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Primary Characterization of a Herpesvirus Agent Associated with Kaposi's Sarcoma

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Detection of novel DNA sequences in Kaposi's sarcoma (KS) and AIDS-related body cavity-based, non-Hodgkin's lymphomas suggests that these neoplasms are caused by a previously unidentified human herpesvirus. We have characterized this agent using a continuously infected B-lymphocyte cell line derived from an AIDS-related lymphoma and a genomic library made from a KS lesion. In this cell line, the agent has a large episomal genome with an electrophoretic mobility similar to that of 270-kb linear DNA markers during clamped homogeneous electric field gel electrophoresis. A 20.7-kb region of the genome has been completely sequenced, and within this region, 17 partial and complete open reading frames are present; all except one have sequence and positional homology to known gammaherpesvirus genes, including the major capsid protein and thymidine kinase genes. Phylogenetic analyses using both single genes and combined gene sets demonstrated that the agent is a gamma-2 herpesvirus (genus *Rhadinovirus*) and is the first member of this genus known to infect humans. Evidence for transient viral transmission from infected to uninfected cells is presented, but replication-competent virions have not been identified in infected cell lines. Sera from patients with KS have specific antibodies directed against antigens of infected cell lines, and these antibodies are generally absent in sera from patients with AIDS without KS. These studies define the agent as a new human herpesvirus provisionally assigned the descriptive name KS-associated herpesvirus; its formal designation is likely to be human herpesvirus 8.

Kaposi's sarcoma (KS) is a neoplasm of uncertain histogenesis occurring in both human immunodeficiency virus (HIV)-infected and uninfected persons. Epidemiologic studies suggest that KS has an infectious etiology (6, 40), but extensive studies have previously failed to identify known infectious agents as the cause of KS (22, 42).

Using representational difference analysis, Chang et al. (10) identified two DNA sequences in KS lesions, KS330Bam and KS631Bam, which are homologous to but distinct from gammaherpesviral capsid and tegument protein genes. These sequences were present in over 90% of AIDS-KS lesions examined but were rarely found in non-KS tissues from persons with and without AIDS. Differential detection of these sequences in various tissues from patients with KS suggests that these DNA sequences belong to an exogenous agent rather than the host human genome. These sequences do not hybridize with DNA from Epstein-Barr virus (EBV), human cytomegalovirus, or common opportunistic pathogens of patients with AIDS. In addition to KS tissues, KS330Bam and KS631Bam were found in three AIDS-related body cavity-based B-cell lymphomas. Subsequent studies have shown that the putative virus is consistently present in this rare form of non-Hodgkin's lymphoma but not in a large variety of other AIDS-related or non-AIDS-related lymphomas (8). Two independently established cell lines, BC-1 and HBL-6, from the same body cavity-based B-cell lymphoma tumor (9, 18), are stably infected with both EBV and this putative new virus.

PCR amplification of a 233-bp region of KS330Bam ($KS330_{233}$) has demonstrated that specific PCR products are generally present in KS lesions but not in control tissues from patients without KS (37). These results have been confirmed in controlled studies of all forms of KS tissues from North America (3), Europe (7, 13, 14), Africa (11, 48), and Asia (50). Specific PCR products have been found in 95% of nearly 200 KS tissues examined by these independent studies. Further, these sequences are detectable in at least 52% of peripheral blood mononuclear cell samples from patients with AIDS-KS prior to KS onset compared to only 11 to 13% of control peripheral blood mononuclear cell samples from homosexual male and hemophiliac patients with AIDS who did not develop KS (38, 51a), indicating that infection precedes the onset of disease.

In this paper, we describe the agent's properties that define it as a new human gammaherpesvirus. The virus has been given the trivial descriptive name KS-associated herpesvirus (KSHV); its formal classification is likely to be human herpesvirus 8.

MATERIALS AND METHODS

Sequencing studies. A lambda phage (KS5) from a KS lesion genomic library identified by positive hybridization with KS330Bam (10) was digested with *Bam*HI and *Not*I (Boehringer Mannheim, Indianapolis, Ind.); five fragments were gel isolated and subcloned into Bluescript II KS (Stratagene, La Jolla, Calif.). The entire sequence was determined by bidirectional sequencing at a sevenfold average redundancy by primer walking and nested deletions.

DNA sequence data were compiled and aligned by using ALIGN (IBI-Kodak, Rochester, N.Y.) and analyzed with the Wisconsin Sequence Analysis Package,

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version 8-UNIX (Genetics Computer Group, Madison, Wis.), and the GRAIL Sequence Analysis, Gene Assembly and Sequence Comparison System, version 1.2 (Informatics Group, Oak Ridge, Tenn.). Protein site motifs were identified with Motif (Genetics Computer Group).

Sources of herpesvirus gene sequence comparisons. Complete genomic sequences of three gammaherpesviruses were available: EBV, a herpesvirus of humans (4); herpesvirus saimiri (HVS), a herpesvirus of the New World monkey *Saimiri sciureus* (1); and equine herpesvirus 2 (EHV2) (51). Additional thymidine kinase (TK) gene sequences were obtained for alcelaphine herpesvirus 1 (25) and bovine herpesvirus 4 (31). Sequences for the major capsid protein genes of human herpesvirus 6B and human herpesvirus 7 were from Mukai et al. (39). The sources of all other sequences used are listed in the work of McGeoch and Cook (34) and McGeoch et al. (35).

Phylogenetic inference. Predicted amino acid sequences were used for tree construction based on previous experience with herpesviral phylogenetic analyses (34). Alignments of homologous sets of amino acid sequences were made with the AMPS (5) and Pileup (19) programs. Regions of alignments that showed extreme divergence with marked length heterogeneity, typically terminal sections, were excised. Generally, positions in alignments that contained inserted gaps in one or more sequences were removed before use for tree construction. Phylogenetic inference programs were from the Phylip set, version 3.5c (17), and from the Genetics Computer Group set (19). Trees were built with the maximum-parsimony (MP) and neighbor-joining (NJ) methods. For the NJ method, which utilizes estimates of pairwise distances between sequences, distances were estimated as mean numbers of substitution events per site with Protdist by using the PAM 250 substitution probability matrix of Schwartz and Dayhoff (49). Bootstrap analysis (16) was carried out for MP and NJ trees, with 100 subreplicates of each alignment, and consensus trees were obtained with the program Consense. In addition, the program Protml was used to infer trees by the maximum-likelihood method. Protml was obtained from J. Adachi, Department of Statistical Science, The Graduate University for Advanced Study, Tokyo, Japan. Because of computational constraints, Protml was used only with the four-species CS1 alignment.

Clamped homogeneous electric field (CHEF) gel electrophoresis. Agarose plugs were prepared by resuspending BC-1 cells in 1% low-melting-point agarose (Bio-Rad, Hercules, Calif.) and 0.9% NaCl at 42°C to a final concentration of 2.5×10^7 cells per ml. Solidified agarose plugs were transferred into lysis buffer (0.5 M EDTA [pH 8.0], 1% sarcosyl, proteinase K at a 1-mg/ml final concentration) and incubated for 24 h. Approximately 10^7 BC-1 cells were loaded in each lane. Gels were run at a gradient of 6.0 V/cm with a run time of 28 h 28 min on a CHEF Mapper XA pulsed-field gel electrophoresis apparatus (Bio-Rad), Southern blotted, and hybridized to KS631Bam, KS330Bam (10), and an EBV terminal repeat sequence (43).

