(1989); M. D. Summers and G. E. Smith, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures (Texas Agricultural Station, College Station, TX, 1987)]. Deletions of SHC indicated in Fig. 2C were obtained by PCR and cloned into the same vector. GST-SHC fusion proteins were purified by binding to glutathi- one-agarose [K. Guan and J. E. Dixon, Anal. Biochem. **192**, 262 (1991)]. The bound proteins were then incubated in 20 mM tris-HCL (pH 7.5), 1 mM dithiothreitol (DTT), 100 mM NaCl, 12 mM MgCl₂, 0.5 mCi of $[\gamma^{-32}P]$ ATP (6000 Ci/mmol), and 250 units of cAMP-dependent protein kinase catalytic subunit from bovine heart tissue for 1 hour at room temperature. The beads were then washed extensively and eluted with 10 mM glutathione. The specific activity of all preparations was typically $>1 \times 10^7$ cpm/µg. SDS-PAGE analysis showed a single band at the predicted sizes for the GST-SHC fusion proteins with either Coomassie staining or autoradiography. Immunoprecipitates or portions of cell lysates containing equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose. The filters were blocked for 2 hours at 4°C in nonfat dry milk (5%) in hybridization buffer [20 mM Hepes (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1 mM DTT, and 0.05% Triton X-100]. The filters were then incubated overnight at 4°C in hybridization buffer containing milk (1%) and 2.5×10^5 cpm/ml of ³²P–GST-SHC fusion protein as a probe. The filters were then washed three times in hybridization buffer with milk (1%), dried. and exposed to x-ray film with an intensifying screen for 6 to 36 hours at -70°C

14. Lysate was prepared in hybridization buffer from

2.5 × 10⁷ BAL17 B cells stimulated by cross-linking the B cell antigen receptor as described [T. M. Saxton *et al.*, *J. Immunol.* **153**, 623 (1994)]. The lysate was incubated with approximately 250 ng of GST-SHCΔSH2 protein containing the IHA epitope tag for 1 hour at 4°C. The mixture was then subjected to immunoaffinity chromatography with the use of a monoclonal antibody to IHA covalently linked to agarose beads. The column was washed with 50 column volumes of hybridization buffer and eluted with 2% SDS. Proteins in equal fractions of the starting mixture, column flowthrough, and SDS eluate were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with ³²P-labeled PTB domain protein probe. In B cells, pp145 was seen as a doublet.

- 15. Anti-SHC immunoprecipitates from PDGF-stimulated fibroblasts immobilized on nitrocellulose filters were incubated in 25 mM imidazole (pH 7.0), 50 mM NaCl, 2.5 mM EDTA, 5 mM DTT, acetylated bovine serum albumin (100 μg/ml), and 5 units each of LAR and T cell tyrosine-specific phosphatases for 60 min at 30°C. An equivalent sample was treated identically except that 5 mM sodium orthovanadate was included. The filters were then washed extensively and blotted with ³²P–GST-SHC as above, except that the hybridization buffer included 1 mM sodium orthovanadate.
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Identification of Herpesvirus-Like DNA Sequences in AIDS-Associated Kaposi's Sarcoma

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Representational difference analysis was used to isolate unique sequences present in more than 90 percent of Kaposi's sarcoma (KS) tissues obtained from patients with acquired immunodeficiency syndrome (AIDS). These sequences were not present in tissue DNA from non-AIDS patients, but were present in 15 percent of non-KS tissue DNA samples from AIDS patients. The sequences are homologous to, but distinct from, capsid and tegument protein genes of the Gammaherpesvirinae, herpesvirus saimiri and Epstein-Barr virus. These KS-associated herpesvirus-like (KSHV) sequences appear to define a new human herpesvirus.

Kaposi's sarcoma is the most common neoplasm occurring in persons with AIDS; approximately 15 to 20% of AIDS patients develop this neoplasm, which rarely occurs in immunocompetent individuals (1). Epidemiologic evidence indicates that AIDS-associated KS (AIDS-KS) may have an infectious etiology. Gay and bisexual male AIDS patients are approximately 20 times more likely than hemophiliac AIDS patients to develop KS, and KS may be associated with specific sexual practices among gay men with AIDS (2). KS is uncommon among adult AIDS patients infected through heterosexual or parenteral human immunodeficiency virus (HIV) transmission, or among pediatric AIDS patients infected through vertical HIV transmission (3). Agents suspected of causing KS include cytomegalovirus (CMV), hepatitis B virus, human herpesvirus 6 (HHV6), HIV, and Mycoplasma penetrans (4). Extensive investigations, however, have not demonstrated an etiologic association between any of these agents and AIDS-KS (5). Noninfectious environmental agents, such as nitrite inhalants, also have been proposed to play a role in KS tumorigenesis (6).

To search for foreign DNA sequences belonging to an infectious agent in AIDS-KS, we used representational difference

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analysis (RDA) to identify and characterize unique DNA sequences in KS tissue that are either absent or present in low copy number in nondiseased tissue obtained from the same patient (7). This method can detect adenovirus genome added in single copy to human DNA, but has not been used to identify previously uncultured infectious agents. RDA is performed by making simplified "representations" of genomes from diseased and normal tissues obtained from the same individual through polymerase chain reaction (PCR) amplification of short restriction fragments. The DNA representation from the diseased tissue is then ligated to a priming sequence and hybridized to an excess of unligated, normal-tissue DNA representation (8). Only unique sequences found in the diseased tissue that have priming sequences on both DNA strands are preferentially amplified during subsequent rounds of PCR amplification. This process can be repeated with different ligated priming sequences to enrich the sample for unique DNA sequences that are found only in the tissue of interest.

