

Monoclonality or Oligoclonality of Human Herpesvirus 8 Terminal Repeat Sequences in Kaposi's Sarcoma and Other Diseases

Jean-Gabriel Judde, Vincent Lacoste, Josette Brière, Eric Kassa-Kelembho, Emmanuel Clyti, Pierre Couppié, Carmen Buchrieser, Micheline Tulliez, Jacques Morvan, Antoine Gessain

Background: Infection with human herpesvirus 8 (HHV8), also termed Kaposi's sarcoma (KS)-associated herpesvirus, is associated with all forms of KS, with primary effusion lymphoma (PEL), and with some forms of multicentric Castleman's disease (MCD), but the pathogenic role of HHV8 in these tumors and the clonal nature of KS are still unclear. The purpose of this study was to examine whether the number of terminal repeats (TRs) contained in the fused TR region of HHV8 could be used as a marker of clonality in HHV8-associated tumors. **Methods:** Pulsed-field gel electrophoresis (PFGE) and multiple-probe Southern blot analysis of the HHV8 TR region were performed on high-molecular-weight DNA obtained from tumoral KS, PEL, and MCD lesions. **Results:** These analysis showed that the fused TR region contains a large but variable number of TR units (ranging from 16 to 75) and that the viral genome is present as extrachromosomal circular DNA in these tumors *in vivo*, with occasional ladders of heterogeneous linear termini reflecting lytic replication. All PEL tumors and PEL-derived cell lines as well as some KS tumors contained monoclonal or oligoclonal fused TR fragments; however, the TR region appeared polyclonal in MCD tumors and in a few KS lesions. **Conclusion:** Several KS and PEL lesions are monoclonal expansions of a single infected cell, suggesting that HHV8 infection precedes tumor growth and thus supporting an etiologic role of latent HHV8 in these proliferations. Our finding that nodular KS lesions display all possible patterns of clonality supports the model according to which KS begins as a polyclonal disease with subsequent evolution to a monoclonal process. [J Natl Cancer Inst 2000;92:729-36]

Human herpesvirus 8 (HHV8) is a novel member of the γ -herpesvirus family and the first known human rhadinovirus (1,2). Among close homologues of HHV8, rhesus macaque rhadinovirus (3), herpesvirus saimiri (4), and Epstein-Barr virus (EBV) (5) are all associated with tumors in monkeys and humans. HHV8 infection is linked with three human proliferative disorders: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and some forms of multicentric Castleman's disease (MCD). Support for an etiologic role of HHV8 in these pathologic conditions is mostly based on epidemiologic and molecular data. It is especially strong for KS, where HHV8 sequences have been detected regularly (in nearly 100% of KS lesions examined), whether in the setting of acquired immunodeficiency syndrome (AIDS) or in the classical and endemic forms of KS (6,7). Geographic areas of high HHV8 seroprevalence also have the highest incidence of KS, and an increase in anti-HHV8 antibody titers precedes the outgrowth of KS in homosexual AIDS pa-

tients (6,7). The epidemiologic association between HHV8 and PEL is not as strong because of the rarity of this condition (8); moreover, for MCD, only a subset involving a plasma cell variant is consistently associated with HHV8 (9).

Despite these associations and the accumulation of data on the function of individual viral gene products, the pathogenic role of HHV8 in these diseases is still unclear. Immunohistochemistry and *in situ* hybridization studies have shown that, in KS lesions, the virus is present in spindle cells, the presumed tumoral component of KS (10,11). The presence of circular HHV8 genomes in PEL cell lines and KS tissues is diagnostic of a latent herpesvirus infection (12,13), and the expression of HHV8 lytic genes is usually detected in only a small fraction of cells in KS, PEL, and MCD lesions (7). These features are in agreement with a transforming role of latent HHV8, as is the case for EBV (14). Although the genome of HHV8 contains a number of potentially transforming genes, including cytokines, chemokines, and an interleukin 8 (IL-8) receptor homologue, many of them are part of the lytic program of gene expression and, therefore, are not expressed in the majority of infected tumor cells (7). Among latent genes that are consistently expressed, viral cyclin-D (v-cyc-D), a viral homologue of cellular cyclin D (encoded by open reading frame [ORF] 72) (15), and viral FLIP (v-FLIP), a homologue of the cellular FLICE (Fas-associated death domain-like interleukin 1 β -converting enzyme) inhibitory protein (encoded by ORF K13/ORF 71) (16,17), are potential candidates for HHV8-induced transformation. However, since KS is often viewed as a reactive hyperplasia where dysregulated production of cytokines and angiogenic factors probably plays an important role, both latent and lytic modes of HHV8 infection could be relevant to the pathogenesis (18,19). Understanding the pathogenic role of HHV8 in PEL is further complicated by the fact that PEL cells are often coinfected with EBV (8,20).

Clonality, a central issue in understanding the pathogenesis of cancer, has been examined in PEL and MCD by the analysis of

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the structure of their immunoglobulin (Ig) genes. While all PEL tumors have clonal Ig gene rearrangements (8,20), the majority of MCD lesions appear to represent polyclonal B-cell proliferations (21). The assessment of clonality in KS, however, has been more difficult because of its mixed cellular characteristics without Ig gene rearrangements. Several studies (22–27) have used X-linked mosaicism analysis to examine clonality in KS lesions from female patients, but they have yielded conflicting data. These conflicting data may be due to the fact that this assay can be influenced by the degree of infiltration of the tumor with nonmalignant cells. An earlier study (28) has shown that, as with other γ -herpesviruses, the long unique region (LUR) of the linear HHV8 virion DNA is flanked by variable numbers of terminal repeats (TRs), each of approximately 800 base pairs (bp) in length, which are joined to form circular viral episomes in latently infected cells. This suggests that the size of the fused HHV8 TR region could provide a molecular marker of clonality in HHV8-infected tumor cells (2,12,13). This approach has been used previously for EBV to demonstrate that EBV-associated nasopharyngeal carcinomas were monoclonal tumors (29) and to show that EBV infection preceded the monoclonal expansion of Burkitt's lymphoma and non-Hodgkin's lymphoma cells (30). Here we have analyzed the structure of HHV8-fused TRs and have examined their size heterogeneity in a panel of HHV8-associated tumors.