TPA induction of genome replication. Late-log-phase BC-1 cells (5×10^5 cells per ml) were incubated with various amounts of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co., St. Louis, Mo.) for 48 h, cells were then harvested and washed with phosphate-buffered saline (PBS), and DNA was isolated by chloroform-phenol extraction. DNA concentrations were determined by measuring the UV absorbence; 5 μg of whole-cell DNA was quantitatively dot blot hybridized in triplicate (Manifold I; Schleicher & Schuell, Keene, N.H.). KS631Bam, EBV terminal repeat, and beta-actin sequences were random primer labeled with ^{32}P (15). Specific hybridization was quantitated on a Molecular Dynamics PhosphorImager 425E.

Cell cultures and transmission studies. BC-1 and HBL-6 (a gift of Riccardo Dalla-Favera) were obtained from an AIDS-related body cavity-based lymphoma as previously described (9, 18). Cells were maintained at 5×10^5 cells per ml in RPMI 1640 with 20% fetal calf serum (FCS; Gibco-BRL, Gaithersburg, Md.) and periodically examined for continued KSHV infection by PCR and dot blot hybridization. The T-cell line Molt-3 (a gift from Jodi Black, Centers for Disease Control and Prevention), Raji cells (American Type Culture Collection, Rockville, Md.), P3H3 cells (a subline of P3HR-1; a gift of George Miller), and RCC-1 cells were cultured in RPMI 1640 with 10% FCS. Owl monkey kidney cells (American Type Culture Collection) were cultured in minimal essential medium with 10% FCS and 1% nonessential amino acids (Gibco-BRL).

To produce the RCC-1 cell line, 2×10^6 Raji cells were cultivated with 1.4×10^6 BC-1 cells in the presence of 20 ng of TPA per ml for 2 days in chambers separated by Falcon 0.45-μm-pore-size filter tissue culture inserts to prevent contamination of Raji cells with BC-1 cells. Demonstration that RCC-1 cells were not contaminated with BC-1 cells was obtained by PCR typing of HLA-DR alleles (30) (Raji and RCC-1, DRβ1*0310 and DRβ3*02; BC-1, DRβ104*07 and DRβ4*01) and confirmed by flow cytometry to determine the absence (Raji and RCC-1) or presence (BC-1) of epithelial membrane antigen, a cell surface marker variably expressed among different lymphoma cell lines. Clonal sublines of RCC-1 were obtained by dilution in 96-well plates to 0.1 cell per well in a mixture of RPMI 1640, 20% FCS, and 30% T-STIM culture supplement (Collaborative Biomedical Products, Bedford, Mass.). Subcultures were examined to ensure that each was derived from a single cluster of growing cells.

In situ hybridization was performed with a previously described 25-bp oligomer located in open reading frame 26 (ORF26) (10) which was 5′ labeled with fluorescein (Operon, Alameda, Calif.) and hybridized to cytospin preparations of BC-1, RCC-1, and Raji cells by the methods of Lungu et al. (32). Slides were directly visualized both by UV microscopy and by incubating slides with anti-fluorescein-alkaline phosphatase-conjugated antibody (Boehringer Mannheim), allowing immunohistochemical detection of bound probe. Positive-control hybridization was performed using a 26-bp 4,7,2′,7′-tetrachloro-6-carboxy fluorescein-labeled EBV DNA polymerase gene oligomer (Applied Biosystems, Foster City, Calif.) which was visualized by UV microscopy only, and negative-control hybridization was performed using a 25-bp 5′-fluorescein-labeled herpes simplex virus type 1 (HSV-1) α47 gene oligomer (Operon) which was visualized in a manner similar to that used for the KSHV ORF26 probe. All nuclei of BC-1, RCC-1, and Raji cells appropriately stained with the EBV hybridization probe, whereas no specific staining of the cells occurred after hybridization with the HSV-1 probe.

The remaining cell lines used in transmission experiments were pelleted and resuspended in 5 ml of filtered (0.22- or 0.45-μm pore size) BC-1 tissue culture supernatant for 16 h. BC-1 supernatants were either from unstimulated cultures or from cultures stimulated with 20 ng of TPA per ml. No difference in transmission to recipient cell lines was noted with various filtration or stimulation conditions. Fetal cord blood lymphocytes were obtained from heparinized fresh postpartum umbilical cord blood after separation on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) gradients and cultured in RPMI 1640 with 10% FCS. Adherent recipient cells were washed with sterile Hanks' buffered salt solution (Gibco-BRL) and overlaid with 5 ml of BC-1 medium supernatant. After incubation with BC-1 medium supernatant, cells were washed three times with sterile Hanks' buffered salt solution and suspended in fresh media. Cells were subsequently rewashed three times every other day for 6 days and grown for at least 2 weeks prior to DNA extraction and testing. PCR to detect KSHV infection was performed using nested and unnested primers from ORF26 and ORF25 as previously described (10, 38).

Indirect immunofluorescence assay. AIDS-KS sera were obtained from ongoing cohort studies (provided by Scott Holmberg, Thomas Spira, and Harold Jaffe, Centers for Disease Control and Prevention, and Isaac Weisfuse, New York City Department of Health). Sera from patients with AIDS-KS were drawn between 1 and 31 months after initial KS diagnosis, sera from intravenous drug user and homosexual or bisexual controls were drawn after non-KS AIDS diagnosis, and sera from HIV-infected hemophiliac controls were drawn at various times after HIV infection. Immunofluorescence assays were performed using an equal-volume mixture of goat anti-human immunoglobulin G-fluorescein isothiocyanate conjugate (Molecular Probes, Eugene, Oreg.) and goat anti-human immunoglobulin M-fluorescein isothiocyanate conjugate (Sigma Chemical Co.) diluted 1:100 and serial dilutions of patient sera. Endpoint titers were read blindly, and specific immunoglobulin binding was assessed by the presence or absence of a specular fluorescence pattern in the nuclei of the plated cells. To adsorb cross-reacting antibodies, 20 μl of serum diluted 1:10 in PBS, pH 7.4, was adsorbed with 1×10^7 to 3×10^7 paraformaldehyde-fixed P3H3 cells for 4 to 10 h at 25°C and removed by low-speed centrifugation. P3H3 cells were induced prior to fixation with 20 ng of TPA per ml for 48 h, fixed with 1% paraformaldehyde in PBS for 2 h at 4°C, and washed three times in PBS prior to adsorption. HBL-6 does not express EBV latent membrane protein (LMP) or EBV nuclear antigen 2 (EBNA2) detectable by monoclonal antibodies (PE2 and CS1-4; Dako, Carpinteria, Calif.). Thus, P3H3 is an appropriate cell type for adsorption of cross-reactive antibodies against EBV in HBL-6; P3H3 cells were induced to ensure representation of both latent and lytic-phase EBV antigens in the preparation used for serum adsorption.

Nucleotide sequence accession number. The sequence of KS5 has been submitted to GenBank under accession number U40377.

RESULTS

Sequence analysis of a 20.7-kb KSHV DNA sequence. To demonstrate that KS330Bam and KS631Bam are genomic fragments from a new and previously uncharacterized herpesvirus, a lambda phage clone (KS5) derived from an AIDS-KS genomic DNA library was identified by hybridization to the KS330Bam sequence (10). The KS5 insert was subcloned after *Not*I-*Bam*HI digestion into five subfragments, and both strands of each fragment were sequenced across junctions by primer walking or nested deletion with a sevenfold average redundancy. The KS5 sequence is 20,705 bp in length and has a G+C content of 54.0%. The observed/expected CpG dinucleotide ratio is 0.92, indicating no overall CpG suppression in this region.

