Published in final edited form as:

Arch Virol. 2011 August ; 156(8): 1323–1333. doi:10.1007/s00705-011-0985-0.

Rapid and quantitative assessment of KSHV LANA-mediated DNA replication

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA) mediates DNA replication of terminal repeat (TR) DNA to enable viral episome persistence in latently infected cells. Southern blotting is routinely used to detect LANA-replicated DNA. We developed and validated a real-time PCR assay for TR-associated DNA and compared it with Southern blot analysis. Both PCR and Southern blot detected LANA-replicated DNA, but the PCR assay was more rapid and did not require radioisotope. PCR detection at 24 and 72 hours post-transfection demonstrated rapid loss of transfected TR DNA. LANA, and to a lesser extent a moderately deficient LANA mutant, reduced the rate of DNA loss through addition of replicated TR DNA and reduction in the loss of non-replicated DNA, the latter of which is consistent with LANA's nuclear segregation function. Therefore, this work develops a rapid, sensitive, and quantitative PCR (qPCR) assay to detect KSHV LANA-replicated DNA and demonstrates that LANA reduces TR DNA loss after transfection through replication and nuclear partitioning of TR DNA.

Introduction

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a double-stranded DNA virus that belongs to the family *Herpesviridae*, subfamily *Gammapherpesvirinae*, genus *Rhadinovirus*, species *Human herpesvirus 8* according to the International Committee on Taxonomy of Viruses classification. KSHV is associated with primary effusion lymphoma, multicentric Castleman's disease and KS [5, 6, 27, 33]. Viral infection in these tumor cells is predominantly latent, and the KSHV genome persists as multiple copies of extrachromosomal circular DNA (episomes) [5, 10]. In order to persist in cells, the KSHV genome must replicate and segregate to daughter cells during each cell division. Latency-associated nuclear antigen (LANA), one of a few viral genes expressed during latent infection, is necessary and sufficient for KSHV episome persistence in the absence of other viral genes [1, 2]. C-terminal LANA binds its recognition sequence in KSHV terminal repeat (TR) DNA to mediate DNA replication. LANA also segregates viral DNA to progeny nuclei by tethering the KSHV genome to host chromosomes [2, 8, 9, 11–13, 16, 25]. N-terminal LANA is essential for chromosome association through interactions with histones H2A/H2B [3, 4, 29] and also is critical for DNA replication [3, 16]. In addition to binding TR DNA, C-terminal LANA also associates with mitotic chromosomes [18, 19, 22], although mutations that severely diminish C-terminal

chromosome association but do not affect DNA binding do not reduce TR DNA replication [19].

Transient replication assays in uninfected cells are used to assess LANA-mediated DNA replication. In these assays, TR DNA is introduced by transfection into cells stably expressing LANA or into cells that are co-transfected with a LANA expression vector [13, 16, 21, 25, 26]. Importantly, the TR-containing plasmid is cloned in *Dam* methylase-positive *Escherichia coli*, which results in methylated adenines at GATC sequences. Two to three days post-transfection, low-molecular-weight DNA is isolated from transfected cells and analyzed for a loss of methylation at *DpnI* restriction enzyme sites (GATC). *DpnI* only cleaves at its recognition site when the adenine is methylated. However, this methylation is lost upon DNA replication in mammalian cells, resulting in resistance to *DpnI* cleavage and allowing detection of replicated DNA. Typically, the *DpnI*-resistant, replicated plasmid is separated from non-replicated, *DpnI*-sensitive, TR DNA using agarose gel electrophoresis and detected by Southern blot after transfer to a membrane. A real-time PCR approach to quantitate papillomavirus E1/E2 DNA replication has been described [34]. Real-time PCR was also used to detect replication of the KSHV minimal replicator element, a ~70-bp sequence in each TR, by amplification of the sequence flanking a *DpnI* site present in the plasmid's puromycin cassette [17, 35]. In this study, we have developed a sensitive, rapid, and quantitative assay to detect LANA-mediated TR DNA replication using real-time PCR and compared this assay with Southern blot analysis.

Materials and methods

Plasmids

We constructed p8TR-gB (Fig. 1) for use in real-time PCR detection of replicated TR-associated DNA. p8TR [3] contains eight KSHV TR copies and was modified by the insertion of a 138-bp DNA fragment into the *HindIII* site to generate p8TR-gB (Fig. 1). The inserted fragment contains a modified herpes simplex virus 1 (HSV-1) glycoprotein B/UL27 (gB) (GenBank accession number S65444) sequence that has been used as a target of real-time PCR to differentiate HSV-1 from HSV-2 [7, 28]. Three *DpnI* sites (GATC) were introduced within gB nucleotides 518-605, and the sequence was flanked by restriction enzyme sites for *ClaI*, *XbaI*, *EcoRV* and *HindIII*. The oligonucleotides to generate this insert were designed using Assembly PCR Oligo Maker (<http://startrek.ccs.yorku.ca/~pjohnson/AssemblyPCROligomaker.html>) and assembled according to Rydzanicz et al. [31]. Briefly, 0.28 μ M oligonucleotides gB-1, gB-2, gB-3 and gB-4 (Table 1) were incubated with 0.2 mM dNTPs and 3 units of Vent DNA polymerase (NEB) in a final volume of 100 μ L of 1x Thermopol buffer (NEB). This mixture was heated at 94°C for 7 minutes, followed by 8 cycles of amplification (94°C for 1.5 minutes, 54°C for 2 minutes, 72°C for 3 minutes) and one elongation step at 72°C for 5 minutes (Stratagene Robo-cycler Gradient 96). Next, 1 μ L of the product of the PCR reaction was incubated with 1.7 μ M oligonucleotides gB-5 and gB-6 (Table 1), 0.2 mM dNTPs and 3 units of Vent DNA polymerase in 100 μ L 1x Thermopol buffer. The mixture was subjected to one cycle at 94°C for 5 minutes followed by 25 cycles of amplification (94°C for 30 seconds, 54°C for 2 minutes, 72°C for 1.5 minute) and one elongation step at 72°C for 5 minutes. The amplified DNA was separated by gel electrophoresis and purified using a Qiaquick Gel Extraction Kit (QIAGEN). The fragment was digested with *HindIII* and inserted into the *HindIII* site of p8TR, and the sequence was confirmed.

