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RAPID COMMUNICATION

# The Latency-Associated Nuclear Antigen Tethers the Kaposi's Sarcoma-Associated Herpesvirus Genome to Host Chromosomes in Body Cavity-Based Lymphoma Cells

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Viruses that establish latent infection must maintain their DNA in the host nucleus through many cellular generations. Here we identify a novel mechanism by which the gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) may achieve this persistence in latently infected body cavity-based lymphoma (BCBL) cells. We find that KSHV genomic DNA is associated with host chromosomes and colocalizes with the latency-associated nuclear antigen (LANA). Furthermore, a region at the left end of the KSHV genome binds strongly to LANA and can colocalize to the host chromosomes with LANA. Additionally, we found that LANA associates with histone H1 in KSHV-infected BCBL cells. We propose that this chromosomal association of the KSHV genome is mediated by LANA and involves a tethering mechanism by which viral episomes are linked to host chromatin through simultaneous interaction with host chromosomal proteins including histone H1 and *cis*-acting KSHV DNA elements. This strategy may be employed by other viruses in establishment of latency in the infected cells. 

*Introduction.* Kaposi's sarcoma was initially described by Moritz Kaposi as a multifocal vascular tumor affecting elderly men of Mediterranean or Eastern European origin. More recently, this neoplasm has become prevalent in immunocompromised patients, such as transplant recipients on immunosuppressive therapy and AIDS patients, and it has become one of the leading common cancers among AIDS patients (*1*). The relationship of the disease to geography and immunocompromised patients led to the suspicion of an infectious agent in Kaposi's sarcoma pathogenesis. This suspicion was supported when Kaposi's sarcoma-associated herpesvirus (KSHV)[2] or human herpesvirus 8 (HHV8) was identified through PCR-based studies of tumor samples from AIDS patients with Kaposi's sarcoma (*2*). Subsequent studies have shown that the virus is of the *Gammaherpesviridae* family, bearing sequence similarity to herpesvirus saimiri (HVS) and Epstein–Barr virus (EBV) (*3*). Although there is increasing epidemiologic data associating the virus with human disease, little is known about the biology of this new gammaherpesvirus.

Indirect immunofluorescence studies of the latently infected body cavity-based lymphoma (BCBL) cell line with serum from Kaposi's sarcoma (KS) patients reveals a characteristic punctate pattern of nuclear immunofluorescence due to the presence of what was termed the latency-associated nuclear antigen (LANA) (*4*). LANA is detected in the majority of cells in a KS lesion as well as in cell lines derived from body cavity lymphomas (*4, 5*). LANA is encoded by orf73 of KSHV and is expressed as a latency-associated protein in the infected cell (*4*). An analysis of the LANA amino acid sequence reveals several acidic and proline/glutamine-rich regions as well as a zinc finger DNA-binding domain (*6, 7*). These structural motifs suggest that LANA may act as a transcription factor; however, it is possible that like EBNA1, LANA may be involved in maintenance of the viral episome in infected cells. LANA appears to be consistently detected in KSHV-infected cells and may be one of only a few KSHV proteins expressed in the latently infected cell (*5, 8*). Therefore the ability of LANA to support persistence of the KSHV genome may be a crucial function in the establishment of latency. Little is known regarding the mechanism and establishment of KSHV latency. In this report we have investigated the possible mechanism by which LANA supports KSHV persistence in latently infected cells.

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[2] Abbreviations used: KSHV, Kaposi's sarcoma-associated herpesvirus; HHV8, human herpesvirus 8; IP, immunoprecipitation; FISH, fluorescence *in situ* hybridization; EBV, Epstein–Barr virus; EBNA, Epstein–Barr virus nuclear antigen; BCBL, body cavity-based lymphomas.

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*Results. LANA and KSHV episomes localize to host metaphase chromosomes in a similar pattern in KSHV-infected cells.* To determine whether LANA has a role in KSHV DNA persistence analogous to EBNA1 in EBV we conducted fluorescence *in situ* hybridization (FISH) and immunofluorescence studies to detect viral DNA and LANA protein on the metaphase chromosomes. Viral cosmids were used as probes on metaphase chromosomes prepared from a KSHV-positive/EBV-negative cell line, BC-3 (*9*), derived from a body cavity lymphoma and the EBV- and KSHV-negative B cell line BJAB was used as a control. Analysis of nuclei from BC-3 cells with the KSHV cosmid probe showed a punctate pattern throughout the nucleus consistent with greater than 30 episomes for each nucleus examined (Fig. 1a). Similarly, when nuclei from KSHV-infected BC-3 were stained with antibodies against LANA and detected by FITC-conjugated secondary antibodies a punctate pattern was also observed throughout the nuclei (Fig. 1b). These results indicated that LANA and KSHV could be associated with the host metaphase chromosomes. Metaphase spreads were then prepared from BC-3 cells and stained as before with KSHV-labeled cosmid probe and human serum recognizing LANA. Although viral DNA was detected in symmetrical patterns achieved by replication of a chromatid to which virus has integrated, it was not consistently seen from one spread to another, suggesting that the signal was associated with the host chromosomes in a random fashion (Fig. 1d). Furthermore, when similar chromosome spreads derived from the same KSHV-positive BC-3 cells were probed with a human serum adsorbed with B cell antigens that preferentially recognize LANA (Fig. 1e), LANA signals localized to the host metaphase chromosomes in a pattern strikingly similar to that of KSHV-specific DNA hybridization (compare Figs. 1d and 1e). This chromosome-associated pattern of immunofluorescence was not evident in metaphase spreads probed with normal human serum from a KSHV-negative adult patient, previously adsorbed with B cell antigens from an EBV-negative cell line, BJAB, as well as an EBV-positive line, B958 (Fig. 1f). Additionally, no signal was observed with KSHV cosmid probe on metaphase spreads from KSHV- and EBV-negative BJAB chromosomes (Fig. 1c). This suggests that the signal is specific for LANA and is not due to a nonspecific signal from the polyclonal human serum (compare Figs. 1e and 1f). Therefore LANA and KSHV are both associated with chromosomes, which is consistent with the notion that LANA may have a function in maintaining KSHV DNA in the infected cells. Moreover, these results also indicate that LANA may have the ability to bind to specific sequences on the KSHV genome and can potentially colocalize with the KSHV genome on the metaphase spreads.

*LANA displays preferential binding to different regions of KSHV DNA in vitro.* To ascertain whether LANA had the capacity to bind specific sequences in KSHV DNA, [$^{32}$P]dCTP-radiolabeled probes spanning the viral genome (Fig. 2b) were incubated with *in vitro* translated LANA–myc fusion protein, followed by immunoprecipitation with anti-myc antibodies. To detect probes specifically bound to LANA, immunoprecipitates were quantified by liquid scintillation counting. Binding of LANA to DNA was expressed as a percentage of total probe coimmunoprecipitated with LANA–myc (Fig. 2b shows a schematic of the regions of KSHV used in this assay). The results of this experiment, shown in Fig. 2a (left panel) demonstrated that LANA most preferentially bound a region of the KSHV genome referred to as Z6, located within the first 34 kb at the left end of the viral genome (*3*). Figure 2a is representative of the mean of separate experiments in which Z6 bound to LANA–myc more favorably than other regions of the KSHV genome. Interestingly, the unique region Z8 in the middle of the viral genome and Z2 also bound, albeit less preferentially to the immunoprecipitated Z6–LANA–myc complex (Fig. 2a). Similar to EBNA 1, an EBV protein important for maintenance of EBV episomes, LANA may bind several different sites in the KSHV genome, suggesting multiple functional roles of LANA in KSHV latent infection (*10–13*).

