The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus interacts preferentially with the terminal repeats of the genome *in vivo* and this complex is sufficient for episomal DNA replication

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The genome of Kaposi's sarcoma-associated herpesvirus (KSHV) persists in latently infected cells as a circular episome. The latency-associated nuclear antigen (LANA) has been shown to tether viral DNA fragments to chromosomes and is proposed to maintain the KSHV genome. In order to identify the *in vivo*-binding sites for LANA on the whole KSHV genome and to analyse the function of this protein–DNA interaction, different *in vivo* systems have been developed. Chromatin immunoprecipitation experiments using three different cell lines latently infected with KSHV demonstrated that LANA binds preferentially and directly to the terminal repeats (TRs) but not to other regions of the viral chromosome *in vivo*. In contrast, *in vitro* LANA–DNA binding was much less specific. To identify autonomously replicating sequences within the KSHV genome, BCBL-1 cells were transfected with cosmids representing the entire genome. Cosmid Z2, consisting of the right end of the unique region and TRs, persisted as an episome in short-term assays. Long term, stable episome replication was observed with constructs derived from Z2 containing TRs only. LANA expression constructs containing a variable number of TRs replicated stably as episomes in uninfected cells. A 424 bp subfragment of the 801 bp TR could mediate episome replication. These studies show that LANA is a *trans*-acting protein that binds preferentially to TRs *in vivo* and these two elements are sufficient for episome replication. These results also suggest that the LANA expression plasmids reported here could be utilized as episomal vectors in a manner similar to Epstein–Barr virus-based vectors.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) (Chang *et al.*, 1994), also referred to as human herpesvirus-8, is a gamma-2 subgroup herpesvirus. Its genome consists of approximately 140 kb of unique sequences flanked by 20–30, 801 bp tandem terminal repeats (TRs) of non-coding DNA (Russo *et al.*, 1996). In latently infected cells, the repetitive ends of the viral genome join and these circular structures persist as multi-copy episomes in the nucleus of infected primary effusion lymphoma (PEL)-derived cell lines (Cesarman *et al.*, 1995a, b; Renne *et al.*, 1996a).

Latent replication of Epstein–Barr virus (EBV), a gamma-1

subgroup herpesvirus, provided a model for the maintenance of KSHV episomes at the initial stages of this work. Three components are required for persistence of episomal EBV DNA. EBV nuclear antigen 1 (EBNA-1) interacts specifically with DNA and transactivates two *cis*-acting elements (Chittenden *et al.*, 1989; Reisman *et al.*, 1985; Sugden *et al.*, 1985; Yates *et al.*, 1984, 1985). We hypothesized that KSHV encodes *cis*- and *trans*-acting elements analogous to those described in EBV.

KSHV does not encode a homologue of EBNA-1 but a nuclear protein designated latency-associated nuclear antigen (LANA), which is expressed in all PEL cells (Kedes *et al.*, 1997; Moore *et al.*, 1996; Rainbow *et al.*, 1997). It was reported that LANA co-localizes with viral episomes (Ballestas *et al.*, 1999; Cotter & Robertson, 1999), suggesting that LANA may function in episomal maintenance.

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Ballestas *et al.* (1999) also showed that uninfected B cells expressing LANA support episome replication of a cosmid and subclones derived from the left end of the genome containing unique sequences as well as TRs. Most recently, Lim *et al.* (2002) demonstrated that LANA supports the transient replication of plasmid DNA containing at least two copies of the TR. These investigators, however, did not extend their search for stable autonomously replicating sequence (ARS) activity and *in vivo* LANA binding to the entire genome, and left open the possibility that other regions of the viral genome may also be important in the maintenance of latent episomes.

As LANA is thought to be involved in episome maintenance, it is probably a sequence-specific DNA-binding protein. Furthermore, LANA has been shown to interact with different chromatin-associated cellular proteins, such as the retinoblastoma protein, RING3, p53 and mSin3 (Radkov *et al.*, 2000; Platt *et al.*, 1999; Friborg *et al.*, 1999; Krithivas *et al.*, 2000), and may have other functions that require DNA-binding activity. Binding to p53 and the retinoblastoma protein can possibly regulate virus transcription, similar to what has been shown for EBNA-1 (Wysokenski & Yates, 1989). Ballestas & Kaye (2001) as well

as Garber *et al.* (2001) showed *in vitro* LANA–TR binding and mapped out minimum interaction sequences using electromobility shift assays with nuclear extracts of cells transfected with LANA and labelled oligonucleotides from the TRs. Thus, these studies did not address the LANA–TR interaction in living cells, whether it is direct or indirect, and the interaction of LANA with other parts of the viral genome. Since *in vitro* biochemical approaches do not permit the examination of protein–DNA binding in living cells and can neither reflect the entire specificity nor quantify these interactions, we have set up novel experimental systems for the study of *in vivo* LANA–DNA binding.

The work presented here shows that the region of the viral chromosome that binds LANA the most selectively and abundantly *in vivo* is the TR and this interaction is a direct DNA–protein contact. Consistent with these findings, genetic approaches show that plasmids containing only TRs can replicate stably in cells expressing LANA.

