In Vitro Establishment and Characterization of Two Acquired Immunodeficiency Syndrome–Related Lymphoma Cell Lines (BC-1 and BC-2) Containing Kaposi's Sarcoma–Associated Herpesvirus-Like (KSHV) DNA Sequences

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Two unique DNA fragments were recently identified in over 90% of acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma tissues. Sequence analysis suggests that these fragments belong to a previously unidentified human herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV). These fragments have also been identified in a subset of non-Hodgkin's lymphomas in human immunodeficiency virus-positive patients; specifically, in body cavity-based lymphomas (AIDS-BCBLs). We have established two cell lines derived from AIDS-BCBLs, BC-1 and BC-2, which retain the KSHV sequences, and have used them to further characterize this putative viral genome. In this report, we demonstrate that the KSHV sequences represent a portion of a much larger DNA molecule that is located predominantly in the nucleus of the infected cells. In situ hybridization of metaphase spreads made from both of these cell lines show these sequences in episomal structures. Their presence in the cells as large nuclear episomes supports previous sequence homology data suggesting that KSHV belongs to the herpesvirus family. These cell lines provide a continuous source of KSHV sequences. Thus, they represent an important tool for future studies of this recently described human herpesvirus that may be involved in the pathogenesis of Kaposi's sarcoma and some AIDS-related non-Hodgkin's lymphomas.

RECENT EVIDENCE suggests the presence of a novel infectious agent in Kaposi's sarcoma (KS). Two DNA fragments, KS330Bam and KS631Bam, with significant homology to gammaherpesviruses, are present in a very high proportion of KS tissues. In contrast, control tissues from non-acquired immunodeficiency syndrome (AIDS) patients without KS are largely negative.[1,2] These sequences have also been identified in eight non-Hodgkin's lymphomas (NHLs) occurring in human immunodeficiency virus (HIV)-positive individuals, all of which are body cavity-based lymphomas (AIDS-BCBLs).[3] These lymphomas appear to represent a distinct group of B-cell NHLs with a strikingly similar and unusual constellation of clinical, morphologic, immunophenotypic, and molecular genetic characteristics that distinguishes them from the vast majority of AIDS-related lymphomas.[4-6] These features include exclusive or dominant involvement of the pleural, pericardial, and/or abdominal cavities as lymphomatous effusions, usually in the absence of any identifiable tumor mass throughout the clinical course, an indeterminate immunophenotype, and a B-cell genotype based on the presence of clonal immunoglobulin gene rearrangements. Furthermore, in contrast with most AIDS-related B-cell NHLs,[7,8] the AIDS-BCBL cases analyzed so far usually contain Epstein-Barr virus (EBV) and consistently lack c-myc gene rearrangements.[3,4,6,9]

Sequence analysis of the KS330Bam and KS631Bam fragments, as well as of cloned flanking regions,[1] has shown homology to two viruses in particular: EBV[10] and herpesvirus saimiri (HVS).[11] Both of these viruses are members of the *gammaherpesvirinae* subfamily of herpesvirus, which members are characterized by their ability to replicate in lymphoblastoid cells.[12] The degree of homology, with amino acid identities in the range of 30% to 50%, is consistent with these sequences belonging to a novel member of the same family, which we refer to as Kaposi's sarcoma-associated herpesvirus (KSHV). It is well known that EBV immortalizes B cells in vitro and is associated with certain malignant lymphomas, including Burkitt's lymphoma, some AIDS-related lymphomas, the post-transplantation lymphoproliferative disorders, and Hodgkin's disease.[13] HVS is a virus of squirrel monkeys (*Saimiri sciureus*) that can be isolated from peripheral blood mononuclear cells of healthy animals but causes fulminant T-cell lymphomas in New World primates other than its natural hosts.[14] HVS is also capable of transforming human T lymphocytes to continuous growth in vitro.[15] Thus, the two herpesviruses most homologous to KSHV have the ability to latently infect peripheral blood lymphocytes of their natural host, immortalize lymphocytes in vitro, and lead to the development of malignant lymphomas. This finding, in conjunction with the presence of KSHV sequences in all AIDS-BCBLs analyzed thus far, suggests that these sequences may play a pathogenic role in the development of some malignant lymphomas. We report the in vitro establishment of two AIDS-BCBL cell lines and their use to determine the cellular localization and general structure of the KSHV sequences.

MATERIALS AND METHODS

Pathologic Samples

The cell lines BC-1 and BC-2 were derived in the laboratory of one of the investigators (D.M.K.) from previously reported cases lymphoma 1 and 2.[3] Case 1 also corresponds to patient 1 reported by Chadburn et al,[9] and case 2 corresponds to patient 1 reported by Knowles et al.[14] These cases also correspond to two of the three

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AIDS-related lymphomas found to be KSHV sequence-positive as reported by Chang et al.[1] The principal clinical, morphologic, immunophenotypic and genotypic characteristics of both cases have been reported.[3]

Malignant effusion samples were collected during the course of standard diagnostic procedures under sterile conditions. Mononuclear cells were isolated from the effusion samples by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation. Cells were cryopreserved in liquid nitrogen in RPMI solution containing 10% dimethyl sulfoxide (DMSO) and 40% fetal calf serum at −170°C.