We then further analyzed Z6 for identifying a more localized region within this large molecule by digesting the cosmid with *Not*I to release the insert followed by digestion with *Bam*HI for the left 10-kb *Not*I fragment and a partial *Hin*dIII for the right *Not*I 24-kb fragment of Z6. The resulting five fragments were then labeled and bound to LANA–myc. The mean of counts of duplicate experiments from the immunoprecipitation plotted in Fig. 2a (right panel) revealed that a fragment containing the terminal repeats and a region of approximately 1.8 kbp from the left end of the genome within Z6 gave the strongest binding activity. This binding activity was approximately 5- to 10-fold greater than the rest of the Z6 fragments (see Fig. 2a, right panel). These results strongly indicate that the potential *cis*-acting DNA element mediating LANA binding to the KSHV genome and so tethering the KSHV genome to the chromosome may lie within a short span of KSHV DNA approximately 1.8 kb at the left end of the KSHV genome and one copy of the terminal repeat. It should be noted that other regions of the genome do bind with relative affinity to LANA, indicating the potential for a multifunctional role for LANA. Further work is under way to investigate the binding to these other regions and the potential for additional roles of LANA.

*LANA and KSHV DNA colocalize to metaphase chromosomes in KSHV infected cells.* To determine whether LANA can colocalize with KSHV DNA, metaphase chromosome spreads were generated from cells prepared by reducing the time of fixing to only 1 h but maintaining the

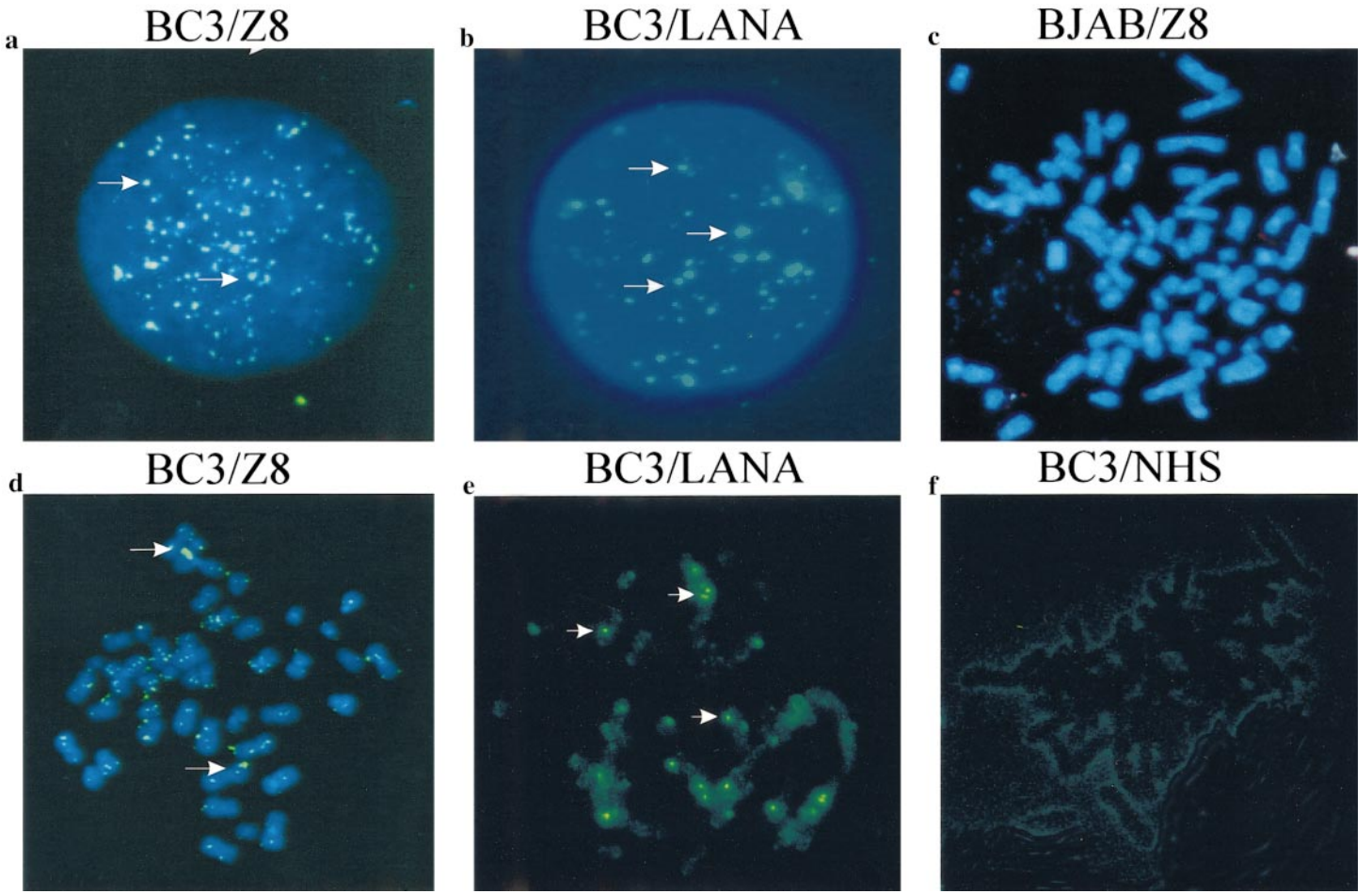


FIG. 1. The KSHV genome is associated with metaphase chromosomes in a pattern similar to LANA. (a) A nucleus of BC-3 KSHV-infected body cavity-based lymphoma cells stained with the labeled KSHV cosmid probe Z8 and detected with FITC-conjugated secondary antibody. (b) A similar BC-3 nucleus stained with anti-LANA human polyclonal serum for detection of LANA in the infected cells. Note the green signals indicating LANA staining in a punctate pattern similar to that seen for the KSHV probe seen in a. (c) A metaphase chromosome spread from the KSHV/EBV-negative cell line BJAB probe with a labeled KSHV cosmid probe Z8. Note that no signal was seen on any of the metaphase chromosomes in c. In both cases seen in a and b, a punctate pattern of fluorescence for the LANA signal as well as the KSHV-labeled probe was detected by the secondary FITC-conjugated secondary antibody against LANA antibodies and the digoxigenin-labeled probe, respectively. (d) Metaphase chromosomes from BC-3 cells hybridized with the Z8 KSHV cosmid DNA as probe, counterstained with DAPI. The multiple green signals as indicated by white arrows on the metaphase spread indicate specific hybridization of the KSHV genome. No consistently symmetrical pattern, which usually indicates viral integration, was detected. (e) Another set of metaphase chromosomes from BC-3 cells incubated with human polyclonal serum reactive against LANA. LANA signals were detected with FITC-conjugated secondary antibody. White arrows show multiple regions of focused green fluorescence in a punctate pattern. These regions indicate where LANA associates with the host chromosomes. (f) The control metaphase chromosome spread using KSHV negative human sera adsorbed against B cell and EBV antigens as a control. The outline of the metaphase chromosome spread shows no specific signal, which is distinct from that seen in e, where specific green focused signals are associated with the chromosomes. All chromosome spreads were analyzed at 1000× original magnification with the appropriate fluorescent filters on an Olympus AX70 or BX60 microscope.