ORF analysis identified 15 complete ORFs with coding regions ranging from 231 to 4,128 bp in length and two incomplete ORFs at the termini of the KS5 clone which were 135 and 552 bp in length (Table 1). The coding probability of each ORF was analyzed by using GRAIL 2 and CodonPreference, which identified 17 regions having excellent to good protein coding probabilities. Each region is within an ORF containing a ho-

TABLE 1. Locations, features, and relative homologies of KS5 ORFs compared with translation products of HVS, EHV2, and EBV

ORF[c]	KSHV genome: Position of[d]: Start	ATG	Stop	TATA	poly(A)	No. of aa encoded	Gene homolog in[a]: HVS ORF	HVS %I, %S	EHV2 ORF	EHV2 %I, %S	EBV ORF	EBV %I, %S	Function[b]
ORF20	20093	20153				184	ORF20		ORF20		BXRF1		
ORF21	20433	20343	18601		18684	580	ORF21	32, 50	ORF21	31, 51	BXLF1	28, 50	TK
ORF22	18628	18613	16421	18685	16414	730	ORF22	35, 55	ORF22	31, 52	BXLF2	26, 48	gH
ORF23	15207	15210	16424	14955	16422	404	ORF23	33, 57	ORF23	34, 56	BTRF1	31, 51	
ORF24	12846	12948	15206	11641	16422	752	ORF24	45, 66	ORF24	41, 58	BcRF1	38, 57	
ORF25	13018	12949	8819	13256	8849	1,376	ORF25	65, 81	ORF25	63, 79	BcLF1	56, 75	MCP
ORF26	8805	8793	7876	13256	6987	305	ORF26	58, 76	ORF26	46, 70	BDLF1	49, 73	VP23
ORF27	7867	7855	6983	7419	6987	290	ORF27	29, 49	ORF27	20, 44	BDLF2	19, 43	
ORF28	6737	6737	6375	6830	5274	120							
ORF29b	5032		6363	4507	6359	443	ORF29b	64, 83	ORF29b	68, 82	BDRF1	60, 76	SG
ORF30	5183	5102	4869	5340	4362	77	ORF30	33, 55	ORF30	38, 56	BDLF3.5	30, 53	
ORF31	4968	4962	4288	5340	4362	224	ORF31	43, 63	ORF31	38, 64	BDLF4	36, 58	
ORF32	4357	4321	2957	5340	3019	454	ORF32	30, 52	ORF32	32, 51	BGLF1	27, 47	
ORF33	3069	2964	2026	3020	1653	312	ORF33	36, 58	ORF33	33, 56	BGLF2	32, 52	
ORF29a	746	1049	1987			312	ORF29a	53, 68	ORF29a	52, 68	BGRF1	41, 57	SG
ORF34	1062	1050	67	3020		327	ORF34	42, 59	ORF34	29, 60	BGLF3	33, 55	
ORF35			138		54	45	ORF35		ORF35		BGLF3.5		

[a] %I, percentage of aligned amino acid identity; %S, percentage of aligned similar amino acids.
[b] gH, glycoprotein H; VP23, virion protein; SG, putative DNA packaging spliced gene.
[c] The nomenclature used for KSHV ORFs is relative to the HVS ORF nomenclature. ORF20 and ORF35 are incomplete ORFs.
[d] TATA, location of suspected upstream TATA elements (TATTAA, TATAAA, or TATAAT); poly(A), polyadenylation signal (AATAAA or ATTAAA).

molog to a known herpesvirus gene, with the exception of one ORF located at the genome position corresponding to ORF28 in HVS. Codon preference values for all of the ORFs were higher across predicted ORFs than in noncoding regions when using a codon table composed of KS5 homologs to the conserved herpesvirus major capsid protein (MCP), glycoprotein H, TK, and the putative DNA packaging protein (ORF29a, ORF29b) genes.

The translated sequence of each ORF was used to search GenBank and EMBL databases with BLASTX and FastA algorithms (2, 41). All of the putative KS5 ORFs, except one, have sequence and colinear positional homology to ORFs from gamma-2 herpesviruses, especially HVS and EHV2. Because of the high degree of colinearity and amino acid sequence similarity between KSHV and HVS, KSHV ORFs have been named according to their HVS positional homologs (e.g., KSHV ORF25 is named after HVS ORF25).

The KS5 sequence spans a region which includes three of the seven conserved herpesvirus gene blocks (Fig. 1) (12). ORFs present in these blocks include genes which encode herpesvirus virion structural proteins and enzymes involved in DNA metabolism and replication. Amino acid identities between KS5 ORFs and HVS ORFs range from 30 to 60%, with the conserved MCP ORF25 and ORF29b genes having the highest percentage of amino acid identity to homologs in other gammaherpesviruses (Table 1). KSHV ORF28, which has no detectable sequence homology to HVS or EBV genes, has positional homology to HVS ORF28 and EBV BDLF3. ORF28 lies at the junction of two gene blocks (Fig. 1); these junctions tend to exhibit greater sequence divergence than intrablock regions among herpesviral genomes (20). Two ORFs with sequence homology to the putative spliced protein packaging genes of HVS (ORF29a and ORF29b) and HSV1 (UL15), were identified. The KS330Bam sequence is located within KSHV ORF26, whose HSV-1 counterpart encodes VP23, is a minor virion structural component.

For every KSHV homolog, the HVS amino acid similarity spans the entire gene product, with the exception of ORF21, the TK gene. The KSHV TK homolog contains a proline-rich domain at its amino terminus (nucleotides 20343 to 19636; amino acids [aa] 1 to 236) that is not conserved in other herpesvirus TK sequences, while the carboxyl terminus (nucleotides 19637 to 18601; aa 237 to 565) is highly similar to the corresponding regions of HVS, EHV2, and bovine herpesvirus 4 TK. A purine binding motif with a glycine-rich region found in herpesviral TK genes, as well as other TK genes, is present in the KSHV TK homolog (GVMGVGKS; aa 260 to 267).

The KS5 translated amino acid sequences were compared with the PROSITE Dictionary of Protein Sites and Patterns (Amos Bairoch, University of Geneva, Geneva Switzerland) with the computer program Motifs. Four sequence motif matches were identified among KSHV hypothetical protein sequences. These matches included (i) a cytochrome *c* family heme-binding motif in ORF33 (CVHCHG; aa 209 to 214) and ORF34 (CLLCHI; aa 257 to 261), (ii) an immunoglobulin and major histocompatibility complex protein signature in ORF25 (FICQAKH; aa 1024 to 1030), (iii) a mitochondrial energy transfer protein motif in ORF26 (PDDITRMRV; aa 260 to 268), and (iv) the purine nucleotide binding site identified in ORF21. The purine binding motif is the only motif with obvious functional significance. A cytosine-specific methylase motif present in HVS ORF27 is not present in KSHV ORF27. This motif has been hypothesized to play a role in the methylation of episomal DNA in cells persistently infected with HVS (1).

