cells carrying latent-state human EBV genomes was prepared by pronase and phenol extraction followed by ethanol precipitation, dialysis, and treatment with Chelex for direct PCR amplification and sequencing (Fig. 6). Among the six EBV strains analyzed (including four B subtypes and one A subtype based on parallel analysis of their EBNA-1 gene sequence patterns [38]), we found almost exactly the same limited level of nucleotide variability (i.e., 9 of 650 bp, or 1.5% overall) as that observed among the HHV-8 strains (10 of 515 bp, or 1.9%). Although the data do not yet allow us to answer whether we can distinguish between typical EBV subtype A versus subtype B patterns within the lytic cycle BNRF1 gene, they do provide evidence that HHV-8 strains may be just as well established and diverged in the human population as is EBV.

Biological role and distribution of HHV-8. There is considerable debate at present about whether HHV-8 is a typical ubiquitous human herpesvirus that is carried as widespread latent infections in all human populations or is instead a relatively rare pathogenic agent of either human or nonhuman origin. If it behaves as a typical herpesvirus within its natural host, HHV-8 should be highly infectious by aerosol or cell-to-cell contact, with most individuals becoming infected (probably asymptomatically) at a very early age, followed by the establishment of a latent state in some specialized cell type or location. Like EBV, it might usually only be associated with occasional tumors or other disease situations under specific rare circumstances, and like all other human herpesviruses, it might also be expected to become reactivated by immunosuppression. Alternatively, HHV-8 infection may be common only in geographical areas associated with classical and endemic KS, or it may have very limited infectability, perhaps being transmitted only sexually or as infected cells, for example. Under these circumstances, the virus might be a relatively recent acquisition of nonhuman origin that would be likely to show very little strain variability. Although they are generally expected to have highly adapted asymptomatic interactions within their natural host species, and all available evidence points to herpesvirus evolution as having been predominantly or exclusively concordant with cospeciation of their mammalian hosts (28, 29), there are sometimes devastating consequences after accidental infection of a closely related species (e.g., rhesus B virus in humans and squirrel monkey HVS in marmosets). On balance, the epidemiological occurrence of KS in up to 25% of male homosexual AIDS patients, but much lower rates (2 to 3%) in AIDS patients in other risk categories, correlates with recent estimates of serological positivity for HHV-8 (16, 20). Similar evidence for KS being associated with an infectious agent other than HIV in homosexual male AIDS patients (4, 5) and seroconversion to HHV-8 in KS patients (37), together with the tight association of high-level HHV-8 DNA copy number with KS lesions, suggests that HHV-8 is indeed likely to be both an infectious and causative agent of KS, but one that may have a very restricted geographical distribution or transmissibility.

Significance of HHV-8 strain variability. Based on the original 230-bp segment of UL26, greater than 90% of all U.S. AIDS-associated KS and BCBL samples examined so far fit tightly into either the A or B strain subgroups referred to above, but with no more than a 1-bp change each among 19 isolates for group A and 7 isolates for group B (Fig. 2). The apparent lack of intrastrain variation was confirmed by extending the analysis over three larger regions (500 bp of ORF26, 515 bp of ORF75, and 1,500 bp of UPS75) for all 12 distinct genomes examined here (Fig. 1, 3, and 4). The 1.5% overall

Isolate Name [Subtype]		650bp BNRF1 (2534)								(3184)
		2640	2715	2719	2794	2809	2850	2971	3016	3112
1. B95-8	[A]	A	G	C	C	C	A	C	C	T
2. Jijoye	[B]	A	(A)	C	C	C	(G)	C	(A)	(C)
*P3-HR1	[B]	A	(A)	C	C	C	(G)	C	(A)	(C)
3. Namalwa	[?]	(G)	G	C	C	(T)	A	(T)	(A)	(C)
4. AG876	[B]	(G)	G	(T)	(T)	C	A	(T)	C	(C)
5. Raji	[B]	(G)	G	C	C	(T)	A	(T)	C	(C)

*P3-HR1 is a deleted non-transforming subclone of the Jijoye cell line.