Cells, transfection, low-molecular-weight DNA isolation, and immunoblotting

BCBL1 primary effusion lymphoma cells were grown in RPMI medium supplemented with 20% bovine growth serum (Hyclone) and 15 μ g/mL gentamicin. BJAB (uninfected B cell

lymphoma) cells or BJAB cells stably expressing N-terminal FLAG-epitope-tagged LANA [1], N-terminal T7-epitope-tagged LANA or LANA with alanine substitutions at positions 14 and 15 (LANA₁₄TG₁₅) [3] were grown in RPMI medium supplemented with 10% bovine growth serum (Hyclone) and 15 µg/mL gentamicin. Stable LANA expression was maintained with hygromycin B selection (200 units/ml) (Calbiochem), and BJAB cells stably expressing the hygromycin resistance gene [3] (used for the experiment shown in Fig. 5) were also maintained with hygromycin B selection. Three to four days prior to transfection, cells were maintained in log phase of growth by splitting each day to a concentration of 0.4×10^6 cells/mL. From here forward, BJAB cells stably expressing N-terminal FLAG-epitope-tagged LANA are referred as WT-1, and BJAB cells stably expressing N-terminal T7-epitope-tagged LANA are referred as WT-2 and WT-3. WT-3 is a subclone of WT-2. Two independently derived BJAB cell lines stably expressing LANA₁₄TG₁₅ [3] are referred to as TG-1 and TG-2.

Ten million cells were transfected with 5 µg or 0.5 µg of p8TR-gB using the Amaxa Nucleofactor II program O-17 in 150 µl of room-temperature solution V (Amaxa). Immediately after transfection, cells were seeded in 6-well plates in a final volume of 5 ml of supplemented RPMI medium. Twenty-four hours post-transfection, cells were expanded to 25-cm² flasks, and 5 million cells were harvested for low-molecular-weight DNA isolation by the Hirt method [15].

For Hirt DNA extraction, cells were lysed in 1 mL lysis buffer (0.6% SDS, 10 mM EDTA, 10 mM, Tris-HCl pH 7.5, 50 µg/ml RnaseA) per 5 million cells and incubated at 37°C for 2 hours. The reaction was then adjusted to contain 1 M NaCl and incubated overnight at 4°C. After a 30-minute centrifugation at $11,000 \times g$ at 4°C, DNA was extracted once with phenol:chloroform (1:1), twice with chloroform:isoamyl alcohol (24:1), ethanol precipitated, and the DNA pellet was washed with 70% ethanol, air-dried and resuspended in buffer (10 mM Tris, pH8, 0.1 mM EDTA). The remaining cells were split daily to keep them in log-phase growth. Seventy-two hours after transfection, 10–15 million cells were harvested, and Hirt DNA was again isolated. For immunoblot assays, proteins were resolved by SDS/PAGE in 6% gels, LANA was detected using immune serum, and tubulin was detected using an anti-alpha-tubulin monoclonal antibody B-5-1-2 (Sigma).

DNA digestion and real-time PCR

Primers and fluorescent probes for real-time PCR were designed using Primer Express software (PE Applied Biosystems) (Table 2, Fig. 1). For the PCR reaction, 300 nM primers gB-F and gB-R, 250 nM fluorescent probe and Taqman Universal master mix (Applied Biosystems) were combined in a final volume of 25 µl. PCR reactions were performed using an ABI 7300 Real-Time PCR System (Applied Biosystems) (1 of cycle 50°C for 2 minutes; 1 cycle of 95°C for 10 minutes; and 40 cycles of 95°C for 30 seconds and 60°C for 1 minute).

To detect total p8TR-gB, 2 µg of Hirt DNA was digested with 30 U of *EcoRV* (NEB) in NEB buffer #2 in a 50-µl reaction overnight at 37°C, followed by heat inactivation of *EcoRV* at 80°C for 30 min. PCR amplification was performed in triplicate using 3.75 µl of the *EcoRV* digestion as template. Amplified DNA was quantified using a standard curve from a p8TR-gB plasmid dilution series. To detect replicated p8TR-gB, 6 µg of Hirt DNA was digested overnight at 37°C with 60 U *DpnI* in NEB buffer #2 in a 50-µl reaction volume, and this was followed by exonuclease III (*ExoIII*) (NEB) treatment (120 U) for 30 min at 37°C to reduce background due to incompletely *DpnI*-digested DNA [34]. To detect non-replicated p8TR-gB, 6 µg of Hirt DNA was digested overnight at 37°C with 60 U *MboI* in NEB buffer #2 in a 50-µl reaction volume, and this was followed by *ExoIII* (120 U) digestion for 30 min. After *ExoIII* heat inactivation of the *DpnI* or *MboI* digestions at 70°C

for 30 minutes, final volume of the digestions were increased to 60 µl using NEB buffer #2, and the DNA was digested with 80 U of *EcoRV* at 37°C for at least 6 hours. *EcoRV* was then heat-inactivated at 80°C for 30 min. PCR amplification was performed in triplicate with 4.5 µl of each digestion as a template.