The initial round of amplification-hybridization from KS and excess normal-tissue DNA resulted in a diffuse banding pattern (Fig. 1, lane 2), but four bands at approximately 380, 450, 540, and 680 base pairs (bp) were identifiable after the second amplification-hybridization (Fig. 1, lane 3). These bands became discrete after a third round of amplification-hybridization (Fig. 1, lane 4). Control RDA, performed by hybridizing DNA extracted from AIDS-KS tissue against itself, produced a single band at ~ 540 bp (Fig. 1, lane 5). The four KS-associated bands (designated KS330Bam, KS390Bam, KS480Bam, and KS631Bam after digestion of the two flanking 28-bp ligated priming sequences with Bam HI) were gel purified.

KS390Bam and KS480Bam Southern (DNA) hybridized nonspecifically to both KS and non-KS human tissues and were not further characterized. The remaining two RDA bands, KS330Bam and KS631Bam, were cloned and sequenced (9). KS330Bam

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is a 330-bp sequence with a 51% G:C content (Fig. 2A), and KS631Bam is a 631-bp sequence with a 63% G:C content (Fig. 2B).

nine codon (bp 633) and

stop codon (bp 1548) in

Fig. 1. Agarose gel electrophoresis of RDA products from AIDS-KS tissue and nondiseased tissue. RDA was performed on DNA extracted from KS skin tissue and unaffected normal skin tissue obtained at autopsy from a homosexual man with AIDS-KS (8). Lane 1 shows the initial PCR-amplified genomic representation of the AIDS-KS DNA after Bam HI digestion. Lanes 2 to 4 show that subsequent cycles of ligation, amplification, hybridization, and digestion of the RDA products resulted in amplification of discrete bands at 380, 450, 540, and 680 bp. RDA of the extracted AIDS-KS DNA performed against itself resulted in a single band at 540 bp (lane 5). Bands at 380 bp and 680 bp correspond to KS330Bam and KS631Bam, respectively, after removal of 28-bp priming sequences. Bands at 450 and 540 bp (KS390Bam and KS480Bam,

Both KS330Bam and KS631Bam code for amino acid sequences with homology to herpesviral polypeptides (10). KS330Bam



respectively) hybridized nonspecifically to both KS and non-KS human DNA. Lane M is a molecular size marker.

is 51% identical by amino acid homology to a portion of the ORF26 open reading frame encoding the capsid protein VP23 of herpesvirus saimiri (11), a gammaherpesvirus that causes fulminant lymphoma in New World monkeys. This fragment is also 39% identical to the amino acid sequence encoded by the corresponding BDLF1 ORF of Epstein-Barr virus (EBV) (12). The amino acid sequence encoded by KS631Bam has homology to the tegument protein (ORF75) of herpesvirus saimiri and to the tegument protein of EBV (ORF BNRF1,p140). KS631Bam is not significantly homologous to corresponding sequences of other herpesviruses.

