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Molecular Polymorphism of Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Latent Nuclear Antigen: Evidence for a Large Repertoire of Viral Genotypes and Dual Infection with Different Viral Genotypes

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Molecular polymorphism was found in Kaposi's sarcoma-associated herpesvirus (KSHV) latent nuclear antigen (LNA), mapped to the internal repeat domain of the encoding orf73 gene, and used to develop a novel genotyping technique, KSHV LNA genotyping (KVNA-typing). KVNAtype was stable during latent and lytic viral replication in cell culture and in humans. Diverse KVNAtypes were identified in 43 specimens: 6 KSHV cell lines and 6 Kaposi's sarcoma (KS) and 4 primary effusion lymphoma (PEL) tumor samples from the United States, 15 KS tumor samples from Italy, and 12 KS tumor samples from Zambia. A single KVNAtype was detected in each of 41 specimens, and 2 KVNAtypes were detected in each of 2 KS specimens. Multifocal KS from 3 patients showed the same single KVNAtype at all sites in each patient. These results demonstrate a large repertoire of KSHV genotypes and suggest that the development of most KSs and PELs is associated with a single viral genotype.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is a blood-borne, sexually transmitted gammaherpesvirus that is etiologically associated with all clinical forms of Kaposi's sarcoma (KS) and several other lymphoproliferative diseases. Extensive serologic and DNA-based epidemiologic studies have demonstrated that KSHV primarily infects persons at high risk for developing KS (e.g., homosexuals and recipients of iatrogenic organ transplants). Direct evidence has not been shown for person-to-person transmission of KSHV, and the risk factors for such transmission remain unclear. Epidemiology and transmission studies will require an efficient viral genotyping technique that can precisely distinguish virus isolates.

Current KSHV genotyping techniques are based on multiple point mutations within several viral lytic genes and have enabled classification of KSHV isolates into a limited number of subtypes [1, 2]. Three KSHV subtypes (A, B, and C) were identified from AIDS-associated KS (AIDS-KS) and primary effusion lymphoma (PEL) samples from the United States, classical KS samples from the Mediterranean region, and endemic KS samples from Africa; DNA sequencing was done of polymerase chain reaction (PCR) products amplified from lytic genes orf25, orf26, and orf75 [1]. A fourth subtype (SA) was found in iatrogenic KS in Saudi Arabia [3]. Similarly, single-strand conformation polymorphism analysis of a 210-bp PCR product from the orf26 gene has been used to classify KSHV isolates into 4 main groups (A, B, C, and D) and 1 miscellaneous group [2]. Geographic clustering of KSHV subtypes has been demonstrated by use of these grouping systems [1-6]. Recently, patients with type A infection [1] were shown to be more likely than patients with types B and C infection to develop more aggressive KS tumors with frequent mucosal or visceral lesions [5]. However, no clear correlation of a KSHV subtype with a particular disease or disease progression has been identified.

Analysis of the sequence divergence of the genes used for current KSHV genotyping (orf25, orf26, and orf75) revealed a 1.5% overall sequence variability among the KSHV isolates examined [1]. The limited sequence divergence and the small number of distinct groups identified [1, 2] by these genotyping techniques limit their application in KSHV epidemiologic studies of person-to-person transmission and its associated risk fac-

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Informed consent was obtained from all participants, and the human experimentation guidelines of the US Department of Health and Human Services, of the University of Texas Health Science Center at San Antonio, of the New York Hospital-Cornell Medical Center (New York, NY), Columbia-Presbyterian Hospital (New York, NY), of the Luigi Sacco Hospital (Milan, Italy), and of the University Teaching Hospital (Lusaka, Zambia) were followed.

The protocol and consent forms were approved by the institutional review boards of the participating institutions.

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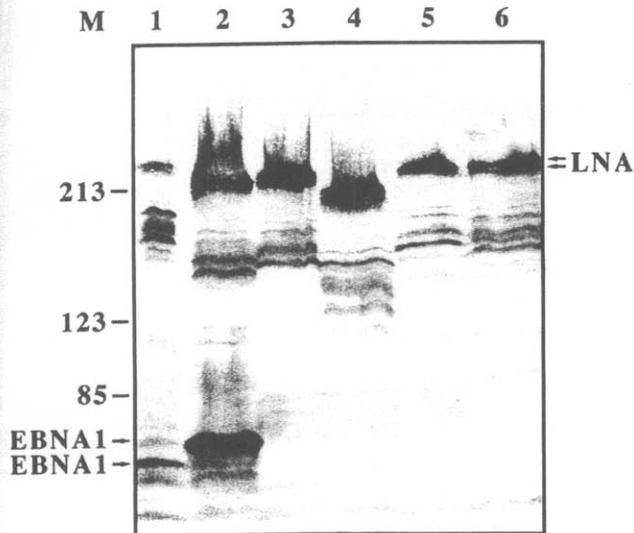


Figure 1. Kaposi's sarcoma-associated herpesvirus (KSHV) latent nuclear antigen (LNA) molecular mass polymorphism in KSHV-infected cell lines as demonstrated by immunoblotting with serum from patient with AIDS-related Kaposi's sarcoma. Lanes 1–6 correspond to cell lines BC-1, BC-2, BC-3, PK-1, BCP-1, and BCBL-1, respectively. M, markers. LNA is doublet of 2 high-molecular-mass proteins, ranging from 204/212 (PK-1) to 226/234 (BC-1) kDa. Epstein-Barr virus (EBV) EBNA1 was also detected in EBV-positive cell lines BC-1 and BC-2.

tors. Additional technical concerns of these genotyping techniques include potential bias because of the examination of a small number or portion of viral gene(s), infidelity of *Taq* DNA polymerase, and heterogeneity in the coding region of the target genes that might prevent PCR primer recognition and sequence determination if dual or multiple KSHV genotypes are present in the same specimen.

Among all the human herpesviruses, KSHV is most closely related to Epstein-Barr virus (EBV) [7]. Development of EBV-associated malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease, is often associated with latent virus infection and the expression of viral

latent antigens. The variability of latent genes among EBV isolates is much greater than that of other viral genes, with amino acid variability up to 45% for EBNA-2, allowing the identification and characterization of individual isolates [8–11]. In analogy to EBV, KSHV latent antigens are likely to have important functions in latent virus infection and cellular transformation as well as greater sequence variability that could be used for viral genotyping.