Phylogenetic analysis of KSHV. Amino acid sequences translated from the KS5 sequence were aligned with corresponding sequences from other herpesviruses. On the basis of the level of conserved aligned residues and the low incidence of introduced gaps, the amino acid alignments for ORFs 21, 22, 23, 24, 25, 26, 29a, 29b, 31, and 34 were suitable for phylogenetic analyses.

To demonstrate the phylogenetic relationship of KSHV to other herpesviruses, a single-gene comparison of ORF25 (MCP) homologs from KS5 and 12 members of the family *Herpesviridae* (Fig. 2A) was made. The 13 available MCP amino acid sequences are large (1,376 aa residues for the

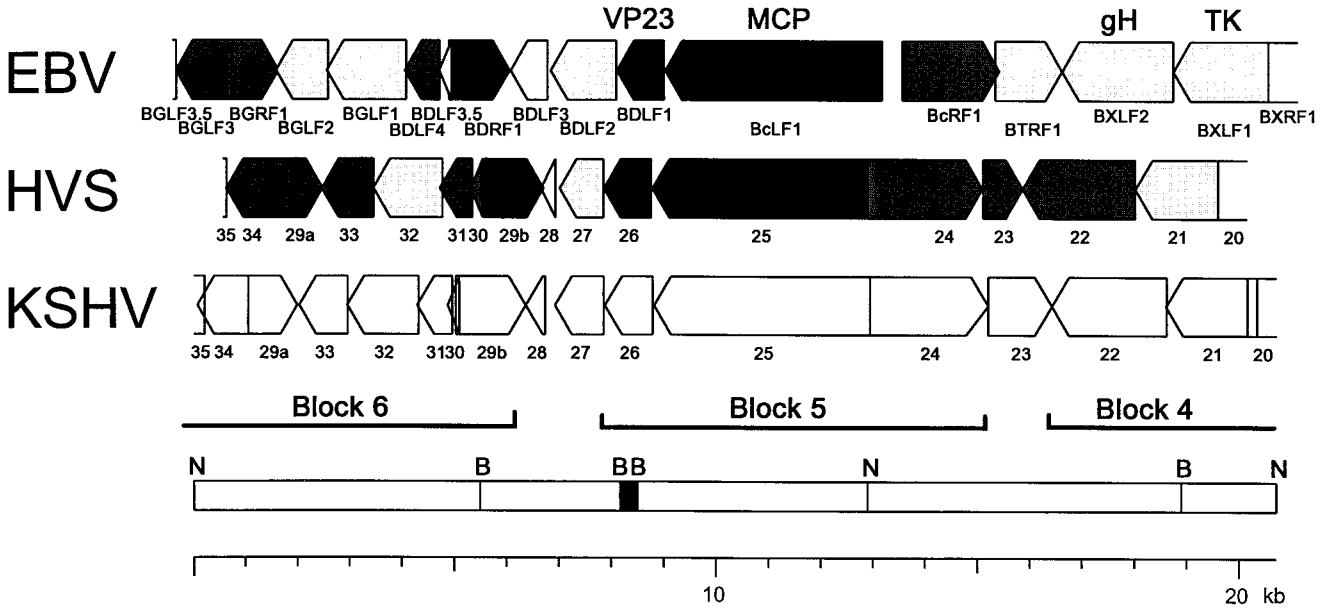

FIG. 1. Genetic map of KS5, a 20.7-kb lambda phage clone insert derived from a human genomic library prepared from an AIDS-KS lesion. Seventeen partial and complete ORFs are identified with arrows denoting reading frame orientations. Comparable regions of the EBV and HVS genomes are shown for comparison. Levels of amino acid similarity between KSHV ORFs are indicated by shading of EBV and HVS ORFs (black, over 70% similarity; dark gray, 55 to 70% similarity; light gray, 40 to 54% similarity; white, no detectable homology). Domains of conserved herpesvirus sequence blocks (12) and locations of restriction endonuclease sites used in subcloning are shown beneath the KSHV map (B, *Bam*HI site; N, *Not*I site). The small *Bam*HI fragment (black) in the VP23 gene homolog corresponds to the KS330Bam fragment generated by representational difference analysis (11) which was used to identify the KS5 lambda phage clone.

KSHV homolog), and alignment required only a low level of gapping; however, the overall similarity between viruses is relatively low (35). The MCP set gave stable trees with high bootstrap scores and assigned the KSHV homolog to the gamma-2 sublineage (genus *Rhadinovirus*), containing HVS, EHV2, and bovine herpesvirus 4 (23, 35, 46). KSHV was most closely associated with HVS. Similar results were obtained for single-gene alignments of TK and UL15 (ORF29) sets (not shown) but with lower bootstrap scores so that among gamma-2 herpesvirus members, branching orders for EHV2, HVS, and KSHV were not resolved.

To determine the relative divergence between KSHV and other gammaherpesviruses, alignments for the nine genes listed above were concatenated to produce a combined gammaherpesvirus gene set (CS1) containing EBV, EHV2, HVS, and KSHV amino acid sequences. The total length of CS1 was 4,247 residues after removal of positions containing gaps introduced by the alignment process in one or more of the sequences. The CS1 alignment was analyzed by the maximum-likelihood method, giving the tree shown in Fig. 2B, and by the MP and NJ methods used with the aligned herpesvirus MCP sequences. All three methods identified KSHV and HVS as sister groups, confirming that KSHV belongs in the gamma-2 sublineage with HVS as its closest known relative. It was previously estimated that divergence of the HVS and EHV2 lineages may have been contemporary with divergence of the primate and ungulate host lineages (35). The results for the CS1 set suggest that HVS and KSHV represent a lineage of primate herpesviruses and that on the basis of the distance between KSHV and HVS relative to the position of EHV2, the divergence between the HVS and KSHV lines is ancient.

Genomic studies of KSHV. CHEF electrophoresis performed on the parental lymphoma tissue and BC-1 cells embedded in agarose plugs demonstrated the presence of a non-integrated KSHV genome as well as a high-molecular-weight species (Fig. 3). KS631Bam (Fig. 3) and KS330Bam (not shown) specifically hybridized to a single CHEF gel band comigrating with 270-kb linear DNA standards in the tumor and in an early passage of BC-1 which diminishes with continued passage. The majority of hybridizing DNA was present in a diffuse band at the well origin in early-passage cells; a low-intensity high-molecular-weight band was also present immediately below the origin (Fig. 3, arrow). The early-passage filter was stripped and probed with an EBV terminal repeat sequence (43), yielding a 150- to 160-kb band corresponding to linear EBV DNA (27). The high-molecular-weight EBV band may correspond to either circular or concatemeric EBV DNA (27).

The phorbol ester TPA induces replication-competent EBV to enter a lytic replication cycle (52). To determine if TPA induces replication of KSHV and EBV in BC-1 cells, these cells were incubated with various concentrations of TPA for 48 h (Fig. 4). Maximum stimulation of EBV occurred at 20 ng of TPA per ml, which resulted in an eightfold increase in hybridizing EBV genome. Only a 1.3- to 1.4-fold increase in KSHV genome abundance occurred after incubation with 20 to 80 ng of TPA per ml for 48 h. Additional studies are being conducted to determine whether other agents are capable of preferentially inducing production of KSHV virions.