Regions adjacent to KS330Bam were cloned and sequenced from a KS-tissue

Fig 2 Nucleotido eo											
ig. 2. Nucleotide se-	Α	10	20	30	40	50	60	70	80	90	100
quences of the 1853-bp		AACCIGCGAG	ATAATICCCA	CGCCGGTCAC	ATCTGACGTT	GCCTATTTCC	AGACCCCCAG	CAACCCCCGG	GGGCGTGCGG	CGTCGGTCGT	GTCGTGTGAT
flanking region that in-		GCTTACAGTA	ACGAAAGCGC	AGAGCOTTTG			160	170	180	190	200
cludes KS330Bam de-	BcLF1-ORF25	210	220	230	240	250	AGACCCCGCG	IACGAAIGCC 270	GGICCACCAA	CAACCCGTGG	GCTTCGCAGC
rived from a KC laster	homolog	GTGGCTCCCT	CGGCGACGTG	CTATACAATA	TCACCTTTCG	CCAGACTGCG	CTGCCGGGCA	TGTACAGTCC	TTGTCGGCAG	290 TTCTTCC2C2	ACCAACACAT
rived from a KS lesion		310	320	330	340	350	360	370	380	390	400
genomic library (A) and the		TATGCGGTAC	AATAGGGGGT	TGTACACTTT	GGTTAATGAG	TATTCTGCCA	GGCTTGCTGG	GCCCCCCCCC	ACCAGCACTA	CAGACCTCCA	GTACGTCGTG
KS631Bam sequence de-		410	420	430	440	450	460	470	480	490	500
rived from KS tissue by		GTCAACGGTA	CAGACGTGTT	TTTGGACCAG	CCTTGCCATA	TGCTGCAGGA	GGCCTATCCC	ACGCTCGCCG	CCAGCCACAG	AGTTATGCTT	GCCGAGTACA
		510	520	530	540	550	560	570	580	590	600
RDA (B). KS330Bam (A) is		STOP610	GCAGACACAC 620	GUULUAGTAU 630	ACAIGGGCCA	GTATCTCATT	GAAGAGGTGG	CGCCGATGAA	GAGACTATTA	AAGCTCGGAA	ACAAGGTGGT
underlined and Bam HI re-		GTATTAGCTA	ACCOTTCTAG	CGTTGGCTAG	TCATGGCACT	CGACAAGAGT	000 100 100 100 100 100 100 100	670 ACTITICACCTIC	680 CAGACTECTURE	690 CCTCATCAAC	700
striction sites (GGATCC)		710	720	730	740	750	760	770	780	790	1000000000
		TCAGTCAAAA	ATAGGGAGCG	TACTGCCGCT	CGGAGATTGC	CACCGTTTAC	AAAATATACA	GGCATTGGGC	CTGGGGTGCG	TATGCTCACG	TGAGACATCT
are double-underlined. A		810	820	830	840	850	860	870	880	890	900
reading frame composed	BDLF1-ORF26	CCGGACTACA	TCCAAATTAT	GCAGTATCTA	TCCAAGTGCA	CACTCGCTGT	CCTGGAGGAG	GTTCGCCCGG	ACAGCCTGCG	CCTAACGCGG	ATGGATCCCT
of the first 607 nucleotides	nomorog	910	920	930	940	950	960	970	980	990	1000
(bp 1 to 607, stop codop in		1010	1020	AACGTATATG		TCAGTGGGAC	AGCAACACCC	AGCTAGCAGT	GCTACCCCCA	TTTTTTAGCC	GAAAGGATTC
		CACCATTGTG	CTCGAATCCA	ACGGATTTGA	CCCCGTGTTC	CCCATCOTCC	TUBU	1070	PVu CCTINTECTICS	II site 109	0 1100
bold) is homologous to the		1110	1120	1130	1140	1150	1160	1170	1180	1190	<u>GGTGTACCAC</u> 1200
COOH-termini of the ma-		ATCTACTCCA	AAATATCGGC	CGGGGCCCCG	GATGATGTAA	ATATGGCGGA	ACTTGATCTA	TATACCACCA	ATCTCTCATT	TITO	ACATATCGTC
ior capsid protein open		1210	1000							1 24 1 1 1 1 1 1 1 1 1 1 1 1 1	
		1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
		TGGACGTAGA	CAACACGGAT	1230 <u>CC</u> ACGTACTG	1240 CCCTGCGAGT	1250 GCTTGACGAT	1260 CTGTCCATGT	1270 ACCTTTGTAT	1280 CCTATCAGCC	1290 TTGGTTCCCA	1300 GGGGGTGTCT
reading frames ORF25 of		TGGACGTAGA 1310	<u>CAACACGGAT</u> 1320	1230 <u></u> ACGTACTG 1330	1240 CCCTGCGAGT 1340	1250 GCTTGACGAT 1350	1260 CTGTCCATGT 1360	1270 ACCTTTGTAT 1370	1280 CCTATCAGCC 1380	1290 TTGGTTCCCA 1390	1300 GGGGGTGTCT 1400
herpesvirus saimiri and		TGGACGTAGA 1310 CCGTCTGCTC	CAACACGGAT 1320 ACGGCGCTCG	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA	1240 CCCTGCGAGT 1340 CAGGCATCCT	1250 GCTTGACGAT 1350 CTGACAGAGG	1260 CTGTCCATGT 1360 TGTTTGAGGG	1270 ACCTTTGTAT 1370 GGTGGTGCCA	1280 CCTATCAGCC 1380 GATGAGGTGA	1290 TTGGTTCCCA 1390 CCAGGATAGA	1300 GGGGGTGTCT 1400 TCTCGACCAG
herpesvirus saimiri and BcLF1 of Epstein-Barr vi-		TGGACGTAGA 1310 CCGTCTGCTC 1410 TTGACCGTCC	CAACACGGAT 1320 ACGGCGCTCG 1420 CACATGACAT	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440	1250 GCTTGACGAT 1350 CTGACAGAGG 1450	1260 CTGTCCATGT 1360 TGTTTGAGGG 1460	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470	1280 CCTATCAGCC 1380 GATGAGGTGA 1480	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500
herpesvirus saimiri and BcLF1 of Epstein-Barr vi-		TGGACGTAGA 1310 CCGTCTGCTC 1410 TTGAGCGTCC 1510	CAACACGGGAT 1320 ACGGCGCTCG 1420 CAGATGACAT 1520	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430 CACCAGGATG 1530	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 CGCGTCATGT 1540	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP	1260 CTGTCCATGT 1360 TGTTTGAGGG 1460 TCAGAGTCTC 1560	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT 1570	1280 CCTATCAGCC 1380 GATGAGGTGA 1480 TTAATCTTGG	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500 CACGTGTATG
herpesvirus saimiri and BcLF1 of Epstein-Barr vi- rus (EBV). An open read-		TGGACGTAGA 1310 CCGTCTGCTC 1410 TTGAGCGTCC 1510 CCTACTCGGC	CAACACGGAT 1320 ACGGCGCTCG 1420 CAGATGACAT 1520 AGAGACTTTG	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430 CACCAGGATG 1530 GCGGCCTCCT	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 CGCGTCATGT 1540 GTTGGTATTC	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP CCCACGCTAA	1260 CTGTCCATGT 1360 TGTTTGAGGG 1460 TCAGAGTCTC 1560 CGATTTGAAG	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT 1570 CGGGGGGGGT	1280 CCTATCAGCC 1380 GATGAGGTGA 1480 TTAATCTTGG 1580	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG 1590 CTCATATTCT	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500 CACGTGTATG 1600
herpesvirus saimiri and BcLF1 of Epstein-Barr vi- rus (EBV). An open read- ing frame from bp 633 to		TGGACGTAGA 1310 CCGTCTGGCTC 1410 TTGAGCGTCC 1510 CCTACTCGGC 1610	CAACACGGAT 1320 ACGGCGCTCG 1420 CAGATGACAT 1520 AGAGACTTTG 1620	1230 <u></u>	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 CGCGTCATGT 1540 GTTGGTATTC 1640	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP CCCACGC TAA 1650	1260 CTGTCCATGT 1360 TGTTTGAGGG 1460 TCAGAGTCTC 1560 CGATTTGAAG 1660	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT 1570 CGGGGGGGGT 1670	CCTATCAGCC 1380 GATGAGGTGA 1480 TTAATCTTGG 1580 ATGGCGTCAT 1680	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG 1590 CTGATATTCT 1690	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500 CACGTGTATG GTCGGTTGCA 1700
herpesvirus saimiri and BcLF1 of Epstein-Barr vi- rus (EBV). An open read- ing frame from bp 633 to 1550 is homologous to		TGGACGTAGA 1310 CCGTCTGCTC 1410 TTGAGCGTCC 1510 CCTACTCGGC 1610 AGGACGGATG	CAACACGGAT 1320 ACGGCGCTCG 1420 CAGATGACAT 1520 AGAGACTTG 1620 ACGGCCTCGT	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430 CACCAGGATG 1530 GCGGCCTCCT 1630 CTGTGAAGTC	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 CGCGTCATGT 1540 GTTGGTATTC 1640 TCCCTGCGTG	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP CCCACGC TAA 1650 GAGGTAGGAA	1260 CTGTCCATGT 1360 TGTTTGAGGG 1460 TCAGAGTCTC 1560 CGATTTGAAG 1660 AAAAACTACC	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT 1570 CGGGGGGGGT 1670 GTCTACCTGC	1280 CCTATCAGCC 1380 GATGAGGTGA 1480 TTAATCTTGG 1580 ATGGCGTCAT 1680 CGGACACTGA	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG 1590 CTGATATTCT 1690 ACCCTGGGTG	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500 CACGTGTATG GTCGGTTGCA 1700 GTAGAGACCG
herpesvirus saimiri and BcLF1 of Epstein-Barr vi- rus (EBV). An open read- ing frame from bp 633 to 1550 is homologous to OBE26 gene of herpesvi-		TGGACGTAGA 1310 CCGTCTGCTC 1410 TTGAGCGTCC 1510 CCTACTCGGC 1610 AGGACGGATG 1710	CAACACGGAT 1320 ACGGCGCCCG 1420 CAGATGACAT 1520 AGAGACTTG 1620 ACGGCCTCGT 1720	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430 CACCAGGATG 1530 GCGGCCTCCT 1630 CTGTGAAGTC 1730	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 CGCGTCATGT 1540 GTTGGTATTC 1640 TCCCTGCGTG 1740	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP CCCACGC TAN 1650 GAGGTAGGAA 1750	1260 CTGTCCATGT 1360 TGTTTGAGG 1460 TCAGAGTCTC 1560 CGATTTGAAG 1660 AAAACTACC 1760	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT 1570 CGGGGGGGGT 1670 GTCTACCTGC 1770	1280 CCTATCAGCC 1380 GATGAGGTGA 1480 TTAATCTTGG 1580 ATGGCGTCAT 1680 CGGACACTGA 1780	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG 1590 CTGATATTCT 1690 ACCCTGGGTG 1790	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500 CACGTGTATG 1600 GTCGGTTGCA 1700 GTAGAGACCG 1800
herpesvirus saimiri and BcLF1 of Epstein-Barr vi- rus (EBV). An open read- ing frame from bp 633 to 1550 is homologous to ORF26 gene of herpesvi-		TGGACGTAGA 1310 CCGTCTGGCTC 1410 TTGAGCGTCC 1510 CCTACTCGGC 1610 AGGACGGATG 1710 ACGCCATCAA	CAACGCGAT 1320 ACGGCGCTCG 1420 CAGATGACAT 1520 AGAGACTTTG 1620 ACGGCCTCGT 1720 AGACGCCTTC	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430 CACCAGGATG 1530 GCGGCCTCCT 1630 CTGTGAAGTC 1730 CTCAGCGACG	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 CGCGTCATGT 1540 GTTGGTATTC 1640 TCCCTGCGTG 1740 GGATCGTGGA	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP CCCACGCTAA 1650 GAGGTAGGAA 1750 TATGGCTCGA	1260 CTGTCCATGT 1360 TGTTGAGGG 1460 TCAGAGTCTC CGATTTGAAG 1660 AAAAACTACC 1760 AAGCTTCATC	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT CGGGGGGGGT 1670 GTCTACCTGC 1770 GTGTGCTGCCT	1280 CCTATCAGCC 1380 GATGAGGTGA 1480 TTAATCTTGG 1580 ATGGCGTCAT 1680 CGGACACTGA 1780 GCCCTCAAAT	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG 1590 CTGATATTCT 1690 ACCCTGGGTG 1790 TCTCACAACG	1300 GGGGGTGTCT 1400 TCTCGACCAG 1500 CACGTGTATG GTCGGTTGCA 1700 GTAGAGACCG 1800 GCTTGAGGAT
herpesvirus saimiri and BcLF1 of Epstein-Barr vi- rus (EBV). An open read- ing frame from bp 633 to 1550 is homologous to ORF26 gene of herpesvi- rus saimiri and BDLF1		TGGACGTAGA 1310 CCGTCTGCTC 1410 TTGAGCGTCC 1510 CCTACTCGGC 1610 AGGACGGATG 1710 ACGCCATCAA 1810	CAACACGRAT 1320 ACGGCGCTCG 1420 CAGATGACAT 1520 AGAGACTTG 1620 ACGGCCTCGT 1720 AGACGCCTTC 1820	1230 <u>CC</u> ACGTACTG 1330 TGCGGCACGA 1430 CACCAGGATG 1530 GCGGCCTCT 1630 CTGTGAAGTC 1730 CTCAGCGACG 1830	1240 CCCTGCGAGT 1340 CAGGCATCCT 1440 GCGGTCATGT 1540 GTTGGTATTC 1640 TCCCTGCGTG GGATCGGTGGA 1840	1250 GCTTGACGAT 1350 CTGACAGAGG 1450 TCTCCTATCT STOP CCCACGCTAA 1650 GAGGTAGGAA 1750 TATGGCTCGA 1850	1260 CTGTCCATGT 1360 TGTTGAGG 1460 TCAGAGTCTC 1560 CGATTTGAAG 1660 AAAAACTACC 1760 AAGCTTCATC	1270 ACCTTTGTAT 1370 GGTGGTGCCA 1470 AGTTCTATAT 1570 CGGGGGGGGG GTCTACCTGC 1770 GTGGTGCCCT	1280 CCTATCAGCC GATGAGCTGA 1480 TTAATCTTGG 1580 ATGGCGTCAT 1680 CGGACACTGA 1780 GCCCTCAAAT	1290 TTGGTTCCCA 1390 CCAGGATAGA 1490 CCCCAGACTG 1590 CTGATATTCT 1690 ACCCTGGGTG 1790 TCTCACAACG	1300 GGGGGTGTCT 1400 CTCGACCAG 1500 CACGTGTATG 600 GTCGGTTGCA 1700 GTAGAGACCG 1800 GCTTGAGGAT

