(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 $MgCl_2$, 0.5 mCi of [γ-^{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 × 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 $MgCl_2$, 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 ^{32}P–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 × 10^7 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 ^{32}P-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 ^{32}P–GST-SHC as above, except that the hybridization buffer included 1 mM sodium orthovanadate.
16. We thank P. P. Di Fiore and B. Knudsen for the Eps 15 and C3G antibodies, respectively, and W. J. Fantl, J. A. Escobedo, D. Schneider, and T. Quinn for reviewing the manuscript. Supported by NIH grants K11 HL02714 and R01 HL32898 and by the Daiichi Research Center.

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

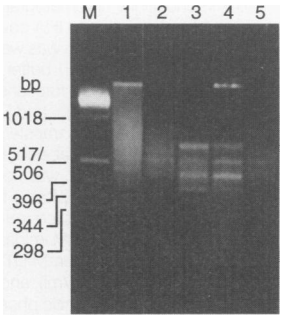

Fig. 1. Agarose gel electrophoresis of RDA products from AIDS-KS tissue and nondiseased tissue. RDA was performed on DNA extracted from KS skin tissue and unaffected normal skin tissue obtained at autopsy from a homosexual man with AIDS-KS (*8*). Lane 1 shows the initial PCR-amplified genomic representation 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, respectively) hybridized nonspecifically to both KS and non-KS human DNA. Lane M is a molecular size marker.

