JOURNAL OF VIROLOGY, Nov. 1996, p. 8151–8154 Vol. 70, No. 11
0022-538X/96/$04.00+0

The Size and Conformation of Kaposi's Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) DNA in Infected Cells and Virions

ROLF RENNE, MICHAEL LAGUNOFF, WEIDONG ZHONG, AND DON GANEM*

Howard Hughes Medical Institute and Departments of Microbiology and Medicine, University of California, San Francisco, California 94143

Received 16 May 1996/Accepted 31 July 1996

The genome of a novel human herpesvirus has been detected in specimens of Kaposi's sarcoma (KS) and in several AIDS-related lymphoproliferative disorders. Here we examine the size and genomic conformation of the DNA of this virus (known as KS-associated herpesvirus or human herpesvirus 8) in latently and lytically infected cells and in virions. Pulsed-field gel electrophoresis of viral DNA shows that the viral genome is similar in size to those of other gammaherpesviruses (160 to 170 kb). As with Epstein-Barr virus, KS-associated herpesvirus DNA is stably maintained in latently infected B cells as episomal monomer circles and induction from latency is associated with the selective accumulation of linear genomic forms.

Kaposi's sarcoma (KS) is the leading neoplasm of patients with the acquired immune deficiency syndrome (7, 8). Recent evidence has linked a novel human herpesvirus, termed KS-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8), to this tumor. KSHV sequences were first identified within AIDS-KS biopsies by representational difference analysis (5), and this and subsequent work (1, 14, 17) has shown that virtually all AIDS-related KS specimens harbor this viral genome. KSHV DNA is also present in human immunodeficiency virus-negative forms of KS (6, 12, 14) and in lymphoid cells of human immunodeficiency virus-positive patients with or at risk for this malignancy. KSHV infection precedes the development of KS, and prior KSHV infection appears to be associated with an increased risk for KS development (20). Within an individual KS tumor, KSHV selectively infects the so-called spindle cells, endothelium-derived cells thought to be central to the pathogenesis of the lesion (2, 19). Thus, at present, KSHV is considered a strong candidate to be an etiologic cofactor in this important human neoplasm.

In addition to its association with KS, KSHV/HHV-8 DNA has also been found in a rare form of AIDS-related B-cell lymphomas (called a body cavity-based lymphoma [3]) and in another lymphoproliferative lesion, that of multicentric Castleman's disease (18). Body cavity-based lymphomas grow as ascites tumors in pleural or peritoneal cavities and are often (but not always) coinfected with Epstein-Barr virus (EBV) (3, 4). These data indicate that HHV-8 can enter and persist within lymphoid cells, an inference strongly supported by phylogenetic analysis of the viral DNA sequence. Recently, the DNA sequence of 21 kbp of KSHV has been determined by Moore et al. (15); computer-assisted homology searches reveal that the two closest viral relatives of KSHV are herpesvirus saimiri and EBV, which are both members of the lymphotropic (gamma) subgroup of herpesviruses.

Little is known at present of the biology of KSHV and its replication, owing in part to the paucity of cell culture systems for viral growth. We have recently reported two KSHV-positive cell lines derived from body cavity-based lymphomas; one of these cell lines (BCBL-1) contains KSHV genomes in the absence of EBV, whereas the other (BCBL-2) is dually infected with both agents. In unstimulated BCBL-1 cells, only a small subset of viral genes is expressed, suggesting a latent infection. Treatment of BCBL-1 with phorbol esters (e.g., 12-*O*-tetradecanoyl phorbol-13-acetate [TPA]), however, rapidly induces lytic growth of KSHV, with the accumulation of large quantities of progeny virus in the medium (16). (BCBL-2 cells, by contrast, do not produce KSHV following TPA induction, although EBV is efficiently induced.) This system makes possible detailed characterization of events in viral replication. Here we report an analysis of the genome size and conformation of KSHV DNA during latent and lytic infection in this cell culture system.