Transmission studies. Prior to determining that the agent was likely to be a member of *Herpesviridae* by sequence analysis (10), BC-1 cells were cultured with Raji cells, a nonlytic EBV-transformed B-cell line, in chambers separated by a 0.45-μm-pore-size tissue culture filter. Recipient Raji cells generally demonstrated rapid cytolysis, suggesting transmission of a cytotoxic component from the BC-1 cell line. Cytolysis was apparently not due to KSHV virion amplification, since increased production of KSHV genome was not detected in the cellular debris by dot blotting (data not shown). One Raji line cultured in 10 ng of TPA per ml for 2 days underwent an initial period of cytolysis before recovery and resumption of logarith-

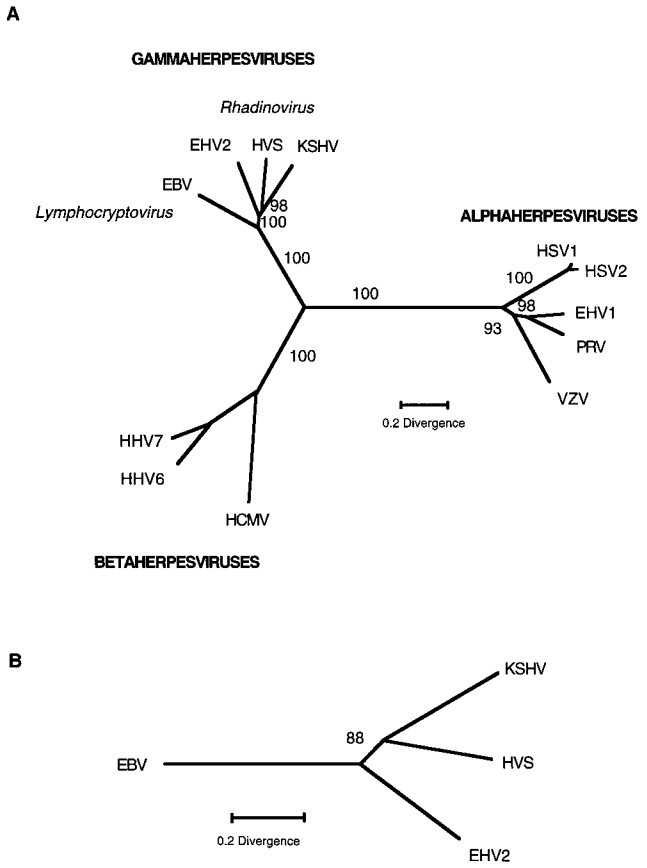

FIG. 2. Phylogenetic trees of KSHV based on comparison of aligned amino acid sequences between herpesviruses for the MCP gene and for a concatenated nine-gene set. (A) The comparison of MCP sequences was obtained by the NJ method and is shown in unrooted form, with branch lengths proportional to divergence (mean number of substitution events per site) between the nodes bounding each branch. Comparable results (not shown) were obtained by MP analysis. The number of times (of 100 bootstrap samplings) that the division indicated by each internal branch was obtained is shown next to each branch; bootstrap values below 75 are not shown. (B) The phylogenetic tree of gammaherpesvirus sequences based on a nine-gene set CS1 (see text) demonstrates that KSHV is most closely related to the gamma-2 herpesvirus sublineage, genus *Rhadinovirus*. The CS1 amino acid sequence was used to infer a tree by the Protml maximum-likelihood method; comparable results (not shown) were obtained with the NJ and MP methods. The bootstrap value for the central branch is marked. On the basis of the MCP analysis, the root must lie between EBV and the other three species. Abbreviations: PRV, pseudorabies virus; VZV, varicella-zoster virus; HCMV, human cytomegalovirus; HHV6 and HHV7, human herpesviruses 6 and 7.

mic growth. This cell line (RCC-1) is a monoculture derived from Raji cells uncontaminated by BC-1 cells, as determined by PCR amplification of HLA-DR sequences and flow cytometry.

RCC-1 has remained positive for the $KS330_{233}$ PCR product for >6 months in continuous culture (approximately 70 passages), but KSHV was not detectable by dot or Southern blot hybridization at any time. In situ hybridization, however, with a 25-bp KSHV ORF26-derived oligomer was used to demonstrate persistent localization of KSHV DNA to RCC-1 nuclei. As indicated in Fig. 5A and C, nuclei of BC-1 and RCC-1 (from passage ~65) cells had detectable hybridization with the ORF26 oligomer, whereas no specific hybridization occurred with parental Raji cells (Fig. 5B). KSHV sequences were detectable in 65% of BC-1 cells and 2.6% of RCC-1 cells under these conditions. In addition, 45 monoclonal cultures were subcultured by serial dilution from RCC-1 at passage 50; of these, eight (18%) clones were PCR positive by $KS330_{233}$. While PCR detection using unnested $KS330_{233}$ primer pairs was lost by passage 15 in each of the clonal cultures, persistent KSHV genome was detected in five clones by using two more-sensitive nonoverlapping nested PCR primer sets (38), suggesting that KSHV genome is lost over time in RCC-1 and its clones.

Low but persistent levels of $KS330_{233}$ PCR positivity were found for one of four Raji, one of four Bjab, two of three Molt-3, one of one owl monkey kidney cell, and three of eight human fetal cord blood lymphocyte cultures after inoculation with filtered (0.2- to 0.45-μm pore size) BC-1 supernatants. Among the PCR-positive cultures, PCR-detectable genome was lost after 2 to 6 weeks and multiple washings. Five fetal cord blood lymphocyte cultures developed cell clusters characteristic of EBV-immortalized lymphocytes and were positive for EBV by PCR using EBER primers (29); three of these cultures were also initially $KS330_{233}$ positive. None of the recipient cell lines had detectable KSHV genome by dot blot hybridization.

Serologic studies. Indirect immunofluorescence antibody assays (IFA) were used to assess the presence of specific antibodies against the KSHV- and EBV-infected cell line HBL-6 in the sera from patients with AIDS-KS and control patients with HIV infection or AIDS. HBL-6 was substituted for BC-1 for reasons of convenience; preliminary studies showed no

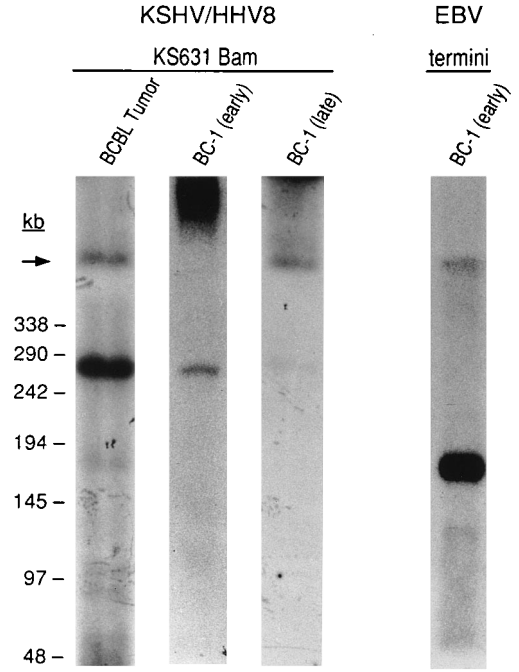

FIG. 3. CHEF gel electrophoresis of the parental body cavity-based lymphoma (BCBL), early-passage BC-1, and late-passage BC-1 DNA hybridized to KS631Bam. The early-passage BC-1 filter was also stripped and rehybridized with an EBV terminal repeat probe. BC-1 is a naturally transformed B-cell line derived from a body cavity-based non-Hodgkin's lymphoma which is coinfected with EBV and KSHV (9). KS631Bam hybridizes to a band at 270 kb as well as to a diffuse band at the origin in the tumor and in the early-passage cells. This band is diminished in late-passage cells. The EBV terminal sequence hybridizes to a 150- to 160-kb band, consistent with the linear form of the genome. Both KS631Bam (arrow) and an EBV terminal sequence hybridize to high-molecular-weight bands immediately below the origin, indicating possible concatemeric or circular DNA (27). The high-molecular-weight KS631Bam hybridizing band reproduces poorly but is visible on the original autoradiographs.