в 10 20 30 40 50 60 GGATCCGCTG GCAGGTGGGC GCGCACCTCG TCGGGTAGCT TGGAGACAAA CAGCTCCAGG 70 80 90 100 110 120 CCAGTCCGCG GCGCTAGCGC CTGCAGGTGC CTCACCACCG GGGCCGGGTC ATGCGATCTG 130 140 150 160 170 180 TTTAGTCCGG AGAAGATAGG GCCCTTGGCA AGCCGCTGAA CCAGCTCCAG GGTCTCCAAG 190200210220230240ATGCGCACCGCGTTGTCGGAGCTGTCGCGATAGAGGTTAGGGTAGGTGTCCGGTCCGTCC 250 260 270 280 290 300 GTGGGCTCAA ACCTGCCCAG ACACCACCT GTCTGCTGGG GGATCATCCT TCTCAGGGAG 310 320 330 340 350 360 ATGCATTCTT TGGAAGTAGT GGTAGAGATG GAGCAGACTG CCAGGGCGTT GCCAGGAGTG 370 380 390 400 410 420 GTGGCGATGG TGCGCACCGT TTTTAAGAAA CCCCCCAGGG TGGGGACTCC CGCTCCCTGC 430 440 450 460 470 480 AGCATCTCGG CCTGCTGTAC GTCCTTGGCG AATATGCGAC GAAATCGGCT GTGCGCACGG 490 500 510 520 530 540 GGTCCCAGGG CCGGTCCGGT GGCATACAGG CCGGTGAGGG CCCCTGGGT CTGTCCGCCT 550 560 570 580 590 600 GGAAACAGGG TGCTGTGAAA CAACAGGTTG CCAAGGCCGC GAATACCCCT CTGCACGCTG 610 620 630 CTGTGGACGT GGGTGTATGC TCCGT<u>GGATC C</u>