MATERIALS AND METHODS

Tumor Samples and DNA Preparation

KS cutaneous lesion samples were obtained by punch biopsy, KS or MCD lymph nodes were surgically removed, and PEL pleural or ascitic effusion samples were collected by needle aspiration. MCD spleen lesions were obtained following splenectomy. Written informed consent was obtained from each subject or from his or her guardian. Tumor biopsy specimens were stored either in liquid nitrogen or at -80°C . The BC-1, BC-2, BC-3, and BCBL-1 PEL cell lines were obtained from the American Type Culture Collection (Manassas, VA). The Cra-BCBL cell line was established in the laboratory of the Unité d'Oncologie Virale, Département des Rétrovirus, Institut Pasteur, and will be described in detail elsewhere (31). Cell lines were maintained in RPMI-1640 medium supplemented with 15% fetal calf serum, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol at 37°C in an atmosphere of 95% air and 5% CO_2 . For extraction of high-molecular-weight DNA, cultured cells were washed twice in phosphate-buffered saline and resuspended in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 50 mM NaCl, 0.5% sodium dodecyl sulfate (SDS), and 200 $\mu\text{g}/\text{mL}$ proteinase K. Tumor biopsy specimens were minced and resuspended in lysis buffer. Samples were then incubated in lysis buffer at 56°C with shaking for 6 hours. The DNA was deproteinized by three consecutive extractions with phenol, phenol/chloroform (1:1 vol/vol), and chloroform; it was then precipitated with isopropanol, washed with ethanol, and resuspended in buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA. The high molecular weight and the integrity of DNA samples were verified by gel electrophoresis and ethidium bromide staining of aliquots of undigested DNA.

Electrophoresis and Southern Blotting

Genomic DNA (10–30 μg) was digested at 65°C with 5 U/ μg of *TaqI* (Roche Diagnostics, Meylan, France). To ensure complete DNA digestion, we added restriction endonuclease three times over a 24-hour period and verified the completeness of the digests by ethidium bromide staining of the gels. For regular gel electrophoresis, *TaqI*-digested DNA samples were subjected to electrophoresis on 0.7% agarose gels in $1\times$ Tris borate EDTA (TBE) buffer along with *HindIII*-digested λ DNA as a size marker. For pulsed-field gel electrophoresis (PFGE), *TaqI*-digested DNA samples were subjected to electrophoresis on 1% agarose gels in $0.5\times$ TBE buffer. PFGE was performed on a 120° Clamped Homogenous Electrical Field DR-II System (Bio-Rad Laboratories, Hercules, CA) for 26 hours at 14°C . The voltage was set at 6 V/cm, with an impulsion time ramping linearly from 1 second to 2 seconds. High-molecular-weight DNA

marker (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) was used as the size marker. Following electrophoresis, gels were incubated for 30 minutes in 0.5 M NaOH–1.5 M NaCl and then for 30 minutes in 3 M sodium acetate (pH 5.0), after which they were transferred by capillarity onto Biodyne A nylon membranes (Pall Corporation, New York, NY). DNA was cross-linked to the membranes by exposure to UV light in a UV Stratalinker (Stratagene Cloning Systems, La Jolla, CA) and incubated for 24 hours in hybridization buffer containing $6\times$ saline sodium phosphate EDTA (SSPE), 0.1% SDS, $5\times$ Denhardt's solution, 50% deionized formamide, and 100 $\mu\text{g}/\text{mL}$ fragmented salmon sperm DNA at 42°C (prehybridization). Hybridization was performed for 24–48 hours at 42°C in the same buffer after the addition of heat-denatured radiolabeled probes. The hybridized membranes were washed first for 1 hour in $2\times$ SSPE and 0.1% SDS at 65°C and then for 10 minutes in $0.2\times$ SSPE and 0.1% SDS at 65°C before being exposed to phosphor screens and analyzed in a phosphorimager (Molecular Dynamics, Amersham-France SA, Les Ulis, France).