A

BcLF1-ORF25 homolog

BDLF1-ORF26 homolog

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          10         20         30         40         50         60         70         80         90        100
AACCTGCGAG ATAATTCCCA CGCCGGTCAC ATCTGACGTT GCCTATTTCC AGACCCCCAG CAACCCCCGG GGGCGTGCGG CGTCGGTCGT GTCGTGTGAT
         110        120        130        140        150        160        170        180        190        200
GCTTACAGTA ACGAAAGCGC AGAGCGTTTG TTCTACGACC ATTCAATACC AGACCCCGCG TACGAATGCC GGTCCACCAA CAACCCGTGG GCTTCGCAGC
         210        220        230        240        250        260        270        280        290        300
GTGGCTCCCT CGGCGACGTG CTATACAATA TCACCTTTCG CCAGACTGCG CTGCCGGGCA TGTACAGTCC TTGTCGGCAG TTCTTCCACA AGGAAGACAT
         310        320        330        340        350        360        370        380        390        400
TATGCGGTAC AATAGGGGGT TGTACACTTT GGTTAATGAG TATTCTGCCA GGCTTGCTGG GGCCCCCGCC ACCAGCACTA CAGACCTCCA GTACGTCGTG
         410        420        430        440        450        460        470        480        490        500
GTCAACGGTA CAGACGTGTT TTTGGACCAG CCTTGCCATA TGCTGCAGGA GGCCTATCCC ACGCTCGCCG CCAGCCACAG AGTTATGCTT GCCGAGTACA
         510        520        530        540        550        560        570        580        590        600
TGTCAAACAA GCAGACACAC GCCCCAGTAC ACATGGGCCA GTATCTCATT GAAGAGGTGG CGCCGATGAA GAGACTATTA AAGCTCGGAA ACAAGGTGGT
     STOP610        620        630     START 640        650        660        670        680        690        700
GTATTAGCTA ACCCTTCTAG CGTTGGCTAG TCATGGCACT CGACAAGAGT ATAGTGGTTA ACTTCACCTC CAGACTCTTC GCTGATGAAC TGGCCGCCCT
         710        720        730        740        750        760        770        780        790        800
TCAGTCAAAA ATAGGGAGCG TACTGCCGCT CGGAGATTGC CACCGTTTAC AAAATATACA GGCATTGGGC CTGGGGTGCG TATGCTCACG TGAGACATCT
         810        820        830        840        850        860        870        880        890        900
CCGGACTACA TCCAAATTAT GCAGTATCTA TCCAAGTGCA CACTCGCTGT CCTGGAGGAG GTTCGCCCGG ACAGCCTGCG CCTAACGCGG ATGGATCCCT
         910        920        930        940        950        960        970        980        990       1000
CTGACAACCT TCAGATAAAA AACGTATATG CCCCCTTTTT TCAGTGGGAC AGCAACACCC AGCTAGCAGT GCTACCCCCA TTTTTTAGCC GAAAGGATTC
        1010       1020       1030       1040       1050       1060       1070                  Pvu II site 1090      1100
CACCATTGTG CTCGAATCCA ACGGATTTGA CCCCGTGTTC CCCATGGTCG TGCCGCAGCA ACTGGGGCAC GCTATTCTGC AGCAGCTGTT GGTGTACCAC
        1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
ATCTACTCCA AAATATCGGC CGGGGCCCCG GATGATGTAA ATATGGCGGA ACTTGATCTA TATACCACCA ATGTGTCATT TATGGGGCGC ACATATCGTC
        1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
TGGACGTAGA CAACACGGAT CCACGTACTG CCCTGCGAGT GCTTGACGAT CTGTCCATGT ACCTTTGTAT CCTATCAGCC TTGGTTCCCA GGGGGTGTCT
        1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
CCGTCTGCTC ACGGCGCTCG TGCGGCACGA CAGGCATCCT CTGACAGAGG TGTTTGAGGG GGTGGTGCCA GATGAGGTGA CCAGGATAGA TCTCGACCAG
        1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
TTGAGCGTCC CAGATGACAT CACCAGGATG CGCGTCATGT TCTCCTATCT TCAGAGTCTC AGTTCTATAT TTAATCTTGG CCCCAGACTG CACGTGTATG
        1510       1520       1530       1540      STOP1560       1570       1580       1590       1600
CCTACTCGGC AGAGACTTTG GCGGCCTCCT GTTGGTATTC CCCACGCTAA CGATTTGAAG CGGGGGGGGT ATGGCGTCAT CTGATATTCT GTCGGTTGCA
        1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
AGGACGGATG ACGGCCTCGT CTGTGAAGTC TCCCTGCGTG GAGGTAGGAA AAAAACTACC GTCTACCTGC CGGACACTGA ACCCTGGGTG GTAGAGACCG
        1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
ACGCCATCAA AGACGCCTTC CTCAGCGACG GGATCGTGGA TATGGCTCGA AAGCTTCATC GTGGTGCCCT GCCCTCAAAT TCTCACAACG GCTTGAGGAT
        1810       1820       1830       1840       1850
GGTGCTTTTT TGTTATTGTT ACTTGAAAAT TGTGTGTACC TAGCCCTGTT CTG.
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B

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          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

         190        200        210        220        230        240
ATGCGCACCG CGTTGTCGGA GCTGTCGCGA TAGAGGTTAG GGTAGGTGTC CGGTCCGTCC

         250        260        270        280        290        300
GTGGGCTCAA ACCTGCCCAG ACACACCACT 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 CCCCCTGGGT CTGTCCGCCT

         550        560        570        580        590        600
GGAAACAGGG TGCTGTGAAA CAACAGGTTG CCAAGGCCGC GAATACCCCT CTGCACGCTG

         610        620        630
CTGTGGACGT GGGTGTATGC TCCGTGGATC C
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Fig. 2. Nucleotide sequences of the 1853-bp flanking region that includes KS330Bam derived from a KS lesion genomic library (**A**) and the KS631Bam sequence derived from KS tissue by RDA (**B**). KS330Bam (A) is underlined and Bam HI restriction sites (GGATCC) are double-underlined. A reading frame composed of the first 607 nucleotides (bp 1 to 607, stop codon in bold) is homologous to the COOH-termini of the major capsid protein open reading frames ORF25 of herpesvirus saimiri and BcLF1 of Epstein-Barr virus (EBV). An open reading frame from bp 633 to 1550 is homologous to ORF26 gene of herpesvirus saimiri and BDLF1 gene of EBV [start methionine codon (bp 633) and stop codon (bp 1548) in 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 $KS330_{233}$ (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 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).