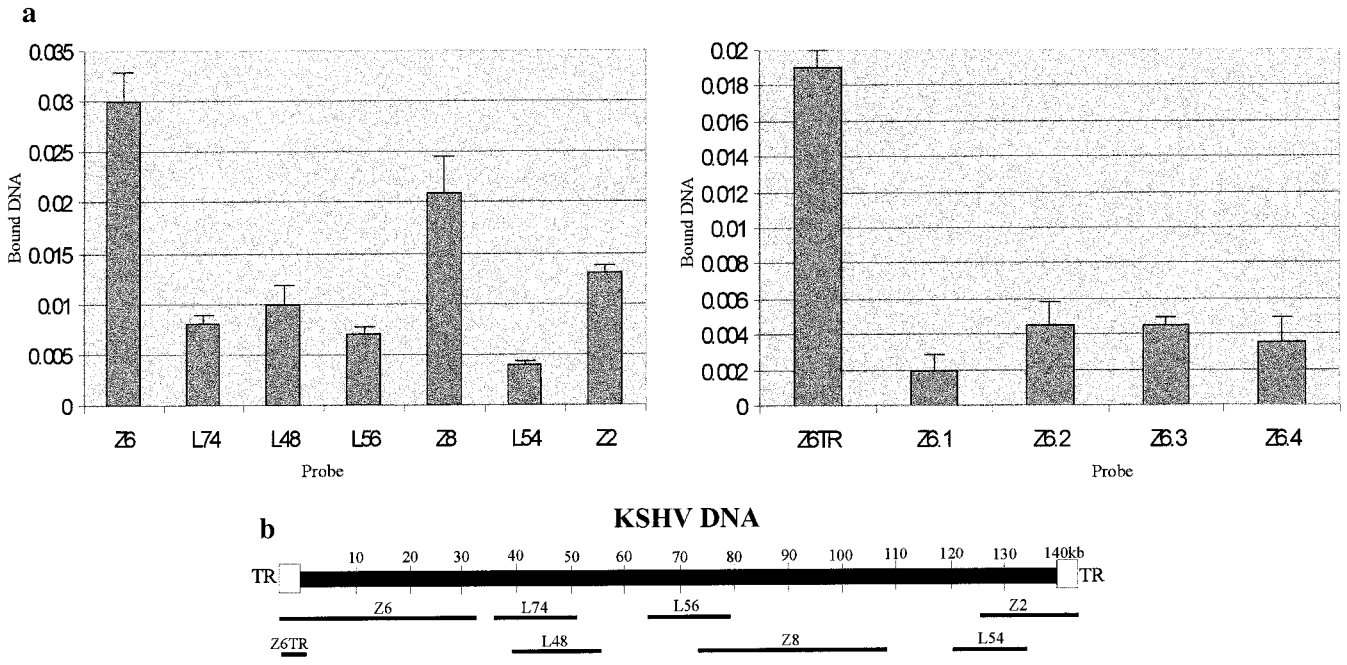


FIG. 2. LANA preferentially binds to specific regions of the KSHV genome. (a) *In vitro* translated LANA–myc fusion proteins were incubated with [$^{32}$P]dCTP-labeled KSHV DNA probes and then coimmunoprecipitated with anti-myc antibody (9E10 from ATCC). Extent of DNA binding is expressed as a ratio of bound DNA to unbound labeled DNA. Z6 binds preferentially to LANA; however, Z8 as well as Z2 also demonstrated significant levels of binding activity. (Right) Z6 insert was purified from vector and then digested into subfragments with *Bam*HI (left 10 kb) and *Hin*dIII (right 24 kb). One of the fragments that includes a copy of the terminal repeats at the left end of the genome and four other subfragments in Z6 were incubated with LANA. The results indicate strong binding to a fragment within Z6 located at the left end of the genome that includes a copy of the terminal repeat. (b) Schematic depicting location of KSHV cosmid and lambda probes within the 140-kb KSHV genome (Z denotes cosmid clone, L denotes lambda clone) (*3*). Note the location of regions showing preferential binding of LANA within the Z6 cosmid. Z6TR indicates a region with the strongest affinity to LANA. The genome is shown as a long unique region flanked by terminal repeats similar to members of the gammaherpesvirus family (*7*).

stringent washes in the DNA hybridization step. This retains chromosome-associated proteins that would be lost by the typical overnight fixation used in standard FISH protocols but keeps the stringency of the signal minimizing nonspecific signals (compare Figs. 1d and 1e with Figs. 3b and 3c). These spreads were probed with KSHV DNA and amplified via a tyramide-based fluorochrome deposition (NEN Life Sciences) for increased sensitivity, followed immediately by anti LANA immunofluorescence in an effort to colocalize viral DNA and LANA protein at host metaphase chromosomes. When both signals from the KSHV probe and the anti-LANA

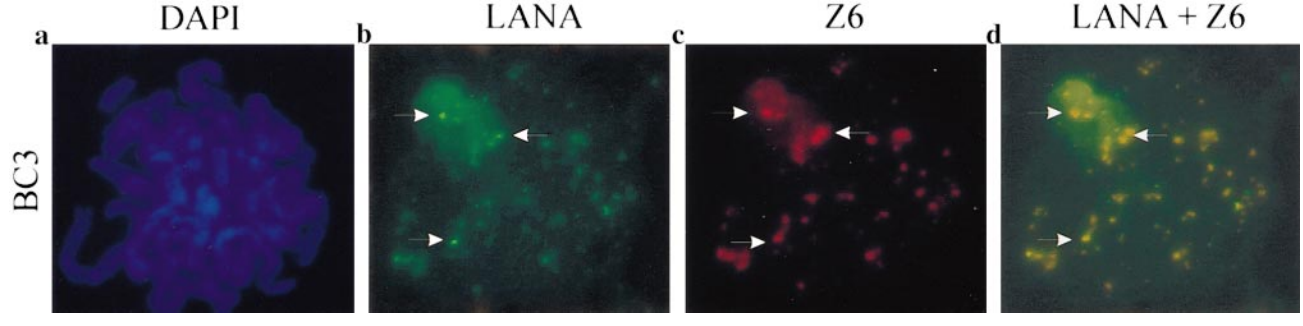


FIG. 3. KSHV DNA colocalizes with LANA to host chromosomes in BC-3 cells. (a–d) BC-3 colocalization of LANA and Z6 probe. Metaphase chromosome spreads of BC-3 cells were probed with Z6-labeled probe and anti-LANA antibody showing specific staining on the chromosome spreads. (a) The representative metaphase chromosome spread for the colocalization experiment stained with DAPI. (b) Metaphase spread for detection of LANA with anti-LANA immunofluorescence; (c) metaphase spreads for detection of KSHV DNA. (d) Signals from both the DNA probe and the anti-LANA serum merged on the same metaphase chromosome demonstrating colocalization at the same positions. (a–d) The BC-3 metaphase chromosomes hybridized with the Z6-specific KSHV DNA probe, followed by anti-LANA immunofluorescence and DAPI counterstaining. (d) Specific signals from the KSHV probe and anti-LANA antibodies tightly colocalize to host chromosomes in the latently infected body cavity-based lymphoma cell line BC-3 (*20*). All metaphase chromosome spreads were analyzed at 1000× original magnification with fluorescent filters on an Olympus AX70 microscope.