Probes Used for Southern Blotting

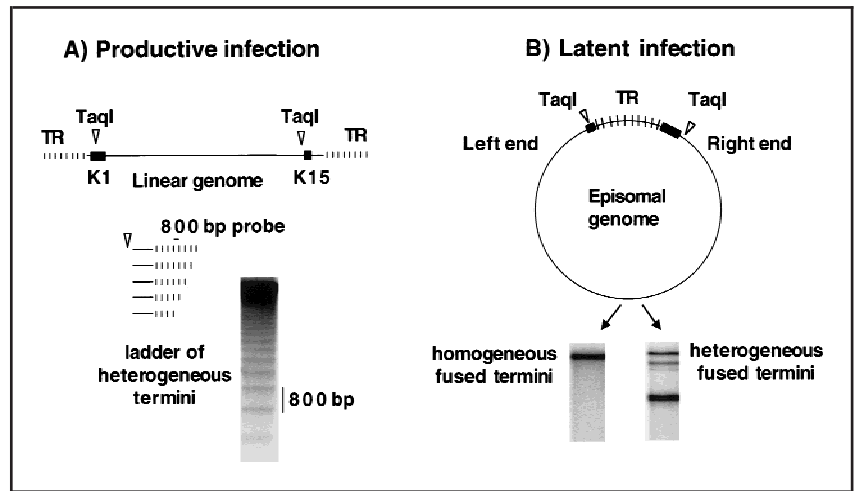
Plasmid P-71 contains the 803-bp HHV8 *NorI* repeat cloned into the Bluescript vector and was provided by Dr. Yuan Chang (Columbia University, New York, NY). The left end probe (LE) and right end probe of the P subtype (RE-P) were obtained by polymerase chain reaction (PCR) with the use of DNA from the BCBL-1 cell line as template and the following primers: LE-S (i.e., sense), 5'-CTACTAATTTTCAAAGGCGGGGTT; LE-AS (i.e., antisense), 5'-TGTTTGGACAAATGGGCAAAGATA; RE-P-S, 5'-CATGTGGACCAGCAT-GTTTGTCTCAT; and RE-P-AS, 5'-CAGCTGTAACAATTTACGAGCCTT. For the right end probe of the M subtype (RE-M), we used DNA from the BC-1 cell line as template with primers RE-M-S (5'-ATGTATTGCCTTGGCAGTTG-GAG) and RE-M-AS (5'-AACGACACAAGTCTGCTCTCTCGG). The resulting 873-bp LE DNA fragment (coordinates 381–1254 of the sequence in GenBank under accession number U86667), 829-bp RE-P DNA fragment (coordinates 3041–3870 of the sequence in GenBank under accession number U85269), and 1369-bp RE-M DNA fragment (coordinates 135087–136455 of the complete BC-1 sequence in GenBank under accession number U75698) were cloned in the pCRII vector (Invitrogen Corp., Carlsbad, CA). Inserts were released from the vectors by digestion with appropriate restriction enzymes and gel purified. For probe radiolabeling, 100 ng of insert DNA was labeled with [^{32}P]deoxycytidine triphosphate with the use of the Prime-It Rmt random priming kit (Stratagene Cloning Systems) and purified with Chroma Spin-30 columns (Clontech Laboratories, Inc., Palo Alto, CA).

Principle of TR Analysis

Panels A and B of Fig. 1 depict the two different forms assumed by γ -herpesviruses during their life cycle. Productive infection, also called lytic phase, ends up with the release of infectious virions and death of the host cell. During this lytic phase, viral genomes are replicated by a rolling-circle mechanism producing long concatemers that are then cleaved randomly within the TR region before packaging. This process produces linear genomes with varying numbers of direct tandem TRs at each terminus. If the DNA of productively infected cells (or of purified virions) is digested with a restriction enzyme (*TaqI* for HHV8) that cuts the viral genome close to but not within the TR region and is hybridized after Southern blotting to a probe corresponding to the TR DNA sequence, a ladder of bands is observed representing heterogeneous terminal fragments varying in molecular weight by increments of the size of each TR unit (Fig. 1, A).

After a latent infection is established, the linear termini are fused at the TR region to produce circular genomes that will be replicated and maintained as multiple episomal copies in the infected cell (Fig. 1, B). In this case, the same Southern blot analysis will generate fragments corresponding to the fused TR region. Because of the variability in the number of TR units incorporated during circularization, independent infection events can be identified as differently sized TR fragments. With EBV, it was shown that, at a low multiplicity of infection, a single form of fused TR region is detected in the clonally expanded progeny of a single infected cell (homogeneous fused termini in Fig. 1, B). Conversely, the presence within a tumor of multiple fused TR fragments heterogeneous in size indicates infection of either an already expanded clonal population or a polyclonal population (heterogeneous fused termini in Fig. 1, B) (29).

Fig. 1. Principle of terminal repeat (TR) Southern blot analysis in herpesvirus-infected cells. **A)** Lytic infection characterized by the production of linear genomes with variable numbers of direct tandem TRs at each terminus. The human herpesvirus 8 (HHV8) genome is about 170 kilobases long, and the *TaqI* sites closest to the TR region are located within open reading frame (ORF) K1 at the left end and close to ORF K15 at the right end. Hybridization of *TaqI*-digested DNA (from sample PEL-2) with a TR probe generates a ladder of heterogeneous termini. **B)** Latent infection with episomal HHV8. TR hybridization of *TaqI*-digested DNA reveals single fused TR fragment in a monoclonal population. Several bands corresponding to multiple fused TR fragments are seen in an oligoclonal or polyclonal population (DNA from sample Cra-Jo and DNA from sample PEL-2 were used, respectively). bp = base pairs.



RESULTS

Structure of HHV8-Fused Termini in Tumor Samples

High-molecular-weight genomic DNA from tumor biopsy specimens was digested with *TaqI*, subjected to electrophoresis by regular gel electrophoresis, and analyzed by Southern blotting with the use of a probe homologous to the TR sequence.

Fig. 2, A, shows a representative Southern blot analysis of several PEL, MCD, and KS biopsy specimens. The viral load appeared much higher in the two PEL samples (lanes 1 and 2) than in the MCD (lanes 3–6) and KS (lanes 7–9) samples, probably because PEL effusion samples consist of a great majority of HHV8-positive tumor cells, whereas KS and MCD biopsy specimens contain a substantial proportion of non-HHV8-infected cells. A ladder of fragments below the major band in the second PEL sample (lane 2) had the typical appearance of a replication ladder, with an approximately 800-bp-size difference between each band. An extra band could be seen in two samples (lanes 1 and 7), suggesting either viral genomic rearrangements or oligoclonal infections (*see below* for the method used to discriminate between these possibilities).