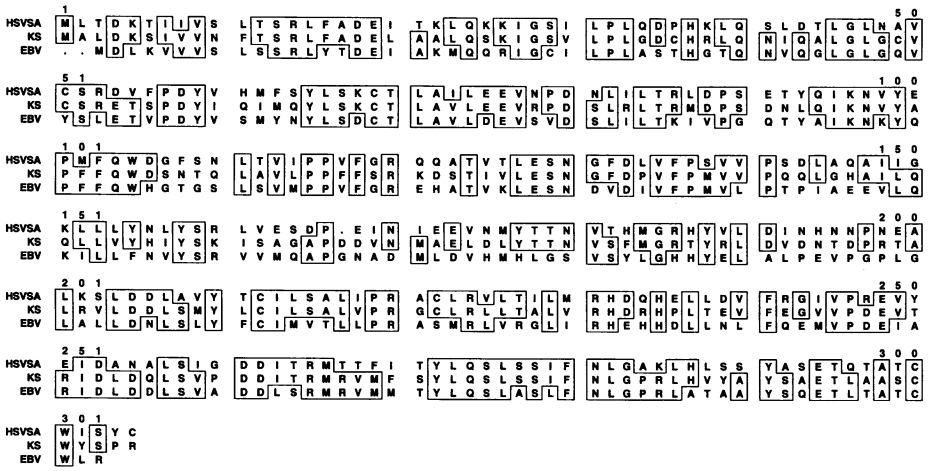

Fig. 3. Comparison of protein sequences encoded by ORF26 from herpesvirus saimiri (HVS), and BDLF1 from EBV, to the protein encoded by the KS-associated DNA open reading frame. Regions of amino acid identity between KS and HVS, or KS and EBV are outlined (*24*).

The polypeptide encoded by the KS-associated 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*).

Table 1. Southern blot hybridization for KS330Bam and KS631Bam and by PCR amplification for $KS330_{233}$ 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‡	0 (0)	0 (0)	0 (0)
Non-AIDS lymph nodes	7	0 (0)	0 (0)	0 (0)
Vascular tumors	5§	0 (0)	0 (0)	0 (0)
Opportunistic infections	13‖	0 (0)	0 (0)	0 (0)
Consecutive surgical biopsies	49¶#	0 (0)	0 (0)	0 (0)

*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 KS631Bam and by PCR amplification for the $KS330_{233}$ amplicon. Comparison of AIDS-KS KS330Bam, KS631Bam, and $KS330_{233}$ 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, KS631Bam, and $KS330_{233}$ positivity were 22.8, $P = 3 \times 10^{-6}$; 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 KS330Bam, KS631Bam, and $KS330_{233}$ 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 hairy-cell leukemias, 2 monocytoid B-cell lymphomas, 1 follicular small cleaved–cell lymphoma, 1 Burkitt's lymphoma, and 1 plasmacytoma. §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 $KS330_{233}$.

To determine the specificity of KS330Bam and KS631Bam for AIDS-KS, these sequences were random-primed, ^{32}P-labeled, 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 $KS330_{233}$. 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 $KS330_{233}$ (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 $KS330_{233}$ appeared 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 $KS330_{233}$ 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 $KS330_{233}$. 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 inadequate PCR amplification was not the reason for lack of detection of $KS330_{233}$ in the control tissues. Thus, $KS330_{233}$ was found in all 25 amplifiable tissues with microscopically detectable AIDS-KS, but rarely occurred in non-KS tissues, including tissues from AIDS patients. Additional DNA samples from EBV-infected lymphocytes and from *M. penetrans* (ATCC #55252), a candidate KS agent (*19*), were negative for $KS330_{233}$. Several KS-tissue DNA samples tested with EBV-specific and mycoplasmata-specific consensus PCR primers were also negative (*20*).