bold]. A Pvu II site at bp 1086 (bold) marks the junction between 1.1- and 3-kb fragments cloned from the KS genomic library. The primer set for $\mathrm{KS330}_{233}$ (bp 987 to 1006 and bp 1200 to 1219) and the internal probe used to detect the PCR amplification product (bp 1078 to 1102) are italicized.

DNA genomic library prepared from a single patient (13). This extended the contiguous sequence flanking both sides of KS330Bam to 1853 bp (Fig. 2A). A complete open reading frame at bp 633 to 1550, which included the KS330Bam sequence, was confirmed to be homologous to the ORF26 and BDLF1 open reading



Fig. 3. Comparison of protein sequences encoded by ORF26 from herpesvirus saimiri (HVS), and BDLF1 from EBV, to the protein encoded by the KS-associated DNA open reading frame. Regions of amino acid identity between KS and HVS, or KS and EBV are outlined (*24*).

Table 1. Southern blot hybridization for KS330Bam and KS631Bam and by PCR amplification for KS330₂₃₃ in human tissues from individual patients.

Tissue type	n	No. positive by KS330Bam DNA hybridization (%)	No. positive by KS631Bam DNA hybridization (%)	No. positive by KS330 ₂₃₃ PCR (%)
AIDS-KS	27*	20 (74)	21 (78)	25 (93)
AIDS lymphomas	27†	3 (11)	3 (11)	3 (11)
AIDS lymph nodes	12	3 (25)	3 (25)	3 (25)
Non-AIDS lymphomas	29‡	O (O)	0 (0)	O (O)
Non-AIDS lymph nodes	7	O (O)	O (O)	O (O)
Vascular tumors	5§	0 (0)	0 (0)	0 (0)
Opportunistic infections	13	O (O)	0 (0 <u>)</u>	O (O)
Consecutive surgical biopsies	49¶#	0 (0)	O (O)	O (O)