Overall, although a few TR fragments appeared smaller than others (Fig. 2, A; lanes 7 and 9), the majority of TR fragments appeared to migrate as single large molecules of similar size (around 35 kilobases [kb]). A similar hybridization pattern was

obtained in other Southern blot experiments (data not shown). Since, in this size range, regular gel electrophoresis does not provide sufficient resolution to separate closely spaced DNA fragments, we developed a PFGE protocol optimized to separate 10- to 60-kb-long DNA fragments. Comparative results of the two electrophoretic methods applied to one PEL and one KS sample are shown in Fig. 2, B. While the TR fragments migrated as single bands during regular gel electrophoresis, they were clearly resolved into four or five distinct bands by PFGE. These data show that, with the use of regular gel electrophoresis, heterogeneous TR fragments could appear as being homogeneous as a result of band compression. As seen in Fig. 2, B, the PFGE procedure could separate 40-kb-long DNA fragments differing in size by about 1 kb, which is equivalent to a single repeat unit. PFGE was, therefore, used systematically throughout the rest of this study.

So that we could confirm that the DNA fragments hybridizing to the TR probe represented fused TRs, membranes were hybridized successively with the TR probe and with probes representing unique DNA sequences from the left end (LE) or right end (RE) of the HHV8 genome (Fig. 3, bottom). Since the LE and RE probes hybridize to sequences located between the TR region and the first *TaqI* restriction site, they will generate hybridization patterns identical to those obtained with the TR probe if the bands detected correspond to intact fused TR frag-

Fig. 2. Comparison of regular gel electrophoresis (RGE) and pulsed-field gel electrophoresis (PFGE) for the Southern blot analysis of human herpesvirus 8 (HHV8)-fused terminal repeats (TRs). **A)** TR Southern blot hybridization of a panel of primary effusion lymphoma (PEL) (lanes 1–2), multicentric Castlemann's disease (MCD) (lanes 3–6), and Kaposi's sarcoma (KS) (lanes 7–9) tumor biopsy specimens following RGE. DNA was digested with *TaqI*. The molecular-weight marker was an *HindIII* digest of λ DNA. Bands representing fused TRs are indicated by **black dots**. **B)** *TaqI*-digested DNA from one PEL tumor (PEL-2) and one KS lesion (KS-2) was subjected to electrophoresis with the use of either RGE (left) or PFGE (right) and analyzed by Southern blotting with a TR probe. **Black arrowheads** indicate fused TR fragments. The migration of the high-molecular-weight DNA marker (Life Technologies, Inc.) is shown on the **right**.

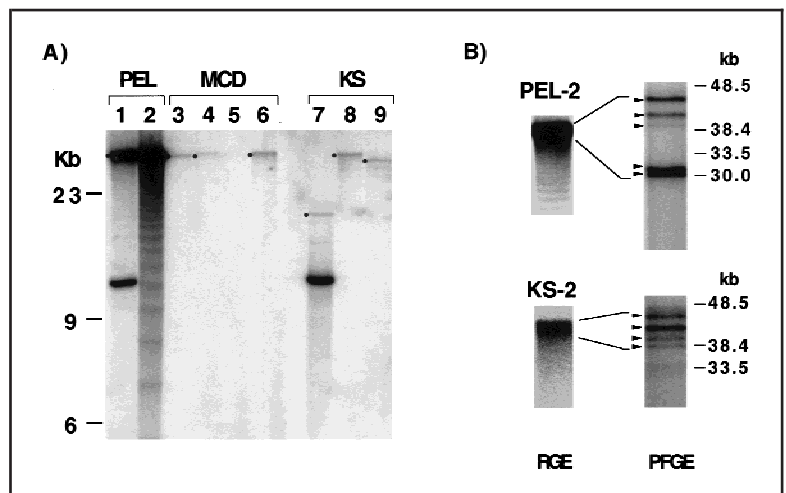
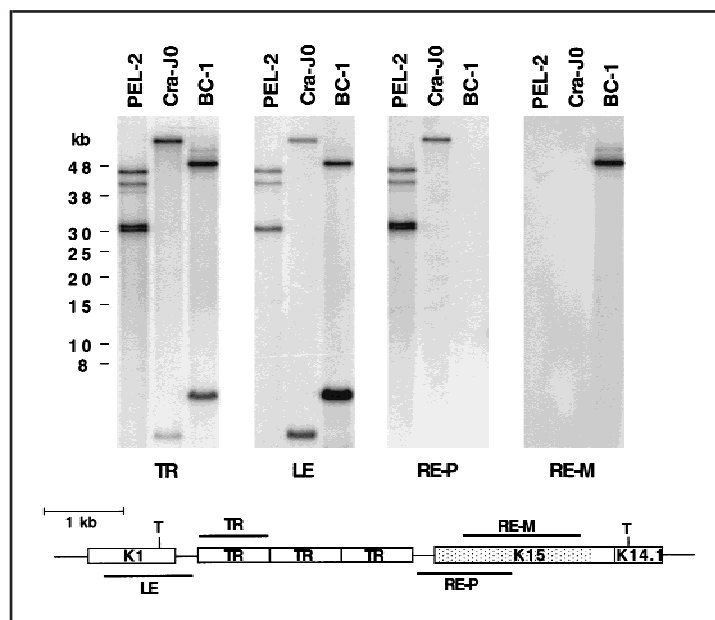