METHODS

KSHV cosmids and subclones. KSHV DNA fragments from BC-1 cells were cloned into SuperCos cosmids (Stratagene), as described
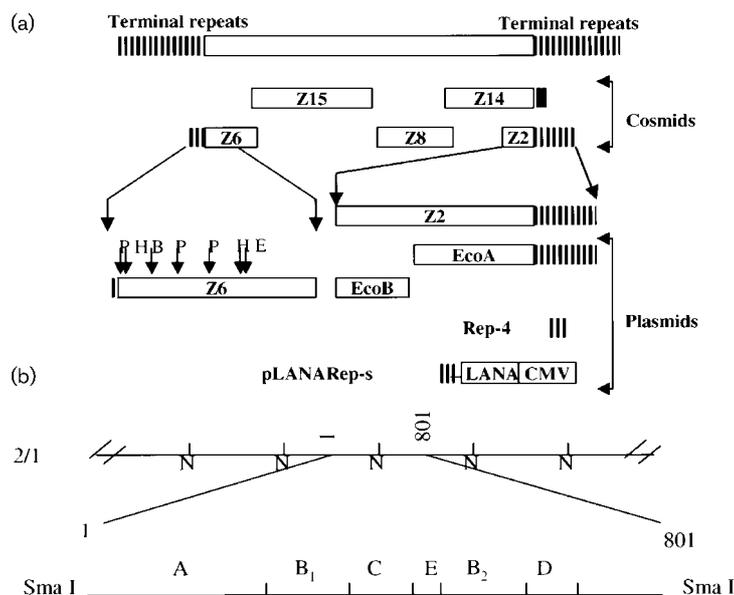
Fig. 1. Schematic representation of the KSHV genome, cosmids and plasmids used in this study. (a) Schematic representation of cosmid and plasmid KSHV clones used in transfection and LANA–KSHV DNA-binding experiments and restriction enzyme patterns of cosmid DNA preparations. Map positions of the clones were determined by sequencing the ends of inserts and by restriction analysis. Z2, 129 669–TRs; Z6, TRs–33 391; Z8, 72 904–107 974; Z14, 107 694–TRs; Z15, 31 234–70 569; Eco A, 132 227–TRs; Eco B, 129 669–132 227; Rep-4, four tandem TRs; pLANARep, pBKCMV-LANA expression plasmid containing TRs. The position of recognition sites for the following restriction enzymes is indicated on the enlarged map of Z6: P, *Pst*I (cuts at 126, 2632 and 4492 bp); H, *Hinc*II (cuts at 217 and 5911 bp); B, *Bgl*II (cuts at 1301 bp); and E, *Eco*RI (cuts at 6189 bp from the left TR–UR junction). (b) Schematic representation of the four TRs (801 nt each) cloned into the *Not*I site of the SuperCos vector to construct the Z2 subclone, 2/1 and diagram of a single TR unit. N represents the unique *Not*I sites at nt 383 (GenBank accession no. U75699) (Russo *et al.*, 1996). One TR unit is magnified in the bottom panel. *Sma*I sites are marked with vertical lines. In the diagram, fragments are labelled A–E in descending size. Two fragments of the same size are labelled B_1 and B_2.

previously (Russo *et al.*, 1996). Fig. 1 illustrates some of the cosmids and plasmids used in this study. Sequencing and restriction analysis showed that cosmid Z2 is located between nt 129 669 and the TRs and contains about 12 units of repeats. Cosmids Z6 and Z14 have four and two units of TRs, respectively. Z2 cosmid fragments were subcloned into the polylinker of SuperCos. Eco A is from 132 227 to the TRs and contains 12 repeat units. Eco B co-ordinates are 129 669–132 227. Rep-4 contains four 801 bp repeat units obtained by partial *Not*I digestion of Z2.

Construction of LANA expression vectors. The open reading frame (ORF) encoding LANA was subcloned from cosmid Z14 and into the pBKCMV (Stratagene) vector and strong protein expression was demonstrated after transient transfection and Western blotting (data not shown).

To generate the pLANARep expression plasmids, one, three and four TR units were inserted into the unique *Mlu*I site of the pBKCMV-LANA expression vector (Fig. 1). Subfragments of the TRs were also cloned into the *Mlu*I site of pBKCMV-LANA.

Cell lines, cell culture, transfection methods and drug selection. BCBL-1, BC-1 and BC-3 cell lines (Cesarman *et al.*, 1995a; Kedes *et al.*, 1997; Renne *et al.*, 1996b), and Jurkat, Ramos and 293 cell lines were obtained from the ATCC. Lymphoid cell lines were cultured in RPMI 1640 and 293 cells were cultured in DMEM, both media containing 10 % foetal calf serum and antibiotics. Lymphoid cells (10^7 cells) were transfected with 10 μg cosmid DNA by electroporation, as described previously (Lund *et al.*, 1997). Transient and stable transfections into 293 and COS cells were done using lipofection with the GenePORTER reagent (Gene Therapy Systems), according to the manufacturer's protocol.

Episome replication assays. Episomal DNA from transfected cells was recovered by Hirt extraction (Hirt, 1967) and was digested by either *Dpn*I or *Mbo*I. Plasmid DNA amplified in *dam* methylase-positive *Escherichia coli* is efficiently digested by *Dpn*I but not by *Mbo*I, while DNA replicated in mammalian cells is resistant to *Dpn*I but sensitive to *Mbo*I. The product of the digestion reactions was either transformed into *E. coli* or subjected to electrophoresis and Southern blotting. Positive *E. coli* transformants were counted and plasmid DNA prepared from these clones was partially sequenced and/or digested with restriction enzymes. Southern blots were probed with vector DNA labelled to a specific activity of approximately 1×10^9 c.p.m. μg^{-1}. The detection limit of this assay was 0·1–1·0 molecules per cell.

Another assay was based on the detection of episomes released from intact cells by *in situ* lysis in the wells of vertical Gardella agarose gels, as described previously (Gardella *et al.*, 1984). Large intact plasmids such as herpesvirus genomes or plasmids larger than about 15 kb have a slower electrophoretic mobility than any linear DNA, including cellular DNA, and form a band between the loading well and the linear cellular DNA band. Specific plasmids were detected on Southern blots, whereas cellular DNA was visualized by ethidium bromide staining. The sensitivity of this assay is 0·1–1·0 copies per cell.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays of *in vivo* formaldehyde or UV cross-linked cells were done essentially as described (Gilmour *et al.*, 1991; Orlando *et al.*, 1997). Briefly, cells were cross-linked with exposure to either 1 % formaldehyde or UV light and washed. Nuclear extracts were prepared and sonicated to an average chromatin size of 400 bp. To assess the efficiency of LANA immunoprecipitations with a rabbit polyclonal antibody, UK163 (Zhu *et al.*, 1999), immunoprecipitates were subjected to Western blotting using a human antiserum from a KS patient (a kind gift of K. Nagy, National Institute of Dermato-Venereology, Department of AIDS and Human Retroviruses, Budapest, Hungary).

Under the conditions used, 5 μg of rabbit antibody UK163 immunoprecipitated about 50 % of the total LANA protein from cross-linked extracts. For ChIP assays, nuclear extracts were incubated with 5 μg of anti-LANA rabbit antibody at 4 °C overnight. As a negative control, a rabbit polyclonal antibody to herpesvirus saimiri ORF 1 protein has been used (Medveczky *et al.*, 1993). Immune complexes were collected with protein A–Sepharose beads and washed under high stringency washes. Protein–DNA complexes were eluted and the cross-links were reversed. DNA was prepared from these samples after proteinase K treatment, phenol/chloroform extraction and ethanol precipitation, labelled with random priming and used as probes for Southern blots.