As an internal control for consistency between Hirt DNA preparations, contaminating cell DNA, which regularly occurs during Hirt DNA extraction, was detected by quantitative PCR amplification of the cell CD21 promoter region in the experiments shown in Fig. 5. For the PCR reaction, 600 nM primers (CD21forward GTGAGTCTGAGCCAA AGAGTG and CD21reverse ACACGTGCTAGTTCTG AGC) that amplify a 136-bp fragment were combined with 1x Power SYBR Green PCR Master Mix (Applied Biosystems) and 60 ng of Hirt DNA in a volume of 20 µL. PCR reactions were performed using the same conditions as above, and a final dissociation stage confirmed the expected melting point. Similar Ct numbers of ~25 were obtained in all cases, confirming that similar amounts of Hirt DNA were used for digestions.

Southern blot

For Southern blot analysis, 5 µg of Hirt DNA isolated at 72 hours post-transfection was digested overnight at 37°C with 40 U *EcoRV* to linearize p8TR-gB. Double digestion of 20 µg of Hirt DNA with *EcoRV* (80 U) and *DpnI* (70 U) was also performed overnight at 37°C. Alternatively, 20 µg of Hirt DNA was digested with 70 U *DpnI* overnight, followed by *ExoIII* digestion (400 U) for 30 minutes. After heat inactivation at 70°C for 30 minutes, *ExoIII*-treated samples were digested with 80 U *EcoRV* to linearize the *DpnI*-resistant p8TR-gB. The complete digestions were separated in a 0.8% agarose gel, transferred to a nylon membrane, and detected by Southern blotting with a ³²P-radiolabeled TR probe. Quantitation of the linearized *EcoRV*- or *EcoRV/DpnI*-digested p8TR-gB was performed using a PhosphorImager SI (Molecular Dynamics).

Results

Development of a PCR assay to quantitatively assess LANA-mediated DNA replication

We developed a real-time PCR assay to assess LANA-mediated DNA replication in a rapid and quantitative fashion, since the typical approach, Southern blot analysis, is cumbersome, requires a radioisotope, and is time-consuming. First, we constructed an appropriate target plasmid for PCR amplification. C-terminal LANA binds to KSHV TR DNA to mediate DNA replication. KSHV TRs are extremely GC-rich (84.5% GC), making PCR of this sequence difficult, and each TR contains only a single *DpnI* site. We modified p8TR [3], which contains 8 copies of the 0.8-kb TR sequence, to facilitate its use in a PCR-based assay to detect LANA-mediated DNA replication. A region of the HSV-1 gB sequence that has been used as a real-time PCR target [7, 28] was altered by introduction of three *DpnI* GATC restriction sites and inserted adjacent to the TRs in p8TR to generate p8TR-gB (Fig. 1). The introduction of the *DpnI* sites allows detection of LANA replicated p8TR-gB DNA, since *DpnI* cleaves at its restriction site when it is methylated after growth in Dam-methylase-positive bacteria but not after LANA-mediated replication in mammalian cells. Therefore, only LANA-replicated p8TR-gB is expected to be PCR-amplified after *DpnI* digestion. Since incomplete *DpnI* digestion can be problematic in a sensitive PCR assay, we used three *DpnI* sites in the qPCR target sequence to reduce the risk of amplification in the setting of incomplete *DpnI* digestion, since cleavage at any of the three *DpnI* sites will abolish amplification (Fig. 1).

We conducted experiments to compare the efficiency of PCR detection of circular, covalently closed p8TR-gB with that of linearized plasmid. Ten fold dilutions of p8TR-gB

plasmid were amplified directly by PCR, either after linearization with *Bgl*III or after linearization with *Eco*RV. Digestion with *Eco*RV, which cuts between the inserted modified gB sequence and the TRs (Fig. 1), enhanced PCR detection of the plasmid DNA compared to uncut or *Bgl*III-linearized plasmid (Table 3) by ~2–3 Ct units. These results suggested that the close proximity to the GC-rich TRs decreased PCR amplification and detection of the target sequence. For this reason, *Eco*RV digestion was subsequently performed prior to all PCR amplifications.

Detection of replicated DNA by Southern blot analysis

To serve as a comparison for the real-time PCR assay, we performed traditional Southern blot analysis to detect LANA-mediated replicated DNA. Uninfected BJAB B lymphoma cells, BJAB cells stably expressing wild type LANA (either N-terminal FLAG epitope tagged or N-terminal T7 epitope tagged), or two independently derived BJAB cell lines stably expressing LANA with alanine substitutions at residues 14 and 15 (LANA₁₄TG₁₅) [3] were transfected with p8TR-gB. Analysis of protein expression showed that all of the stable cell lines expressed LANA at similar levels, with the exception of TG-2, which expressed LANA₁₄TG₁₅ at a modestly higher level (Fig. 2A). LANA₁₄TG₁₅ is moderately diminished in DNA replication, chromosome association, and episome persistence [3, 4]. Seventy-two hours post-transfection, low-molecular-weight DNA was purified from cells using the Hirt method [15] and digested with *Eco*RV to linearize p8TR-gB (Fig. 2A, lanes 5 and 9–13), with *Eco*RV and *Dpn*I to assess replication (Fig. 2A, lanes 6 and 15–19), or with *Dpn*I, *Exo*III, and *Eco*RV to assess replication. Digestion with *Exo*III was also included to parallel the PCR digestion protocol (Fig. 2A, lanes 7 and 21–25).