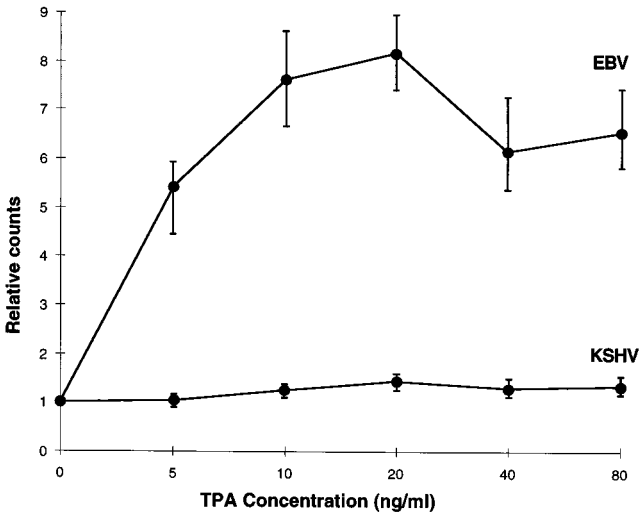

FIG. 4. Induction of KSHV and EBV replication in BC-1 with increasing concentrations of TPA. Each determination was made in triplicate after 48 h of TPA incubation, and hybridization was standardized to the amount of cellular DNA by hybridization to beta-actin. The figure shows the mean and range of relative increase in hybridizing genome for EBV and KSHV induced by TPA compared to the level in uninduced BC-1. TPA at 20 ng/ml induced an 8-fold increase in EBV genome (upper line) at 48 h, compared to only a 1.4-fold increase in KSHV genome (lower line). Despite the lower level of KSHV induction, increased replication of KSHV genome after induction with TPA concentrations over 10 ng/ml was reproducibly detected.

significant differences in IFA results between HBL-6 and BC-1. To remove cross-reactive antibodies against EBV and lymphocyte antigens, sera were adsorbed with paraformaldehyde-fixed EBV-infected P3H3 (a subline of P3HR-1) cells. P3HR-1 has an EBNA2 deletion preventing expression of EBNA2 and LMP1 antigens. However, neither EBNA2 nor LMP1 is expressed by HBL-6 as detected by monoclonal antibodies (7b), indicating that EBV in P3H3 may have an antigenic profile similar to that of HBL-6 and is an appropriate cell line for adsorption studies of HBL-6 using human sera.

HBL-6 cells have diffuse immunofluorescence staining with most unadsorbed sera from patients with KS and control patient sera, suggesting nonspecific antibody binding (Fig. 6A and C). After adsorption with paraformaldehyde-fixed, TPA-induced P3H3 to remove cross-reacting antibodies, KS patient sera generally showed specular nuclear staining at high titers while this staining pattern was absent from control patient sera (Fig. 6B and D). Staining was localized primarily to the nucleus, but weak cytoplasmic staining was also present at low serum dilutions.

With unadsorbed sera, the initial endpoint geometric mean titers (GMT) against HBL-6 cell antigens for the sera from patients with AIDS-KS (GMT = 1:1,153; range, 1:150 to 1:12,150) were higher than for sera from control, non-KS patients (GMT = 1:342; range, 1:50 to 1:12,150; $P = 0.04$) (Table 2). While patients with AIDS-KS and HIV-infected homosexual or bisexual and intravenous drug user control patients had similar endpoint titers to HBL-6 antigens (GMT = 1:1,265 and 1:1,578, respectively), hemophiliac AIDS patient titers were lower (GMT = 1:104) (Table 2). Both case and control patient groups had elevated unadsorbed IFA titers against the EBV-infected cell line P3H3.

The difference in endpoint GMTs between case and control titers against HBL-6 antigens increased after adsorption with P3H3. After adsorption, the case GMT declined to 1:780 and the control GMT declined to 1:81 ($P = 0.00009$). Similar results were obtained by using BC-1 instead of HBL-6 cells. Further, a similar pattern of serum reactivity was found after adsorption with paraformaldehyde-fixed Raji cells (data not shown). High specific titers were also seen with sera from a homosexual male patient with KS without HIV infection who was in complete remission for KS for 9 months at the time of testing (HBL-6 titer, 1:450; P3H3 titer, 1:150). Paired sera taken 8 to 14 months prior to KS onset and after KS onset were available for three patients with KS: KS patients 8 and 13 had eightfold rises and patient 10 had a threefold fall

FIG. 5. In situ hybridization with an ORF26 oligomer to BC-1, Raji, and RCC-1 cells. Hybridization to nuclei of KSHV-infected BC-1 cells (A) but not to uninfected Raji cells (B) occurred. (C) RCC-1, a Raji cell line derived by cultivation of Raji cells with BC-1 cells in communicating chambers separated by a 0.45-μm-pore-size filter, shows rare cells with positive hybridization to the KSHV ORF26 probe.

FIG. 6. Representative example of IFA staining of HBL-6 with sera from patients with AIDS-KS and control sera from HIV-infected patients without KS. Both sera from patients with AIDS-KS (A) and sera from controls (B) show homogeneous staining of HBL-6 at 1:50 dilution. (C) After adsorption with paraformaldehyde-fixed P3H3 to remove cross-reacting antibodies directed against lymphocyte and EBV antigens, antibodies from sera from patients with AIDS-KS localize to HBL-6 nuclei. (D) P3H3 adsorption of control sera eliminates immunofluorescent staining of HBL-6.

in P3H3-adsorbed BC-1 titers from preonset sera to post-KS sera.

DISCUSSION

These studies demonstrate that specific DNA sequences found in KS lesions by representational difference analysis (10) belong to a newly identified human herpesvirus. Our current studies define this agent as a human gamma-2 herpesvirus that can be continuously cultured in naturally transformed, EBV-coinfected lymphocytes from AIDS-related body-cavity based lymphomas.

Sequence analysis of the KS5 lambda phage insert provides clear evidence that the KS330Bam sequence is part of a larger herpesvirus genome. KS5 has a 54.0% G+C content, which is considerably higher than that of the corresponding HVS region (34.3% G+C). While there is no CpG dinucleotide suppression in the KS5 sequence, the corresponding HVS region has a 0.33 expected/observed CpG dinucleotide ratio (1). The CpG dinucleotide frequency in herpesviruses varies from global CpG suppression among gammaherpesviruses to local CpG suppression in the betaherpesviruses, which may result from deamination of 5′-methylcytosine residues at CpG sites resulting in TpG substitutions (24). CpG suppression among herpesviruses (24, 33, 47) has been hypothesized to reflect coreplication of latent genomes in actively dividing host cells, but whether KSHV is primarily maintained by a lytic replication cycle in vivo is unknown.