GST–LANA–DNA pull-down assay. Of each cosmid, Z2, Z6, Z8, Z14 and Z15, 2 μg DNA was cut with *Eco*RI to release the viral DNA insert from the vector. DNA was then sonicated to an average size of 400 bp. Baculovirus-expressed GST–LANA was purified as described earlier (Zhu *et al.*, 1999) and incubated with these DNA fragments in DNA-binding buffer (DBB) (20 mM HEPES pH 7·9, 100 mM KCL, 1 mM MgCl$_2$, 0·1 mM EDTA, 1 mM DTT, 12 % glycerol and 1 mg BSA ml^{-1}) with rotation at room temperature for 1 h. GST–LANA was pulled down with glutathione beads (Sigma) and washed four times with DBB. LANA-bound DNA fragments were eluted from the beads, digested with proteinase K, phenol/chloroform extracted, ethanol precipitated and used on Southern blots as probes.

RESULTS

Cosmid Z2 and its subclones persist in BCBL-1 cells

To map *cis*-acting ARS element(s) in the KSHV genome, we chose BCBL-1 cells derived from a KSHV-positive PEL. All factors required for episome replication in *trans* are expressed in these cells. We tested replication of the cosmids depicted in Fig. 1(a) in BCBL-1 cells using Gardella gels. Five individual BCBL-1 cultures were transfected with equal amounts of DNA from the five cosmids selected with G418 and analysed for persisting episomes. Fig. 2(a) shows that cultures transfected with the Z2 cosmid for 10 days contained episomal circular DNA. The range of expected episomal DNA was determined by analysis of DNA bands detected by ethidium bromide staining prior to Southern blotting (Fig. 2c); the top band corresponds to the loading well containing cellular DNA that failed to enter the gel and the band close to the bottom represents linear cellular DNA. The episomal band detected in Z2-transfected cells was also broad, suggesting that some of the molecules had rearranged. At 30 days after transfection, the Z2 episomal DNA was still present but was much sharper (Fig. 2b). No other cosmids persisted as episomes. These results were highly reproducible and similar findings were noted in five other experiments.

To localize further the ARS activity of cosmid Z2, three subclones (shown in Fig. 1a) were transfected into BCBL-1 cells and selected with G418. Episome replication of plasmids was assayed by two methods.

G418-resistant, transfected BCBL-1 cells were cultured for 2 months and purified episomal DNA was digested with
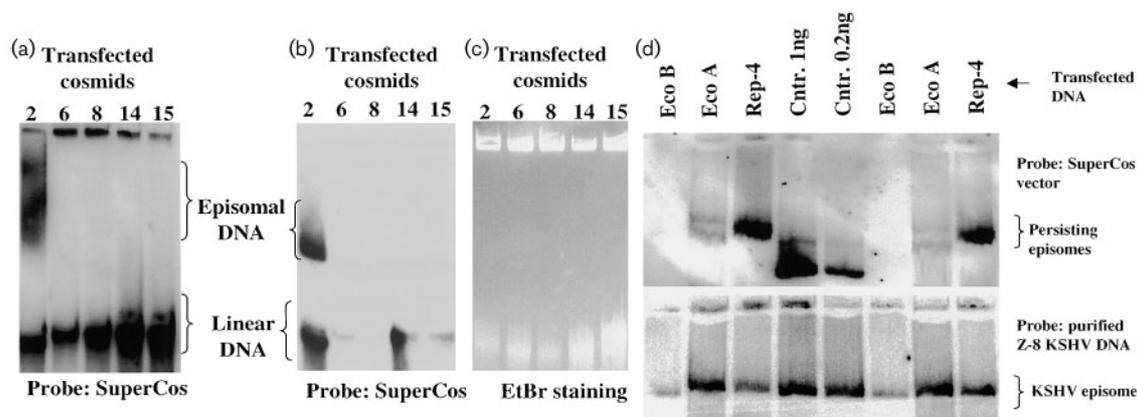
Fig. 2. Only cosmid Z2 and its subclones containing TRs persist as an episome in transfected BCBL-1 cells. A 10 µg sample of DNA purified from each cosmid was transfected into BCBL-1 cells and cells were cultured for 10 (a) or 30 (b, c) days. To determine the physical state of the transfected sequences and to distinguish between episomal and linear DNA, we used the agarose gel method described previously by Gardella *et al.* (1984). (c) An ethidium bromide-stained gel of (b). DNA was transferred to nitrocellulose and the Southern blot was hybridized with cloning vector SuperCos probe (a, b). Brackets indicate the range of episomal and linear DNA, as determined from ethidium bromide-stained gel images. Large episomal DNA bands run between the loading well and linear cellular DNA. A 10 µg sample of DNA purified from each plasmid was transfected into BCBL-1 cells and G418-resistant cell lines were selected. At 2 months after transfection, 1×10^6 cells were analysed for free episomal DNA using the agarose gel method described previously by Gardella *et al.* (1984). DNA was transferred to nitrocellulose (d). The Southern blot was hybridized with the cloning vector SuperCos probe (top) and with purified Z8 KSHV probe containing the unique KSHV sequences (bottom). The positions of bands corresponding to transfected episome replicating plasmid and the resident KSHV genome are indicated.

either *Mbo*I or *Dpn*I, which digest replicated or non-replicated DNA, respectively. *E. coli* was transformed with digested DNA and colonies were counted. Table 1 shows that Eco A- or Rep-4- but not Eco B-transfected BCBL-1 cells contain plasmids resistant to *Dpn*I but sensitive to *Mbo*I, indicating episome replication in these cells. Transfected BCBL-1 cultures were grown for 9 months (corresponding to 142 cell divisions). Table 1, experiment 3, shows that several plasmids were recovered from Eco A- and Rep-4- but not from Eco B-transfected cultures.

As a second approach, BCBL-1 cultures transfected stably with the Z2 subclones were analysed for free episomes 2 months after transfection using Gardella gels. Fig. 2(d, top blot) shows that the SuperCos vector probe hybridized with DNA migrating in the range of episomal DNA in

Eco A- and Rep-4-transfected cultures. Eco B-transfected cultures did not contain any detectable episomes. To exclude the possibility that transfected plasmids integrated into the resident KSHV episome through recombination and were replicating as part of the viral genome, the same blot was hybridized with a purified Z8 KSHV DNA probe, which contains the unique KSHV DNA and so should hybridize with the resident KSHV genome. Fig. 2(d, bottom blot) shows that the Z8 KSHV probe detected new bands in all lanes that migrated more slowly. Thus, these data showed unambiguously that the resident genome and transfected episomal DNA replicated independently in BCBL-1 cells as episomes. The Eco A and Rep-4 plasmids persisted as episomes and were detected by Gardella gels 9 months after transfection (data not shown).