FIG. 6. DNA sequence variability of the equivalent N-terminal region of the EBV homolog of HHV-8 UL75 (block II). A summary of the results of PCR DNA sequence analysis of six distinct EBV-positive lymphoblastoid cell lines over a 631-bp segment of the BNRF1 gene is given. Positions 2534 to 3184 represent nucleotides downstream from the ATG initiator codon of BNRF1 and are approximately equivalent to the homologous region encompassing the 515-bp segment of HHV-8 ORF75 that was evaluated in Fig. 3. Again, only variable positions are shown, with nucleotides that differ from the prototype EBV type A pattern of isolate B95-8 enclosed in circles.

variability seen among HHV-8 isolates within the three gene blocks analyzed so far (38 of 2,500 positions) appears somewhat small compared to the generally quoted values among isolates of other herpesviruses of 3% average DNA sequence divergence for HSV isolates, 4% divergence for EBV isolates, and 5% divergence for HCMV isolates (18). However, different herpesvirus genes evidently evolve at very different rates, and the results are highly dependent on the levels of amino acid conservation required to maintain functional integrity. Therefore, the only numbers that have any real value are comparisons of the same regions of the equivalent genes among different viruses from within at least the same subclass as we have done here for ORF75 of HHV-8 and BNRF1 of EBV.

In the case of EBV, the classification into distinct A and B subtypes depends primarily on large differences among their latency genes, especially EBNA-2, which seems to be driving the biological divergence with 45% amino acid variability, although significant and diagnostic differences are also apparent in the less highly diverged EBERs, EBNA-3C and EBNA-1 proteins. LMP is also highly variable, but appears to be evolving independently of the nuclear latency gene patterns. Finally, nothing is known about possible EBV variability in lytic cycle genes. Similarly, in HVS, although the three subgroups display major differences in their left-hand-end transforming regions, little is known about the level of variability in other parts of the genome. In HHV-6, isolates are divided into A or B subtypes based on an average of 5% nucleotide differences in known lytic cycle genes, together with greater variations in the MIE locus.

We do not wish to imply that the three HHV-8 subgroups (or strains) described here necessarily represent an equivalent level of divergence to that seen in the A and B EBV subtypes, which are based primarily upon characteristic differences among the latent-state nuclear proteins. Admittedly, as we have shown here, differences among lytic cycle genes between the two EBV subtypes also appear to be small (only 1.9% at the DNA level), but it is not possible at present to address the issue of potential equivalent subtype variation for latent-state HHV-8 genes, since none have yet been identified.

Based on our finding that all 12 HHV-8 genomes that we tested fell into one of three distinct subgroups, the strain variation analysis suggests that HHV-8 is probably an ancient and well-established human virus, but one that is nevertheless relatively rare in most human populations and that has been geographically isolated in several small subpopulations until very recently. The alternative possibility that it has been acquired from the same exogenous nonhuman host species source on at least three separate occasions appears remote. Either way, the AIDS epidemic is now providing opportunities to scramble some of the differences by recombination. The finding of identical novel C strain genomes in all six lesions tested of the patient with aggressive disseminated disease raises that possibility that it may be possible in the future to test for clonality of the KS tumor cells at the different sites by terminal heterogeneity analysis of the type applied successfully to EBV genomes in tumor samples (33).

Evidently, the A strains of HHV-8 predominate in areas associated with classical KS, whereas the B and C strains are more prevalent in Africa. All three strains are represented among American AIDS patient samples and in BCBL cell lines. However, most significantly, many HHV-8 genomes from East Coast U.S. AIDS samples are virtually identical over the entire 2,500 bp tested, which includes a region encompassing a large segment from the relatively poorly conserved N terminus and the adjacent upstream noncoding block of the ORF75 gene. Therefore, these results support the notion that most HHV-8 genomes occurring in U.S. AIDS patients in particular derive from only a very small number of separate virus isolates, with a great preponderance (perhaps more than 75%) being of just a single distinct A genome substrain. This pattern is very distinct from the usual spectrum of variable and unrelated isolates found for the other ubiquitously distributed herpesviruses that usually can be distinguished from each other easily by restriction enzyme digest patterns. Although it will be important to determine whether the classical Mediterranean KS-associated viruses may also display equally limited variability and to resolve whether or not additional strains or subgroups occur, it seems most likely at present that just a very small number of distinct infectious exogenous isolates have been transmitted horizontally within the AIDS epidemic.

These studies were funded by DHEW research grants U01 CA37314, P30 CA06973 (pilot project supplement), and R01 CA73585 to G.S.H. from the National Institutes of Health.

We thank Marshall Horwitz (Einstein College of Medicine, New York); Richard Ambinder, Tom Horn, Dave Sidranski, and Tom Quinn (all of Johns Hopkins School of Medicine); and Kuan-Teh Jeang (National Institutes of Health) for providing clinical samples for analysis. We also gratefully acknowledge a gift of the HBL6 cells from Patrick Moore and Yuan Chang (Columbia College of Medicine, New York). Beverly Lambe provided technical expertise, and Pamela Wright and Sarah Heaggans assisted in preparation of the manuscript.