Fig. 3. Multiple-probe Southern blot analysis of human herpesvirus 8 (HHV8)-fused terminal repeats (TRs). *TaqI*-digested DNA from two primary effusion lymphoma (PEL) tumor biopsy specimens (PEL-2 and Cra-J0) and one PEL cell line (BC-1) was subjected to electrophoresis by pulse-field gel electrophoresis. Following Southern blot hybridization with a TR probe, the membrane was rehybridized successively with probes LE (i.e., left end probe), RE-P (i.e., right end probe of the P subtype), and RE-M (i.e., right end probe of the M subtype). A linear representation of the HHV8 genome is shown **at the bottom**. The size and location of each probe are indicated (further details are provided in the "Materials and Methods" section). The locations of the *TaqI* restriction sites closest to the TR region are indicated. The shaded portion of open reading frame K15 corresponds to the highly divergent sequence of the P and M viral subtypes. kb = kilobase.



ments. Fig. 3 shows this analysis applied to two PEL tumor biopsy specimens and one PEL cell line. An identical hybridization pattern was obtained with the three probes for all of the large fragments, except for one of two closely migrating bands just above 30 kb in sample PEL-2, which did not hybridize to probe LE. This band may correspond to a TR fragment in which a mutation created a new *TaqI* site near the junction between the TR and the left end of the genome, thus preventing hybridization of the LE probe to the TR fragment. Note that two different RE probes were used because of the existence of two viral subtypes, P and M, with an almost totally divergent sequence in this region of the genome (32). Thus, probe RE-P is homologous to the P subtype and hybridized to samples PEL-2 and Cra-J0, whereas probe RE-M is homologous to the M subtype and hybridized to sample BC-1 (Fig. 3).

In samples Cra-J0 and BC-1, two small fragments of about 5 and 7 kb hybridized only with the TR and LE probes, but not with the RE probe, suggesting that they do not represent intact fused terminal regions. Indeed, in a previous study (2), the same 7-kb band was found in BC-1 and was thought to result from the insertion of a duplicated region of the LUR within the TR region, about 7 kb away from the left end of the genome. The 5-kb fragment seen in sample Cra-J0 probably represents a similar rearrangement by duplication of a left end portion of the LUR and its insertion in the TR region. The fact that, in each sample, the upper band hybridized with the LE probe rules out the possibility that the rearrangements simply resulted from a mutation generating a new *TaqI* recognition sequence within the TR region. Such multiple hybridization analysis confirms that HHV8 DNA is present in tumors as extrachromosomal circular episomes and permits the identification of rearrangements such as *TaqI* restriction fragment length polymorphisms occurring in the TR region or insertions of duplicated portions of the LUR in the TR region.

Clonality of the HHV8 TR Region in PEL Biopsy Specimens and Cell Lines

Genomic DNA was obtained from five PEL cell lines and a panel of tumor biopsy specimens previously found to be positive

for HHV8 by PCR analysis (33,34). A total of four PEL, 26 KS, and 10 MCD biopsy samples were analyzed by either regular gel electrophoresis, PFGE, or both methods. Table 1 summarizes patients' clinical data, the nature of the biopsy material, and the results of the TR analysis for the samples that gave informative results by PFGE analysis

The four PEL lesions (three pleural effusions and one ascitic effusion) had clonal Ig gene rearrangements as determined by PCR and/or Southern blot analysis (data not shown) and gave easily detectable TR hybridization signals after PFGE (Fig. 4, A). Two samples (Cra-J0 and PEL-3) contained a single fused TR fragment of 60 and 44 kb, respectively, while the other two samples (PEL-2 and PEL-4) contained heterogeneous fused TR fragments. Sample PEL-2 showed five TR bands ranging from 31 to 46 kb, whereas sample PEL-4 showed two TR bands (a major one of 38 kb and a minor one of 19 kb). The DNA ladder coming down from the 19-kb TR band in PEL-4 indicates that this viral form is undergoing lytic replication. Sample PEL-2 was also found to contain lytically replicating HHV8, which is visible on a longer exposure in Fig. 4, A, and in a regular gel electrophoresis experiment (Fig. 2, A; lane 2).

Among the five PEL cell lines studied, four (Cra-BCBL, BC-1, BC-2, and BCBL-1) contained a monoclonal episome. A very faint TR band visible above the major TR fragment in sample BC-1 may, however, correspond to a second episomal form present in a small fraction of the cells. The BC-3 cell line contained two episomal species, with TR regions of 46 and 29 kb, and also had replicative linear HHV8 DNA. Viral genomic rearrangements were observed in three cell lines and in one tumor biopsy specimen. The two 5- and 7-kb bands in the Cra-J0 tumor and the BC-1 cell line have been described above (Fig. 3). The Cra-J0-derived Cra-BCBL cell line showed a TR pattern identical to that of the parent tumor, with the same 5-kb rearranged band (Fig. 4, A and B). The 12-kb fragment in the BCBL-1 cell line hybridized with the RE probe but not with the LE probe, whereas the upper 52-kb band hybridized with all of the probes (data not shown). This observation suggests the insertion in the TR of a portion of the right end of the LUR, 12 kb away from the right end of the genome. All PEL tumors were

Table 1. Pulse-field gel electrophoresis analysis of human herpesvirus 8 terminal repeats (TRs) in PEL, KS, and MCD tumors*