Table 1. Number of *E. coli* clones obtained from transfected cells

DNA was isolated from BCBL-1 cells stably transfected for 2 or 9 months with plasmids as indicated. DNA was digested with *Dpn*I or *Mbo*I and *E. coli* was transformed with DNA corresponding to 2×10^5 cells. In experiment 3, DNA was not digested with any restriction enzyme.

Transfected plasmid	*Dpn*I (2-month-culture)		*Mbo*I (2-month-culture)		No enzyme (9-month-culture)
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 3
Eco A	81	273	0	8	851
Eco B	0	3	0	0	0
Rep-4	271	71	0	0	322

Data from two different types of experiments indicated conclusively that plasmids containing TR elements replicated stably and autonomously in BCBL-1 cells.

To evaluate whether any viral protein is required for the ARS activity observed in BCBL-1 cells, various cosmids and plasmids (Fig. 1) were also transfected into the uninfected lymphoblastoid cell lines Jurkat and Ramos or into the human embryonic kidney cell line 293. In five independent experiments after G418 selection, we could not detect any persisting episomes after Gardella gel analysis (data not shown). Thus, virus factor(s) are necessary to achieve episome replication of TRs.

LANA is sufficient to support the stable replication of TRs in 293 cells

As LANA may be involved in the maintenance of KSHV episomes, we investigated whether LANA could support the maintenance of plasmids containing TRs. A vector expressing LANA and also containing various numbers of TRs at a unique *Mlu*I site was constructed using the pBKCMV expression vector. The single TR unit was cloned as a *Not*I fragment and as a *Sau*3A fragment. Subclones encoding different smaller parts of the TR were also inserted into the LANA expression vector for replication assays. 293 cells were stably transfected with the constructs and controls (pBKCMV-LANA without repeats and empty vector) using G418 selection. Clones resistant to G418 were tested for episome replication of the transfected plasmids. Purified episomal DNA was digested with either *Dpn*I or *Mbo*I. Fig. 3(top panel) shows that cells transfected with LANA expression vectors encoding TRs, but not the ones without them, all contained episomal DNA replicated by the mammalian replication machinery. Fig. 3(bottom panel) shows ethidium bromide staining of the gel, indicating equal loading of DNA. LANA expression vectors containing only a single repeat unit replicated efficiently; however, the number of episomal DNA copies was much lower than in cells transfected with vectors containing three or more repeat units. The DNA sequence necessary for LANA-mediated replication was localized further using subclones encoding different parts of the TR to the region between bp 548 and 171 of the repetition unit (data not shown).

LANA binds TRs with high selectivity *in vivo*

To determine which region of the KSHV genome binds LANA *in vivo*, we used ChIP assays. KSHV-infected cells, treated with formaldehyde to cross-link DNA to binding proteins and sonicated cell extracts were immunoprecipitated with a specific anti-LANA antibody, or, for a control, with non-specific antibody. Immunoprecipitated DNAs were purified and used as probes on Southern blots to identify LANA-bound KSHV DNA.

Representative results of ChIP assays using three different PEL cell lines are shown in Fig. 4. First, we compared visually the distribution of hybridization signals (Fig. 4a–c)

with the ethidium bromide-stained gel (Fig. 4e) representing all of the DNA fragments. We concluded that the LANA-specific antibody selectively immunoprecipitated an 801 bp DNA fragment. Several mapping experiments and DNA sequencing showed that the 801 bp fragments correspond to the TRs. These fragments are encoded by cosmids Z2, Z6 and Z14 and present as supermolar bands indicated by the ethidium bromide image of Z2 and Z6 (Fig. 4e). This apparently selective immunoprecipitation of TR by the LANA antibody was the case with all three different KSHV-harbouring cell lines BCBL-1, BC-1 and BC-3 (Fig. 4a–c). No significant DNA contamination due to the procedure occurred, as we did not observe notable hybridization with probes obtained with immunoprecipitations using a non-specific antibody (Fig. 4a–c). In the control experiment, we used unselected total cellular DNA from BCBL-1 cells as a probe. Fig. 4(c) shows that repetitive DNA bands hybridized with only slightly stronger binding than those of unique DNA fragments, indicating that the immunoprecipitated DNA contained much higher concentrations of TRs than unique DNA. Taken together, these results suggested significant and specific *in vivo* LANA binding to the TR.

To evaluate and confirm the apparent specificity of the Southern blot results of Fig. 4, we compared the data quantitatively. This was accomplished by measuring and comparing intensities of unique and repetitive radioactive bands using the PhosphorImager and the ImageQuant program. For example, we compared relative intensities of cosmid Z14 unique fragment A with that of the TR. The ratio between the signals of the TR and the Z14 A fragment for the immunoprecipitated BCBL-1 DNA was 34·4, and with the control unselected probe it was 0·7. Thus, in the case of the BCBL-1 cell line, LANA binds the TR *in vivo* at least 50-fold more than a unique Z14 A fragment of the virus genome. Similar data were obtained when BC-1 and BC-3 cells were analysed.