antibody were superimposed it became evident that both signals colocalize to the chromosomes in BC-3 (Fig. 3d). Spreads of BJAB cells did not result in any specific signal as before in Figs. 1d and 1e (data not shown). The observation that no signal was seen in the BJAB spreads indicated that the signals seen in BC-3 are specific for a KSHV-encoded or a KSHV-induced cellular protein and is most likely due to that of the latency-associated antigen since the polyclonal human serum used was previously adsorbed with B cell as well as EBV-infected B cell antigens, therefore increasing the specificity for the KSHV latent antigen LANA (*3, 4*). These results strongly suggest that LANA and KSHV DNA can colocalize to the host metaphase chromosome in the KSHV-infected BC-3 cells. Moreover, we have shown that LANA binds preferentially to regions of the KSHV genome. Therefore it would be interesting to determine whether these regions can be targeted to the metaphase chromosomes in the presence of LANA.

*Cis-acting DNA elements in Z6 plus LANA are sufficient for chromosomal localization in BJAB transfected cells.* The previous data provided the basis for further experiments in which we tested the hypothesis that regions of KSHV DNA that display preferential binding to LANA may contain *cis*-acting elements that can cooperate with LANA to confer chromosome localization of the KSHV genome. BJAB cells were cotransfected with Z6 or Z8 cosmid DNA along with an expression plasmid construct of the LANA–myc fusion protein under the control of the CMV IE promoter. Immuno-FISH analysis was then carried out on these cells, using the appropriate cosmids that were transfected as probes followed by immunofluorescence with anti-myc monoclonal antibody to improve specificity for detection of the LANA–myc protein not obtained with the polyclonal human sera. Analysis of these transfected cells revealed the colocalization of Z6 but not Z8 viral DNA with LANA to the host chromosomes (Figs. 4a–4c and 4j–4l). Furthermore, this colocalization was dependent on the presence of LANA protein, as the same probe did not localize to host chromosomes when cotransfected with empty myc vector (Figs. 4d–4f). The number of signals seen in these spreads was less than that seen in Figs. 1a and 1b where multiple DNA and protein signals were seen. We elected to increase the stringency of washes to minimize nonspecific associations frequently seen in these assays. These data indicate that LANA and *cis*-acting elements in Z6 are sufficient for chromosome localization of KSHV DNA. Furthermore, while Z8 appeared to display a significant affinity for LANA protein in the *in vitro* binding assay (see Fig. 2a), this interaction did not result in chromosomal localization. Additionally, LANA did not localize to the chromosomes itself, suggesting a requirement for a complex to stabilize KSHV and LANA to the host chromosomes. In these experiments approximately 20% of the cells were usually transfected and in screening the prepared chromosome spreads 5–10% had signals for KSHV DNA. In the spreads where positive signals were seen with KSHV, LANA signals were also seen in every instance. However, no signal was seen with Z8 or LANA in Z8 plus LANA transfected cells although LANA was expressed in all BJAB transfected cells. These results may indicate a consequence of LANA's ability to bind regions of the KSHV genome outside of the maintenance origin and could be potentially involved in transcription or other regulatory events required for the long-term survival of KSHV in the infected cell.

*LANA interacts with the nucleosome-associated histone H1 linker protein.* The results of these experiments prompted us to hypothesize that LANA tethers KSHV episomes through interactions with *cis*-acting elements at Z6 to host chromosomes potentially through its association with chromosomal proteins. Therefore, coimmunoprecipitation experiments were conducted to determine whether LANA specifically interacts with nucleosome-associated histone proteins that could allow for a biochemical mechanism by which the KSHV genome is tethered to host chromosomes. Initially, histone H1 was chosen since we hypothesized that H1 would be more easily accessible to tethering than the core histones H2, H3, and H4, which would be tightly bound within the chromatin structure. Immunoprecipitates generated from Western blot analysis of anti-H1 immunoprecipitates using anti-LANA polyclonal human serum (Fig. 5a) revealed a 222- to 234-kDa band comigrating in lysate and immunoprecipitation lanes in BC-1 and BC-3 but not in the KSHV-negative BJAB cells (compare lanes 1 and 3 with lanes 4 and 6 and lanes 7 and 9 in Fig. 5a). This result suggests association of LANA with linker histone H1 in KSHV-infected cells. Similarly, Western blot for LANA from immunoprecipitates with anti-H3 and -H4 antibodies indicated that no specific signal for LANA was seen by Western blot. These results suggest that LANA may be associated with the chromosome linker histone and not the core histones.

To determine whether LANA can interact directly with histone H1 we performed an *in vitro* experiment in which *in vitro* translated LANA–myc tagged protein was incubated with crude histone extracts and immunoprecipitated with anti-myc antibodies. The immunoprecipitates were then fractionated by SDS–PAGE. Anti-H1 Western blot analysis of these immunoprecipitates revealed a 30- to 32-kDa doublet in the crude histone input lane (10 $\mu$g of crude histones) comigrating with an identical band in the histone plus LANA–myc lane, but not in control lanes where crude histones or LANA alone was immunoprecipitated (Fig. 5b). This suggests that LANA interacts directly with histone H1 probably through its large acidic domains (*3, 4*). It should be noted that Western blots for H3 and H4 from similar *in vitro* experiments did not show any specific signals (data not shown). There is precedence for the H1 association since one previous report