We examined KSHV latent genes K12, *orf72*, and *orf73* and identified molecular polymorphism in *orf73*, which encodes an immunodominant latent nuclear antigen (LNA) [12]. LNA is a doublet of two high-molecular-mass proteins, p226/234, previously identified by immunoblotting and characterized as viral LNAs in KSHV-infected cell lines [13]. LNA is also the major component of the latency-associated nuclear antigen revealed by indirect immunofluorescence assays [14, 15]. LNA molecular polymorphism was initially observed in 2 KSHV-infected cell lines, BC-1 and BCP-1, when we developed the LNA-immunoblot assay for KSHV infection [13, 14].

In this report, we extended the observation of LNA molecular polymorphism to 6 KSHV-infected cell lines, 33 KS lesions, and 4 PELs and mapped the polymorphic region to the *orf73* internal repeat domain. A novel KSHV genotyping technique, KVNAtyping, which is based on LNA molecular polymorphism, has been developed and used to identify and characterize KSHV genotypes (isolates) from KSHV-infected cell lines and KS and PEL tumor samples from the United States, Italy, and Zambia.

Materials and Methods

Patient enrollment, tissue collection, and sample preparation. KS specimens from patients in the United States were generously provided by Yuan Chang and Patrick Moore (Columbia University, New York City), and KS specimens from patients in Italy were generously provided by Carlo Parravicini and Mario Corbellino (Luigi Sacco Hospital, Milan). Multifocal lesions from African patients with KS were obtained from the Dermatovenereology Clinic, University Teaching Hospital (Lusaka, Zambia). PEL specimens were either provided by Ethel Cesarman or obtained from

Table 1. Properties of Kaposi's sarcoma-associated herpesvirus (KSHV)-infected cell lines and molecular polymorphism of KSHV latent nuclear antigen (LNA).

Cell line	Source	EBV coinfection	TPA inducibility ^a	Copies of KSHV genome	Age of cell line (years)	LNA-II PCR size (bp)	Estimated size of <i>orf73</i> (bp)	Apparent MW of LNA	Reference
BC-1	PEL	+	+	40–80	5	1898	3486	226/234	[16]
BC-2	PEL	+	+	20–40	3	1600	3188	212/220	[16]
BC-3	PEL	–	++++	15–30	3	1650	3238	214/222	[17]
PK-1	PEL	–	++	15–30	2	1350	2938	204/212	Gao et al., unpublished data
BCP-1	PBMC	–	+	60–120	4	1755	3343	222/230	[14]
BCBL-1	PEL	–	++	15–30	4	1755	3343	222/230	[18]

NOTE. EBV, Epstein-Barr virus; PCR, polymerase chain reaction; bp, base pairs; MW, molecular mass (in kilodaltons); PEL, primary effusion lymphoma; PBMC, peripheral blood mononuclear cells.

^a Higher inducibility is indicated by more +.