Establishment of Cell Lines and Cell Culture Procedures

The cryopreserved cells were thawed and plated at a concentration of 2×10^6/mL on top of a layer of heterologous feeder cells. For this purpose, peripheral blood lymphocytes from a normal donor were irradiated with 50 Gy and plated at a concentration of 1.5×10^6 per well in 24-well plates 24 hours before the addition of the lymphoma cells directly to these cultures. Because the AIDS-BCBL cells contain EBV, superinfection with this virus was not used for their establishment. Cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum at 37°C in the presence of 5% CO_2. Cell lines were passaged every 3 to 4 days after one to two cell doublings. Cell viability and evidence of cell lysis was evaluated by examination in a phase contrast microscope in the presence of Trypan blue.

Immunophenotypic Analysis

The immunophenotype of the BCBL tumor cells and cell lines was determined by direct and indirect immunofluorescent flow cytometry using the FACScan fluorescent activated cell sorter (Becton-Dickinson, Mountain View, CA) as previously described.[4,5,9] The monoclonal antibodies used include leukocyte common antigen (LCA; CD45), BerH2, Ki-1 (CD30), epithelial membrane antigen (EMA; Dako Corp, Santa Barbara, CA), B4 (CD19), B1 (CD20), B2 (CD21; Coulter Immunology, Hialeah, FL), Leu1 (CD5), Leu14 (CD22), Leu20 (CD23; Becton-Dickinson Immunocytometry Systems, Mountain View, CA), and T3 (CD3; United Biomedical Inc, Hauppauge, NY). Antisera to kappa and lambda immunoglobulin light chains were obtained from Dako.

DNA Extractions

Genomic DNA was extracted from mononuclear cell suspensions by a previously reported salting-out procedure not requiring organic extraction.[16] To determine the nuclear versus cytoplasmic localization of KSHV DNA, we disrupted the plasma membranes by resuspending the cells in a solution of 20 mmol/L Tris (pH 7.9), 3 mmol/L $MgCl_2$, and 2 mmol/L $CaCl_2$, incubating 20 minutes on ice, and adding NP40 to 0.5%. The nuclei were then separated from the cytoplasmic fraction by centrifugation at 1,500*g* for 15 minutes. The cytoplasmic fraction was recentrifuged at 2,500*g*, and the pellet was discarded to eliminate residual nuclei. Subsequently, the microsomal fraction was separated from the cytoplasm by centrifugation at 17,000*g* for 30 minutes. The three fractions—nuclear, microsomal, and cytoplasmic—were then extracted with phenol/chloroform. We used the method described by Hirt[17] to determine whether the KSHV DNA was of relatively high or low molecular weight. Essentially, the cells were lysed with 0.6% sodium dodecyl sulfate (SDS) and 1 mol/L NaCl; after centrifugation at 17,000*g* for 30 minutes, the pellet containing the high-molecular-weight DNA was treated with Proteinase K, and both the pellet and supernatant were extracted with phenol/chloroform. Using this method, unintegrated small viral DNA molecules should be found in the supernatant.

Oligonucleotide Primers and Probes

All the oligonucleotides used for polymerase chain reaction (PCR) amplification in this study were synthesized by the solid-phase triester method. Sequences of oligonucleotides used for amplification and sequencing of the $KS330_{233}$ region have been previously reported.[1] Primers used for amplification and sequencing of flanking regions were derived from published sequences[1] and are underscored in Fig 1.

Direct Sequencing of PCR

PCRs were performed as previously described.[1] DNA sequencing for the $KS330_{233}$ fragment and flanking region was performed on the two positive lymphomas from which the cell lines were obtained. PCR products were directly sequenced using the Taq DyeDeoxy terminator cycle sequencing system with an ABI 373A automated DNA sequencer (Applied Biosystems Inc, Foster City, CA). The two strands and two independent PCRs were sequenced to exclude mismatches caused by polymerase mistakes.