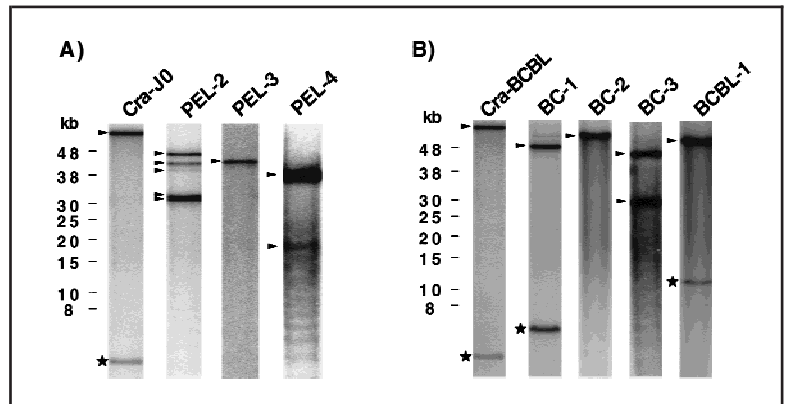
Sample	Age, y	Sex	HIV status†/risk factor	Biopsy specimen	TR analysis	
					No. of fused TR bands‡	Lytic replication ladder
PEL-1	38	M	+ /homosexual	Pleural effusion	1	–
PEL-2	36	M	+ /homosexual	Ascitic fluid	5	+
PEL-3	42	M	+ /homosexual	Pleural effusion	1	–
PEL-4	44	M	+ /homosexual	Pleural effusion	2	+
KS-1	75	M	–/HTLVI	Cutaneous	3	–
KS-2	42	M	+ /homosexual	Lymph node	4	–
KS-3	32	F	+ /epidemic	Cutaneous	1	–
KS-4	78	M	–/–	Cutaneous	1	–
KS-5	38	F	+ /epidemic	Cutaneous	2 + smear	–
KS-6	48	M	+ /epidemic	Lymph node	3	–
MCD-1	68	M	–/–	Lymph node	Multiple + smear	–
MCD-2	37	M	+ /homosexual	Spleen	1 + smear	–
MCD-3	32	M	+ /epidemic	Lymph node	ND	–
MCD-4	47	F	+ /transfusion	Lymph node	ND	–
MCD-5	35	M	+ /IVDU	Spleen	ND	–

*Abbreviations used: PEL = primary effusion lymphoma; KS = Kaposi's sarcoma; MCD = multicentric Castlemann's disease; M = male; F = female; HTLVI = human T-cell leukemia virus type I; IVDU = intravenous drug user; ND = not detected.

†For human immunodeficiency virus (HIV) status, + stands for HIV seropositive and – stands for HIV seronegative.

‡Smear indicates heterogeneous fused termini that were not resolved as distinct bands.

Fig. 4. Pulsed-field gel electrophoresis separation and terminal repeat (TR) Southern blot analysis of primary effusion lymphoma (PEL) samples. **A)** PEL tumoral effusions. **B)** PEL cell lines. **Arrowheads** indicate bands corresponding to human herpesvirus 8-fused TR fragments. **Stars** indicate bands corresponding to rearrangements. Note the presence of lytic replication DNA ladders in samples PEL-4 and BC-3. kb = kilobase.



co-infected with monoclonal EBV, as determined by Southern blot analysis (data not shown). BC-1 and BC-2 are EBV positive (35), whereas BC-3 and BCBL-1 are EBV negative (36,37). Thus, there was no relationship between the presence of EBV and the clonal pattern of HHV8 in PEL samples.

Clonality of the HHV8 TR Region in KS and MCD Biopsy Specimens

A total of 26 KS biopsy samples were analyzed. Histologically, 14 were nodular skin lesions and two were infiltrated lymph nodes; all 16 had a medium to abundant spindle cell content. Ten other lesions contained few spindle cells. The six lesions that gave detectable hybridization signals by PFGE were among the 16 spindle cell-rich lesions (38%). A monoclonal TR pattern was found in two samples (KS-3 and KS-4), while three other samples (KS-1, KS-2, and KS-6) showed an oligoclonal TR pattern with two to four distinct TR bands (Fig. 5). The KS-5 sample gave a more intricate pattern, with two distinct bands together with a smear. In only two additional samples, a clear band was detected at around 35 kb by regular gel electrophoresis (e.g., KS-8 in Fig. 2, A), but these samples exhibited no band after PFGE (data not shown). We interpret this finding as a loss of signal intensity resulting from the separation of multiple

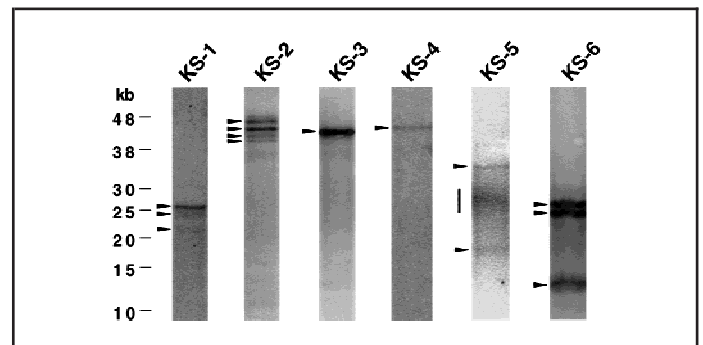


Fig. 5. Pulsed-field gel electrophoresis (PFGE) separation and terminal repeat (TR) Southern blot analysis of Kaposi's sarcoma (KS) samples. **Arrowheads** indicate bands corresponding to human herpesvirus 8-fused TR fragments. **Vertical line** indicates diffuse hybridization signal corresponding to multiple bands.

bands, and these samples were thus scored as polyclonal. PFGE analysis could not be performed because there was not enough DNA left from sample KS-7, which showed a strong signal after regular gel electrophoresis in Fig. 2, A.