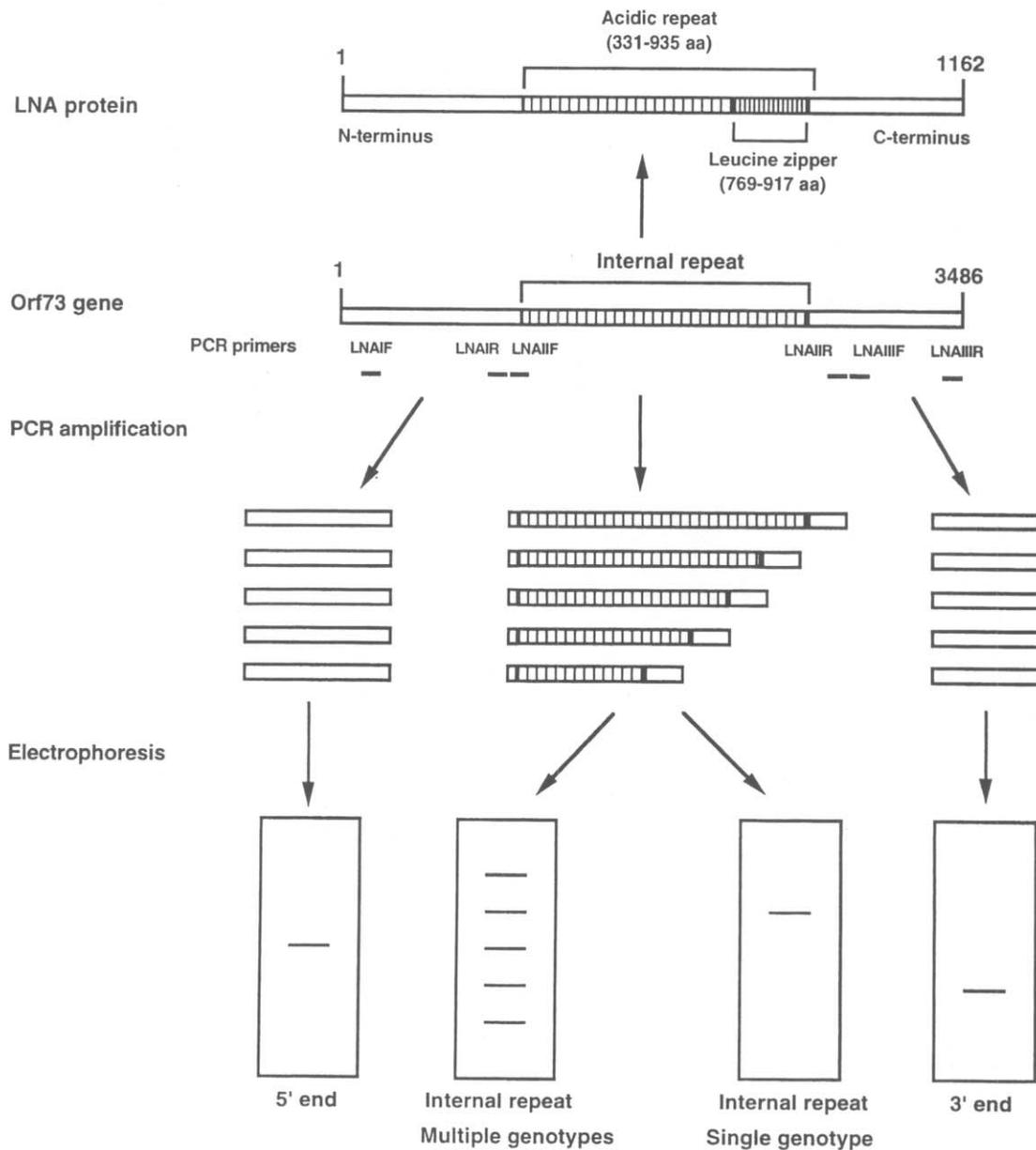


Figure 2. Schematic diagram of Kaposi's sarcoma-associated herpesvirus (KSHV) latent nuclear antigen (LNA)-encoding orf73 gene and polymerase chain reaction (PCR) primers used for mapping of orf73 molecular polymorphism and KSHV genotyping. PCR amplification of both N- and C-terminal domains of orf73 gene results in invariant single band regardless of specimens examined. Amplification of internal repeat domain of orf73 gene produced single band in cell population infected with single viral genotype and multiple bands in cell population infected with multiple viral genotypes.

the University Hospital of the University of Texas Health Science Center at San Antonio (UTHSCSA). Patients with AIDS-KS or PEL were enrolled from UTHSCSA, Columbia-Presbyterian Hospital and New York Hospital-Cornell Medical Center (New York City), Luigi Sacco Hospital, and University Teaching Hospital (Lusaka).

KS lesions were biopsied during regular clinical examinations, and the diagnosis of KS was confirmed by histopathologic ex-

amination. PEL specimens were collected from abdominal ascites of 3 human immunodeficiency virus (HIV)-infected patients and 1 patient without HIV who had previously had KS. All clinical specimens were fresh-frozen immediately after collection and stored at -70°C . DNA was extracted with phenol-chloroform, ethanol-precipitated, resuspended in deionized distilled water at $100\text{ ng}/\mu\text{L}$, and stored at 4°C .

Cell lines and cell culture. BC-1, BC-2, and BC-3 are cell lines

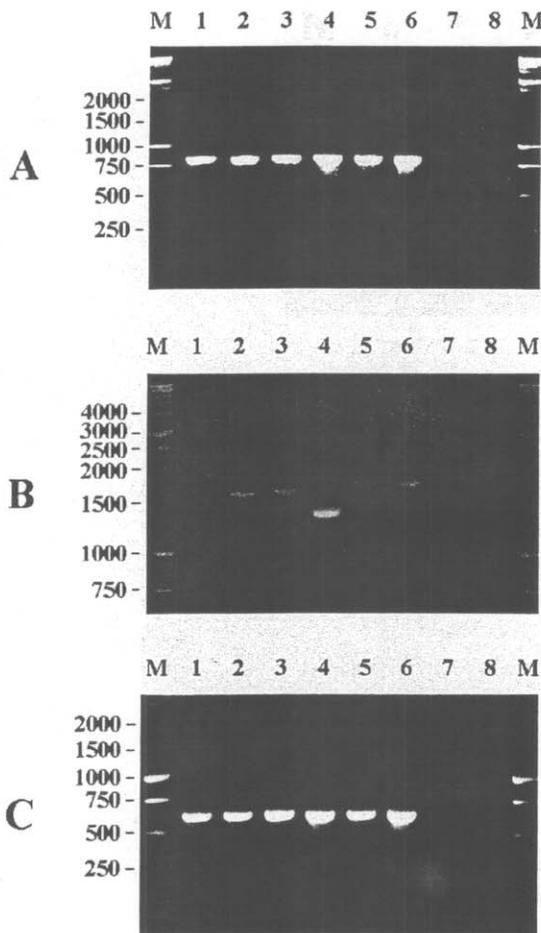


Figure 3. Mapping of Kaposi's sarcoma-associated herpesvirus (KSHV) orf73 gene molecular polymorphism. DNA extracted from KSHV-infected cell lines was amplified by polymerase chain reaction (PCR) for N-terminal domain (A), internal repeat domain (B), and C-terminal domain (C). Lanes: 1-6, KSHV-infected cell lines BC-1, BC-2, BC-3, PK-1, BCP-1, and BCBL-1, respectively; 7, KSHV-negative, Epstein-Barr virus-infected control cell line P3HR-1; 8, water control; and M, DNA standard size markers in base pairs. PCR amplification of both N- and C-terminal domains of orf73 gene produced invariant single band in all cell lines examined. Single band of internal repeat domain with variant sizes was detected in each cell line examined.

previously established from AIDS-related PEL [16, 17]. PK-1 is a cell line newly established in our laboratory from an AIDS-related PEL (Gao et al., unpublished data). BCP-1 was previously isolated from peripheral blood mononuclear cells from an HIV-uninfected patient with PEL [14]. BCBL-1 has been described elsewhere [18] and was obtained from the National Institutes of Health AIDS Reagent Program (Baltimore, MD). BC-1 and BC-2 are dually infected with KSHV and EBV, whereas BC-3, BCP-1, BCBL-1, and PK-1 are infected with KSHV but not EBV. P3HR-1, an EBV-infected cell line, and Ramos, an EBV-negative cell line, from Burkitt's lymphomas were obtained from American Type Culture Collection (Rockville, MD). All cell lines were cultured with RPMI

1640 supplemented with 10% fetal bovine serum. TPA induction of KSHV lytic replication has been described elsewhere [7].

LNA immunoblotting. LNA immunoblotting was done with whole cell extracts from KSHV-infected cell lines as described elsewhere [13].