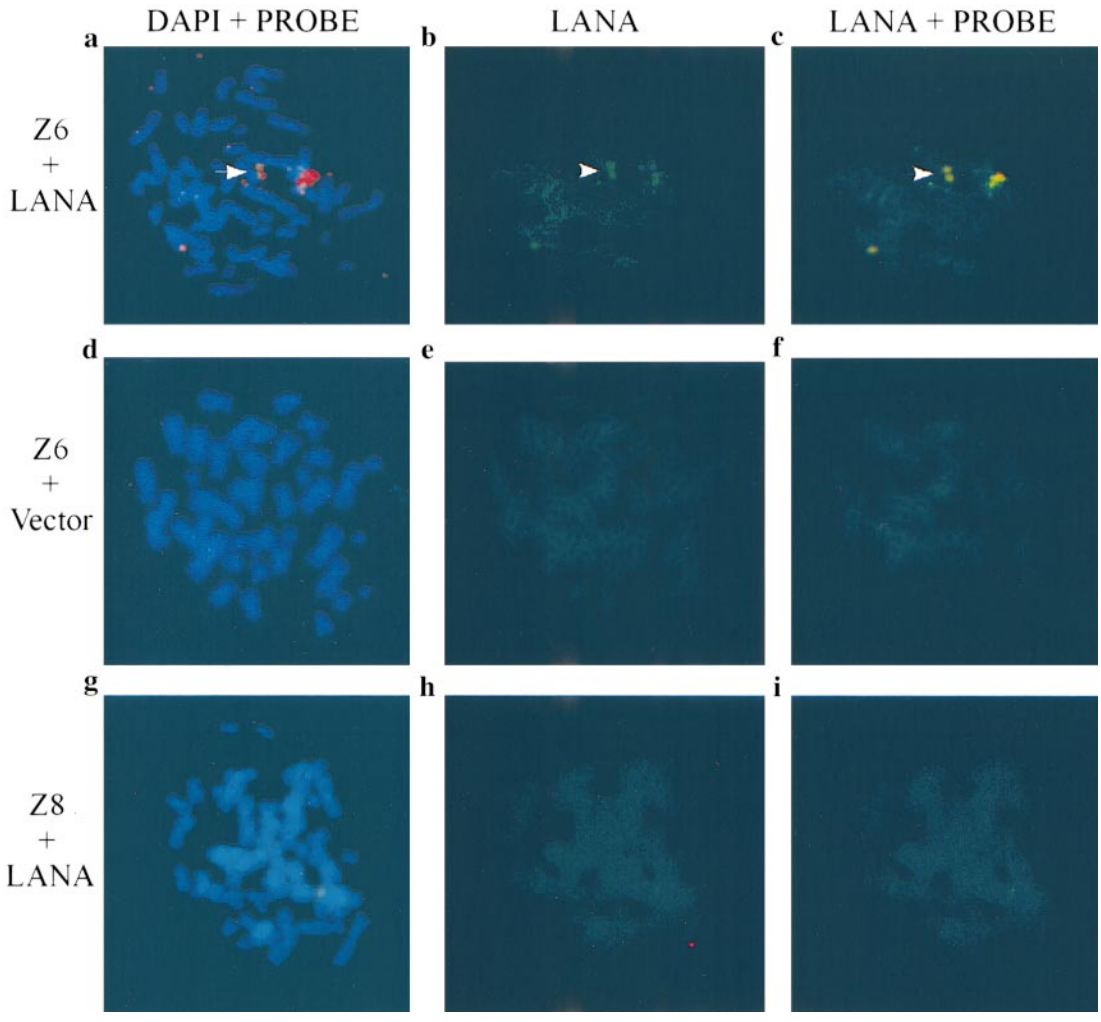


FIG. 4. *Cis*-acting elements within the Z6 but not Z8 regions of the KSHV genome and LANA are sufficient to confer colocalization of KSHV viral DNA and LANA to host chromosomes. BJAB cells were cotransfected with Z6 + LANA-myc (a–c), Z6 + empty myc vector (d–f), or Z8 + LANA-myc (g–i). Metaphase chromosomes were prepared from transfected cells and probed for the specific KSHV DNA cosmid transfected in each experiment, followed by immunofluorescence with an anti-myc antibody specific for LANA-myc. (a–c) The Z6 cosmid localizes to chromatin in the presence of LANA. (a) DAPI with signal from the Z6 probe, (b) green fluorescence indicating LANA–myc signals, and (c) colocalization of Z6 and LANA–myc. (d–f) The similar probe, Z6, does not localize to metaphase chromosomes in the absence of LANA. (g–i) Z8 does not localize to metaphase chromosomes in the presence of LANA. No signal was seen for LANA, which does not seem to target the chromatin structure in the absence of a specific DNA element within Z6. It is also possible that LANA expression may be more diffuse in this experiment and was removed during the procedural phase of this experiment, suggesting nonspecific association. This transfection was done multiple times with no positive signal for either Z8 or LANA–myc on the metaphase chromosome spreads in all fields on multiple slides. All chromosome spreads were analyzed at 1000× original magnification with fluorescent filters on an Olympus AX70 microscope.

by Sugden and colleagues identified histone H1 as a target for EBNA1 in a yeast screen (*14*). Cumulatively, these results support our hypothesis that LANA associates with histone H1 in the KSHV-infected cells. Given the acidic nature of the LANA protein (*3, 4*), it is possible that LANA may also interact with other chromosomal proteins; however, further immunoprecipitation experiments from KSHV-infected cells and with crude nuclear extracts using specific antibodies to other nucleosome-associated antigens will prove interesting if LANA associates with other nucleosome-associated proteins. It is clearly possible that histone H1 may not be the only chromosomal protein associated with LANA and further experiments will have to be done to address associations with many other chromosome-associated antigens, as more reagents become available. The ability of histone H1 to function as a linker histone (*15*) could be an important factor in providing the accessibility for LANA to tether the KSHV genome to the chromosome. These data clearly demonstrates that LANA interacts with histone H1 and associates with H1 in KSHV-infected body cavity-based lymphoma cells.

*Discussion.* Taken together, these data indicate that the KSHV genome is tethered to the host chromosome through its interactions with LANA. It was further demonstrated that LANA binds preferentially to *cis*-acting