Stronger relative signals were obtained not only for TR but also for two other DNA fragments in the *Not* I/*Eco*RI Z6 digest. One of them is a truncated TR unit (labelled as tr in Fig. 4a). The other is the leftmost, 6198 bp *Eco*RI fragment of the unique region of Z6. This fragment is fused to a small part of the TR, even after *Not*I digestion (labelled as Z6 TR–UR junction in Fig. 4a). Since this part of Z6 representing the TR–UR junction was found to hybridize in the anti-LANA chromatin immunoprecipitates, the possibility exists that not only the TR region but also the unique region of Z6 binds LANA *in vivo*. To address this question, we cut the Z6 cosmid with *Not*I and then with *Pst*I, *Eco*RI, *Bgl*II and *Hinc*II, respectively. Southern blotting with immunoprecipitated DNA was then performed. These enzymes cut at different distances from the TR junction (Fig. 1). Fig. 4(f) shows that the immunoprecipitated probe hybridized with the TR fragment and with fragments containing the TR unique region junction (*Eco*RI and *Bgl*II) but not with fragments containing only the unique region
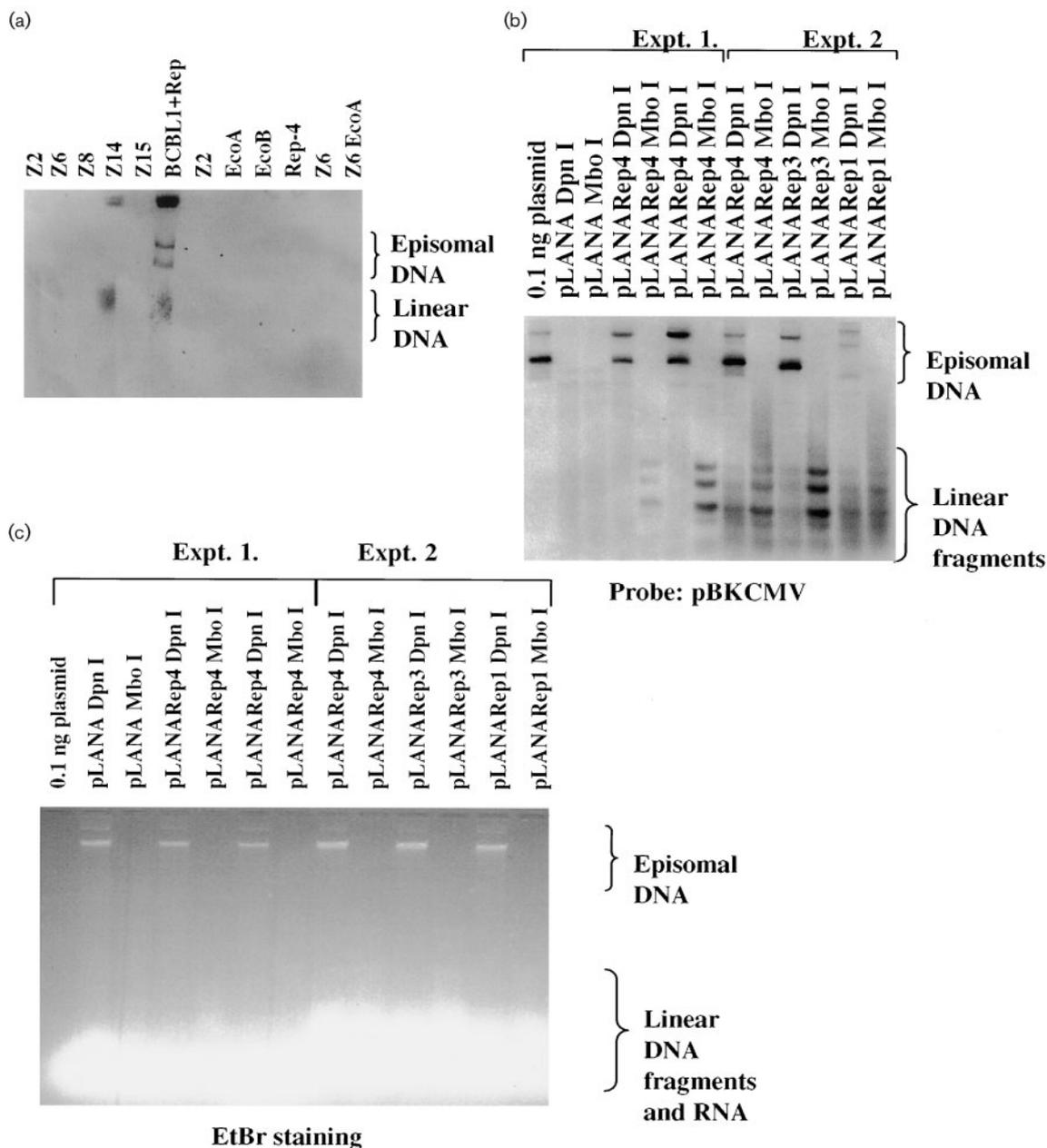
Fig. 3. Cosmid and plasmid clones replicate as episomes in uninfected cells only if LANA is expressed. Plasmid DNA of LANA expression constructs containing one, three and four units of TRs (pLANARep1, pLANARep3 and pLANARep4, respectively), or containing no repeats (pLANA), were transfected in 293 cells. G418-resistant clones were selected and cultured for 1 (expt 2) or 2 months (expt 1). Experiment 1 included three cultures, two with independent transfections with pLANARep4, and one with pLANA, as indicated. Episomal DNA was extracted from cell cultures by the method of Hirt (1967) and DNA samples equivalent to about 1×10^6 cells were digested with *Dpn*I or *Mbo*I. Digested DNA samples and 0·1 ng of undigested pLANARep4 plasmid (leftmost lane, labelled 'plasmid') from an *E. coli* plasmid preparation were separated on 1 % agarose gels, transferred to nitrocellulose and hybridized with pBKCMV vector probe (a). Ethidium bromide staining of the gel indicated equal loading of DNA; this assessment was based on visualization of cellular, most likely mitochondrial, DNA in each sample (b).