PCR amplification of orf73 gene fragments. Mapping of the molecular polymorphic domain of the orf73 gene was done by use of primers designed from three regions of the orf73 gene of BC-1. The primer set LNAIF, 5'-GTAGGAAACGAAACAGGTC-3', and LNAIR, 5'-ATTCTTGGATGCTTCTTCT-3', amplified an 821-bp fragment of the N-terminal domain; the primer set LNAIIF, 5'-GAAGTGGATTACCCTGTTGTTAGC-3', and LNAIIR, 5'-ACTCTTTATGTCATTTCTGTGG-3', amplified a 650-bp fragment of the C-terminal domain; and the primer set LNAIIF, 5'-ATGGGGACAACGAGATTAGC-3', and LNAIIR, 5'-CGACCCGTGCAAGATTATG-3', amplified an 1898-bp fragment of the internal repeat domain. All samples were tested for amplifiability by use of primers specific for human β -actin gene [19].

Each PCR reaction was done in a final volume of 25 μ L, including ~100 ng of genomic DNA, 100 pmol of each primer, 2 U of *Taq* DNA polymerase, 100 μ M each of dNTP, 1.5 mM MgCl₂, 50 mM potassium chloride, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100. PCR amplification was done at 94°C for 6 min (1 cycle); 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (35 cycles); and 72°C for 5 min (1 cycle).

PCR products were separated by electrophoresis on 0.8%–1.5% agarose gels, stained with ethidium bromide, and visualized under

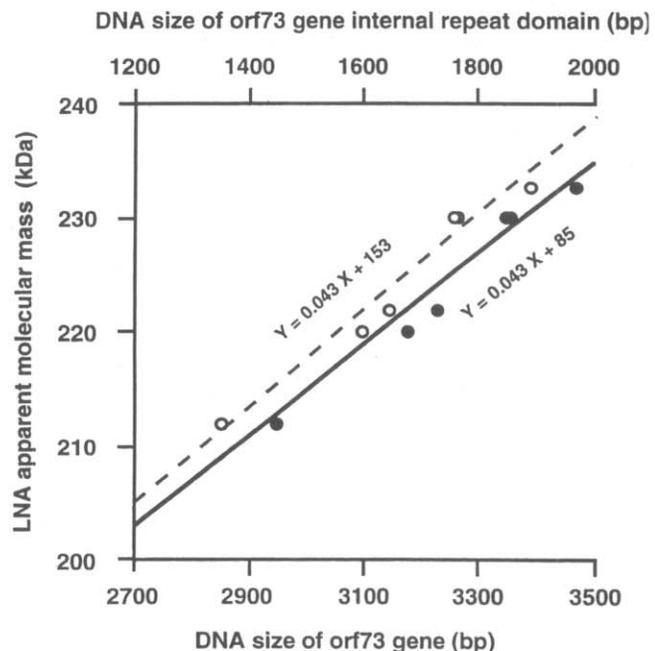


Figure 4. Linear correlation of experimentally derived Kaposi's sarcoma-associated herpesvirus latent nuclear antigen (LNA) apparent molecular mass (kDa) with experimentally derived sizes of orf73 gene (solid line, $r^2 = .96$, $P = .0006$) and its internal repeat domain (dashed line, $r^2 = .96$, $P = .0006$); bp, base pairs.

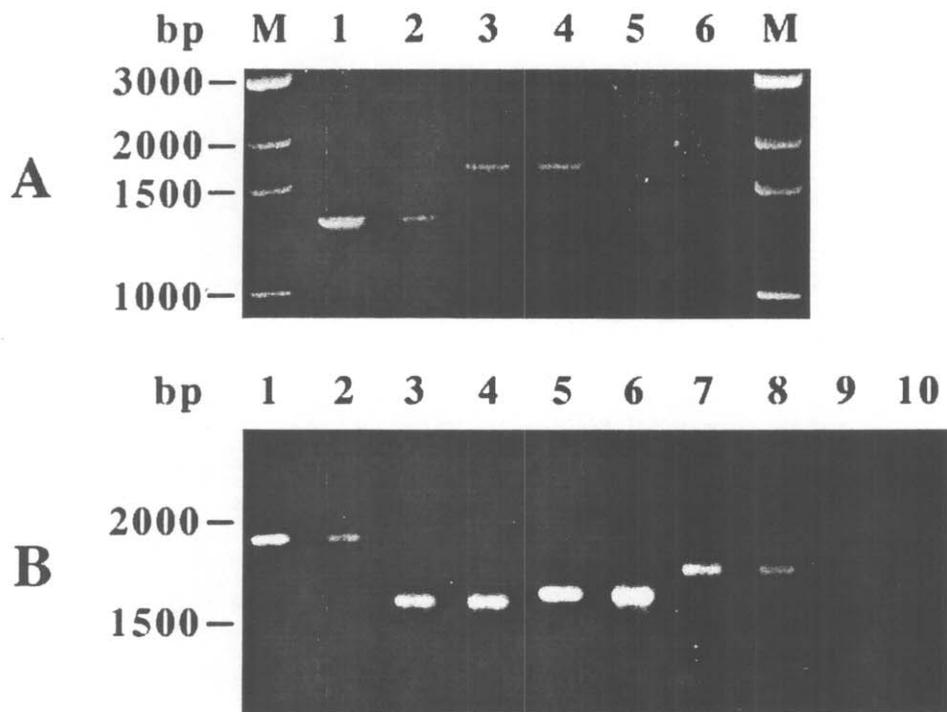


Figure 5. Stability of Kaposi's sarcoma-associated herpesvirus (KSHV) orf73 internal repeat domain. *A*, DNA size of orf73 internal repeat domain is stable after long-term in vitro cell culture. DNA samples isolated from cells of <5 passages after initial cell line isolation (lanes: 1, PK-1; 3, BCP-1) and cells after long-term cell culture (lanes: 2, PK-1, 121 passages in 1 year; 4, BCP-1, 366 passages in 3 years) were amplified by polymerase chain reaction for orf73 internal repeat domain. Lanes: 5, negative control cell line P3HR-1; 6, water control; M, markers. *B*, DNA size of orf73 internal repeat domain is stable during both latent and lytic viral replication. Uninduced (odd lanes) and TPA-induced cells (even lanes) of each KSHV-infected cell line (lanes: 1 and 2, BC-1; 3 and 4, BC-2; 5 and 6, BC-3; 7 and 8, BCBL-1) shared same size of orf73 internal repeat domain band. Lanes 9 and 10 correspond to uninduced and TPA-induced KSHV-negative P3HR-1 cells; bp, base pairs.