To determine the genome size of KSHV, we performed clamped homogeneous electric field gel electrophoresis on BCBL-1 and BCBL-2 cells. Cells were embedded in agarose at a concentration of approximately 2.5×10^7 cells per ml. For cell lysis, agarose plugs were incubated in lysis buffer containing 0.5 M EDTA, 1% Sarkosyl, and proteinase K to a final concentration of 1 mg/ml at 37°C for 48 h. Approximately 10^6 cells were loaded in each lane. Additionally, we similarly examined partially purified viral particles concentrated by ultracentrifugation from the supernatant of TPA-treated BCBL-1 cells as previously described (16). Gel electrophoresis was carried out at a gradient of 6 V/cm (switch time linearly ramped from 6 to 24 s over 28 h) on a clamped homogeneous electric field mapper, model XA (Bio-Rad). DNA was transferred to a nylon membrane and hybridized either to a KSHV-specific or an EBV-specific radiolabeled probe. The KSHV-specific probe was derived from the major capsid gene, while the EBV-specific probe spanned *oriP* and EBNA-1 coding sequences. In TPA-treated BCBL-1 cells, the KSHV probe hybridized specifically to a band migrating between 150- and 200-kbp linear DNA standards (Fig. 1A, left panel, lane 1). A signal of the same size was detectable in lane 3 where purified virions had been loaded. No KSHV DNA signal at this position was visible in untreated BCBL-2 cells, though a KSHV-specific signal appeared trapped in the wells of this gel (Fig. 1A, left panel, lane 2). When an identical filter was hybridized to the EBV-specific probe (Fig. 1A, right panel, lane 2), BCBL-2 cells showed a single band of the expected size (174 kb); since the mobility of this band was slightly slower than that of KSHV in BCBL-1, we

* Corresponding author. Phone: (415) 476-2826. Fax: (415) 476-0939. Electronic mail address: ganem@socrates.ucsf.edu.

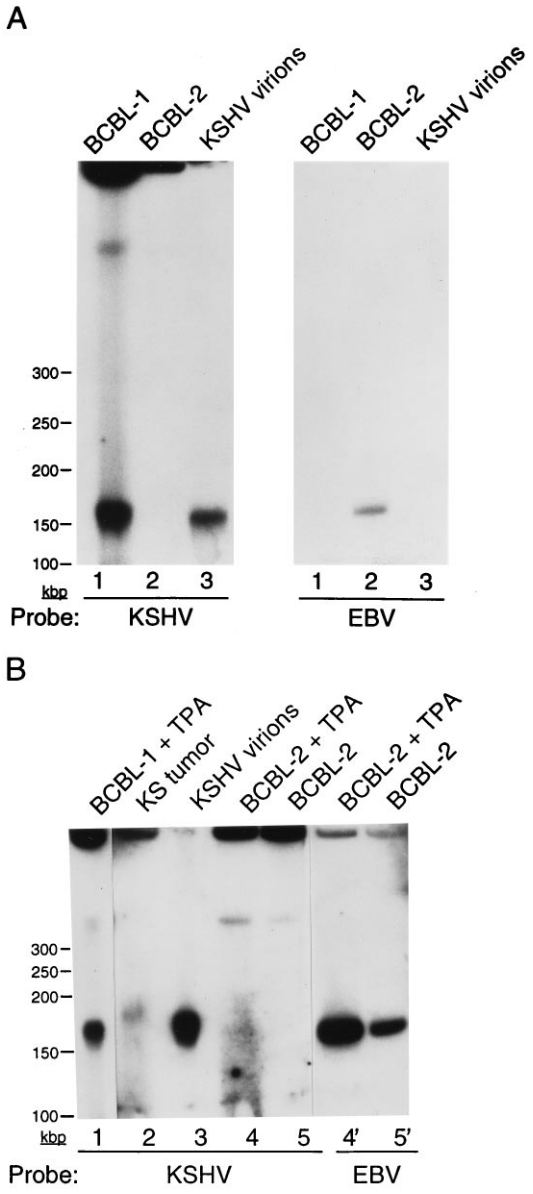

FIG. 1. (A) Determination of the genome size of KSHV by pulsed-field gel electrophoresis. BCBL-1 cells and BCBL-2 cells were embedded in agarose plugs prior to incubation in lysis buffer. Approximately 10^6 cells were loaded in each well and electrophoresed with a clamped homogeneous electric field mapper apparatus, model XA (Bio-Rad). After transfer to nylon membranes, filters were hybridized in Church buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 M $NaHPO_4$ [pH 7.2], 7% SDS) at 65°C for 12 h to ^{32}P-labeled probes specific for the major and minor capsid genes of KSHV (left panel) or to an EBV-specific probe corresponding to the EBNA-1 coding region and *oriP* (that is, EBV *Bam*HI-K and *Bam*HI-C) (right panel). Washes were carried out in Church wash (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) for 1 h at 65°C. Lanes 1, BCBL-1 cells treated with TPA for 48 h; lanes 2, BCBL-2 cells, similarly induced with TPA; lanes 3, purified virions from the supernatant of TPA-treated BCBL-1 cells. (B) Analysis of KSHV genomes in BCBL-2 cells and a KS tumor. The indicated cells were loaded into each well and electrophoresed as described above; following transfer to nylon membranes, samples were hybridized to ^{32}P-labeled probes specific for KSHV (lanes 1 to 5) or EBV (lanes 4′ and 5′). Lane 1, DNA from TPA-induced BCBL-1 cells; lane 2, DNA from a primary KS tumor; lane 3, DNA from partially purified virions secreted from TPA-induced BCBL-1 cells; lanes 4 and 4′, DNA from TPA-induced BCBL-2 cells; lanes 5 and 5′, DNA from uninduced BCBL-2 cells. Molecular size markers are noted at the left.

estimated the genome size of KSHV/HHV-8 to be ca. 160 to 170 kb.