Southern Blot Analysis

Aliquots (5 or 10 μg) of genomic DNA were digested with the appropriate restriction endonucleases according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN), electrophoresed in 0.8% agarose gels, denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern.[18] In cellular fractionation experiments, 1 μg of DNA from nuclear, cytoplasmic, and microsomal fractions was digested and loaded onto the corresponding lane; in the Hirt extraction experiments, 5 μg of DNA were used from both the pellet and the supernatant fractions. The filters were hybridized as previously described[19] to probes that had been ^{32}P-labeled by the random primer extension method.[20] Autoradiography was performed at −70°C for 2 to 16 hours. The presence of KSHV sequences was determined by hybridization of *Bam*HI-digested DNAs to ^{32}P-labeled KS330Bam and KS631Bam probes.[1] The immunoglobulin heavy chain gene was investigated by hybridization of *Eco*RI- and *Hin*dIII-digested DNAs to an immunoglobulin heavy chain gene joining region (J_H) probe.[21] The presence and clonality of EBV was investigated by hybridization of *Bam*HI-digested DNAs to a probe (TR) that detects EBV genomic termini.[22]

In Situ Hybridization of Metaphase Spreads

Metaphase spreads were prepared from the BC-1 and BC-2 cell lines by conventional methods. For fluorescence in situ hybridization (FISH), a bacteriophage λ clone, SGL1, containing a KSHV 12-kb insert was used as a probe. This clone was obtained by screening a lymphoma 1 genomic library with the KS631Bam fragment, and was shown to contain only KSHV sequences by hybridization experiments. SGL1 DNA was labeled by nick translation with biotin 11-deoxyuridine triphosphate (dUTP) (BRL, Gaithersburg, MD). FISH and detection of the hybridized probe were performed as described.[23] Images were captured by a cooled CCD Camera (Photometrics, Tucson, AZ) and analyzed using Smartcapture Imaging System (Vysis, Framingham, MA).

RESULTS

Confirmation of KSHV Sequences in the AIDS-BCBL Tumor Cells

We performed PCR amplification and sequencing of a 965-bp region of KSHV spanning the KS330Bam fragment to confirm the presence and identity of these sequences in the tumor cells from cases lymphoma 1 and 2 (Fig 1). Our

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                 ->                                  START
KS          1    TGTATTAGCT AACCCTTCTA GCGTTGGCTA GTCATGGCAC TCGACAAGAG TATAGTGGTT AACTTCACCT CCAGACTCTT
BCBL-1      1    TGTATTAGCT AACCCTTCTA GCGTTGGCTA GTCATGGCAC TCGACAAGAG TATAGTGGTT AACTTCACCT CCAGACTCTT
BCBL-2      1    TGTATTAGCT AACCCTTCTA GCGTTGGCTA GTCATGGCAC TCGACAAGAG TATAGTGGTT AACcTCACCT CCAGACTCTT

KS         81    CGCTGATGAA CTGGCCGCCC TTCAGTCAAA AATAGGGAGC GTACTGCCGC TCGGAGATTG CCACCGTTTA CAAAATATAC
BCBL-1     81    CGCTGATGAA CTGGCCGCCC TTCAGTCAAA AATAGGGAGC GTACTGCCGC TCGGAGATTG CCACCGTTTA CAAAATATAC
BCBL-2     81    CGcTGAcGAA CTGGCCGCCC TTCAGTCAAA AATAGGGAGC GTACTGCCGC TCGGAGATTG CCACCGTTTA CAAAATATAC

KS        161    AGGCATTGGG CCTGGGGTGC GTATGCTCAC GTGAGACATC TCCGGACTAC ATCCAAATTA TGCAGTATCT ATCCAAGTGC
BCBL-1    161    AGGCATTGGG CCTGGGGTGC GTATGCTCAC GTGAGACATC TCCGGACTAC ATCCAAATTA TGCAGTATCT ATCCAAGTGC
BCBL-2    161    AGGCATTGGG CCTGGGGTGC GTATGCTCAC GTGAGACATC TCCGGACTAC ATCCAAATTA TGCAGTATCT ATCCAAGTGC

KS        241    ACACTCGCTG TCCTGGAGGA GGTTCGCCCG GACAGCCTGC GCCTAACGCG GATGGATCCC TCTGACAACC TTCAGATAAA
BCBL-1    241    ACACTCGCTG TCCTGGAGGA GGTTCGCCCG GACAGCCTGC GCCTAACGCG GATGGATCCC TCTGACAACC TTCAGATAAA
BCBL-2    241    ACACTCGCTG TCCTGGAGGA GGTTCGCCCG GACAGCCTGC GCCTAACGCG GATGGATCCC TCTGACAACC TTCAGATAAA
                                                                                              ->
KS        321    AAACGTATAT GCCCCCTTTT TTCAGTGGGA CAGCAACACC CAGCTAGCAG TGCTACCCCC ATTTTTTAGC CGAAAGGATT
BCBL-1    321    AAACGTtTAT GCCCCCTTTT TTCAGTGGGA CAGCAACACC CAGCTAGCAG TGCTACCCCC ATTTTTTAGC CGAAAGGATT
BCBL-2    321    AAACGTtTAT GCCCCCTTTT TTCAGTGGGA CAGCAACACC CAGCTAGCAG TGCTACCCCC AcTTTTTAGg CGAAAGGATT