UV illumination. The DNA sizes of the PCR products were calculated by use of the Multi-Analyst PC Software for Bio-Rad's Image Analysis Systems (Bio-Rad, Hercules, CA). DNA samples from KSHV-negative cell lines (P3HR-1 or Ramos) and deionized water were used as negative controls for the experiments.

To increase the sensitivity of the PCR assay for the orf73 internal repeat domain, PCR products resolved by electrophoresis on an agarose gel were transferred to a nitrocellulose membrane and hybridized with a ^{32}P -labeled probe prepared from the orf73 internal repeat domain PCR product of the BC-1 cell line. Specific hybridization was documented on a GS-525 Molecular Imager System (Bio-Rad).

Determination of viral genome copies. BC-1 was previously determined to have ~40–80 copies/cell of KSHV genome [16]. The genome copies of other KSHV-infected cell lines were determined by dot-blot hybridization using purified KS330 PCR product as standard copy number and probe [7].

Results

LNA molecular mass polymorphism. To examine LNA molecular mass polymorphism, we immunoblotted whole cell protein extracts from 6 KSHV-infected cell lines with serum from a KS patient. As described elsewhere, LNA, represented by a

doublet of two high-molecular-mass proteins [13], was identified in all 6 cell lines examined (figure 1). In addition, LNA molecular mass polymorphism was present in these cell lines. The apparent molecular mass of LNA ranged from 204/212 kDa for cell line PK-1 to 226/234 kDa for cell line BC-1. LNA molecular mass polymorphism did not correlate with cell line source, EBV coinfection, copies of viral genome, or TPA inducibility of viral lytic replication (table 1). Comparison of early-passage cells (5 passages after initial isolation) with those after long-term culture of 2 KSHV-infected cell lines, BCP-1 (366 passages in 3 years) and PK-1 (121 passages in 1 year) cells, did not detect any difference of LNA molecular mass (data not shown) and indicated that LNA molecular mass was stable during long-term in vitro cell culture.

Mapping of LNA molecular mass polymorphism: molecular polymorphism of orf73 internal repeat domain. In the BC-1 cell line, the LNA gene orf73 encodes a 1162-amino acid protein characterized by an internal repeat domain separated into two regions by a glutamine-rich sequence [20]. The first region consists almost exclusively of aspartic and glutamic acid residue repeats, whereas the second glutamic acid-rich region has a leucine zipper structure. The apparent molecular mass of this

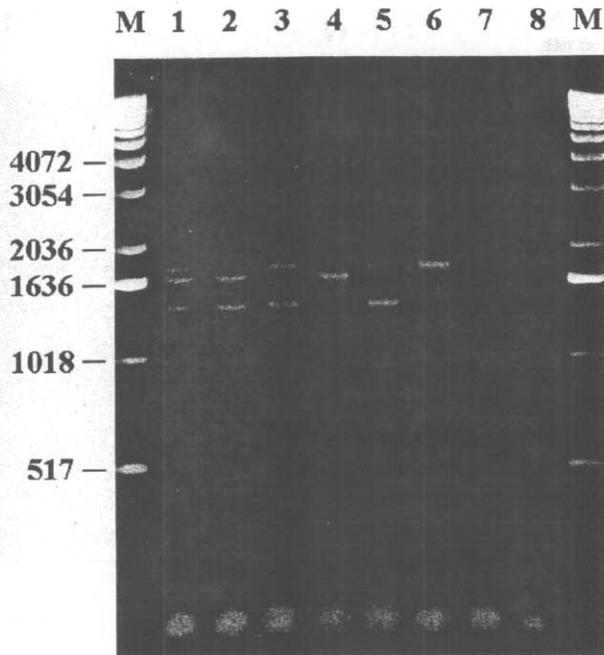


Figure 6. Simultaneous detection of dual or multiple Kaposi's sarcoma-associated herpesvirus (KSHV) genotypes by KSHV latent nuclear antigen genotyping (KVNA typing; novel genotyping technique). Single, dual, or multiple KVNA types were identified in cell cultures that contained 1–3 KSHV-infected cell lines. Lanes: 1, BC-3, PK-1, and BCP-1; 2, BC-3 and PK-1; 3, PK-1 and BCP-1; 4, BC-3; 5, PK-1; 6, BCP-1; 7, negative control cell line P3HR-1; 8, water control; M, markers.

LNA as determined by immunoblotting (234/226 kDa) [13] is much larger than the theoretical molecular mass calculated from the BC-1 orf73 gene (135 kDa) [20]. To map the gene region responsible for the LNA molecular mass polymorphism, we PCR-amplified the N-terminal, internal repeat, and C-terminal domains of the orf73 gene (figure 2). The PCR products of both the N- and C-terminal domains had invariant sizes, whereas those of the internal repeat domain exhibited DNA size polymorphism among the 6 cell lines examined (figure 3).

The sizes of PCR products from the internal repeat domain correlated well with the LNA apparent molecular masses ($r^2 = .96$, $P = .0006$). A linear correlation equation was established to predict the LNA apparent molecular mass on the basis of the size of PCR product amplified from the orf73 internal repeat domain (figure 4). This equation is useful for characterization of LNA in KS specimens because of the abnormal migration pattern of LNA in polyacrylamide gel.

Stability of orf73 internal repeat domain and KVNA typing. The DNA size of the LNA internal repeat domain was stable in BCP-1 and PK-1 cell lines during long-term culture (figure 5A), confirming the immunoblotting result. A single orf73 internal repeat domain band was identified in each of the 6 cell lines examined regardless of the age of the cell lines (figure 3B

and table 1), further suggesting the stability of orf73 internal repeat domain during long-term *in vitro* cell culture.