of Z6. We observed no hybridization signal using the control antibody (Fig. 4g). To ensure that the preferential hybridization signals came from the TR only, the same blot was stripped and probed with a gel-purified unit length TR probe (Fig. 4h). This experiment gave a practically identical pattern of hybridization as the anti-LANA ChIP DNA. Taken together, these ChIP experiments show that LANA binds strongly and specifically to the TR *in vivo*.
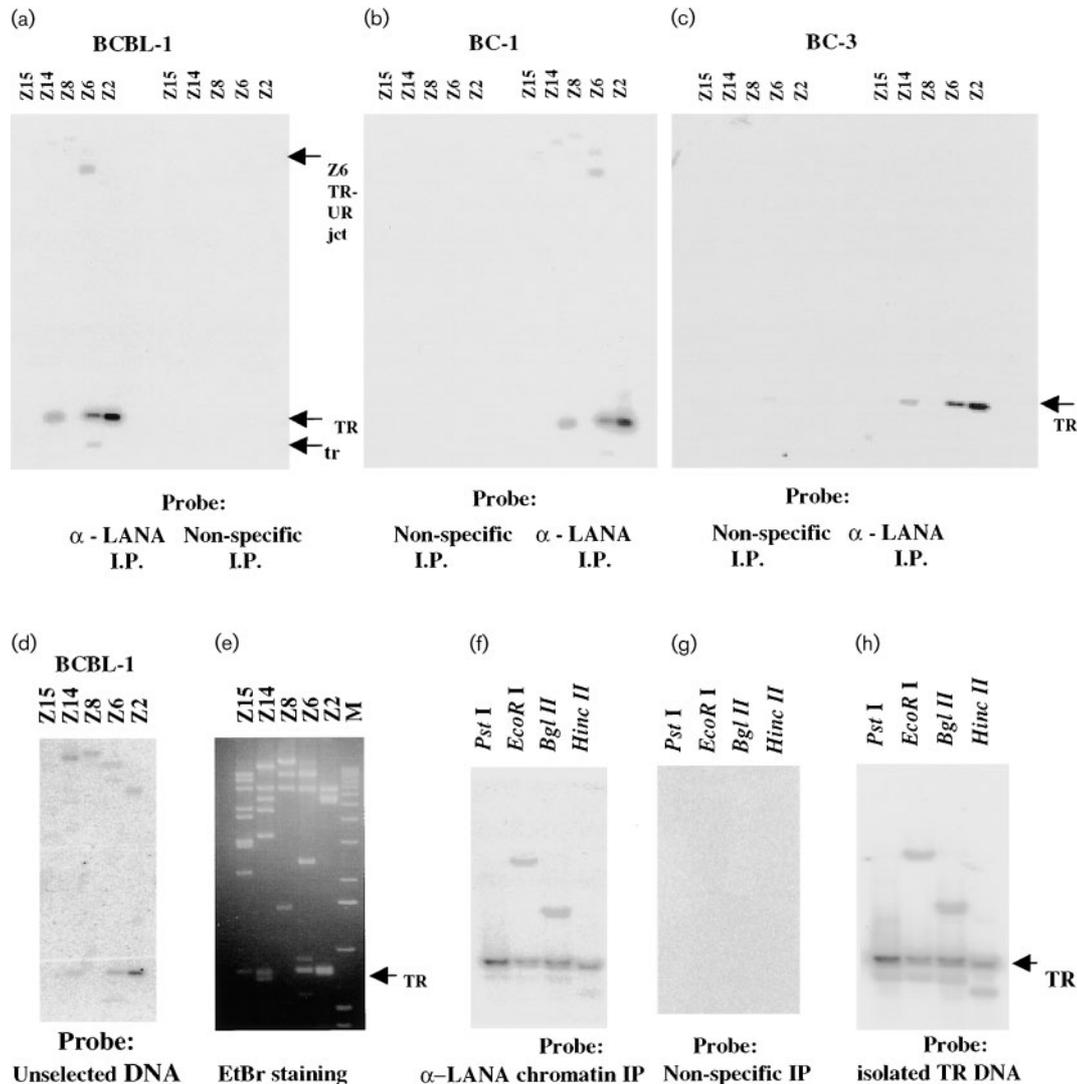
Fig. 4. LANA binds the TRs with high selectivity *in vivo*. Extracts corresponding to $3 \cdot 2 \times 10^6$ formaldehyde-treated BCBL-1 (a), BC-1 (b) and BC-3 (c) cells were immunoprecipitated with anti-LANA antibody or non-specific antibody raised against the ORF 1 protein of herpesvirus saimiri (Medveczky *et al.*, 1993). DNA was purified from these samples and used as probe (a–c). Control, unselected DNA was isolated from the same extract of BCBL-1 cells without immunoprecipitation and used as probe (d). Southern blots contained 2 μg DNA of cosmids Z2, Z6, Z8, Z14 and Z15 cut with *Not*I/*Eco*RI and separated on 1 % agarose gels. Ethidium bromide-stained DNA fragments of the original gel are shown in (e). A 2 μg sample of DNA of cosmid Z6 was cut with *Not*I and then with *Pst*I, *Eco*RI, *Bgl*II and *Hinc*II, respectively, and separated on 1 % agarose gels. The blotted DNA fragments were hybridized with anti-LANA ChIP probe from BCBL-1 cells (f) or with control probe obtained with the non-specific antibody raised against the ORF 1 protein of herpesvirus saimiri (g). After stripping, the same filter was hybridized with gel-purified TR DNA probe (h).

GST–LANA binds DNA *in vitro* with slight selectivity to the TRs

To assess the *in vitro* DNA-binding capacity of LANA and to compare it to the *in vivo* ChIP results, DNA pull-down assays were performed using a baculovirus-expressed GST-tagged LANA, as described in Methods. Purified DNA fragments were labelled and used as probes in Southern blots containing *Eco*RI/*Not*I-digested cosmid DNA. Fig. 5 shows the result of a representative set of these assays. GST–LANA bound the DNA fragments effectively (Fig. 5b). However, this DNA binding was not specific for KSHV DNA, as not only the viral but also the vector DNA bands were readily detectable on the Southern blots. Comparing the results of these blots to those from the ethidium
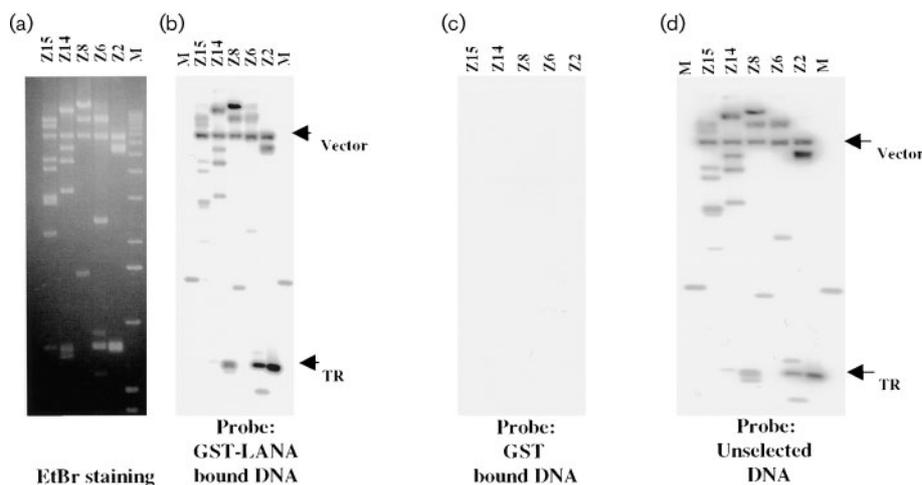
Fig. 5. GST–LANA binds DNA with some selectivity to the TRs. A 2 μg sample of DNA of cosmids Z2, Z6, Z8, Z14 and Z15 were cut with *Not*I/*Eco*RI and separated on 1 % agarose gels. The blotted DNA fragments were hybridized to GST–LANA-bound DNA (b), GST-bound DNA (c) or to the unselected mixture of the *Eco*RI-digested and sonicated cosmid DNA (d). The ethidium bromide-stained gel is also shown (a).

bromide-stained gel (Fig. 5a), it is obvious that each DNA fragment hybridized with the GST–LANA-bound probes. This DNA-binding activity was not due to the binding of DNA fragments to the GST protein or to the glutathione beads, as baculovirus-expressed GST protein did not show DNA-binding activity in this assay (Fig. 5c).