*Includes one AIDS-KS specimen unamplifiable for p53 exon 6 and one tissue which on microscopic examination did not have any detectable KS tissue present. Both of these samples were negative by Southern blot hybridization to KS330Bam and KSS31Bam and by PCR amplification for the KS3302₂₃₃ amplicon. Comparison of AIDS-KS KS330Bam, KS631Bam, and KS330₂₃₃ results to each of the control tissue subgroups is significant [(P < 0.01, 1-tail Fisher's exact test (FET)]. For comparisons between AIDS-KS and AIDS lymphomas, the odds ratios and FET P values for KS330Bam, KS651Barn, and KS330₂₃₃ positivity were 22.8, $P = 3 \times 10^{-5}$, 28, $P = 8 \times 10^{-7}$; and 100, $P < 10^{-7}$, respectively. For comparisons between AIDS-KS and AIDS lymph nodes, the odds ratios and FET P values for KS330Barn, KS631Barn, and KS330₂₃₃ positivity were 8.6, P = 0.006; 10.5, P = 0.004; and 38, $P = 4.7 \times 10^{-5}$, respectively. †Includes 7 small noncleaved-cell lymphomas, and 20 diffuse large-cell and immunoblastic lymphomas. Three of the lymphomas with immunoblastic morphology were positive for KS330Bam and KS631Bam. ±Includes 13 anaplastic large-cell lymphomas, 4 diffuse large-cell lymphomas, 4 small lymphocytic lymphomas-chronic lymphocytic leukemias, 3 hairycell leukemias, 2 monocytoid B-cell lymphomas, 1 follicular small cleaved-cell lymphoma, 1 Burkitt's lymphoma, and 1 plasmacvtoma. §Includes 2 angiosarcomas, 1 hemangiopericytoma, 1 lymph node with vascular transformation, and 1 lymphangioma. Includes 2 cryptococcus, 1 toxoplasmosis, 1 cat-scratch bacillus, 1 CMV, 1 EBV, and 7 acid-fast bacillus-infected tissues. In addition, pure cultures of Mycobacterium avium-complex were negative by Southern hybridization and PCR, and pure cultures of Mycoplasma penetrans and lymphocyte cultures with EBV were negative by PCR (not included). ¶Tissues included skin, appendix, kidney, prostate, hernia sac, lung, fibrous tissue, gallbladder, colon, foreskin, thyroid, small bowel, adenoid, vein, axillary tissue, lipoma, heart, oral mucosa, hemorrhoid, pseudoaneurysm, and fistula track. Tissues were collected from a consecutive series of biopsies on patients without AIDS but with unknown HIV serostatus. #Apparent nonspecific hybridization at approximately 20 kb occurred in four consecutive surgical biopsy DNA samples: one colon and one hernia sac DNA sample hybridized to KS330Bam alone, another hernia sac DNA sample hybridized to KS631Bam alone, and one appendix DNA sample hybridized to both KS330Bam and KS631Bam. These samples did not hybridize in the 330- to 630-bp range expected for these sequences and were PCR negative for KS330233

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frames (55% and 56% matching nucleotide identity, respectively) of herpesvirus saimiri and EBV (11, 12). Significantly lower homologies exist to corresponding proteins of bovine herpesvirus type 4, HHV6, CMV, and human herpesvirus 7 (HHV7).

The polypeptide encoded by the KSassociated DNA open reading frame shows extensive amino acid homology to the proteins encoded by herpesvirus saimiri ORF26 and EBV BDLF1 (Fig. 3). Although it is homologous to these herpesvirus regions, the polypeptide does not match any other known sequence and thus provides evidence for a viral genome related to but distinct from known members of the herpesvirus family. In addition, the 5' end of the 1853-bp sequence (bp 1 to 607) is 66% and 67% identical to corresponding regions of the major capsid protein (MCP) genes of herpesvirus saimiri (ORF25) and EBV (BcLF1), respectively. In both EBV and herpesvirus saimiri genomes, the MCP gene is found immediately adjacent to the BDLF1-ORF26 gene (11, 12). This region also has lower degrees of similarity to MCP genes of other human herpesviruses, including HSV1, VZV, HHV6, CMV, and HHV7 (14).

To determine the specificity of KS330Bam and KS631Bam for AIDS-KS, these sequences were random-primed, ³²Plabeled, and hybridized to Southern blots of DNA extracted from cryopreserved tissues obtained from patients with and without AIDS (15). Twenty of 27 (74%) AIDS-KS DNA specimens hybridized with variable intensity to both KS330Bam and KS631Bam, and one additional KS specimen hybridized only to KS631Bam by Southern blotting (Fig. 4 and Table 1). In contrast to AIDS-KS lesions, only 6 of 39 (15%) non-KS tissues from patients with AIDS hybridized to KS330Bam and KS631Bam. Specific hybridization did not occur with lymphoma or lymph node DNA from 36 persons without AIDS or with control DNA from 49 tissue biopsy specimens obtained from a consecutive series of patients. DNA specimens extracted from vascular tumors and tissues with opportunistic infections common in AIDS were also negative (Table 1). In addition, DNA samples from EBV-infected peripheral blood lymphocytes and pure cultures of Mycobacterium avium-complex were negative as well. Overall, 20 of 27 (74%) AIDS-KS specimens hybridized to KS330Bam and 21 of 27 (78%) AIDS-KS specimens hybridized to KS631Bam, as compared to only 6 of 142 (4%) non-KS human DNA control specimens ($\chi^2 = 85.02$, P < 10⁻⁷ and $\chi^2 = 92.4$, P < 10⁻⁷, respectively).

The sequence copy number in the AIDS-KS tissues was estimated by simultaneous and a hybridization with KS330Bam 440-bp probe for the single-copy constant region of the T cell receptor β gene (16). Samples in lanes 5 and 6 of Fig. 4 showed similar intensities for the two probes, indicating an average copy number of approximately two KS330Bam sequences per cell, whereas remaining KS tissues had weaker hybridization signals for the KS330Bam probe.

These results were confirmed and extended by PCR amplification with primers designed from KS330Bam (Fig. 2A) that amplify a 233-bp subfragment (17) designated KS330₂₃₃. Although Southern blot hybridization detected the KS330Bam sequence in only 20 of 27 KS tissues, 25 of the 27 tissues were positive by PCR amplification for KS330₂₃₃ (Fig. 5A), demonstrating that KS330Bam is present in some KS lesions at levels below the threshold for detection by Southern blot hybridization. The two AIDS-KS specimens that were negative for KS330₂₃₃ ap-

Table 2. Differential detection of KS330Bam, KS631Bam, and KS330₂₃₃ sequences in KS-affected (KS) and unaffected autopsy tissues from four patients with AIDS-KS. Patients A, B, and C were gay males with AIDS and patient D was a female intravenous drug user with AIDS.