KS        401    CCACCATTGT GCTCGAATCC AACGGATTTG ACCCCGTGTT CCCCATGGTC GTGCCGCAGC AACTGGGGCA CGCTATTCTG
BCBL-1    401    CCACCATTGT GCTCGAATCC AACGGATTTG ACCtCGTGTT CCCCATGGTC GTGCCGCAGC AACTGGGGCA CGCTATTCTG
BCBL-2    401    CCACCATTGT GCTCGAATCC AACGGATTTG ACatCGTGTT CCCCATGGTC GTGCCtCAGC AACTGGGGCA CGCTATTCTG
                                                                                  ->
KS        481    CAGCAGCTGT TGGTGTACCA CATCTACTCC AAAATATCGG CCGGGGCCCC GGATGATGTA AATATGGCGG AACTTGATCT
BCBL-1    481    CAGCAGCTGT TGGTGTACCA CATCTACTCC AAAATATCGG CCGGGGCCCC GGATGATGTA AATATGGCGG AACTTGATCT
BCBL-2    481    CAGCAGCTGT TGGTGTACCA CATCTACTCC AAAATATCGG CCGGGGCCCC GGgTGATGTc AATATGGCGG AACTTGATCT
                                                                              <-
KS        561    ATATACCACC AATGTGTCAT TTATGGGGCG CACATATCGT CTGGACGTAG ACAACACGGA TCCACGTACT GCCCTGCGAG
BCBL-1    561    ATATACCACC AATGTGTCAT TTATGGGGCG CACATATCGc CTGGACGTAG ACAACACGGA TCCACGTACT GCCCTGCGAG
BCBL-2    561    ATATACCACC AATGTGTCAT TTATGGGGCG CACATATCGT CTGGACGTAG ACAACACGGA TCCACGTACT GCCCTGCGAG

KS        641    TGCTTGACGA TCTGTCCATG TACCTTTGTA TCCTATCAGC CTTGGTTCCC AGGGGGTGTC TCCGTCTGCT CACGGCGCTC
BCBL-1    641    TGCTTGACGA TCTGTCCATG TACCTTTGTA TCCTATCAGC CTTGGTTCCC AGGGGGTGTC TCCGTCTGCT CACGGCGCTC
BCBL-2    641    TGCTTGACGA TCTGTCCATG TACCTTTGTA TCCTATCAGC CTTGGTTCCC AGGGGGTGTC TCCGTCTGCT CACGGtGCTC

KS        721    GTGCGGCACG ACAGGCATCC TCTGACAGAG GTGTTTGAGG GGGTGGTGCC AGATGAGGTG ACCAGGATAG ATCTCGACCA
BCBL-1    721    GTGCGGCACG ACAGGCATCC TCTGACAGAG GTGTTTGAGG GGGTGGTGCC AGATGAGGTG ACCAGGATAG ATCTCGACCA
BCBL-2    721    GTGCGGaACG ACAGGCATCC TCTGACAGAG GTGTTTGAGG GGGTGGTGCC AGATGAGGTG ACCAGGATAG ATCTCGACCA

KS        801    GTTGAGCGTC CCAGATGACA TCACCAGGAT GCGCGTCATG TTCTCCTATC TTCAGAGTCT CAGTTCTATA TTTAATCTTG
BCBL-1    801    GTTGAGCGTC CCAGATGACA TCACCAGGAT GCGCGTCATG TTCTCCTATC TTCAGAGTCT CAGTTCTATA TTTAATCTTG
BCBL-2    801    GTTGAGCGTC CCAGATGACA TCACCAGGAT GCGCGTCATG TTCTCCTATC TTCAGAGTCT CAGTTCTATA TTTAATCTcG
                                                                                  STOP              <-
KS        881    GCCCCAGACT GCACGTGTAT GCCTACTCGG CAGAGACTTT GGCGGCCTCC TGTTGGTATT CCCCACGCTA ACGATTTGAA GCGGG
BCBL-1    881    GCCCCAGACT GCACGTGTAT GCCTACTCGG CAGAGACTTT GGCGGCCTCC TGTTGGTATT CCCCACGCTA ACGATTTGAA GCGGG
BCBL-2    881    GCCCCAGACT GCACGTGTAT GCCTACTCGG CAGAGACTTT cGCGGCCTCC TGTTGGTATT CCCCACGCTA ACGATTTGAt GCGGG
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Fig 1. Sequence analysis of the $KS330_{233}$ fragment and flanking regions in BCBL cases lymphoma 1 and lymphoma 2. Direct sequencing after PCR of a 965-bp region, which comprises the entire open reading frame (start and stop codons indicated) homologous to EBV BDLF1 and HVS ORF26, was performed. Primers used for sequencing are underscored, and their orientation is shown by arrows. The region corresponding to the $KS330_{233}$ fragment previously reported[1,3] is italicized. Sequences obtained from cases lymphoma 1 and lymphoma 2 PCR are compared with previously reported sequences from a KS DNA.[1] Differences in the nucleotide sequences are shown in bold lower case letters.