To evaluate if GST–LANA binds certain segments of the KSHV genome preferentially, the hybridization signals of the Southern blots were quantified using the ImageQuant program. The signal intensities of the KSHV DNA fragments have been normalized to that of the vector DNA that served as an internal control for non-specific DNA binding. For example, when GST–LANA-bound double-stranded DNA was used as a probe, the Z2 TR fragment : vector ratio was 1·379. The TR : vector ratio in the control experiment (Fig. 5d) was 0·327. Thus, GST–LANA binds the TR DNA approximately 4-fold more than a non-specific DNA fragment in this assay.

LANA binds TRs *in vivo* in a direct DNA–protein interaction

Formaldehyde effectively cross-links proteins to DNA, but, besides DNA–protein cross-links, it also creates protein–protein covalent linkages. Thus, it was not clear if LANA binds DNA directly or indirectly through another bridging protein. To address this question, a ChIP assay with *in vivo* UV cross-linking has been carried out. The UV method only detects the direct protein–DNA interactions (Ausubel *et al.*, 1995; Gilmour *et al.*, 1991). Fig. 6 shows the result of such an experiment. Similar to the formaldehyde cross-linking experiments, the major hybridization signal was found with the TR. No signal was detected with the non-specific control immunoprecipitation. Nevertheless, the intensity

of the TR signal was less strong than that seen with formaldehyde cross-linking. This weaker reaction is probably due to the fact that UV irradiation is a specific but ineffective method for protein–DNA cross-linking (Ausubel *et al.*, 1995; Gilmour *et al.*, 1991). Thus, this experiment has shown that at least a part of the LANA–DNA interaction is a direct contact.
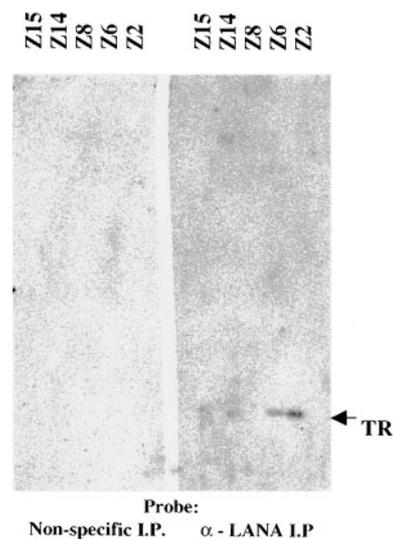
Fig. 6. LANA binds the TRs directly. Extracts corresponding to $3·2 \times 10^6$ UV-irradiated BCBL-1 cells were immunoprecipitated (I.P.) with anti-LANA antibody (right panel) or non-specific antibody (left panel). DNA samples purified from the immunoprecipitates were labelled and used as probe on Southern blots, as described in the legend to Fig. 4(a–d).

Preferential binding sites in the TR for LANA cannot be detected *in vivo*

We asked the question if some sequences within the TR contain specific binding sites for LANA. If there is a strong preference for LANA to bind a certain small region within the TR, this preferential site can be mapped by anti-LANA ChIP assays. TR DNA was digested with *Sma*I, electro-blotted and probes from anti-LANA ChIP assays and non-selected DNA were used on Southern blots. Fig. 1(b) shows a schematic diagram of the KSHV TRs containing *Sma*I sites.

Fig. 7 shows a representative set of ChIP experiments performed with formaldehyde (a) and UV (b) cross-linked probes as well as with the control probe (c). Fig. 7(d) shows hybridization data using the non-specific control antibody and the original ethidium bromide-stained gel is shown in (e). Results showed strong hybridization of the anti-LANA ChIP probe to all *Sma*I fragments within the TR. Although there were slight differences in the relative intensities of the individual DNA fragments, we could not detect an obvious preferential *in vivo* LANA-binding site. Results were rather consistent with multiple LANA binding along the whole TR. As found previously, no hybridization occurred with the control probe.

DISCUSSION

To identify ARS elements within the KSHV genome, genomic libraries representing the entire genome were transfected into BCBL-1 cells and replication was observed with cosmid Z2 and its subclones containing TRs but no unique sequences. These plasmids persisted very stably as episomes in BCBL-1 cells or uninfected cells expressing LANA without rearrangement or integration in the viral genome. The importance of TRs in episome replication was also shown in cells not infected with KSHV. In 293 cells, we found that terminal TRs persisted stably only in the presence of the LANA protein. These observations and data published by others (Ballestas *et al.*, 1999; Ballestas & Kaye, 2001) show clearly that LANA alone is sufficient as a *trans*-acting factor to maintain KSHV DNA as episomes. In addition, the work presented here shows that only TRs are required as *cis*-acting sequences and other parts of the viral genome do not have ARS activity.

Interestingly, ARS activity correlated with the number of repeats present in various plasmids. In 293 cells, we estimated that plasmids containing three or four repeats yielded at least 100 copies of episomes per cell, while the clone containing a single repeat unit yielded only about 10 copies per cell. These data suggest that multiple repeats provide higher copy numbers and are in agreement with data published recently using a transient DNA replication assay (Lim *et al.*, 2002).