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

Table 2. Differential detection of KS330Bam, KS631Bam, and $KS330_{233}$ 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	KS631Bam	$KS330_{233}$
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	–	–	–

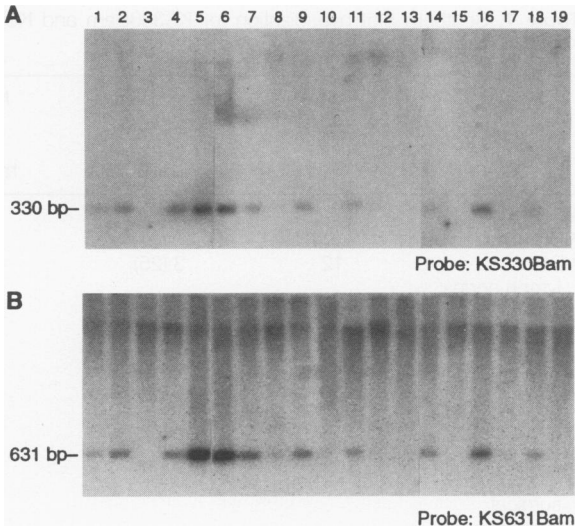

Fig. 4. Hybridization of ^{32}P-labeled KS330Bam (**A**) and KS631Bam (**B**) sequences obtained by RDA to a representative panel of 19 DNA samples extracted from KS lesions and digested with Bam HI. KS330Bam hybridized to 11 of the 19 and KS631Bam hybridized to 12 of 19 DNA samples from the AIDS-KS 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.

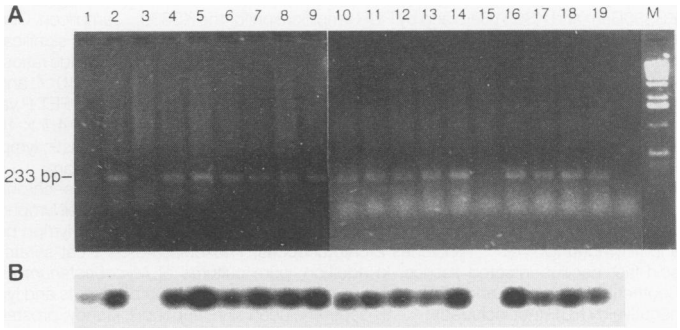

Fig. 5. PCR amplification of the 19 KS-derived DNA samples shown in Fig. 4, using the $KS330_{233}$ primers shown in Fig. 2. (**A**) shows the agarose gel of the amplification products from 19 KS DNA samples (lanes 1 to 19), and (**B**) shows specific hybridization of the PCR products to a ^{32}P end-labeled 25-bp internal oligonucleotide (Fig. 2) 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 $KS330_{233}$. Lane 20 is a negative control and lane M molecular size marker.

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 $KS330_{233}$, 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.

To show that KS330Bam and 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 μg) extracted from both the KS lesion and unaffected tissue were separately digested to completion with Bam HI (20 U/μg) at 37°C for 2 hours, and 2 μg of digestion fragments were ligated to NBam12 and NBam24 priming sequences [primer sequences described in (*7*)]. Thirty cycles of PCR amplification were performed to amplify "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 μg) were hybridized to 20 μg 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 subjected 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 procedure 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 sequences 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, ~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.
14. Use of BLASTX (*10*) for local alignment of the translated 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 ($6.1 \times e^{-103}$, 70%), EBV ($2.0 \times e^{-99}$, 67%), bovine herpesvirus type 4 ($1.6 \times e^{-85}$, 73%), HHV1 ($5.0 \times e^{-26}$, 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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17. 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 μg of genomic DNA, 50 pmol of each primer, 1 U of *Taq* polymerase, 100 μM 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′-ACAGGGCTGGTTGCCCAGGGT-3′; P6-3, 5′-AGTTGCAAACCAGACCTCAG-3′ [G. Gaidano *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5413 (1991)].
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23. L. Kingsley, unpublished data.
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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Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma

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