results show that the KSHV sequences in both of these cases are very similar to the sequence previously reported from a case of KS,[1] differing by only three and 14 nucleotides in lymphomas 1 and 2, respectively. This result suggests that the sequences obtained by PCR from the malignant lymphomas correspond to the same agent previously identified in KS. Furthermore, some nucleotide differences were identified, consistent with the presence of independent isolates in the two lymphomas and the case of KS, making contamination unlikely.

Establishment of the BCBL Cell Lines

Two AIDS-BCBL cell lines were established from two different patients with lymphomatous effusions. These cell lines, BC-1 and BC-2, were derived from lymphoma cases 1 and 2, respectively.[3] The main characteristics of these neoplasms have been previously reported.[3] After cultures were started, only large tumor cells were visible at about day 30 in both cases. Both cell lines have a doubling time of 48 hours to 72 hours. Cells maintained at a concentration of 0.5 $\times$ 10^6/mL to 1.5 $\times$ 10^6/mL had a viability of greater than 80% and showed little evidence of lysis. Cells of both cultured BCBL cell lines tend to grow in small clusters. BC-1 and BC-2 cells have been grown for more than 150 and 50 passages, respectively.

Characterization of the BCBL Cell Lines

Immunophenotypic analysis. Table 1 shows the results of the comparative immunophenotypic analysis performed on BC-1 and BC-2 cells and the original pathologic specimens from which they were derived. These results demonstrate that the immunophenotype of the cell lines is generally comparable with the parental tumor. One exception is the expression of CD23 by the original lymphoma 2 tumor cells

Table 1. Summary of Immunophenotypic Profiles of Two AIDS-BCBLs and the Corresponding Cell Lines

Antigen	Lymphoma 1 Tumor Cells	BC-1 Cell Line	Lymphoma 2 Tumor Cells	BC-2 Cell Line
CD45	>90	>99	>90	>99
CD19	—	—	—	—
CD20	—	—	—	—
CD21	—	—	—	—
CD22	—	—	—	—
CD23	ND	—	>90	—
Ig	—	—	—	—
CD3	—	—	—	—
CD5	—	—	—	—
CD30	>90	>99	—	—
EMA	>90	>99	ND	>99

If positive, the percentage of positive cells is given; —, negative.
Abbreviations: EMA, epithelial membrane antigen; ND: not determined.

but not by the BC-2 cells. Although the expression of CD23 was not studied in the lymphoma 1 tumor cells because of insufficient clinical material, it is not expressed in the BC-1 cells, similar to BC-2. CD23 is a B cell-associated activation antigen that is superinduced by EBV.[24] The mechanism responsible for the loss of CD23 expression in these cell lines remains to be elucidated. The results of immunophenotypic analysis also show that our cultures contain a monotonous population of neoplastic cells, where over 99% of the cells were positive for the expressed antigens. Thus, methods such as limiting dilution to obtain pure cell populations were unnecessary.

Genotypic analysis. To define the clonality of the established BCBL cell lines and their derivation from the original neoplasm, Ig heavy chain gene rearrangement analysis was performed. These studies demonstrated that BC-1 and BC-2 cells display clonal Ig gene rearrangements, which in BC-1 were identical to those seen in the corresponding pathologic specimen (Fig 2A). Direct comparison of the Ig gene rearrangement pattern of BC-2 and the original tumor was not performed because of insufficient DNA from the latter. We also studied the cell lines and the original tumors for the presence and clonality of EBV. This virus was present in both cell lines in similar copy number and with the same number of terminal repeats as in the original tumor cells (Fig 2B). Thus, both our cultures contained the same clonal population as the tumor cells from which they were established.

Comparison of restriction pattern of KSHV in lymphoma 1 tumor cells, cell line, and KS. We performed Southern blot analysis for KSHV sequences using DNA obtained from the original BCBL-1 specimen and the cell line derived from this case to determine whether this virus is maintained with respect to copy number and structure in spite of multiple passages. DNA for these experiments was obtained from BC-1 cells after approximately 100 passages. We were unable to analyze BCBL-2 in the same manner because of insufficient DNA from the original tumor cells. We included DNA obtained from KS tissue in these studies as a control and to assess at this level the degree of identity of KSHV between KS and BCBL. These results are shown in Fig 3. They demonstrate that although KSHV sequences were present in approximately 60-fold higher copy numbers in the lymphoma than in the KS DNA, the cell line had only slightly lower copy numbers than the original neoplasm. The restriction pattern was very similar when the BC-1 DNA was compared with the original tumor cells, except for a strong extra band in the cell line using *Pst* I restriction enzyme and the KS631Bam probe. This band may represent a polymorphism acquired in a proportion of the cells during passage. Other, fainter bands are seen with *Hin*dIII and probe KS330Bam in the original tumor cells, as well as with *Pst* I and KS330Bam in the cell line, which may be due to minor polymorphisms or partial DNA digestions (although the latter is unlikely as this experiment has been repeated with identical results). The restriction pattern is also very similar to that seen in the KS tissue, with the exception of the pattern seen with *Pst* I and probe KS627Bam and an additional band seen with *Pvu* II and probe KS330Bam, confirming once again the presence of a very similar, although not identical, organism.