In lysates of both BCBL-1 and BCBL-2 cells, additional hybridization signals were seen. A strong signal was visible at the origin of the gel and presumably derived from unlysed cells. A second band, migrating in the 800- to 1,000-kb region of the gel, was also detectable. This band was also visible in the ethidium bromide-stained agarose gel, strongly suggesting that it represented large fragments of host chromosomal DNA; the KHSV signal in this region therefore likely represents trapping of viral DNA in this network of cellular sequences, although the formal possibility of viral DNA integration into host DNA cannot be ruled out by these data (see below). Similar trapped episomal DNA species have been observed for EBV in a comparable analysis of EBV-infected cells (10).

Since all of these HHV-8-positive samples were of lymphoid origin, we also performed a similar analysis on biopsy material from a KS lung tumor. This seemed particularly important in light of earlier suggestions that the KSHV genome might be as large as 270 kb (15). As shown in Fig. 1B, a band roughly comigrating with that of purified virions (160 to 170 kb) is also detectable in the KS lung lesion (Fig. 1B, compare lanes 2 and 3). The slightly slower mobility of this gel is most likely to be due to trapping of KSHV episomes in the chromosomal background of the tumor material; however, we cannot exclude the possibility that KSHV isolates from KS tumors and BCBL-1 cells differ slightly in size. We note, however, that the viral DNA in the KS tumor (Fig. 1B, lane 2) is clearly less than ca. 180 kb in size.

In addition, we analyzed BCBL-2 cells before and after TPA treatment. Because KSHV was not detectable in BCBL-2 cells in the previous experiment (Fig. 1A, lane 2), we loaded approximately 2.5×10^6 cells in each well. Even after longer exposure times, no KSHV-specific signal could be detected before and after induction with TPA (Fig. 1B, lanes 4 and 5). An identical filter hybridized to an EBV-specific probe showed a strong increase of EBV DNA after TPA treatment in BCBL-2 cells (Fig. 1B, lanes 4′ and 5′). This indicates that the failure of KSHV to be induced in this line is not due to a defect in the cellular signalling pathway for TPA. We do not know why induction of KSHV is poor in this line but note that similar behavior has been reported for other EBV-positive B-cell lines (4). This raises the possibility that EBV infection interferes with KSHV induction from latency, but many other explanations for this phenomenon are possible.

Next, we sought to examine the conformation of viral DNA within latently and lytically infected BCBL-1 cells. Because pulsed-field gel electrophoresis cannot distinguish linear from circular DNA molecules (10) we used a native agarose gel system originally described by Gardella et al. (9) to analyze the forms of KSHV DNA in BCBL-1 cells. BCBL-1 cells, which were more than 95% viable (as judged by trypan blue exclusion), were resuspended in sample buffer A (15% Ficoll, 40 μg of RNase A per ml, and 0.01% bromphenol blue in TBE buffer [Tris-borate-EDTA]) at a concentration of 2×10^7 cells per ml. Approximately 5×10^5 cells were loaded in slots of a vertical 1% agarose gel (120 by 120 by 2.5 mm). For cell lysis, samples were carefully overlaid with 100 μl of lysis buffer (5% Ficoll, 1% sodium dodecyl sulfate [SDS], 1 mg of pronase per ml, 0.05% xylene cyanol green), and electrophoresis was first carried out at 0.8 V/cm for 3 h and then at 7.5 V/cm for 12 h. All steps were carried out at 4°C. After electrophoresis, gels were stained with ethidium bromide and the DNA was transferred to a nylon membrane and hybridized to a KSHV-specific radiolabeled probe.