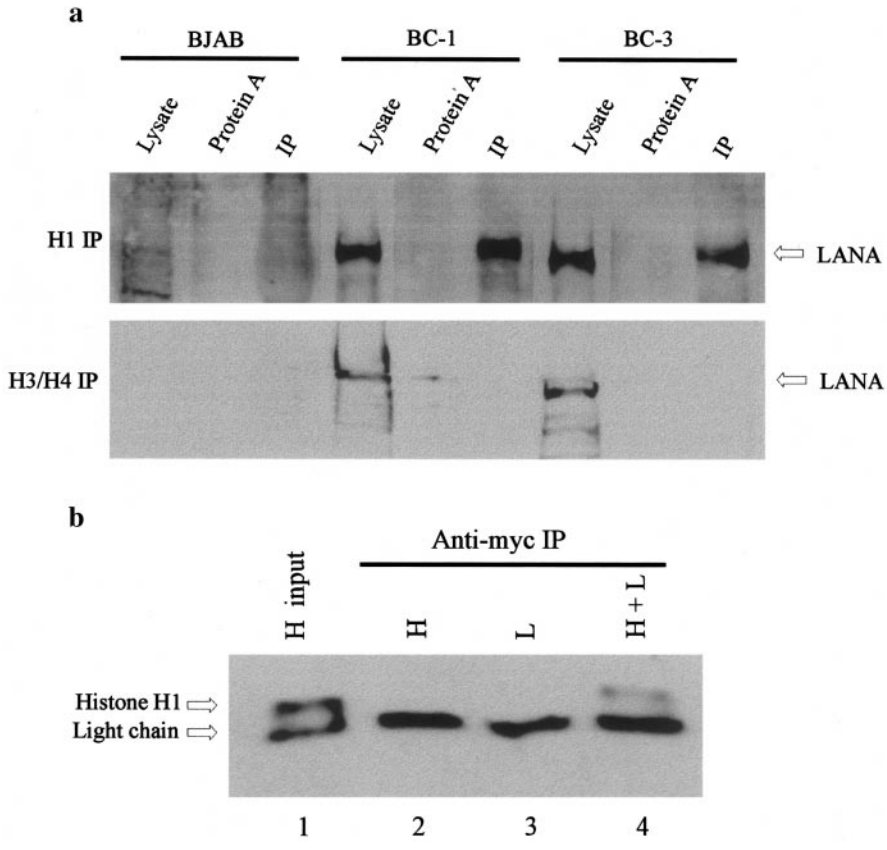


FIG. 5. The KSHV encoded LANA protein interacts with nucleosomal-associated histone H1 protein. (a) LANA associates with histone H1 in KSHV-infected body cavity lymphoma cells BC-1 and BC-3 as demonstrated by immunoprecipitation with anti-H1 monoclonal antibody and Western blot with human serum with activity against LANA. Lysates from BJAB, BC-3, and BC-1 cells were precleared with Protein A–-Sepharose beads, incubated with monoclonal H1 antibodies, and precipitated by centrifugation after the addition of more Protein A–Sepharose beads. These immunoprecipitates were fractionated by SDS–PAGE and Western blot analysis using a polyclonal human serum reactive to LANA. Arrow on the right indicates the position of the 222- to 234-kDa LANA protein seen in the lysate lane of BC-1 and BC-3 (lanes 4 and 7) but not in the KSHV-negative BJAB lanes (lane 1). LANA was immunoprecipitated in BC-1 and BC-3 cells (lanes 6 and 9) but not in the KSHV negative BJAB cells (lane 3). (Bottom) Results of immunoprecipitation experiments with anti-H3 and -H4 antibodies (Upstate Biotechnology). Immunoprecipitates were fractionated on a 6% SDS–PAGE gel and then transferred to nitrocellulose membrane. The membrane containing bound proteins was then incubated with human polyclonal serum for detection of LANA. As demonstrated LANA signal was clearly detected in the lysate lanes of the KSHV-infected BC-1 and BC-3 cell lines. No signals were seen in the immunoprecipitate lanes in either BC-1 or BC-3. (b) LANA interacts with histone H1 *in vitro.* Crude histones (50 $\mu$g) were incubated with *in vitro* translated LANA–myc fusion protein and immunoprecipitated with anti-myc antibodies followed by Protein G–Sepharose beads and centrifugation. Immunoprecipitates were fractionated by 10% SDS–PAGE and transferred to a 0.45-$\mu$m nitrocellulose membrane followed by Western blot analysis with anti-H1 monoclonal antibody. Arrows on the left indicates the migration of histone H1 doublet in lane 1 with 10 $\mu$g of crude histones; the lower H1 band is comigrating with immunoglobulin light chain (compare lanes 1 and 4). The H1 signal detected in lane 1 is also seen in lane 4 where LANA–myc and crude histones are present. No signal was seen in the lanes where only crude histones or LANA–myc was immunoprecipitated with anti-myc monoclonal antibody. H input, 10 $\mu$g of crude histones; H, crude histones alone; L, *in vitro* translated LANA–myc alone; H+L, crude histones plus LANA–myc.

elements located within the Z6, but also to Z8 and Z2 KSHV cosmid probes and that chromatin localization depends on the presence of both LANA and Z6, but not Z8. Further mapping of the binding region within Z6 indicates that this *cis*-acting element is located close to the left end of the KSHV genome. Moreover, the immunoprecipitation data show that LANA binds host histone H1 in body cavity-based lymphoma-derived cells and in crude histone extracts *in vitro.* This interaction has two potentially important consequences. First, it provides the first biochemical link between KSHV episomes and a host chromatin protein that may thereby confer persistence of the KSHV genome in daughter cells. Second, because histone H1 has increasingly been regarded as a modulator of eukaryotic transcription (*15*), LANA may influence expression of histone H1 transcriptional targets through modulation of histone H1 transcriptional activities. These interactions lead us to assert a model describing a novel mechanism of viral persistence, summarized in Fig. 6. In this model, the KSHV encoded LANA tethers the KSHV episome to host chromosomes through interaction with the linker histone H1 and specific *cis*-acting elements located within the Z6 region of the genome. Additional experiments in our laboratory have

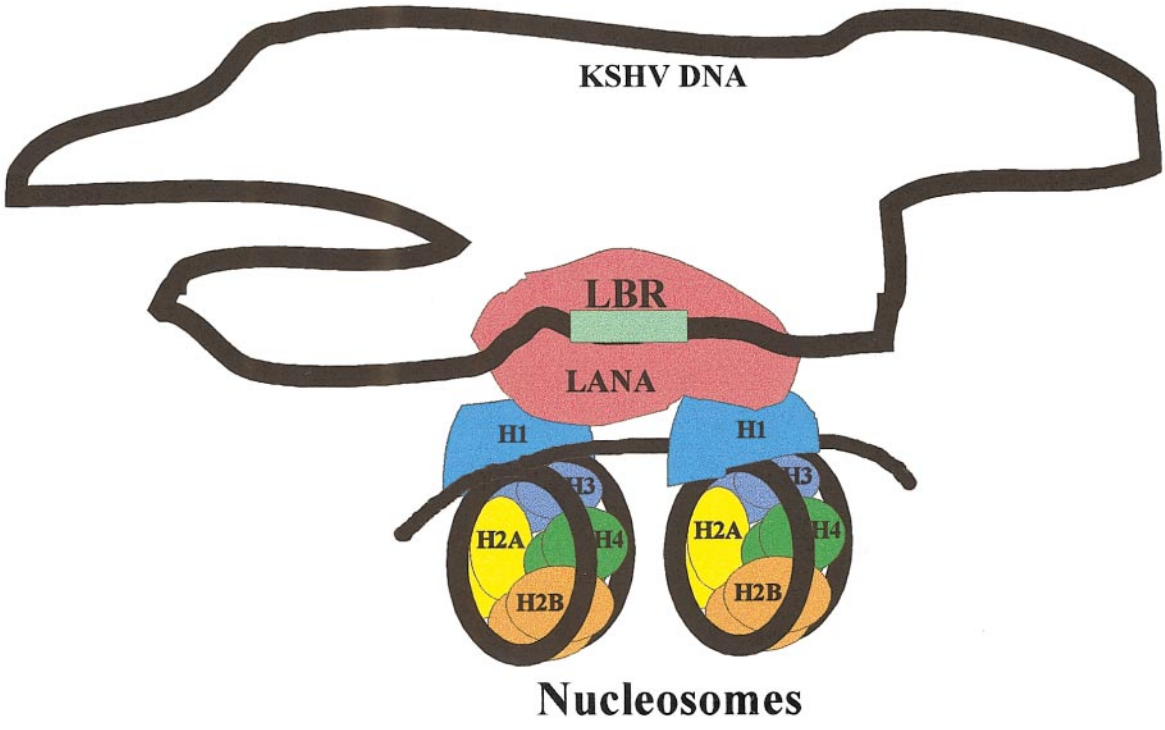


FIG. 6. Schematic of a proposed mechanism of persistence in KSHV-infected body cavity-based lymphoma cells. LANA binds to specific *cis*-acting DNA elements on the KSHV genome depicted here as LBR (LANA-binding region) and simultaneously binds the chromosomal linker protein histone H1 and potentially other as yet unidentified chromosomal proteins. This association allows LANA to tether the KSHV episomal DNA to host chromosomes and thereby ensures viral persistence in daughter cells through successive rounds of cell division. This mechanism may be a common strategy utilized by most latent viruses in the infected cell.

indicated that Z2 may also be capable of tethering to host chromosomes, suggesting a possible *cis*-acting element within the TR element. However, Z2 contains an unsequenced region approximately 3 kbp in size to the extreme right end of the unique sequence (*3*). Moreover, LANA also demonstrated some degree of binding to Z8 but had no ability to tether this fragment of the KSHV to the host chromosome. This indicates some other potential functional role of LANA besides the ability to link KSHV to host chromosomes. Further analysis would be required to determine the LANA-binding elements within Z2 and Z8. These results may shed light on other roles of LANA in KSHV-infected body cavity-based lymphoma cells.