Molecular Characterization of KSHV Sequences

Nuclear versus cytoplasmic localization of KSHV sequences. To investigate the intracellular DNA localization of the KSHV sequences, we isolated DNA from nuclear, microsomal, and cytoplasmic fractions after cell lysis and centrifugation. The different fractions were digested with *Bam*HI and analyzed by Southern blot hybridization with the KS330Bam probe (Fig 4A). Most of the hybridization signal was seen in the nuclear fraction, suggesting that KSHV has a predominantly nuclear localization, a feature consistent with a herpesvirus. As a control, the same filters were rehybridized with a probe for EBV, and the hybridization signal was similarly obtained in the nuclear fraction (not shown).

Hirt extraction of KSHV DNA. A method for selective extraction of low-molecular-weight DNA was established by Hirt[17] to purify polyoma virus DNA. Hybridization to the KS330Bam probe occurred predominantly in the pellet, containing the high-molecular-weight DNA fraction, and only fainter hybridization is seen in the supernatant, containing the low-molecular-weight DNA (Fig 4B). Similar results were obtained when these filters were rehybridized with a probe for EBV (not shown). These results suggest that most of the KSHV DNA is either integrated or of a relatively large size.

In situ hybridization of metaphase spreads. We performed in situ hybridization of metaphase spreads prepared from the BC-1 and BC-2 cells to determine whether the KSHV sequences were integrated or episomal (Fig 5). No specific integration site was identified, consistent with episomal DNA. In addition, multiple signals were detected in each cell, consistent with previous quantitative data suggesting that 40 to 80 copies of KSHV are present per cell in AIDS-BCBLs.[3]

DISCUSSION

This report describes the in vitro establishment of two AIDS-BCBL cell lines, BC-1 and BC-2. AIDS-BCBLs rep-

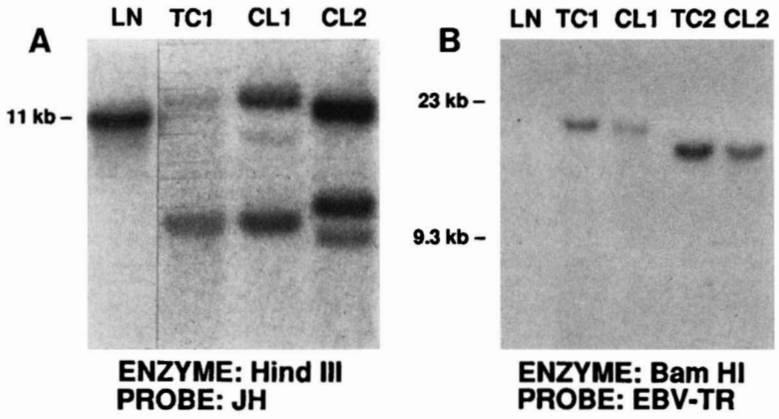

Fig 2. Southern blot hybridization analysis for Ig heavy chain gene rearrangements and for EBV termini heterogeneity. (A) Ten micrograms of DNA from a normal lymph node (LN), used as a germline control, lymphoma 1 tumor cells (TC1), and BC-1 (CL1) and BC-2 (CL2) cell lines, as indicated, were digested with *Hin*dIII and hybridized to an Ig heavy chain joining region (J_H) probe. The lane labeled LN shows a germline band at 11 kb (dash), while the lymphoma 1 tumor cells, BC-1 cells, and BC-2 cells have clonal rearrangement bands. The same rearrangement pattern is seen in case lymphoma 1 tumor cells and the corresponding cell line. (B) Five micrograms of DNA from a normal lymph node (LN), used as a negative control, lymphoma 1 tumor cells (TC1), BC-1 cells (CL1), lymphoma 2 tumor cells (TC2), and BC-2 cells (CL2), as indicated, were digested with *Bam*HI and hybridized to a DNA probe specific for the fused termini of the EBV genome. The location of the 23-kb and 9.4-kb size markers is shown (dashes). Both BCBL tumor cells and corresponding cell lines showed solitary strong bands, indicative of clonal EBV infection. The same pattern is seen in each of the cell lines as compared with the original tumor cells.

resent a clinicopathologically distinct subset of AIDS-related malignant lymphomas that, among other distinguishing features, are characterized by the presence of KSHV sequences.[3] The similarity of these cell lines to the tumor cells from which they were derived has been demonstrated by immunophenotypic and immunogenotypic studies. An important feature of the AIDS-BCBL cell lines is the retention of essentially unchanged KSHV sequences in spite of numerous passages.