The 20,705-bp KS5 fragment has 17 protein-coding regions, 15 of which are complete ORFs with appropriately located TATA and polyadenylation signals and 2 of which are incomplete ORFs located at the phage insert termini. Sixteen of these ORFs correspond by sequence and colinear positional homology to 15 previously identified herpesvirus genes, including the highly conserved spliced gene. The conserved positional and sequence homologies for KSHV genes in this region are consistent with the possibility that the biological behavior of the virus is similar to that of other gammaherpesviruses. For example, identification of a TK-like gene on KS5 implies that the agent is potentially susceptible to TK-activated DNA polymerase inhibitors and like other herpesviruses possesses viral genes involved in nucleotide metabolism and DNA replication (44). The presence of MCP and glycoprotein H gene homologs suggests that replication-competent virus would produce a capsid structure similar to that of other herpesviruses.

Phylogenetic analyses of molecular sequences show that KSHV belongs to the gamma-2 sublineage of the *Gammaherpesvirinae* subfamily and is thus the first human gamma-2 herpesvirus identified. Its closest known relative on the basis of available sequence comparisons is HVS, a squirrel monkey gamma-2 herpesvirus that causes fulminant polyclonal T-cell lymphoproliferative disorders in some New World monkey species. Data for the gamma-2 sublineage are sparse: only three viruses (KSHV, HVS, and EHV2) can at present be placed on the phylogenetic tree with precision (the sublineage also contains murine herpesvirus 68 and bovine herpesvirus 4 [35]). Given the limitation in resolution imposed by this thin background, KSHV and HVS appear to represent a lineage of primate gamma-2 viruses. Previously, McGeoch et al. (35) proposed that lines of gamma-2 herpesviruses may have originated by cospeciation with the ancestors of their host species. Extrapolation of this view to KSHV and HVS suggests that these viruses diverged at an ancient time, possibly contemporaneously with the divergence of the Old World and New World primate host lineages. Gammaherpesviruses are distinguished as a subfamily by their lymphotrophism (44), and this grouping is supported by phylogenetic analysis based on sequence data (35). The biologic behavior of KSHV is consistent with its phylogenetic designation in that KSHV can be found in in vitro lymphocyte cultures (9, 18) and in in vivo samples of lymphocytes (3).

Since we cannot exclude the possibility that the 270-kb CHEF electrophoresis band is a concatemeric form, we cannot unambiguously estimate the genome size for KSHV. This band

TABLE 2. Indirect immunofluorescence endpoint titers and GMTs in sera from patients with AIDS-KS and control patients with AIDS against HBL-6 and P3H3 prior to and after adsorption with P3H3

Patient type and no.	HIV risk group[a]	Endpoint titer in:			
		Nonabsorbed sera against:		P3H3-absorbed sera against:	
		HBL-6	P3H3	HBL-6	P3H3
AIDS-KS					
1	H/B	4,050	1,350	4,050	50
2	H/B	450	50	450	50
3	H/B	450	450	450	50
4	H/B	450	450	150	<50
5	H/B	4,050	1,350	1,350	150
6	H/B	4,050	1,350	450	50
7	H/B	12,150	450	12,150	150
8	H/B	1,350	1,350	1,350	150
9	H/B	1,350	450	1,350	50
10	H/B	150	150	150	<50
11	H/B	150	450	50	<50
12	H/B	450	450	450	50
13	H/B	1,350	450	1,350	50
14	H/B	4,050	1,350	4,050	50
GMT		1,153	526	780	63
HIV-AIDS (controls)					
1	H/B	150	150	50	50
2	H/B	150	150	50	50
3	H/B	12,150	4,050	150	150
4	H/B	1,350	4,050	150	150
5	H/B	4,050	4,050	450	450
6	IVDU-F	1,350	1,350	150	150
7	IVDU-F	12,150	12,150	450	450
8	Hemo	50	150	<50	<50
9	Hemo	50	50	<50	<50
10	Hemo	150	150	<50	<50
11	Hemo	450	1,350	50	150
12	Hemo	150	450	50	50
13	Hemo	50	50	<50	<50
14	Hemo	50	<50	<50	<50
15	Hemo	150	450	50	50
16	Hemo	150	150	50	50
GMT		342	450	81	87
Kruskall-Wallace *H* value[b]		4.3	0.31	15.4	1.2
P value[b]		0.04	0.6	0.00009	0.30

[a] H/B, homosexual or bisexual male; IVDU-F, female intravenous drug user; hemo, hemophiliac male.
[b] Comparison between log titers for case and control sera.

appears to be a linear form of the genome because other high-molecular-weight bands which may represent circular forms of the genomes are present for both EBV and KSHV in BC-1 cells. The linear form of the EBV genome, associated with replicating and packaged DNA (44), migrates substantially faster than the closed circular form associated with latent viral replication (27). While the 270-kb band appears to be a linear form, it is also consistent with a replicating dimer plasmid since the genome size of HVS is approximately 135 kb. Diminution of the episomal band over time is consistent with a loss of replication-competent virus from BC-1 cells in prolonged culture. We have been unable to identify restriction endonucleases which cleave the KSHV genome sufficiently infrequently to permit distinguishing linear, circular, and/or concatemeric forms. The true size of the genome may be resolved only by ongoing mapping and sequencing studies.

Replication-deficient EBV mutants are common among EBV strains passaged through prolonged tissue culture (26). The EBV strain infecting Raji cells, for example, is a BALF-2-deficient mutant (21); virus replication is not inducible with TPA, and its genome is maintained only as a latent circular form (26, 36). The EBV strain coinfecting BC-1 does not appear to be lytic replication defective because TPA induces eightfold increases in DNA content and has an apparent linear form on CHEF electrophoresis. KSHV replication, however, is only marginally induced by comparable TPA treatment, indicating either insensitivity to TPA induction or that the genome has undergone a loss of genetic elements required for TPA induction. Additional experiments, however, indicate that KSHV DNA can be pelleted by high-speed centrifugation of filtered organelle-free, DNase I-protected BC-1 cell extracts, which is consistent with KSHV encapsidation (36a).

Transmission of KSHV DNA from BC-1 to a variety of recipient cell lines is possible, and KSHV DNA can be maintained at low levels in recipient cells for up to 70 passages. However, detection of virus genome in recipient cell lines by PCR may be due to physical association of KSHV DNA fragments rather than true infection. This appears to be unlikely, given evidence for specific nuclear localization of the ORF26 sequence in RCC-1. If transmission of infectious virus from

BC-1 occurs, it is apparent that the viral genome declines in abundance with subsequent passages of recipient cells. This is consistent with studies of spindle cell lines derived from KS lesions. Spindle cell cultures derived from KS biopsy specimens, pleural effusions, and peripheral blood generally have PCR-detectable KSHV genome when first explanted but rapidly lose viral genome after initial passages (7a, 28), and established spindle cell cultures generally do not have detectable KSHV sequences (3).