A synthesis of data collected over several years reveals that such a mechanism of persistent infection may also be possible in EBV-infected cells. It is well known that EBV episomes and EBNA1 are randomly associated with metaphase chromosomes (*16*). It has long been thought that EBNA1 was necessary for both viral persistence and replication (*12, 13*). However, recent studies strongly suggest that EBNA1 does not recruit the replicative machinery to oriP and may not be required for replication (*14*). EBNA1, however, allows persistence of oriP containing plasmids. Additionally, histone H1 was identified in a one-hybrid screen for proteins that interact with EBNA1 bound to oriP (*14*). Despite all these data, colocalization of EBNA1 and EBV to chromosomes as well as the biochemical basis of their association with chromosomes has not been demonstrated.

Sugden and colleagues hypothesized that EBNA1 binds to specific DNA sequences on EBV and links this bound DNA through its many linking domains by interacting with chromosome-associated proteins (*14*). LANA may exhibit functions analogous to those of EBNA1 in linking KSHV episomes to host chromosomes, thereby ensuring control of copy number, segregation, and persistence in the infected cell. LANA may also function as a viral defense against host strategies that may have evolved to eliminate viral genetic material, similar to the way that EBNA1 prevents overwhelming episomal loss in EBV infection (*14*).

Here we describe the first specific biochemical mechanism by which the KSHV episome is linked or tethered to the host chromosome through the interaction of LANA with histone H1 and possibly other chromosomal proteins with specific *cis*-acting elements of KSHV DNA. We suggest that a similar mechanism may exist for the persistence of EBV and other latent viruses in infected cells.

We are currently in the process of finely mapping the specific *cis*-acting DNA elements within the KSHV genome that confer this chromosomal tethering. Additional experiments will be necessary to determine whether

these interactions exist in other latent viruses including the hepatitis (HBV, HCV) and polyoma (HPV, SV40) families and how they may affect viral replication and distribution of viral genomes during mitosis. Furthermore, these interactions are potentially multifunctional in that they suggest roles in persistence, replication, and transcription. Delineation of the multiple binding regions for LANA on the KSHV genome may provide clues as to the nature of the potential multifunctional roles of LANA. It is also clear that antagonism of these interactions represents a novel therapeutic possibility for eradication of KSHV-associated diseases and other viral infections.

Recently, while our work was in review, Ballestas and colleagues also published a separate report of similar findings in which LANA was found to colocalize with the KSHV genome in BCBL1-infected cells (*17*). Therefore these findings though independent from each other both support the hypothesis that LANA functions in persistence of the KSHV genome in the infected cells.

*Materials and methods. Genomic and cDNA clones, cell culture, and transfection.* Cosmid and lambda clones spanning the KSHV genome (Z2, Z6, Z8 and L48, L54, L56, L74, respectively) were obtained from the National Institutes of Health, AIDS Research and Reference Reagent Program (*3*). Coordinates for these fragments (closest approximation) are: Z6 from the 5′ end of KSHV to 34,613; L74 from 36,243 to 57,672; L47 from 56,496 to 69,547; L56 from 64,788 to 80,444; Z8 from 74,131 to 109,353; L54 from 121,829 to 137,183, and Z2 from 127,780 to the 3′ end of the KSHV genome (James Russo and Patrick Moore, personal communication). ORF73 cDNA was obtained by polymerase chain reaction amplification of DNA using Vent DNA polymerase (NEB) from a body cavity lymphoma-derived cell line, BC-1 (*9, 18*), with the following primers: 5′-GAGAATTCTTATGGCGCCCCCGGGAATG-3′ (sense), 5′-GAGATATCCCTGTCATTTCCTGTGGAGA-3′ (antisense). Fragments were purified, digested with *Eco*RI and *Eco*RV, and cloned into a myc-tagged expression vector, pA3M (*19*). BC-1, BC-3 (*9, 18, 20*) (obtained from ATCC), and BJAB cells (obtained from Elliott Kieff) were grown in RPMI 1640 (Gibco), supplemented with 20% FBS (10% for BJAB), penicillin (25 U/ml), streptomycin (25 $\mu$g/ml), and gentamicin (10 $\mu$g/ml).

For transfections, 15 million BJAB cells were collected and transfected with 50 $\mu$g KSHV cosmid DNA (Z6 or Z8) and 50 $\mu$g LANA–myc plasmid cDNA (or myc vector alone) by electroporation (220 V, 975 $\mu$F) in 400 ml of RPMI 1640 supplemented with 10% FBS and then incubated in 10 ml of the medium. After transfection, cells were incubated for 24 h and then collected for immunofluorescence *in situ* hybridization and/or Western blot analysis.

*Fluorescence in situ hybridization analysis.* Metaphase chromosome spreads for FISH were prepared by standard protocols. Briefly, cells were metaphase arrested with colcemid (10 $\mu$g/ml, Gibco) for 1 h at 37°C and then treated with 0.75 M KCl for 12 min at 37° C, followed by overnight fixation in fresh methanol:acetic acid (3:1) at 4°C. Cells were spread on slides and allowed to age for no less than 72 h. Hybridization was done overnight at 37°C with a digoxigenin-labeled KSHV cosmid probe and then detected with rhodamine- or fluorescein-conjugated anti-digoxigenin antibodies and counterstained with DAPI.

*Preparation of metaphase chromosomes for detection of chromosomal-associated proteins.* Chromosome spreads for immunofluorescence were prepared as above except that fixation time was shortened to 1 h to preserve chromosome-associated antigens. Additional washing steps were included to minimize nonspecific associations and to retain the specific associations with the chromosomes. Therefore care was taken to be as stringent as possible without losing most of the protein signals. Slides were blocked in 20% normal goat serum for 30 min at room temperature, washed in PBS, and incubated in human serum reactive to LANA or normal KSHV-negative human serum overnight at 4°C. Slides were again washed and then incubated in goat anti-human FITC secondary antibody (1:1000) for 1 h at room temperature. Slides were washed, counterstained with DAPI, and coverslipped with antifade for fluorescence microscopy analysis.