Southern blot detection of *Eco*RV-digested Hirt DNA (without *Dpn*I digestion) exhibited differing levels of linearized p8TR-gB DNA. The WT LANA lanes had the highest levels of DNA (Fig. 2B, lanes 9–11, arrowhead), LANA₁₄TG₁₅ had intermediate levels of DNA (Fig. 2B, lanes 12, 13), and control BJAB cells lacking LANA had the lowest level (Fig. 2B, lane 5) (longer exposure in panel at right). Linearized DNA is expected to include all transfected DNA and is sometimes used as an indication of transfection efficiency. Although it is possible that the lower levels of linearized p8TR-gB in BJAB cells and BJAB cells expressing LANA₁₄TG₁₅ might be due to reduced transfection efficiency, this was not the case, since the amount of p8TR-gB DNA detected by PCR from these cells at 24 hours post-transfection was not reduced (discussed below). The more likely explanation is that transfected DNA is lost in cells lacking WT LANA. LANA binds TR DNA and targets it to newly formed nuclei after mitosis by tethering DNA to mitotic chromosomes. In cells lacking LANA or expressing LANA₁₄TG₁₅, which has reduced chromosome-tethering ability [3, 4], p8TR-gB DNA may localize to the cytoplasm after mitosis, where it is expected to be degraded. Further, LANA replicated DNA is expected to add to the total amount of p8TR-gB. Less p8TR-gB will be added by LANA₁₄TG₁₅, due to its deficient replication, and none will be added in the absence of LANA.

As expected, *Dpn*I-resistant, linearized p8TR-gB DNA was detected in all three WT LANA lanes (Fig. 2B, lanes 15–17, arrowhead). Partially replicated (Fig. 2B, lanes 15–17, upper vertical line) and non-replicated DNA (Fig. 2B, lanes 15–17, lower vertical line) was also present and migrated beneath the full-length, linearized p8TR-gB. A reduced amount of *Dpn*I-resistant DNA was detected for LANA₁₄TG₁₅ (Fig. 2B, lanes 18, 19), and no *Dpn*I-resistant DNA was detected in the absence of LANA (Fig. 2B, lane 6). The reduced *Dpn*I-resistant DNA band detected for LANA₁₄TG₁₅ was consistent with previous results [3]. The inclusion of *Exo*III resulted in an absence of partially replicated and non-replicated DNA (Fig. 2B, lanes 21–25) due to *Exo*III digestion at the termini where *Dpn*I had cut. *Exo*III also resulted in somewhat less signal for full-length, linearized p8TR-gB, perhaps due to digestion at nicks that may have occurred during Hirt DNA preparation.

LANA reduces the rate of loss of p8TR-gB DNA from cells

We also investigated the levels of p8TR-gB DNA using real-time PCR. The amount of p8TR-gB DNA present 24 and 72 hours post-transfection was assayed after transfection of BJAB cells or BJAB cells expressing LANA or LANA_{14TG15}. At 24 hours after transfection with 5 µg of p8TR-gB, similar amounts of DNA were present in all transfections, with the exception of LANA_{14TG15} (TG-1), which had ~58 pg compared to ~30 pg for the other transfections (Fig. 3A; results are the average of four experiments). Transfection of BJAB cells or LANA expressing cells (WT-2) with tenfold less p8TR-gB (0.5 µg instead of 5 µg) resulted in about tenfold less DNA detected (~3 pg compared to ~30 pg) (Fig. 3A, right). Therefore, similar amounts of p8TR-gB were present at 24 hours after transfection of cells either expressing or lacking LANA, which is consistent with generally similar transfection efficiencies. Notably, DNA from the experiment shown in Fig. 2 was included in the analysis and had levels similar to those in the other experiments, indicating that decreased transfection efficiencies did not account for the reduced linearized p8TR signal in the BJAB and LANA_{14TG15} lanes.

To investigate p8TR-gB levels over time, DNA quantities were again assessed at 72 hours. After transfection of BJAB cells with 5 µg of p8TR-gB in the absence of LANA, 4.4 pg of DNA was present at 72 hours (Fig. 3B) compared to 30.7 pg in BJAB cells at 24 hours—a 7.0-fold decrease (Fig. 3C). Seventy-two hours after transfection of WT LANA-expressing cells (Fig. 3B), 10.4 pg (WT-1), 9.1 pg (WT-2), and 10.0 pg (WT-3) of p8TR-gB DNA was present, compared to 27.5 pg (WT-1), 36.7 pg (WT-2), and 34.0 pg (WT-3) at 24 hours post-transfection, resulting in 2.6-, 4.0-, and 3.4-fold decreases, respectively (Fig. 3C). LANA_{14TG15} had 8.7 pg (TG-1) and 5.7 pg (TG-2) DNA at 72 hours, compared with 58.3 pg and 27.6 pg at 24 hours, respectively, resulting in 6.7- and 4.8-fold losses at 72 hours (Fig. 3C). Transfection with ten times less DNA (0.5 µg instead of 5 µg) gave similar results, with an 8.0-fold decrease for BJAB but only a 3.7-fold decrease for WT LANA (WT-2) at 72 hours (Fig. 3C). These results indicate a rapid loss of p8TR-gB DNA in all transfections between 24 and 72 hours, but the rate of loss was reduced in the presence of LANA.