All 6 KSHV-infected cell lines maintained relatively consistent copy numbers of KSHV genomes, varying from 15 to 120 copies per cell (table 1). The finding (by PCR amplification) of a single LNA internal repeat band in each of the cell lines examined, regardless of the genomic copy number per cell, indicates that the KSHV genomes in each cell line have a constant number of orf73 internal repeat reiterations. This implies that the progenitor KSHV-infected cell(s) of each cell line was either infected by a single genotype or that one particular viral genotype was preferentially amplified. In contrast, the number of orf73 internal repeat reiterations and the size of the corresponding PCR product varied from cell line to cell line and was characteristic for each KSHV isolate.

With the exception of BC-1, which is under strict control of latent replication [21], the other 5 KSHV-infected cell lines exhibited various degrees of leakage of viral lytic replication (Gao et al., unpublished data). Amplification of a single orf73 internal repeat domain band from each of these 5 cell lines therefore represents amplification of both latent and lytic viral genomes and indicates the stability of the orf73 internal repeat domain under both latent and lytic viral replication. To confirm this result, the orf73 internal repeat domain was amplified from BC-1, BC-2, BC-3, and BCBL-1 cells that were TPA induced into lytic viral replication. Again, a single orf73 internal repeat domain band of the same size as the corresponding noninduced cells was observed for each of these cell lines (figure 5B).

Therefore, the size of the orf73 internal repeat domain, which correlates with the LNA apparent molecular mass, is characteristic of each individual KSHV genotype or isolate and could be used for the identification and characterization of individual KSHV genotypes or isolates. We named this technique KSHV LNA genotyping, or KVNA typing, and we named the KSHV genotype identified by this technique the KVNA type.

Although only one KSHV genotype was identified in each of the 6 KSHV cell lines examined, KVNA typing can detect dual or multiple viral genotypes in individual samples (figure 6). KVNA typing was done with DNA extracted from mixed cell cultures of dual or multiple KSHV-infected cell lines. Dual or triple KVNA types were clearly identified, as expected, from the mixed cell cultures of two or three cell lines, respectively.

KVNA typing of KS and PEL. To determine the diversity of KVNA types in KSHV-infected persons and whether KSHV infection in KS and PEL is associated with single or multiple viral genotypes, we used the KVNA typing technique to examine 24 KS lesions from 8 US patients and 16 Italian patients and 4 PEL specimens from the United States. Using PCR, we detected various KVNA types in 6 of the 8 KS specimens from the United States, 13 of the 16 KS specimens from Italy (figure 7A, C), and all 4 PEL specimens (figure 8). Two KS specimens from the United States (figure 7A, lanes 5 and 8) and 3 KS specimens from Italy (figure 7C, lanes 11, 20, and 21) were

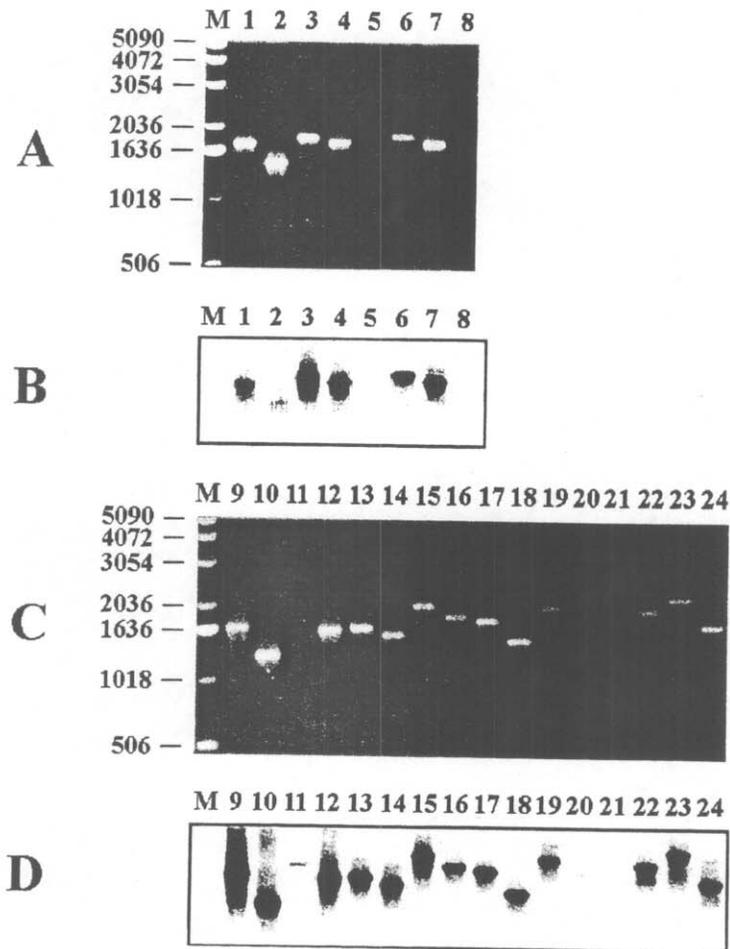


Figure 7. Kaposi's sarcoma-associated herpesvirus latent nuclear antigen genotyping (KVNA typing; novel genotyping technique) in Kaposi's sarcoma (KS) lesions from patients with AIDS-related KS from United States (*A, B*) and Italy (*C, D*). *A, C*, polymerase chain reaction (PCR) products separated by electrophoresis on agarose gels; *B, D*, results of *A* and *C* after Southern hybridization, by use of ^{32}P -labeled probe prepared from BC-1 orf73 internal repeat domain. M, markers. Single KVNA type is identified in each PCR-amplifiable KS specimen examined except specimens 22 and 24, in which dual infection with 2 different KVNA types was detected after Southern hybridization (*D*, lanes 22 and 24). No KVNA type was detected in 3 PCR-unamplifiable samples (*A, B*, lanes 5, 8; *C, D*, lane 21; also see text).

negative by this PCR-based KVNA typing technique, but Southern hybridization of the PCR products with a probe prepared from the BC-1 orf73 internal repeat domain revealed two additional KVNA types from the Italian specimens (figure 7*D*, lanes 11 and 20). PCR amplification of the 3 remaining KVNA-type-negative KS DNA specimens (figure 7, lanes 5 and 8 [from the United States] and lane 21 [from Italy]) with primers designed from human β -actin gene did not detect any signal (data not shown); therefore, these 3 specimens were concluded to be nonamplifiable.