REFERENCES

1. **Adams, V., W. Kempf, M. Schmid, B. Muller, J. Briner, and G. Burg.** 1995. Absence of herpesvirus-like DNA sequences in skin cancers of non-immunosuppressed patients. Lancet **346:**1715–1716.
2. **Ambinder, R. F., C. Newman, G. S. Hayward, R. Biggar, M. Melbye, L. Kestens, E. Van Marck, P. Piot, P. Gigase, P. B. Wright, and T. C. Quinn.** 1987. Lack of association of cytomegalovirus with endemic African Kaposi's sarcoma. J. Infect. Dis. **156:**193–197.
3. **Ambroziak, J. A., D. J. Blackbourn, B. G. Herndier, R. G. Glogan, J. H. Gullett, A. R. McDonald, E. T. Lennette, and J. A. Levy.** 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science **268:**582–583.
4. **Beral, V.** 1991. Epidemiology of Kaposi's sarcoma. Cancer Surv. **10:**5–22.
5. **Beral, V., T. A. Peterman, R. C. Berkelman, and H. W. Jaffe.** 1990. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet **335:**123–128.
6. **Boldogh, P., E. Beth, E.-S. Huang, S. K. Kyalwazi, and G. Giraldo.** 1981. Kaposi's sarcoma. IV. Detection of CMV DNA, CMV RNA and CMNA in tumor biopsies. Int. J. Cancer **28:**469–474.
7. **Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss, and J. J. O'Leary.** 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nature Med. **1:**1274–1278.
8. **Boshoff, C., S. Talbot, M. Kennedy, J. O'Leary, T. Schulz, and Y. Chang.** 1996. HHV8 and skin cancers in immunosuppressed patients. Lancet **347:** 338–339.
9. **Boshoff, C., D. Whitby, T. Hatziioannou, C. Fisher, J. van der Walt, A. Hatzakis, R. Weiss, and T. Schulz.** 1995. Kaposi's-sarcoma-associated herpesvirus in HIV-negative Kaposi's sarcoma. Lancet **345:**1043–1044.
10. **Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N. Engl. J. Med. **332:**1186–1191.
11. **Cesarman, E., P. S. Moore, P. H. Rao, G. Inghirami, D. M. Knowles, and Y. Chang.** 1995. In vitro establishment and characterization of two AIDS-related lymphoma cell lines containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. Blood **86:**2708–2714.
12. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869.
13. **Collandre, H., S. Ferris, O. Grau, L. Montagnier, and A. Blanchard.** 1995. Kaposi's sarcoma and new herpesvirus. Lancet **345:**1043.
14. **Dambaugh, T., K. Hennessey, L. Chamnankit, and E. Kieff.** 1984. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr virus nuclear antigen 2. Proc. Natl. Acad. Sci. USA **81:**7632–7636.
15. **Dupin, N., M. Grandadam, V. Calvez, I. Gorin, J. T. Aubin, S. Havard, F. Lamy, M. Leibowitch, J. M. Huraux, and J. P. Escande.** 1995. Herpesvirus-like DNA sequences in patients with Mediterranean Kaposi's sarcoma. Lancet **345:**761–762.
16. **Gao, S.-J., L. Kingsley, M. Li, W. Zheng, C. Parravicini, J. Ziegler, R.**

Newton, C. R. Rinaldo, A. Saah, J. Phair, R. Detels, Y. Chang, and P. S. Moore. 1996. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nature Med. **2:**925–928.