We have used the BCBL cell lines to assess whether the genomic structure of the KSHV sequences is consistent with previous sequence homology data suggesting they belong to a herpesvirus. Although the human herpesviral genomes range in size from 120 to 230 kilobase pairs and are linear and double-stranded, they circularize immediately after release from capsids into the nuclei of infected cells. Thus, although herpesvirus DNA can sometimes integrate into the mammalian genome, it is usually found as nuclear circular episomes in latently infected cells.[12] We have found that KSHV sequences are present predominantly in the nucleus. Furthermore, these sequences were largely absent from Hirt supernatants. Although this method was established for selective extraction of low-molecular-weight DNA,[17] it has also been used to extract herpesvirus DNA, including that of herpes simplex virus,[25] herpes zoster virus,[26] and human cytomegalovirus,[27] despite their relatively large size. However, only unit-size linear herpesvirus DNA molecules, present in productive infections, have been found in the Hirt supernatants. In contrast, larger replicative structures are retained with the cellular DNA when using this method.[28]

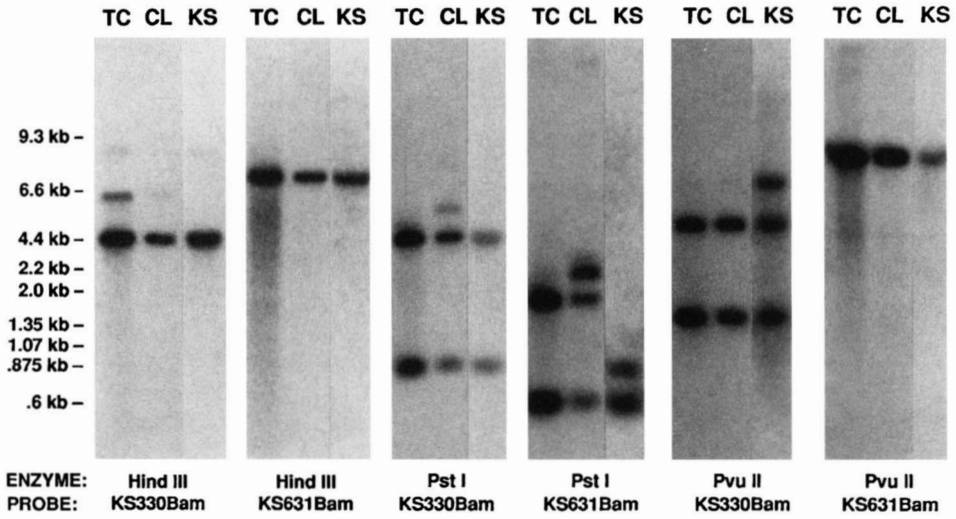

Fig 3. Southern blot hybridization analysis of lymphoma 1 tumor cells (TC), BC-1 cell line (CL), and KS DNA for KSHV sequences. Restriction enzyme analysis was performed with *Hin*dIII, *Pst* I, and *Pvu* II, after which agarose gel electrophoresis and transfer to nitrocellulose filters was performed. Filters were hybridized with ^{32}P-labeled KS330Bam and KS631 Bam probes. Autoradiographs shown represent a 2-hour exposure at room temperature for lanes labeled TC and CL containing DNA extracted from the lymphoma 1 original tumor cells and the BC-1 cell line, respectively, and a 36-hour exposure at −70°C for the lanes labeled KS containing KS DNA.