Figure 2A, lane 1, shows that DNA from KSHV virions (and

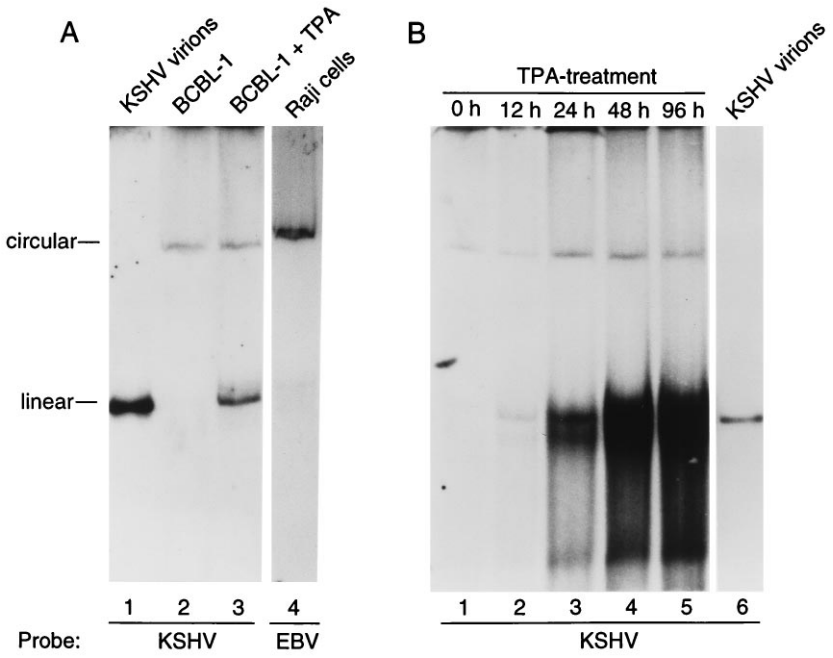

FIG. 2. Native agarose gel electrophoresis of BCBL-1 cell DNA. Approximately 10^6 cells were loaded in each well of a horizontal 1% agarose gel in sample buffer. Samples were overlaid with 100 μl of lysis buffer prior to electrophoresis at 0.8 V/cm for 3 h and then at 7.5 V/cm for 12 h at 4°C. (A) Purified viral DNA (lane 1), BCBL-1 cells (lane 2), and BCBL-1 cells treated with TPA (lane 3) were hybridized to a KSHV-specific probe. As a control for circular DNA, Raji cells were loaded in lane 4 and hybridized to an EBV-specific probe. (B) BCBL-1 cells uninduced or treated with TPA were analyzed on a horizontal agarose gel and electrophoresed as described above at 12, 24, 48, and 72 h after TPA was added. Lane 6 contains purified viral DNA.

therefore representing linear molecules) migrates as a single band. When DNA from uninduced BCBL-1 cells was similarly examined, a single band of much slower mobility was observed (lane 2). This band comigrated with EBV DNA extracted from Raji cells, which are known to harbor only circular episomal forms of EBV DNA (lytic replication in this line is impaired by two lesions in EBV genes essential for lytic growth; the line therefore cannot produce linear EBV DNA) (11). Thus, this species most likely represents circular episomal monomers of KSHV/HHV-8 DNA. When BCBL-1 cells were treated with TPA to induce lytic replication, a second viral DNA species appeared, comigrating with virion DNA (Fig. 2A, lane 3). Figure 2B shows the result of a kinetic analysis of the appearance of these genomic conformers following induction from latency. BCBL-1 cells were harvested before (lane 1) or at various times after (lanes 2 to 5) TPA addition, and their DNAs were analyzed as described above. As early as 12 h after TPA addition, linear DNA species were detectable (lane 2), and these linear DNA molecules accumulated progressively over time (lanes 2 to 5). (At late times, some DNA degradation was evident, likely owing to nucleases released during cell lysis at this point). By contrast, circular KSHV genomes increased only slightly during the first 48 h (while cells were still dividing); thereafter, the concentration of circular DNA was largely unchanged (Fig. 2B). These results are similar to what was observed during the induction of EBV from latency (13) and support the inference that the predominant form of the HHV-8 genome in latency is an extrachromosomal circular episome. Since BCBL-1 cells maintain a stable copy number of KSHV genomes (estimated at ca. 50 genome equivalents per cell) (16), in the face of active cell division, it is likely that, as in EBV, an active system of viral gene products is responsible for maintenance of the KSHV episome.