17. **Giraldo, G., E. Beth, and E.-S. Huang.** 1980. Kaposi's sarcoma and its relationship to cytomegalovirus (CMV). III. CMV DNA and CNMV early antigens in Kaposi's sarcoma. Int. J. Cancer **26:**23–29.
18. **Gompels, U. A., J. Nicholas, G. Lawrence, M. Jones, B. J. Thomson, M. E. D. Martin, S. Efstathiou, M. Craxton, and H. A. Macaulay.** 1995. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. Virology **209:**29–51.
19. **Huang, Y. Q., J. J. Li, M. H. Kaplan, B. Poiesz, E. Katabira, W. C. Zhang, D. Feiner, and A. E. Friedman-Kien.** 1995. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. Lancet **345:**759–761.
20. **Kedes, D. H., E. Oberskalski, M. Busch, R. Kohn, J. Flood, and D. Ganem.** 1996. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nature Med. **2:**918–924.
21. **Kemeny, L., M. Kiss, R. Gyulai, A. Dobozy, E. Adam, and F. Nagy.** 1995. Herpes-like nucleic acid sequences in patients with eastern European sporadic Kaposi's sarcoma. J. Invest. Dermatol. **106:**381.
22. **Knowles, D. M., G. Inghirami, A. Ubriaco, and R. Dalla-Favera.** 1989. Molecular genetic analysis of three AIDS-associated neoplasms of uncertain lineage demonstrates their B-cell derivation and the possible pathogenetic role of the Epstein-Barr virus. Blood **73:**792–789.
23. **Lebbe, C., P. de Cremoux, M. Rybojad, C. Costa da Cunha, P. Morel, and F. Calvo.** 1995. Kaposi's sarcoma and new herpesvirus. Lancet **345:**1180.
24. **Ling, P. D., J. J. Ryon, and S. D. Hayward.** 1993. EBNA-2 of herpesvirus papio diverges significantly from the type A and type B EBNA-2 proteins of Epstein-Barr virus but retains an efficient transactivation domain with a conserved hydrophobic motif. J. Virol. **67:**2990–3003.
25. **Lisitsyn, N., N. Lisitsyn, and M. Wigler.** 1993. Cloning the differences between two complex genomes. Science **259:**946–951.
26. **Lunardi-Iskandar, Y., J. L. Bryant, R. A. Zeman, V. H. Lam, F. Samaniego, J. M. Besnier, P. Hermans, A. R. Thierry, P. Gill, and R. C. Gallo.** 1995. Tumorigenesis and metastasis of neoplastic Kaposi's sarcoma cell line in immunodeficient mice blocked by a human pregnancy hormone. Nature **375:**64–68.
27. **Lundgren, J. D., M. Melbye, C. Pedersen, P. S. Rosenberg, and J. Gerstoft.** 1995. Changing patterns of Kaposi's sarcoma in Danish acquired immunodeficiency syndrome patients with complete follow-up. The Danish Study Group for HIV Infection (DASHI). Am. J. Epidemiol. **141:**652–658.
28. **McGeoch, D. J., and S. Cook.** 1994. Molecular phylogeny of the Alphaherpesvirinae subfamily and a proposed evolutionary timescale. J. Mol. Biol. **238:**9–22.
29. **McGeoch, D. J., S. Cook, A. Dolan, F. E. Jamieson, and E. A. R. Telford.** 1995. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. J. Mol. Biol. **247:**443–458.
30. **Miles, S. A.** 1994. Pathogenesis of HIV-related Kaposi's sarcoma. Cur. Opin. Oncol. **6:**497–502.
31. **Moore, P. S., and Y. Chang.** 1995. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. N. Engl. J. Med. **332:**1182–1185.
32. **Nicholas, J., V. Ruvolo, J. Zong, D. Ciufo, H.-G. Guo, M. S. Reitz, and G. S. Hayward.** 1997. A single 13-kilobase divergent locus in Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) contains nine open reading frames that are homologous to or related to cellular proteins. J. Virol. **71:**1963–1974.
33. **Raab-Traub, N., and K. Flynn.** 1986. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell **47:**883–889.
34. **Rady, P. L., A. Yen, J. L. Rollefson, I. Orengo, S. Bruce, T. K. Hughes, and S. K. Tyring.** 1995. Herpesvirus-like DNA sequences in non-Kaposi's sarcoma skin lesions of transplant patients. Lancet **345:**1339–1340.
35. **Renne, R., W. Zhong, B. Herndier, M. McGrath, N. Abbey, D. Kedes, and D. Ganem.** 1996. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nature Med. **2:**342–346.
36. **Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. d'Agay, J. P. Clauvel, M. Raphael, L. Degos, et al.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood **86:**1276–1280.
37. **Whitby, D., M. R. Howard, M. Tenant-Flowers, N. S. Brink, A. Copas, C. Boshoff, T. Hatzioannou, R. E. Suggett, D. M. Aldam, A. S. Denton, et al.** 1995. Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet **346:**799–802.
38. **Wrightham, M. N., J. P. Stewart, N. J. Janjua, S. D. Pepper, C. Sample, C. M. Ronney, and J. R. Arrand.** 1995. Antigenic and sequence variation in the C-terminal unique domain of the EBV EBNA1. Virology **208:**521–530.