Tissue type	KS330Bam KS6	631Bam	KS330 ₂₃₃
	Patient A		
KS, skin	+	+	+
Skin	+	+	+
Muscle	+	+	+
	Patient B		•
KS. skin	+	+	+
Muscle	_	_	_
Brain		_	_
	Patient C		
KS, stomach	+	+	+
Stomach.	_	_	+
adjacent to KS			
Muscle	-	_	_
Brain	-	_	_
Colon	_	_	_
Heart	_	_	_
Hilar lymph	_	_	_
nodes			
	Patient D		
KS, skin	+	+	+
Skin,	-	-	+
adjacent to KS			
Hilar lymph	_	_	+
node			
Mesenteric	_	_	_
lymph			
node			
Brain	_	_	_
Lung	_	_	_
Stomach	_	_	_
Spleen	_	_	-
Liver	_	_	_
Muscle	-	_	_

peared to be so for technical reasons: One had no microscopically detectable KS tissue in the frozen sample (Fig. 5A, lane 3), and the other (Fig. 5A, lane 15) was negative in the control PCR amplification for the p53 gene (18), indicating either DNA degradation or the presence of PCR inhibitors in the sample. All KS330₂₃₃ PCR products hybridized to a ^{32}P end-labeled 25-bp internal oligomer, confirming the specificity of the PCR (Fig. 5B).

Except for the six non-KS control samples from AIDS patients that were positive by Southern blot hybridization, none of the other 136 non-KS control specimens were positive by PCR for KS330₂₃₃. Overall, DNA samples from 25 (93%) of 27 AIDS-KS tissues were positive by PCR, as compared to 6 (15%) of 39 non-KS lymph nodes and lymphomas from AIDS patients ($\chi^2 = 38.2$, $P < 10^{-6}$), 0 of 36 lymph nodes and lymphomas from non-AIDS patients ($\chi^2 = 55.2$, $P < 10^{-7}$), and 0 of 49 consecutive biopsy specimens ($\chi^2 = 67.7$, $P < 10^{-7}$). All control specimens were amplifiable for p53, indicating that inad-

Fig. 4. Hybridization of ³²P-labeled KS330Bam (A) and KS631Bam (B) sequences obtained by RDA to a representative panel of 19 DNA samples extracted from KS lesions and digested with Bam HI. KS330Bam hybridized to 11 of the 19 and KS631 Barn hybridized to 12 of 19 DNA samples from the AIDS-KS lesions shown. Two cases (lanes 12 and 13) showed faint bands with both KS330Bam and KS631Bam probes after longer exposure. One negative specimen (lane 3) did not have microscopically detectable KS in the tissue specimen. Seven of 8 additional KS DNA samples not shown also hybridized to both sequences.

Fig. 5. PCR amplification of the 19 KS-derived DNA samples shown in Fig. 4, using the KS330₂₃₃ primers shown in Fig. 2. (A) shows the agarose gel of the amplification products from 19 KS DNA samples (lanes 1 to 19), and (B) shows specific hybridization of the PCR products to a ³²P endlabeled 25-bp internal oligonucleotide (Fig. 2)



Of the six control tissues from AIDS patients that were positive by both PCR and Southern hybridization, two patients had KS at other sites, two did not develop KS, and complete clinical histories for the remaining two patients were unobtainable. Three of these tissues were lymph nodes with follicular hyperplasia taken from patients with AIDS. Undetected microscopic KS foci may have been present in these lymph nodes, given the high lifetime occurrence of KS (>50%) in some



Probe: KS631Bam



after transfer of the gel to a nitrocellulose filter. Negative samples in lanes 3 and 15, respectively, lacked microscopically detectable KS in the sample or did not amplify the human p53 exon 6, suggesting that these samples were negative for technical reasons. An additional eight AIDS-KS samples were amplified and all were positive for KS330₂₃₃. Lane 20 is a negative control and lane M molecular size marker.

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risk groups of AIDS patients (21). Alternatively, these lymph nodes may have been asymptomatically infected with, or may have been incubating, the putative agent. The other three positive tissue specimens were a form of B cell immunoblastic lymphoma from AIDS patients. Given the previously noted association between KS and lymphoproliferative disorders (22), it is possible that the putative KS agent is also a cofactor for a subset of AIDS-associated lymphomas. A comparison of AIDS-KS tissues to only lymph node and lymphoma tissues from AIDS patients demonstrates that KS330Bam and KS631Bam remain significantly associated with the KS phenotype when controlling for concurrent AIDS, indicating that HIV disease is not a confounding factor in our analysis. Among only AIDS tissue samples from separate patients (Table 1), over 90% of KS specimens (100% of confirmed and amplifiable KS specimens) were positive for KS330 $_{\rm 233}$, as compared to 15% of lymph node and lymphoma tissues from AIDS patients. These sequences therefore appear to be specifically associated with KS in AIDS patients, although it is not clear whether the presence of these sequences is causal or is an epiphenomenon of KS.

show that KS330Bam and To KS631Bam are not heritable polymorphic DNA markers for KS, we tested multiple unaffected tissue DNA samples from four additional patients with AIDS-KS (Table 2). Whereas KS lesion DNA samples were positive by Southern hybridization and PCR, unaffected tissues were generally negative for these sequences. All other tissues except muscle and unaffected skin from patient A, stomach adjacent to the KS lesion in patient C, and adjacent skin and hilar lymph nodes in patient D were negative. These results are consistent with an infectious process and may represent local and disseminated spread of the putative virus.