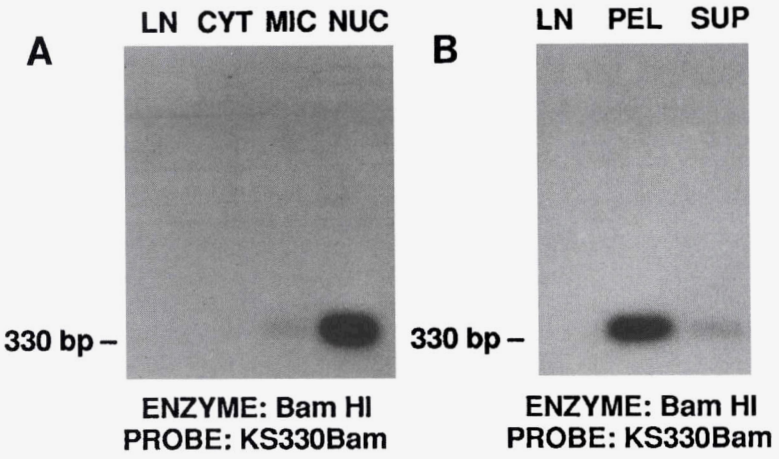

Fig 4. Southern blot analysis for KSHV sequences in BC-1 cells. (A) Hybridization of DNA obtained from different cellular fractions. One microgram of DNA from a normal lymph node (LN), used as a negative control, and from cytoplasmic (CYT), microsomal (MIC), and nuclear (NUC) fractions obtained from cell line BC-1 was used in each lane as indicated. (B) Hybridization of DNA obtained after Hirt extraction from the BC-1 cells. Five micrograms of DNA from a normal lymph node (LN), used as a negative control, and from supernatant (SUP) and pellet (PEL) fractions obtained from cell line BC-1 was used in each lane as indicated. All DNAs were digested with *Bam*HI, electrophoresed, and transferred to nitrocellulose filters. These filters were hybridized with a ^{32}P-labeled KS330Bam probe, and autoradiography was performed for 24 hours (A) or 1 hour (B) at −70°C. Most of the hybridization signal was seen in the nuclear fraction in panel A and in the pellet fraction containing the high-molecular-weight DNA in panel B.

Thus, the scarcity of KSHV DNA in the Hirt supernatants suggests a latent infection by a large, or integrated, viral genome, with a small amount of either mature linear viral DNA or fragmented DNA caused by cellular lysis. The in situ hybridization of metaphase spreads demonstrated KSHV sequences in episomal structures. Thus, we have found that KSHV sequences are present as large nuclear episomes in the BCBL cell lines, consistent with a latent herpesvirus genome.

We have strong circumstantial evidence in support of the hypothesis that KSHV sequences represent a portion of a novel human herpesvirus. However, we have not yet shown transmissibility of KSHV or the presence of KSHV virions. Studies attempting to induce lytic infections and to search for KSHV particles are complicated by the coinfection of these cells with EBV. A small percentage of cells (less than 10%) appear to show viral cytopathic effects, which could be caused by some EBV or KSHV viral replication. Sequences of both viruses are detectable in cell culture supernatants in small amounts that are seen by PCR but not by Southern blot hybridization analysis (unpublished observation, October 1994). These sequences may represent the presence of a small number of mature viral particles or, alternatively, fragments of EBV and KSHV DNA that are released into the supernatant due to cellular lysis. Additional studies using these cell lines are being performed in an attempt to further establish the viral nature of the KSHV sequences. Although KSHV sequences may eventually correspond to human herpesvirus-8 (HHV-8), this nomenclature should be decided on by the International Committee for the Taxonomy of Viruses (ICTV) after its review of the evidence for the herpesviral nature of these sequences.

In conclusion, we have established two AIDS-BCBL cell lines that retain the KSHV sequences present in the parental tumor cells.[3] These cells represent an important biologic reagent for further characterization of the nature of these

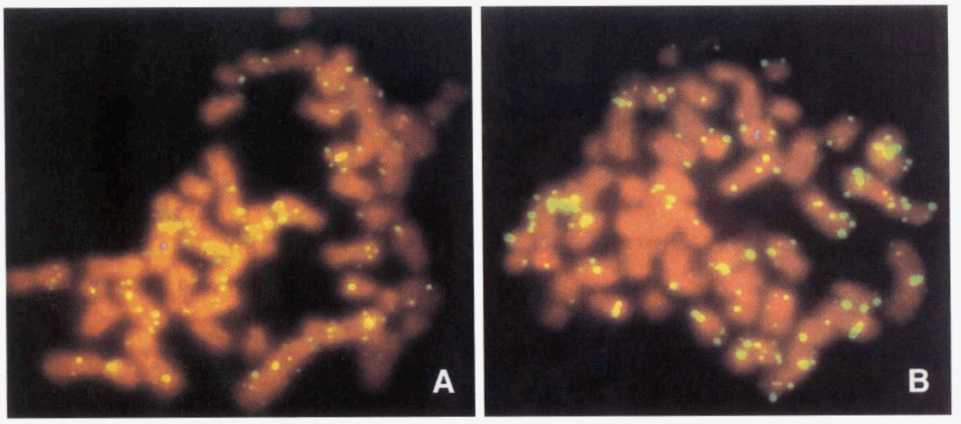

Fig 5. In situ hybridization of metaphase spreads. FISH of KSHV sequences to BC-1 (A) and BC-2 (B) is shown. Green signals show hybridization sites of KSHV sequences in the chromosomes.

sequences. The AIDS-BCBL cell lines should provide a great aid in future experiments aimed at determining whether this putative virus has transforming capabilities and its role in the development of KS as well as a subset of AIDS-related NHLs.

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In vitro establishment and characterization of two acquired immunodeficiency syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences

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