Comparison of Southern blot with real-time PCR detection of p8TR-gB DNA

We quantitatively compared the results from Southern blot and real-time PCR. We compared linearized p8TR-gB DNA detected by Southern blotting with the p8TR-gB detected by real-time PCR in the same experiments at 72 hours post-transfection. The linearized DNA on Southern blots was quantified after collection of signal on a phosphorimager, and the amount was determined by interpolation on a plot of signal from known amounts of p8TR-gB plasmid DNA. The amounts of DNA detected by Southern blot and real-time PCR were then compared after accounting for the amount of input Hirt DNA (Table 4, second column). After transfection with WT LANA, the amount of p8TR-gB DNA detected by PCR was ~3 times (range of 2.84–3.30) greater than that detected by Southern blot. However, after transfection of BJAB cells lacking LANA, 13.18 times more p8TR-gB DNA was detected by real-time PCR than by Southern blot. Similarly, LANA_{14TG15} had significantly more p8TR-gB DNA detected by real-time PCR compared to Southern blot, resulting in ratios of 7.88 and 5.06 (of real-time-PCR- to Southern-blot-detected DNA) for the two different LANA_{14TG15} clones. This higher rate of detection of p8TR-gB by PCR may be at least partially due to degradation of p8TR-gB in the absence of WT LANA. Only the full-length, linearized p8TR-gB band was quantified from the Southern blot, and this does not account for any degraded p8TR-gB DNA migrating below the linear form. In contrast, real-time PCR amplifies only a 96-bp target sequence, which is likely to be present in partially degraded p8TR-gB. Consistent with this possibility, in some experiments, Southern blots had detectable smears migrating below the full-length, linearized p8TR-gB

bands, and the relative intensities of the smear signal to that of the linearized p8TR was disproportionately greater for BJAB cells and BJAB cells expressing LANA_{14TG15} than for BJAB cells expressing LANA (data not shown). Therefore, compared to Southern blot analysis, PCR was more sensitive for detection of total p8TR-gB DNA, especially when LANA was absent or deficient.

Detection of replicated DNA by real-time PCR

We used real-time PCR to assess LANA-replicated DNA at 72 hours after transfection of BJAB cells, BJAB cells expressing LANA, or BJAB cells expressing LANA_{14TG15} with p8TR-gB. Hirt DNA was digested with *DpnI*, followed by *ExoIII* digestion to further digest any p8TR-gB that was incompletely digested with *DpnI*. After transfection with 5 µg p8TR-gB, real-time PCR detected 5.1, 3.0, and 3.6 pg of p8TR-gB from the three WT LANA cell lines, compared to only 0.038 pg detected from BJAB cells lacking LANA, resulting in a 79–134-fold increase in detection from cells expressing LANA (Fig. 4A). Real-time PCR detected 0.58 and 1.03 pg of p8TR-gB DNA after transfection of cells expressing LANA_{14TG15}, which was 15- and 27-fold greater, respectively, than the BJAB control. The intermediate level of *DpnI*-resistant DNA for LANA_{14TG15} was expected due to its reduced replication and chromosome-targeting ability [3, 4]. After transfection with tenfold less p8TR-gB (0.5 µg), real-time PCR detected about tenfold less DNA in both LANA-expressing cells (compare 3.0 pg with 0.35 pg) and control cells (compare 0.038 pg with 0.003 pg), indicating a direct relationship between the amount of transfected and replicated DNA.

To account for differences in transfection efficiencies, results were normalized against the control using total p8TR-gB detected at 24 hours (Fig. 4B). Normalization resulted in 5.9, 2.8, and 3.4 pg of DNA from the three WT LANA cell lines, and 0.34 and 1.2 pg of DNA for the LANA_{14TG15} cell lines, compared to only 0.038 pg detected from BJAB cells lacking LANA. This resulted in a 74–155-fold increase in replicated DNA from cells expressing LANA and 9- and 32-fold increases for cells expressing LANA_{14TG15}. These results were similar to the results obtained without normalization (Fig. 4A).

Quantitative comparison of replicated p8TR-gB DNA detected by Southern blot and PCR

We compared the amount of replicated DNA detected by Southern blot with that detected by real-time PCR after adjusting for the amount of digested DNA used for the Southern blot and for the PCR reactions (Table 4, third and fourth columns). The Southern blot data used for comparison included those obtained using samples digested with *ExoIII*, similar to the PCR samples, and samples without *ExoIII* treatment. The amount of DNA on the Southern blot was determined by interpolation on a plot of signal vs. the amount of p8TR-gB plasmid. In the absence of *ExoIII* digestion prior to Southern blot, the ratios of replicated DNA detected by PCR to that detected by Southern blot were slightly less than one and ranged from 0.71 to 0.98 (Table 4, third column). However, when the DNA was treated with *ExoIII* prior to Southern blotting, similar to the treatment of PCR DNA, the ratios ranged between 0.93 and 1.58. These differences may be due to the fact that *ExoIII* can act at sites of DNA that may have been nicked during the process of Hirt extraction, resulting in somewhat less double-stranded, linearized p8TR-gB.