Among the 10 MCD lymph node or spleen biopsy specimens, seven gave a detectable TR hybridization signal after regular gel electrophoresis. Of these seven samples, five could be analyzed

by both regular gel electrophoresis and PFGE. While a single TR band was visible in all five samples after regular gel electrophoresis (Fig. 6, RGE panel), following PFGE, two samples showed a heterogeneous TR pattern, with multiple bands visible above a smeary background, while the other three samples did not give a detectable signal, again because of the dilution of the signal resulting from the separation of multiple bands (Fig. 6, PFGE panel). Thus, all five MCD lesions appeared to contain polyclonal HHV8 episomes.

DISCUSSION

The data presented here show that HHV8 is present as an extrachromosomal episomal DNA *in vivo* in tumors and that the size of the fused TR region of HHV8 provides a molecular marker to analyze viral and cellular clonality in HHV8-associated tumors. Our initial TR Southern blot studies of *TaqI*-digested tumor DNA revealed a rather uniform hybridization pattern, with a large (>35 kb) single TR-hybridizing fragment present in most samples. A previous study using only regular gel electrophoresis (2) had reported a comparable monomorphic TR hybridization pattern in a few PEL cell lines and in a single KS lesion. However, our results with the use of PFGE showed that the lack of resolution of regular gel electrophoresis in this size range can make DNA fragments differing in length by up to 15 kb appear to migrate as a single band (Fig. 2, B). High-resolution PFGE is, therefore, required to reliably assess the size heterogeneity of the HHV8-fused TR region. As shown above, in a series of nine PEL (five cell lines and four tumor biopsy specimens) and six KS samples, the size of HHV8-fused TR fragments ranged from 13 to 60 kb—corresponding to 16–75 TR units, which is considerably larger than the EBV-fused TR region (one to nine TR units of 500 bp each) (29,30). Such variability in the number of HHV8-fused TRs is compatible with the differences in viral genome size observed in previous PFGE studies (13,36) of intact cell and virion DNA from a few PEL cell lines and one KS tumor sample. This variability is also consistent with the presumed random cleavage within the TR region of linear concatemers at a signal cleavage sequence present within each TR (2) and suggests that successful packaging of

infectious HHV8 virions can tolerate at least 20% variation in genome size.

The presence of monoclonal HHV8 episomes in two cases of four PEL tumoral effusions examined indicates that HHV8 infection preceded the clonal expansion of a single infected cell, which strongly supports an etiologic role of latent HHV8 in these lymphomas. Still, many questions remain with regard to the precise role HHV8 plays in PEL pathogenesis. Since only a very small fraction of individuals infected with HHV8 will ever develop PEL, even in the context of immunosuppression, such as HIV infection and AIDS, it is conceivable that additional factors are involved in lymphomagenesis, such as the occurrence of a specific genetic lesion in the PEL cell precursor or the existence of rare lymphomagenic HHV8 variants. For instance, by analogy with EBV in Burkitt's lymphoma and AIDS-related non-Hodgkin's lymphoma (14), either an occult prelymphoma stage characterized by an HHV8-driven polyclonal B-cell proliferation may just increase the chance of a genetic lesion taking place, or HHV8 may directly cooperate with that genetic lesion to drive the proliferation of the lymphoma cells. However, aside from the frequent presence of mutations in the 5' region of the BCL-6 gene, the importance of which is unknown (38), no proto-oncogene rearrangement has yet been identified in PEL.

Our finding of oligoclonal episomal fused TR fragments in two PEL tumors and one PEL cell line (BC-3) was quite surprising and is difficult to reconcile with their cellular monoclonality. Hybridization with the TR, LE, and RE probes showed that these bands correspond to intact fused TR regions (except for one of five bands in sample PEL-2) and may, therefore, represent multiple infection events. As discussed above, if PEL development requires a particular genetic lesion in addition to HHV8, then the presence of oligoclonal HHV8 episomes in a monoclonal cell population is possible if HHV8 infection and the subsequent expansion of a few infected clones occurred in a monoclonal population of pretumoral cells. It is intriguing, however, that the tumor samples containing oligoclonal HHV8 episomes also contained lytically replicating virus. Although this finding could just be a coincidence, it also suggests that the production of virus inside the lesions may have caused the occasional superinfection of other latently infected cells, thus giving the appearance of oligoclonality. The possibility of superinfection by EBV has been documented previously in EBV-infected cell lines (39,40). Alternatively, heterogeneous fused termini in these PEL lesions and in some KS lesions (*see below*) may represent fused termini of linear concatameric replicative intermediates that could possibly be generated in high copy numbers in cells permissive for lytic replication as described for EBV (41).

Finally, given the large number of HHV8-fused TRs, another possibility to consider is that polymorphic TR fragments would result from rearrangements generated, for instance, by slippage of the cellular DNA polymerase. Since we observed frequent rearrangements suggesting the insertion of a duplicated fragment of the LUR within the TR region (in Cra-J0, BC-1, and BCBL-1), it seems possible that internal deletions or duplications of a number of TRs could also take place. Such TR rearrangement events may not be detected by our multiple-probe hybridization analysis and could be responsible for the apparent polymorphic TR patterns that we observed in some PEL and KS tumors. In this case, the occurrence of monoclonal samples could, in fact,