Infections with the human herpesviruses are generally ubiquitous in that nearly all humans are infected by early adulthood with six of the seven previously identified human herpesviruses (45). Universal infection with EBV, for example, is the primary reason for the difficulty in clearly establishing a causal role for this virus in EBV-associated human tumors. Our serologic studies identified nuclear antigen in BC-1 and HBL-6 cells which is recognized by sera from patients with AIDS-KS but generally not by sera from control patients with AIDS without KS after removal of EBV-reactive antibodies. Since both cases and controls had similar IFA titers against EBV antigens, as indicated by the unadsorbed titers against P3H3 (Table 2), differences in IFA titers to HBL-6 antigens are not likely to be due to cross-reactive antibodies against EBV antigens present in HBL-6 cells. These data are consistent with result of PCR studies of KS and control patient lymphocytes, suggesting that KSHV is not ubiquitous among adult humans but is specifically associated with persons who develop KS. In this respect, it appears to be epidemiologically similar to HSV-2 rather than the other known human herpesviruses. However, an alternative possibility is that elevated IFA titers against BC-1 reflect disease status rather than infection with the virus. It is possible that KS is associated with reactivation of KSHV, resulting in elevated specific titers. The implications of the dually KSHV-EBV-infected cell line HBL-6 not expressing EBV LMP1 antigen requires further investigation since LMP1 is a major EBV transforming gene product (26).

Our data provide evidence for a new human herpesvirus that is present in KS and in a subset of AIDS-related non-Hodgkin's lymphomas. In conjunction with a number of studies that demonstrate a specific association of KSHV with KS, our study indicates that this virus is likely to be an important factor in the pathogenesis of KS.

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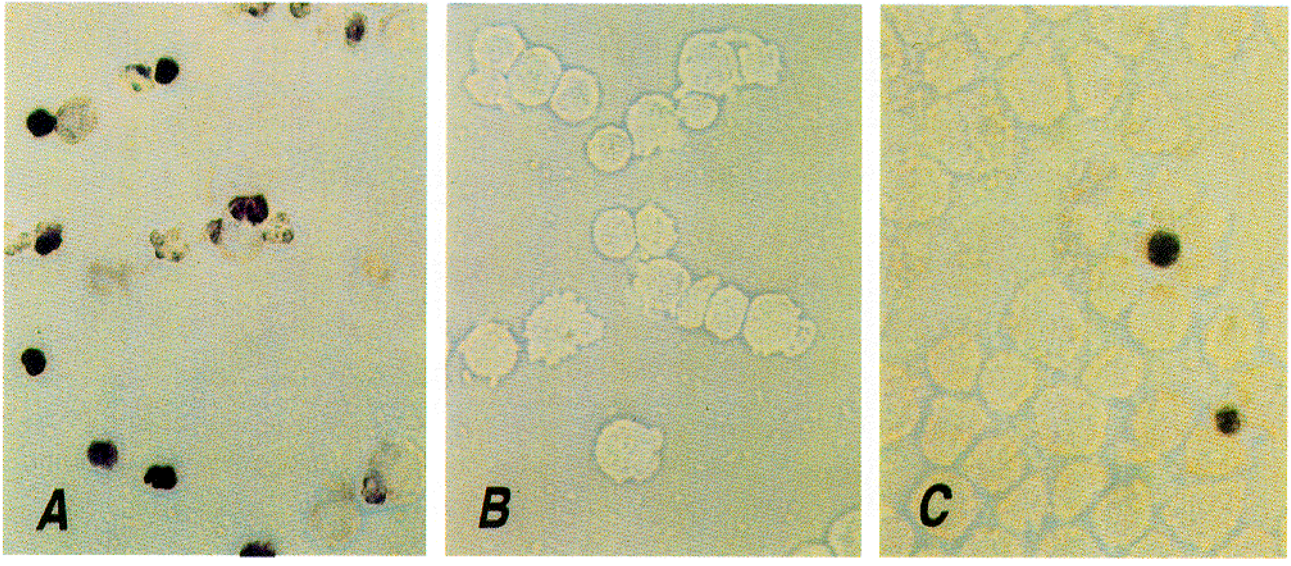
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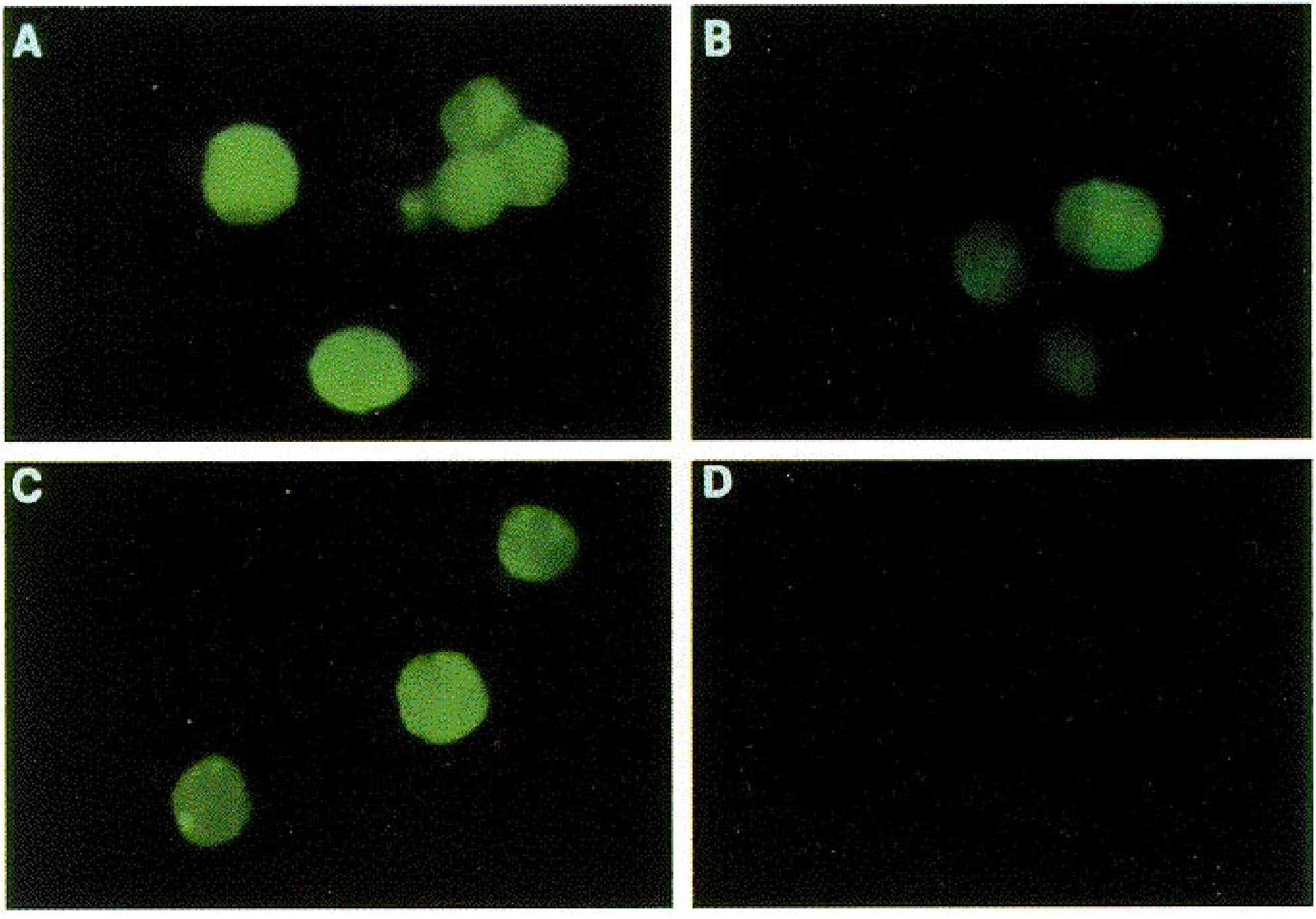
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