LANA reduces the rate of loss of non-replicated p8TR-gB DNA from cells

LANA's reduction in the rate of loss of p8TR-gB DNA between 24 and 72 hours post-transfection compared with that of BJAB cells (Fig. 3) may be due to the addition of replicated p8TR-gB and/or prevention of loss of p8TR-gB due to efficient segregation to daughter nuclei. In the absence of segregation to the nucleus, p8TR-gB is expected to be degraded in the cytoplasm. Prevention of p8TR-gB loss can be determined by assaying for

loss of non-replicated p8TR-gB. We therefore determined both replicated and non-replicated p8TR-gB DNA levels after transfection of BJAB cells or BJAB cells expressing WT-1 or TG-2. Prior to PCR detection, Hirt DNA was digested with either *DpnI*, to detect replicated DNA, or *MboI*, to detect DNA that was not replicated. *MboI* recognizes the same GATC restriction site as *DpnI*, but only digests the DNA when the restriction site is not methylated. This activity contrasts with that of *DpnI*, which only digests the restriction site after *dam* methylation, which occurs in *dam*-positive *E. coli*. This methylation is lost after replication in mammalian cells. Therefore, *MboI*-resistant p8TR-gB DNA has not been replicated, while *DpnI*-resistant DNA p8TR-gB has undergone replication in the BJAB cells. At 72 hours post-transfection, 8.0 pg of *DpnI*-resistant p8TR-gB was detected from LANA-expressing cells, 0.12 pg was detected from BJAB cells, and 1.8 pg was detected from LANA_{14TG15}-expressing cells (Fig. 5A). These levels of replicated p8TR-gB were similar to those detected in Fig. 4, although the BJAB control was modestly higher.

MboI-resistant DNA was detected at 24 and 72 hours post-transfection. At 24 hours, there were similar amounts of *MboI*-resistant p8TR-gB in BJAB cells (36.5 pg) and BJAB cells expressing LANA (40.1 pg) or LANA_{14TG15} (37.6 pg). However, at 72 hours post-transfection, the levels of *MboI*-resistant p8TR-gB were 2.7 pg, 8.9 pg, and 4.2 pg for BJAB cells and BJAB cells expressing LANA or LANA_{14TG15}, respectively (Fig. 5B). Therefore, between 24 and 72 hours, there was a 13.5-fold decrease in the amount of p8TR-gB in BJAB cells, while the decrease was only 4.5-fold in LANA-expressing cells (Fig. 5C). LANA_{14TG15}-expressing cells had an intermediate decrease of 9.0-fold. Comparison of replicated (*DpnI*-resistant) and non-replicated (*MboI*-resistant) p8TR-gB DNA from LANA-expressing cells at 72 hours revealed very similar levels (8.0 pg replicated and 8.9 pg non-replicated). Since both the *DpnI* and *MboI* digestions were treated with *ExoIII* nuclease, which eliminates nicked or partially replicated DNA, these results pertain to *ExoIII*-resistant DNA. In contrast, at 72 hours most of the LANA_{14TG15} DNA was non-replicated (*MboI* resistant) (4.2 pg) rather than replicated (*DpnI* resistant) (1.8 pg). Therefore, LANA, and to a lesser extent, LANA_{14TG15}, both replicated p8TR-gB DNA and reduced the rate of loss of non-replicated p8TR-gB from cells.

Discussion

In this study, we developed and validated a real-time PCR method to quantitatively assess LANA-mediated TR DNA replication and compared this approach to Southern blot analysis. The PCR approach has several strengths. It is more sensitive than the traditional Southern blot method since amounts as low as 0.003 pg can be detected (data not shown); 5 pg of DNA was the limit of detection for our Southern blots (Fig. 2A). Therefore, fewer cells are required using this approach. Furthermore, the real-time PCR method generates results much faster than the Southern blot method (3 hours versus 4–5 days for Southern blot), and many more samples can potentially be analyzed simultaneously using a multiwell plate format for PCR, raising the possibility of high-throughput approaches. Quantitative results are also obtained without the need for radioactivity or time-consuming phosphorimager analysis, which must be performed for Southern blots.

The differing amounts of linearized TR plasmid present on Southern blots between the control (Fig. 2A, lane 5), LANA (Fig. 2A, lanes 9–11), and LANA_{14TG15} lanes (Fig. 2A, lanes 12, 13) differs from previous observations in which the levels were similar [3, 13]. Nucleofection with Amaxa in these experiments may have affected the results, since the cells acidified the medium more quickly than we have previously observed after transfection by electroporation. A rapid recovery of cells after nucleofection may lead to earlier re-entry into the cell cycle and an increased number of cell divisions prior to harvest, which would increase the cumulative effect of absent or inefficient LANA targeting p8TR-gB to the

nucleus, resulting in increased DNA degradation in the absence of WT LANA. In addition, more cell divisions would increase DNA replication, adding to the total TR DNA, in the presence LANA. Furthermore, in some previous experiments, cells were harvested at 48 hours instead of 72 hours [3]. (Forty-eight to 72 hours is the typical time window during which cells are harvested in experiments assessing LANA-mediated replication [13, 16, 21, 25, 26].) The shorter time course would allow fewer cell divisions and less opportunity for both DNA degradation and replication. It is also possible that cells lacking LANA could have had higher transfection efficiencies, resulting in increased TR DNA. Other studies have examined LANA-mediated DNA replication after co-transfection of cells lacking LANA with a LANA expression vector and a TR plasmid [16, 19, 25, 26]. These experiments are intrinsically different from the ones presented here due to the transient nature of LANA expression and an initial period of time during which TR plasmid is present in cells in the absence of LANA. However, it is interesting to note that in a number of experiments [16, 25, 26], the linearized TR plasmid was at lower levels in the absence of LANA compared to in its presence, similar to our observations here.