Although these sequences suggest the presence of a new human herpesvirus in KS lesions, 'a causal link between these sequences and AIDS-KS cannot be established by our retrospective case control study. It is possible that this agent is a common latent virus in humans that preferentially colonizes KS lesions in immunosuppressed patients. Unlike previous studies searching for agents associated with KS, the sequences found in our study were present in all intact KS DNA samples from a large number of patients and were preferentially found in diseased as compared to normal tissues from the same host. Our results have been independently confirmed with 100% concordance in a blinded PCR evaluation with extracted AIDS-KS lesion DNA and non-KS brain DNA from the same patients (23).

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- 8. DNA specimens (10 μ g) extracted from both the KS lesion and unaffected tissue were separately digested to completion with Bam HI (20 U/µg) at gested to completion with particular χ_{μ} 37°C for 2 hours, and 2 μ g of digestion fragments were ligated to NBam12 and NBam24 priming sequences [primer sequences described in (7)]. Thirty cycles of PCR amplification were performed to am plify "representations" of both genomes. After construction of the genomic representations, fragments of DNA between 150 and 1500 bp (Fig. 1, lane 1) were isolated from an agarose gel, and NBam priming sequences were removed by digestion with Bam HI. To search for unique DNA sequences not found in non-KS DNA, a second set of priming sequences (JBam12 and JBam24) was ligated onto only the KS fragments (Fig. 1, lane 1). The ligated KS DNA fragments (0.2 μ g) were hybridized to 20 μ g of unligated, fragments representing normal tissue DNA. A sample of the hybridization product was then subjected to 10 cycles of PCR amplification with JBam24, followed by mung bean nuclease digestion. A sample of the mung bean-treated difference product was then subject ed to 15 more cycles of PCR with the JBam24 primer (Fig. 1, lane 2). Amplification products were redigested with Bam HI, and 200 ng of the digested product was ligated to RBam12 and RBam24 primer sets for a second round of hybridization and PCR amplification (Fig. 1, lane 3). This enrichment pro-cedure was repeated a third time with the JBam primer set (Fig. 1, lane 4). Both the original KS DNA and the DNA from non-KS tissue used in the RDA (Table 2, patient A) were subsequently found to contain the AIDS-KS-specific sequences KS330Bam and KS631Bam, indicating that RDA can be successfully used when the target sequence es are present in unequal copy number in both tissues.
- 9. Gel-purified RDA products were cloned in the pCRII vector through use of the TA cloning system (Invitrogen, San Diego, CA). Sequencing was done with Sequenase version 2.0 (U.S. Biochemical) system according to the manufacturer's instructions. Nucleotide sequences were confirmed with an Applied Biosystems 373A Sequencer in the DNA Sequencing Facilities at Columbia University.
- 10. SwissProt and PIR protein databases were searched for homologous ORF with BLASTX [S. F. Altshul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, J. Mol. Biol. 215, 403 (1990)].
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- 13. KS330Bam was used as a probe to isolate eight cross-hybridizing λ phage clones. DNA from one of these clones was digested with Pvu II, which cuts once within KS330Bam (bp 1084, Fig. 2A) and probed with labeled KS330Bam DNA. Two hybridizing bands, \sim 1.1 kb and 3 kb in length from opposites sides of the Pvu II site, were identified and subcloned. The entire 1.1-kb fragment and 768 bp of the 3-kb fragment were sequenced for homology comparisons.
- Use of BLASTX (10) for local alignment of the trans-14. lated six-frame nucleotide sequence to the NCBI NR database resulted in the following list of herpesviridae MCP alignments, in decreasing order of homology (Poisson probabilities and percentage amino acid identity of major HSP in parentheses): HVS (6.1 × e^{-103} , 70%), EBV (2.0 × e^{-99} , 67%), bovine herpesvirus type 4 (1.6 × e^{-85} , 73%), HHV1 (5.0 × e^{-26} , 40%), equine herpesvirus type 1 (1.9 \times e^{-23} , 41%), VZV (2.0 \times e^{-20} , 46%), suid herpesvirus type 1 (3.5 \times e^{-16} , 61%), HHV6 (1.8 \times e^{-14} , 25%), HHV7 (6.7 \times e⁻¹⁴, 26%), and CMV (3.5 \times e⁻¹³, 27%).
- 15. The tissues, listed in Table 1, were collected from diagnostic biopsies and autopsies between 1983 and 1993 and stored at -70°C. Each tissue sample was from a different patient. Most of the 27 KS specimens were from lymph nodes dissected under surgical conditions, which diminishes possible contamination with normal skin flora. All AIDS-KS specimens were examined microscopically for morphologic confirmation of KS and immunohistochemically for factor VIII. Ulex europaeus, and CD34 antigen expression. One of the AIDS-KS specimens was apparently mislabeled, because KS tissue was not detected on microscopic examination but was included in the KS specimen group for purposes of statistical analysis. Additional clinical and demographic information on the specimens was not collected to preserve patient confidentiality.
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- The conditions for PCR analyses were as follows: 94°C for 2 min (1 cycle); 94°C for 1 min, 58°C for 1 min, 72°C for 1 min (35 cycles); 72°C extension for 5 min (1 cycle). Each PCR reaction used 0.1 μ g of genomic DNA, 50 pmol of each primer, 1 U of Taqpolymerase, 100 μ M of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM tris-HCl (pH 9.0), and 0.1% Triton X-100 in a final volume of 25 µl. Amplifications were carried out in a Perkin-Elmer 480 Thermocycler with 1-s ramp times between steps.
- 18. PCR amplification of the human p53 tumor suppressor gene was used as a control for DNA quality. Sequences of p53 primers derived from published sequences are as follows: P6-5, 5'-ACAGGGCTG-GTTGCCCAGGGT-3'; P6-3, 5'-AGTTGCAAACCA-GACCTCAG-3' [G. Gaidano et al., Proc. Natl. Acad. Sci. U.S.A. 88, 5413 (1991)].
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- 24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln, R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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