In this study, we also mapped and analysed the binding activity of the KSHV LANA protein to the entire viral genome by several methods. *In vivo* DNA binding of LANA was tested by ChIP assays. Using this assay, we have demonstrated in different KSHV-infected cells that LANA binds to the TRs specifically. Mapping of protein–DNA-binding sites with ChIP assays usually requires the PCR amplification of the immunoprecipitated DNA (Orlando, 2000). Most remarkably, this was not necessary in our
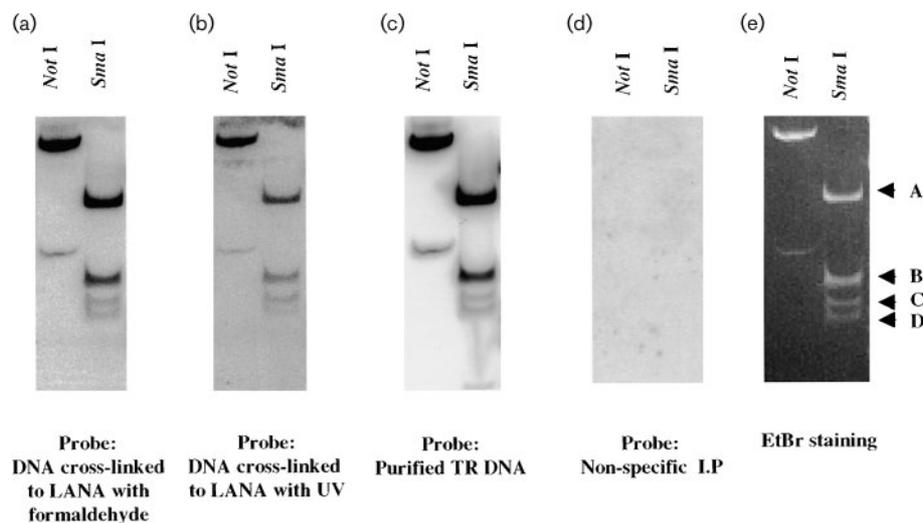
Fig. 7. A single preferential binding site for LANA cannot be detected *in vivo*. DNA of plasmid 2/1 was cut with *Not*I/*Sma*I (0·5 and 1 µg, respectively) and separated on a polyacrylamide gel. Electroblotted fragments were hybridized with anti-LANA ChIP probes prepared from formaldehyde (a) and UV (b) cross-linked BCBL-1 extracts or from gel-purified control TR DNA (c). The result of hybridization with the control probe, obtained with the non-specific antibody, is shown in (d). (e) The ethidium bromide-stained DNA fragments in the original gel are shown.

case, as the results indicated strong hybridization signals with the TR using the anti-LANA-immunoprecipitated DNA probe directly. Several reasons can explain this fact. The copy number of KSHV in latently infected cells is 50–100 and the TR units are present in multiple copies in the viral chromosome. LANA has been shown to multi-merize (Schwam *et al.*, 2000), which gives a further enhancement to the LANA ChIP assay, as formaldehyde treatment makes not only DNA–protein but also protein–protein cross-links.

No LANA-binding sites were detected in the unique region. This does not necessarily mean that such binding sites do not exist, as their signal could be under the level of detection employed in this study. Nevertheless, the results show a much more prominent *in vivo* binding of LANA to the TR than to any other part of the viral genome.

Significant non-specific DNA-binding activity of recombinant LANA was detected by *in vitro* GST pull-down studies. Although recombinant baculovirus-expressed LANA bound the TR more strongly than other fragments, this protein could also bind sequences unrelated to KSHV: for example, DNA of the SuperCos vector. This paradoxical observation can be explained by the effect of other viral or cellular factors that are present in the virus-infected cell but absent in an *in vitro* LANA–DNA-binding assay. Indeed, LANA has been shown to bind several other DNA or chromatin-associated cellular proteins (Platt *et al.*, 1999; Krithivas *et al.*, 2000; Lim *et al.*, 2001; Friborg *et al.*, 1999; Radkov *et al.*, 2000). Alternatively, it is also possible that a complex, chromatin-like packaging of the viral genome *in vivo* confers greater sequence specificity to LANA. Cotter & Robertson (1999) demonstrated the association of LANA with various segments of the genome *in vitro*. This essentially corresponds to our *in vitro* results showing non-specific DNA binding by LANA and the results collectively underline the necessity of the *in vivo* ChIP assay. The finding that LANA has a non-specific DNA-binding activity *in vitro* is not surprising, since this characteristic is a common feature of several proteins, such as p53, that also recognize specific DNA sequences (Bayle *et al.*, 1995).

UV cross-linking experiments presented here have shown that LANA binds the TR DNA directly. Similar to the formaldehyde cross-linking studies, these experiments also indicated preferential TR binding. Attempts to narrow down the LANA-binding region within the TR using the ChIP assay did not detect any predominant LANA-binding region. There are inherent size limitations of mapping with ChIP, since sonication generates 3–500 bp long fragments; nevertheless, results are consistent with at least two or, possibly, multiple LANA-binding sites within the repeats. However, fine mapping of these *in vivo*-binding sites should be attempted by other more sensitive methods.

Other groups have observed co-localization of LANA to large regions of the viral genome (Ballestas *et al.*, 1999; Cotter & Robertson, 1999). These regions, which included

a minimum of three units of the TR plus about 13 kb of unique sequence to the right of the TR, were also shown to be involved in the maintenance of episomal DNA (Ballestas *et al.*, 1999; Cotter & Robertson, 1999). Specific interaction of LANA with the TRs measured by *in vitro* assays was also reported recently (Ballestas & Kaye, 2001; Garber *et al.*, 2001). We comparatively evaluated LANA binding to different regions of the KSHV genome *in vivo* and characterized the nature of this protein–DNA interaction. Our results, consistent with the above-mentioned data, provide the first evidence that LANA is associated specifically with the TRs *in vivo*, demonstrate that the interaction is a direct protein–DNA contact and suggest multiple binding sites within the TR, but exclude other parts of the viral genome as major *in vivo* LANA-binding sites.

Studies on the related tumour virus herpesvirus saimiri also implicate the TRs in episome replication. TRs are essential for the establishment and/or maintenance of herpesvirus saimiri episomes, as demonstrated by construction and analysis of virus mutants lacking intact TRs (Collins *et al.*, 2002). Furthermore, LANA expression vectors encoding TRs replicated as episomes in uninfected cells (Collins *et al.*, 2002), indicating that the basic molecular mechanisms for the maintenance of these viruses are similar.

Taken together, our findings suggest a model in which multimers of the LANA protein complex with each TR unit at the virus episome during latency so that dozens of LANA–DNA complexes are concentrated in a very small area. The high local concentration of LANA complexes may be essential for the association of the episome with chromatin structures. These events explain the LANA-mediated tethering observed by microscopy (Ballestas *et al.*, 1999; Cotter & Robertson, 1999). There is also a likely role for other viral and/or cellular factors in this model. It appears that the LANA–TR interactions are analogous to those described between EBNA-1 and the family of repeats of EBV (Chittenden *et al.*, 1989; Reisman *et al.*, 1985; Sugden *et al.*, 1985; Yates *et al.*, 1984, 1985).

There are still unresolved issues related to the latency of KSHV episomes. Further studies are required to assess the host range of the LANARep plasmids and to understand how TRs promote episome replication. The extremely high $G+C$-containing repeats are unlikely to provide an origin function, since all known origins have a low $G+C$ content to allow for easy unwinding (Challberg & Kelly, 1989). The comprehensive understanding of the molecular mechanisms involved in the latency of gamma-2 herpesviruses can lead to the development of KSHV-based gene delivery vectors and to specific strategies and drugs against virus factors to destroy latent virus.

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