This work indicates that not only does LANA mediate p8TR-gB replication, but it also reduces the rate of loss of transfected, non-replicated p8TR-gB DNA from cells. In the absence of LANA, non-replicated p8TR-gB DNA was lost at a much faster rate from BJAB cells than from LANA-expressing cells (13.5-fold loss between 24 and 72 hours versus a 4.5-fold loss) (Fig. 5C). This finding is consistent with the model that LANA segregation of episomes to the nucleus protects DNA from destruction in the cytoplasm. This effect was prominent despite the fact that some non-replicated p8TR-gB DNA is lost in LANA-expressing cells as a direct result of LANA-mediated replication. LANA_{14TG15} also reduced the rate of non-replicated p8TR-gB loss compared to BJAB cells (9.0-fold loss at 72 hours for BJAB cells expressing LANA_{14TG15}, versus 13.5-fold loss for BJAB cells), but the effect was significantly less than that of LANA. This finding is consistent with LANA_{14TG15}'s reduced chromosome binding and partitioning ability [4].

Consistent with its ability to replicate p8TR-gB and protect non-replicated p8TR-gB from degradation, LANA decreased the rate of loss of total p8TR-gB DNA from cells (Fig. 3). Interestingly, at 72 hours post transfection, similar amounts of replicated and non-replicated p8TR-gB were present in LANA-expressing cells (Fig. 5). The finding that a ~3-fold loss of p8TR-gB occurred in LANA-expressing cells between 24 and 72 hours post-transfection (Fig. 3C) may relate at least in part to an initial excess of transfected p8TR-gB molecules relative to the binding capacity of LANA, resulting in an absence of LANA nuclear targeting for the excess p8TR-gB molecules and leading to degradation. LANA_{14TG15}'s reduced ability to both segregate and replicate KSHV DNA led to intermediate rates of DNA loss of 4.8–6.7-fold at 72 hours (Fig. 3C), greater than the ~3-fold loss with WT LANA, but less than the 7-fold loss in the absence of LANA.

The rapid loss of transfected TR DNA and the finding that LANA reduced this rate of loss, is consistent with other work. A GFP reporter system used to track TR-associated DNA demonstrated a rapid loss of GFP signal and showed that LANA reduced the rate of loss during the first seven days post-transfection [32]. After seven days, the loss of the GFP signal in LANA-expressing cells ceased and remained stable. Similar results were observed with the Epstein-Barr virus (EBV) episome maintenance protein EBNA1, which acts on EBV oriP DNA to mediate episome persistence [24]. After transfection of cells with oriP DNA, a precipitous loss of oriP DNA occurred over a two-week period, but the rate of loss was lower in the presence of EBNA1. Of note, in contrast to newly transfected oriP DNA, oriP episomes established and maintained under drug selection are lost at a much slower rate of 2–4% per cell division after removal of drug selection [20, 24]. The higher stability of

established episomes for both EBV and KSHV are likely due to epigenetic events occurring on the viral episomes [14, 24].

The PCR-based assay described here should be useful for assessing LANA-mediated replication in a rapid and quantitative fashion, without the need for radioisotopes. This assay could be used to determine the relative replication deficiency of LANA mutants and to investigate the effects of cell proteins on LANA replication. Furthermore, this assay could be adapted to assess replication of other viral proteins that mediate DNA replication. In such a case, the TRs could be replaced with the cis-acting DNA element upon which the other viral protein acts.

Acknowledgments

This work was supported by grants (KMK) from the National Cancer Institute (CA082036 and CA082036S1) and from the US Department of Defense (PR093491). Bo Zhao provided advice regarding CD21 promoter amplification.

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Fig. 1.

Schematic diagram of the p8TR-gB plasmid. p8TR-gB was generated by inserting a 138-bp fragment into the *Hind*III site of p8TR [3]. The insert consists of a portion of the HSV-1 gB sequence modified to contain three *Dpn*I (GATC) sites adjacent to eight KSHV terminal repeat elements (8TR). The location of annealing sites for amplification primers (arrows) and fluorescent probes are indicated. “F,” FAM fluorophore; “T,” TAMRA quencher.

Fig. 2. Immunoblot of LANA and Southern blot detection of LANA-mediated DNA replication. (A) Immunoblot with LANA immune serum. KSHV-infected BCBL-1 primary effusion lymphoma cells (BCBL-1), control (BJAB), or BJAB cells stably expressing WT-1, WT-2, WT-3, TG-1, and TG-2 cells are shown. Each lane contains 1.5×10^5 cells. LANA is typically detected as doublet migrating at ~222 kDa and ~234 kDa along with a series of faster-migrating species [23, 30]. Tubulin was also detected from the same blot and is shown beneath. (B) BJAB (control) or BJAB cells stably expressing full-length LANA (WT) or LANA₁₄TG₁₅ were transfected with 5 μ g of p8TR-gB. After 72 h, low-molecular-weight DNA was isolated using the Hirt method, and 5 μ g was digested with *EcoRV*, 20 μ g was digested with *DpnI* and *EcoRV*, and 20 μ g was digested with *DpnI*, *ExoIII*, and *EcoRV*. DNA was resolved in an agarose gel, followed by Southern blotting and detection with the TR probe. Lanes 1–3, the indicated amounts of p8TR-gB plasmid; lanes 5–7, BJAB; lanes 9–11 and lanes 21–23, BJAB cell lines stably expressing LANA (WT-1, WT-2, WT-3). WT-1 is an N-terminal FLAG-epitope-tagged LANA, and WT-2 and WT-3 are N-terminal T7-epitope-tagged LANA. Lanes 12–13 and 24–25, independently derived BJAB cell lines stably expressing LANA₁₄TG₁₅ (TG-1, TG-2). Lane 26, p8TR-gB plasmid double-digested with *DpnI* and *EcoRV*. The arrowhead indicates linearized p8TR-gB. Non-replicated *DpnI*-digested DNA (lower vertical line) and partially replicated *DpnI*-digested DNA (upper vertical line) are indicated. Exposure to film was for 12 hours. The panel at the right shows lanes 5–7 after exposure for 72 hours. This result is representative of three experiments.