A large diversity of KVNA types was identified among these KS and PEL specimens. Except for 2 KS specimens from Italy, individual specimens contained only one distinct KVNA type. The 2 specimens from Italy each contained two KVNA types (figure 7*D*, lanes 22 and 24). In both dually infected specimens,

one of the genotypes predominated, while the other was present in low copy number and was only detectable after Southern hybridization. Broad bands were also observed in 4 other specimens after Southern hybridization (figure 7*B* [lane 3], *D* [lanes 9, 12, and 15]); however, serial exposures of the filters for shorter periods revealed that they were due to the high sensitivity of the assay. Thus, each of these 4 specimens also contained a single KVNA type. These results suggest that the development of most PEL and KS tumors is associated with one predominant viral genotype. The presence of different KVNA types (size variations) shows unambiguously that these PCR products represent true KVNA types from the specimens rather than PCR contamination, which is frequently encountered during use of PCR techniques. Similar to KSHV-infected cell lines (figure 3), each of the KS and PEL specimens that had a definite KVNA-

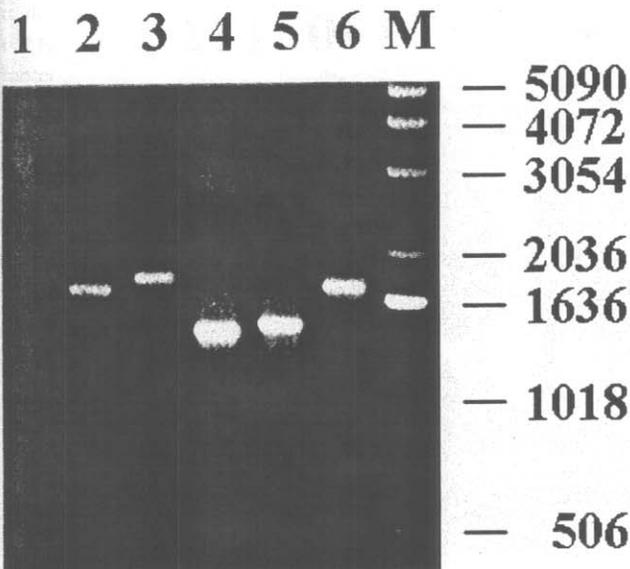


Figure 8. Kaposi's sarcoma-associated herpesvirus (KSHV) latent nuclear antigen genotyping (KVNA typing; novel genotyping technique) in primary effusion lymphoma (PEL). Lanes: 1, negative control cell line P3HR-1; 2, KSHV-positive BC-3 cell line; 3–6, KSHV-positive PEL from United States; M, markers. Lanes 3 and 4 are PEL from which original BCP-1 and PK-1 cell lines were established, respectively. Single KVNA type was detected in each PEL specimen.

type (figures 7, 8) produced an invariant PCR product on the N- and C-terminal domains of the orf73 gene (data not shown).

To determine whether multiple lesions from different sites on individual patients with KS contained single or multiple KVNA types, we examined 3 additional African patients with KS, each with multiple lesions (figure 9). A single identical KVNA type was identified for each patient, suggesting that individual patients with KS also harbor a single predominant KSHV genotype.

The detection of a single orf73 internal repeat domain band in most patient specimens (figures 7–9) indicated that, similar to the case in *in vitro* cell culture (figures 3, 5), KVNA type is stable in patients with KS and PEL. Furthermore, two of the PEL specimens (figure 8, lanes 3 and 4) had the same KVNA types as their respective established cell lines (BCP-1 and PK-1) despite cell isolation and long-term *in vitro* cell culture (figure 3, lanes 5 and 4). While KSHV in most KS spindle cells undergoes latent replication, a small number of these cells also constantly undergoes lytic replication [22, 23], suggesting that, similar to the case for KSHV-infected cell lines, the KVNA type is stable during both latent and lytic viral replication in patients with KS.

The DNA sizes of the orf73 gene for the KS and PEL specimens examined ranged from 2876 to 4023 bp, generating LNA apparent molecular masses of 200/208–250/258 kDa.

Discussion

We have described the molecular polymorphism of KSHV LNA in KSHV-infected cell lines and in KS and PEL lesions and mapped the polymorphic region to the LNA-encoding orf73 gene internal repeat domain. On the basis of LNA molecular polymorphism, we developed a novel viral genotyping technique, KVNA typing, for the identification of KSHV genotypes. The KVNA type is stable during both latent and lytic viral replication in long-term *in vitro* cell culture and in patients with KS and PEL. Therefore, KVNA type represents important characteristics of each individual KSHV genotype or isolate, and KVNA typing is likely to be useful for studies related to KSHV epidemiology and transmission.

KVNA types were identified in 43 of 46 specimens examined, including 6 KSHV cell lines and 33 KS and 4 PEL lesion samples; 3 of the negative KS specimens contained nonamplifiable DNA. Of the 37 KS and PEL specimens that had detectable KVNA types, one KVNA type was detected in each of 35 specimens (95%; figures 7–9), suggesting that most of these malignancies originated from a cell(s) infected with a single KSHV genotype or that a particular viral genotype was preferentially amplified. Therefore, the development of the majority of KS and PEL lesions is likely to be associated with a single dominant KSHV viral genotype that is preserved after selection by various host and environmental factors. None of the 3 patients with KS who had multifocal lesions had evidence of infection with dual or multiple KVNA types (figure 9), suggesting that these patients also harbor a single KSHV genotype.

Simultaneous development of multiple KS lesions in the same patient is very common. The emergence of multiple KS lesions in the same patient could be due to infection with different viral genotypes at different body sites and subsequent simultaneous expansion of multiple genotypes in the patient. However, this hypothesis contradicts our current results. Although the nature of KS remains controversial [24–27], our previous studies (C.S.R.) have suggested that KS is clonal, and multifocal KS lesions from the same patient are likely to arise from a monoclonal population of circulating progenitor cells that home to multiple local sites and proliferate [28, 29]. In this scenario, similar to that for the development of a single lesion, multiple lesions in most patients with KS are also likely to be infected with one viral genotype, which subsequently expands, or with multiple viral genotypes, with just one genotype that dominantly expands.