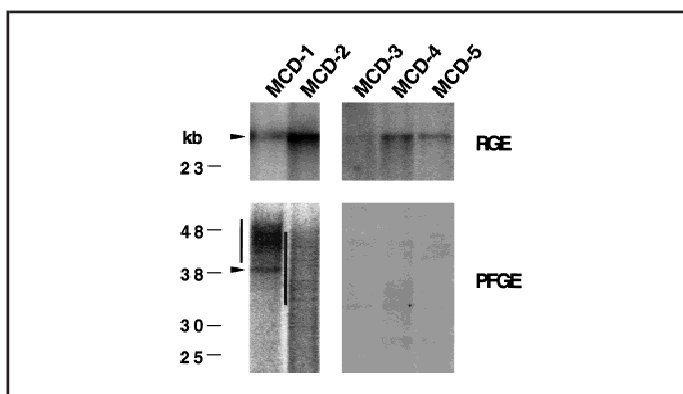


Fig. 6. Terminal repeat (TR) Southern blot analysis of multicentric Castleman's disease (MCD) samples. The same *TaqI*-digested DNA samples were subjected to electrophoresis with the use of regular gel electrophoresis (RGE) (top panels) or pulsed-field gel electrophoresis (PFGE) (bottom panels). Arrowheads indicate bands corresponding to human herpesvirus 8-fused TR fragments. Vertical lines indicate diffuse hybridization signal corresponding to multiple bands. kb = kilobase.

be underestimated. The fact that several PEL cell lines contain monomorphic HHV8 episomes despite prolonged *in vitro* culture and the preservation of the parental Cra-J0 tumor TR pattern in the Cra-BCBL-derived cell line do not give support to this possibility, although it is possible that the TR region is more prone to rearrangements in the tumor microenvironment *in vivo*. We are currently designing experiments to address some of these questions.

KS is a complex tumor of uncertain pathogenesis and clonality, in which the existence of a truly neoplastic component is still controversial. Many features of KS suggest that, at least in its early stage, it is a reactive hyperplasia rather than a true neoplasm (18,42,43). The clonality of KS tumoral lesions has been studied previously by analyzing the pattern of X-chromosome inactivation, specifically by looking at the methylation status of the androgen receptor gene (23,24,27). However, with this technique, a tumor might appear to be polyclonal if it contains enough non-neoplastic infiltrating cells, which is often the case in KS lesions. Perhaps more importantly, this approach relies on the assumption that there is no skewing of the methylation pattern in the tumor progenitor cells, which is unknown for immature or lymphatic endothelium, the presumed KS precursor tissue (44,45). These potential problems may explain why the data from X-inactivation studies of KS clonality have been conflicting. Our viral TR-based clonal analysis of KS does not suffer from these caveats, but it has a lower sensitivity than PCR-based assays, so that only a fraction (38%) of advanced (nodular) KS lesions, those containing a high enough percentage of HHV8-infected cells, gave a detectable hybridization signal.

We identified two of the examined KS lesions that contained a single fused TR fragment. Four other KS biopsy samples showed an oligoclonal TR pattern, with two to four distinct TR bands either alone (in three cases) or together with a diffuse smear (in one case), suggesting the outgrowth of a few dominant clones within a polyclonal lesion in the last case. These data support a direct viral involvement in KS pathogenesis, by showing that individual lesions are monoclonal or oligoclonal expansions of cells latently infected with HHV8. Such data are consistent with a recent report (46) showing that latent HHV8 infection induces a transformed phenotype in immortalized endothelial cells. However, we also found evidence of viral polyclonality in three KS nodular cutaneous biopsy specimens (the mixed oligoclonal/polyclonal case just mentioned and two polyclonal cases in which the TR band detected by regular gel electrophoresis was no longer seen after PFGE). The finding that nodular KS lesions display all possible patterns of clonality supports the model according to which KS begins as a polyclonal disease with subsequent evolution to a monoclonal process (27,43). In this scenario, lytically HHV8-infected cells could play a key role in the pathogenesis of early KS through paracrine mechanisms, whereas latent infection would mark the outgrowth of truly neoplastic clones in advanced lesions. Our TR-based Southern blot approach makes it now feasible to study larger series of KS lesions involving various sites, such as lymph nodes and cutaneous lesions, and, most importantly, to analyze the clonal relationship of multiple advanced KS lesions in the same patient.

The polymorphic TR pattern that we observed in MCD clearly differs from the monoclonal or the oligoclonal patterns found in all PEL and in some KS samples. All of our MCD samples were of the plasma cell type, characterized by a prolif-

eration of B cells, with polyclonal Ig rearrangements in the large majority of the cases (9). A recent immunocytochemical study of HHV8-positive MCD-reactive lymph nodes (45) revealed the expression of the LNA-1 latent HHV8 antigen in about 10%–30% of the cells. The vIL-6 HHV8 IL-6 homologue, which belongs to the lytic class of viral genes, was also found to be expressed, although in a lower percentage of cells (2%–13%) (47), and both hIL-6 and vIL-6 are thought to be major growth factors in this proliferation (19,48,49). The polyclonal HHV8 TR pattern that we observed denotes a process of ongoing infection that would be consistent with the presence of lytically infected cells within the lesions. However, the TR hybridization signal was very weak for MCD samples because of the low percentage of infected cells in the lesions and, thus, would not have allowed the detection of lytic replication ladders. It will be interesting to examine the clonality of HHV8 in the lymphomas that develop occasionally in these patients (50).

In summary, we have shown that the size heterogeneity of HHV8-fused TRs provides a unique molecular tool to study the clonality of HHV8-infected tumors. Our observation that HHV8 infection precedes the clonal expansion of several PEL and KS tumors strongly supports a direct role of latent HHV8 in their pathogenesis and constitutes a definite demonstration of monoclonality in KS lesions. A plausible implication of these findings is that the process leading to the clonal expansion of a single HHV8-infected cell in KS and PEL proliferations is likely to involve additional factors, such as cellular genetic lesions that cooperate with latent viral gene products or mutations in HHV8 resulting in an increased transforming potential.

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NOTES

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