Fig. 3.

Detection of p8TR-gB DNA by real-time PCR. BJAB cells (control) or BJAB cells stably expressing LANA (WT) or LANA₁₄TG₁₅ were transfected with 5 μ g or 0.5 μ g of p8TR-gB. Three cell lines for LANA WT (WT-1, WT-2, WT-3) and two cell lines for LANA₁₄TG₁₅ (TG-1, TG-2) were tested. Hirt DNA was isolated at 24 and 72 hours post-transfection and digested with *EcoRV*. **(A)** Amount of p8TR-gB at 24 hours post-transfection. **(B)** Amount of p8TR-gB at 72 hours post-transfection. **(C)** The amount of p8TR-gB present at 24 hours post-transfection was divided by the amount of DNA present at 72 hours to determine the fold decrease in p8TR-gB DNA. Results are an average of four experiments. Standard deviation is shown for panels **(A)** and **(B)**.

Fig. 4.

Detection of replicated p8TR-gB by real-time PCR. Cells and transfections are as described in Fig. 3. Hirt DNA harvested at 72 hours post-transfection was digested with *DpnI*, then treated with *ExoIII* and *EcoRV*, and amplified by real-time PCR. **(A)** Total amount of replicated p8TR-gB detected at 72 hours post-transfection. **(B)** Amount of replicated p8TR-gB normalized for transfection efficiency. To normalize, the amount of p8TR-gB detected at 24 hours post-transfection for each cell line was divided by the amount present in the BJAB control at 24 hours from that experiment (normalization ratio). Each replicated p8TR-gB result at 72 hours was then divided by the corresponding normalization ratio for that cell line. Results are an average of four experiments, and standard deviation is shown.

Fig. 5.

Detection of replicated and non-replicated p8TR-gB DNA. Hygromycin-resistant BJAB cells (control) or BJAB cells stably expressing LANA (WT-1) or LANA₁₄TG₁₅ (TG-2) were transfected with 5 µg of p8TR-gB. **(A)** To detect replicated p8TR-gB DNA, Hirt DNA was isolated at 72 hours post-transfection and digested with *DpnI*, then treated with *ExoIII* and *EcoRV*, and amplified by real-time PCR. **(B)** To detect non-replicated p8TR-gB DNA, Hirt DNA was isolated at 24 and 72 hours post-transfection, digested with *MboI*, then treated with *ExoIII* and *EcoRV*, and amplified by real-time PCR. **(C)** The fold decrease in non-replicated p8TR-gB DNA is shown from 24 to 72 hours for BJAB, WT-1, and TG-2. The average amounts (from panel **(B)**) of non-replicated p8TR-gB DNA at 24 hours were divided by the average amounts of non-replicated p8TR-gB DNA at 72 hours. Results are an average of three experiments, and standard deviation is shown in panels **(A)** and **(B)**. For **(A)** and **(B)**, normalization for transfection efficiencies was performed as in Fig. 4B, using the total amount of p8TR-gB detected at 24 hours post transfection to generate normalization ratios.

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#01	1. SAY THE LINE AND TRY TO SAY AGAIN WITH THE SAME MEANING
#02	2. CHOOSE THE LINE THAT BEST MEANS THE SAME THING
#03	3. SAY THE LINE AND TRY TO SAY AGAIN WITH THE SAME MEANING
#04	4. CHOOSE THE LINE THAT BEST MEANS THE SAME THING
#05	5. SAY THE LINE AND TRY TO SAY AGAIN WITH THE SAME MEANING
#06	6. CHOOSE THE LINE THAT BEST MEANS THE SAME THING
#07	7. SAY THE LINE AND TRY TO SAY AGAIN WITH THE SAME MEANING
#08	8. CHOOSE THE LINE THAT BEST MEANS THE SAME THING

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Table 2

Oligonucleotides used for real-time PCR

gB-F	5' TCA AGA CCA CCT CCT CCA TGA 3'
gB-R	5' CGG CCC AAC ATA TCG TTG AC 3'
Fluorescent probe	5' FAM-TCG CCC GGC TGA TCG CAG TT-TAMRA 3'

Boxes indicate nucleotides within *DpnI* sites. TAMRA-quencher (6-carboxy-tetramethylrhodamine). FAM-reporter (6-carboxy-fluorescein)

Table 3*Eco* RV digestion of p8TR-gB increases real-time PCR detection

Plasmid DNA copy number in PCR reaction	C _t value for circular plasmid	C _t value for <i>Bgl</i> II-linearized plasmid	C _t value for <i>Eco</i> RV-linearized plasmid
5 × 10 ⁶	18.9	NT	16.8
5 × 10 ⁵	22.2	21.82	19.7
5 × 10 ⁴	25.7	25.6	22.8
5 × 10 ³	29.2	NT	25.8
500	32.5	NT	28.3

NT not tested

Table 4

Comparison of p8TR-gB DNA detected by PCR and Southern blot at 72 hours post-transfection

Cell line	p8TR-gB DNA detected by PCR/ <i>EcoRV</i> -linearized DNA detected by Southern blot	Replicated DNA detected by qPCR/ replicated DNA detected by Southern blot	Replicated DNA detected by qPCR/ replicated <i>ExoIII</i> treated DNA detected by Southern blot
Control	13.18	–	–
WT-1	2.84	0.98	1.58
WT-2	2.88	0.83	1.43
WT-3	3.30	0.95	1.41
TG-1	7.88	0.71	0.93
TG-2	5.06	0.84	1.09