Two KS specimens harbored dual KSHV genotypes (figure 7D, lanes 22 and 24). This is the first report of KSHV dual infection with different viral genotypes (or isolates) in patients with KS. It would be interesting to further determine the biologic significance of such dual infection in the pathogenesis of KSHV-related malignancies. Dual EBV infection with different viral strains or isolates has been described in healthy seropositive individuals and patients with infectious mononu-

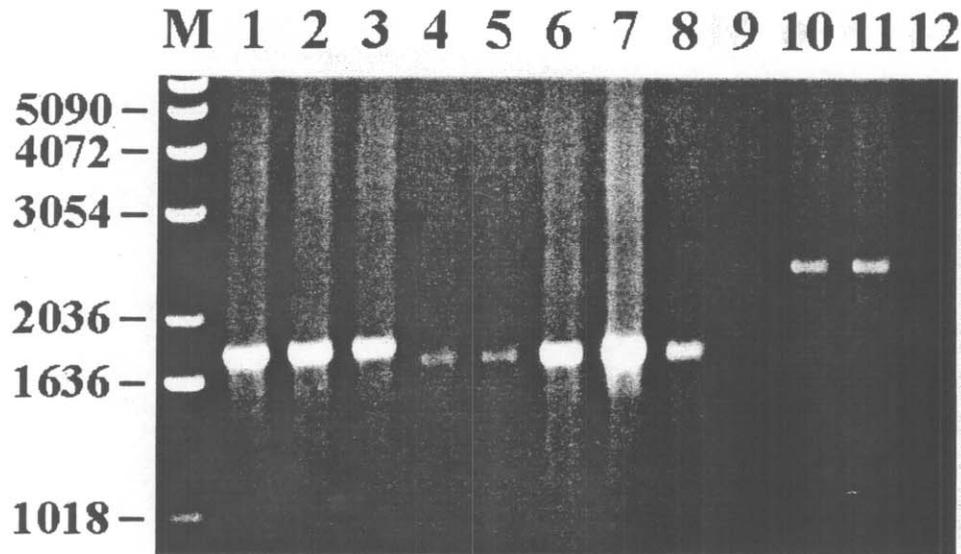


Figure 9. Kaposi's sarcoma-associated herpesvirus latent nuclear antigen genotyping (KVNAtyping; novel genotyping technique) of Kaposi's sarcoma (KS) lesions from 3 African patients with KS, each with multiple lesions. Lanes represent samples from patients: 1–3, patient 1; 4–8, patient 2; 9–12, patient 3; M, markers. One KVNAtype was detected in each patient with KS who developed multiple lesions.

cleosis [30, 31] and in increased frequency in HIV-infected patients [32].

The most commonly used serologic assays for the detection of KSHV infection are LNA based and use one KSHV genotype (or isolate) from either BCP-1 or BCBL-1 cell lines as antigen substrate. The finding of molecular polymorphism in LNA raises concerns about the sensitivity of these serologic assays because certain patients could potentially be infected with KSHV genotypes or isolates that do not have antibody cross-reactivity with the one used for the assays. In fact, a small number of patients with KS, particularly those with AIDS-KS, have seronegative results from LNA-based serologic assays [13–15, 33]. Antibodies to EBV EBNA1 have been detected in patients with AIDS-KS [13, 14], suggesting that the inability to detect KSHV-specific antibodies in these patients is probably due to viral genotype or isolate variations but not to immunosuppression caused by AIDS progression. Simultaneous use of multiple KSHV genotypes or isolates in the serologic assays could potentially resolve this problem.

The *orf73* internal repeat domain is composed of multiple heterogeneous repeat domains interspersed with short nonrepeat sequences. While molecular polymorphism of LNA has been mapped to the internal repeat domain of the encoding *orf73* gene, the mechanism of generating new KVNAtypes is still unknown. Preliminary results from sequence determination of the *orf73* internal repeat domain from the PK-1 cell line indicated that sequence deletions and point mutations in multiple regions are responsible for LNA polymorphism (Zhang et al., unpublished data). Analysis of the DNA sequences of

these domains from more KVNAtypes is likely to shed light on the mechanism underlying the generation of new KSHV genotypes and provide a basis for the development of a system for KVNAtype classification.

Because KVNAtyping can identify a diverse variety of KVNAtypes and the molecular mass polymorphism of their PCR products can be used as an internal control for PCR contamination, this technique is particularly useful for the detection of KSHV infection. Areas of dispute, such as the association of KSHV infection with multiple myeloma, sarcoidosis, central nervous system lymphoma, angiosarcoma, and encephalitis, as well as the occasional detection of KSHV sequences in the general population or in non-KS and non-AIDS control populations in the United States and Europe could potentially be resolved by use of this technique.

Only a very limited number of KSHV genotypes has been identified by use of techniques based on multiple point mutations [1–3]. The estimated genetic divergence of the genes used in these techniques (*orf25*, *orf26*, and *orf75*) is only 1.5% [1]. In contrast to these previous reports, our preliminary results of KVNAtyping of a limited number of KSHV-infected cell lines and KS and PEL lesions (43 in total) suggest the presence of a much greater diversity of KSHV genotypes. The genetic divergence of LNA among KSHV isolates also appears to be much greater than that of viral lytic genes. Recently, *orf-K1*, an early or immediate early viral gene, has also been shown to have high sequence variability among KSHV isolates [34].

LNA is the most abundant latent protein expressed in KSHV-infected cell lines [13, 14], KS and PEL lesions, and malignant

multicentric Castleman's disease cells [12, 35]. LNA has essential function for KSHV episomal replication [36], and the promoter activity of its transcript mimics the expression of cellular cyclin D to regulate cell cycle progression [37]. Therefore, LNA polymorphism is an important biologic marker for KSHV genotyping that could potentially be used to study the epidemiology, diversity, transmission, and biology of KSHV genotypes. KVNAtyping could be used to address important questions, such as the mutation rate and stability of the virus, the geographic origin, distribution, and prevalence of viral genotypes, the interaction of host with viral genotype, the risk factors for viral transmission, and the association of a particular genotype with KSHV-related diseases or disease progression.

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