*In vitro DNA binding.* Cosmids, lambda probes, or isolated DNA fragments of KSHV DNA were radiolabeled with [$^{32}$P]dCTP through a standard nick translation protocol and separated from unincorporated label with NucTrap probe purification columns (Stratagene). LANA–myc protein was generated by *in vitro* translation of LANA–myc cDNA with rabbit reticulocyte lysate (Promega) as per the manufacturer's suggestions. Three microliters of protein was incubated with 3 $\mu$ l labeled KSHV probe at 4°C for 45 min in 50 $\mu$ l of binding buffer (10% glycerol, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; pH 7.9), 150 mM NaCl, 0.5% Nonidet-P40, 250 $\mu$g bovine serum albumin per milliliter, 2 mM dithiothreitol, 10 $\mu$g of aprotinin per milliliter, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu$M leupeptin, 1 $\mu$M pepstatin) (*19*). Simultaneously, 25 $\mu$l Protein G–Sepharose beads were incubated with 50 $\mu$l monoclonal anti-myc antibody (supernatant from 9E10 hybridoma cells) in 350 $\mu$l binding buffer rotating at 4°C for 45 min (*19*). Bound DNA protein complexes were added to prebound Ab–Protein G–Sepharose complexes and incubated for 45 min with continued rotation at 4°C. Complexes were then collected by centrifugation at 15,000 rpm for 1 min at 4°C in a microcentrifuge. Precipitates were washed twice by removal of supernatant, resuspended in 200 $\mu$l binding buffer as above, and centrifuged in a microcentrifuge. Supernatants from washes were pooled and counted in a liquid scintillation counter, as were the corresponding pellets. DNA bound to LANA protein was

then calculated as a fraction of labeled DNA in the pellet over the labeled DNA in the supernatant.

*Preparation of metaphase chromosomes for immuno-FISH double-labeling.* Metaphase chromosome spreads for immuno-FISH colocalization were prepared as above with a 1-h fixation period to preserve chromosome-associated antigens. Hybridization was done overnight at 37°C with a biotinylated KSHV cosmid or lambda probe and then detected with a direct tyramide–rhodamine signal amplification system (NEN Life Sciences), according to the manufacturer's suggestions. This system increases the sensitivity by amplification of the signals at the site deposited, allowing for detection of very low signals. After repeated washes to remove the nonspecific signals the slides were subjected to the immunofluorescence protocol as described above. Slides were washed, counterstained with DAPI, and coverslipped with antifade for fluorescence microscopy analysis.

*Immunoprecipitation and in vitro binding.* BC-1, BC-3, or BJAB cells were lysed in RIPA buffer and precleared by incubation with Protein A–Sepharose as previously described. Immunoprecipitates were generated with monoclonal H1 antibodies (2 $\mu$ g/ml; Upstate Biotechnology), washed four times in RIPA buffer, fractionated by SDS–PAGE, and transferred to 0.45-$\mu$m nitrocellulose membranes. Western blots were performed with human serum reactive to LANA at a 1:500 dilution and standard chemiluminescence detection protocols (Amersham). For *in vitro* binding experiments, 50 $\mu$g of crude histones (type II-A, Sigma) were incubated with 20 $\mu$l of *in vitro* translated LANA–myc fusion protein for 3 h at 4°C. Complexes were precipitated by the addition of anti-myc antibodies and Protein G–Sepharose beads followed by centrifugation. Immunoprecipitates were fractionated by SDS–PAGE and transferred to nitrocellulose membranes for Western blot analysis with monoclonal H1 antibodies.

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## REFERENCES

1. Beral, V., and Newton, R. (1998). Overview of the epidemiology of immunodeficiency-associated cancers. *J. Natl. Cancer Inst. Monogr.* **23,** 1–6.
2. Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**(5192), 1865–1869. [See comments]
3. Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. USA* **93**(25), 14862–14867.
4. Kedes, D. H., Lagunoff, M., Renne, R., and Ganem, D. (1997). Identification of the gene encoding the major latency-associated nuclear antigen of the Kaposi's sarcoma-associated herpesvirus. *J. Clin. Invest.* **100**(10), 2606–2610.
5. Gao, S. J., Kingsley, L., Hoover, D. R., Spira, T. J., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Parry, P., Chang, Y., and Moore, P. S. (1996). Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. *N. Engl. J. Med.* **335**(4), 233–241.
6. Ganem, D. (1997). KSHV and Kaposi's sarcoma: The end of the beginning? *Cell* **91**(2), 157–160.
7. Neipel, F., Albrecht, J. C., and Fleckenstein, B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: Determinants of its pathogenicity? *J. Virol.* **71**(6), 4187–4192.
8. Dittmer, D., Lagunoff, M., Renne, R., Staskus, K., Haase, A., and Ganem, D. (1998). A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **72**(10), 8309–8315.
9. Horenstein, M. G., Nador, R. G., Chadburn, A., Hyjek, E. M., Inghirami, G., Knowles, D. M., and Cesarman, E. (1997). Epstein–Barr virus latent gene expression in primary effusion lymphomas containing Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. *Blood* **90**(3), 1186–1191.
10. Ambinder, R. F., Shah, W. A., Rawlins, D. R., Hayward, G. S., and Hayward, S. D. (1990). Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein–Barr virus DNA. *J. Virol.* **64**(5), 2369–2379.
11. Mackey, D., and Sugden, B. (1997). Studies on the mechanism of DNA linking by Epstein–Barr virus nuclear antigen 1. *J. Biol. Chem.* **272**(47), 29873–29879.
12. Rawlins, D. R., Milman, G., Hayward, S. D., and Hayward, G. S. (1985). Sequence-specific DNA binding of the Epstein–Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. *Cell* **42**(3), 859–868.
13. Yates, J. L., Warren, N., and Sugden, B. (1985). Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells. *Nature* **313**(6005), 812–815.
14. Aiyar, A., Tyree, C., and Sugden, B. (1998). The plasmid replicon of EBV consists of multiple *cis*-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element. *EMBO J.* **17**(21), 6394–6403.
15. Wolffe, A. P. (1997). Histone H1. *Int. J. Biochem. Cell Biol.* **29**(12), 1463–1466.
16. Harris, A., Young, B. D., and Griffin, B. E. (1985). Random association of Epstein–Barr virus genomes with host cell metaphase chromosomes in Burkitt's lymphoma-derived cell lines. *J. Virol.* **56**(1), 328–332.
17. Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* **284**(5414), 641–644.

*18.* Cesarman, E., Moore, P. S., Rao, P. H., Inghirami, G., Knowles, D. M., and Chang, Y. (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**(7), 2708–2714.

*19.* Aster, J. C., Robertson, E. S., Hasserjian, R. P., Turner, J. R., Kieff, E., and Sklar, J. (1997). Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-Jkappa or nuclear localization sequences retain the ability to associate with RBP-Jkappa and activate transcription. *J. Biol. Chem.* **272**(17), 11336–11343.

*20.* Arvanitakis, L., Mesri, E. A., Nador, R. G., Said, J. W., Asch, A. S., Knowles, D. M., and Cesarman, E. (1996). Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) in the absence of Epstein–Barr virus. *Blood* **88**(7), 2648–2654.