These studies reveal that the genome of KSHV/HHV-8 is similar in size to those of its closest viral relatives, herpesvirus saimiri and EBV, and that like EBV the genome is maintained in latency as an episomal monomer circle, at least in B cells. Our estimate of genome size is based on the use of cells harboring a genome that has previously been shown to be competent for lytic replication; the availability of encapsidated virion DNA in sufficient quantities has greatly simplified and clarified this analysis. Earlier studies employing nonpermissive or semipermissive B cells dually infected with EBV identified KSHV/HHV-8 genomic forms estimated to be 250 to 270 kb in size (15), with no smaller forms being evident. However, since no detectable encapsidated virus could be purified from such cells, it is difficult to exclude the possibility that such forms represent multimeric, concatameric, or rearranged forms of the genome. While it is formally possible that our BCBL-1 isolate represents a deletion variant of a putative 270-kb virus, this seems surpassingly unlikely, given (i) its known replication competence (16); (ii) the fact that genomes of similar sizes have been identified in a KS sample; and (iii) the close similarity of its size to those of its gammaherpesvirus relatives, especially herpesvirus saimiri, with whose genome KSHV displays remarkable colinearity (15).

This work was supported by the Howard Hughes Medical Institute.

REFERENCES

1. **Ambroziak, J., D. Blackbourn, B. Herndier, R. Glogan, J. Gullet, A. McDonald, E. Lennette, and J. Levy.** 1995. Herpesvirus-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science **268:**582–583.
2. **Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, A. C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss, and J. J. O'Leary.** 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat. Med. **1:**1274–1278.
3. **Cesarman, E., Y. Chang, P. S. Moore, J. W. Said, and D. M. Knowles.** 1995. Kaposi's sarcoma-associated herpesvirus-like sequences in AIDS-related body cavity-based lymphomas. N. Engl. J. Med. **332:**1186–1191.

4. **Cesarman, E., P. S. Moore, H. Pulirarthy, G. Inghirami, D. M. Knowles, and Y. Chang.** 1995. 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. Blood **86:**2708–2714.
5. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science **266:**1865–1869.
6. **Chuck, S., R. M. Grant, E. Katongole-Mbidde, M. Conant, and D. Ganem.** 1996. Frequent presence of herpesviral-like DNA sequences in lesions of HIV-negative Kaposi's sarcoma. J. Infect. Dis. **173:**248–251.
7. **Ensoli, B., G. Barillari, and R. C. Gallo.** 1991. Pathogenesis of AIDS-associated Kaposi's sarcoma. Hematol. Oncol. Clin. N. Am. **5:**281.
8. **Ganem, D.** 1995. Viruses, cytokines and Kaposi's sarcoma. Curr. Biol. **5:**469–471.
9. **Gardella, T., P. Medveczky, T. Sairenji, and C. Mulder.** 1984. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. J. Virol. **50:**248–254.
10. **Harris, A., and R. Bentley.** 1988. Separation of episomal Epstein-Barr virus from Burkitt's lymphoma host cell DNA in pulse field gels. Nucleic Acids Res. **16:**4172.
11. **Hatfull, G., A. T. Bankier, B. G. Barrell, and P. J. Farrell.** 1988. Sequence analysis of Raji Epstein-Barr virus DNA. Virology **164:**334–340.
12. **Huang, Y.-Q., et al.** 1995. A human herpesvirus-like DNA sequence in various forms of Kaposi's sarcoma. Lancet **345:**759–761.
13. **Liebowitz, D., and E. Kieff.** 1993. Epstein-Barr virus, p. 107–172. *In* B. Roizman, R. J. Whitley, and C. Lopez (ed.), The human herpesviruses. Raven Press, New York.
14. **Moore, P. S., and Y. Chang.** 1995. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. N. Engl. J. Med. **332:**1181–1185.
15. **Moore, P. S., S. J. Gao, G. Dominguez, E. Cesarman, O. Lungu, D. M. Knowles, R. Garber, P. E. Pellett, D. J. McGeoch, and Y. Chang.** 1996. Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. J. Virol. **70:**549–558.
16. **Renne, R., W. Zhong, B. Herndier, M. McGrath, N. Abbey, D. Kedes, and D. Ganem.** 1996. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat. Med. **2:**342–346.
17. **Schalling, M., M. Eukman, E. Kaaya, A. Linde, and P. Bieberfeld.** 1995. A role for a new herpesvirus (KSHV) in different forms of Kaposi's sarcoma. Nat. Med. **1:**707–708.
18. **Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M. F. D'Agay, J. P. Clauvel, M. Raphael, L. Degos, and F. Sigaux.** 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. Blood **86:**1276–1280.
19. **Staskus, K., W. Zhong, K. Gebhardt, H. Wang, R. Renne, B. Herndier, D. Ganem, and A. Haase.** Kaposi's sarcoma-associated herpesvirus genes are expressed predominantly in the endothelial (spindle) tumor cells of Kaposi's sarcoma. Submitted for publication.
20. **Whitby, D., et al.** 1995. Detection of Kaposi's sarcoma-associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet **346:**799–802.