

# Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells

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**MicroRNAs (miRNAs) are an endogenously encoded class of small RNAs that have been proposed to function as key posttranscriptional regulators of gene expression in a range of eukaryotic species, including humans. The small size of miRNA precursors makes them potentially ideal for use by viruses as inhibitors of host cell defense pathways. Here, we demonstrate that the pathogenic human herpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) encodes an array of 11 distinct miRNAs, all of which are expressed at readily detectable levels in latently KSHV infected cells. Individual KSHV miRNAs were expressed at up to 2,200 copies per cell. The KSHV miRNAs are expressed from what appears to be a single genetic locus that largely coincides with an ≈4-kb non-coding sequence located between the KSHV *v-cyclin* and *K12/Kaposin* genes, both of which are also expressed in latently infected cells. Computer analysis of potential mRNA targets for these viral miRNAs identified a number of interesting candidate genes, including several mRNAs previously shown to be down-regulated in KSHV-infected cells. We hypothesize that these viral miRNAs play a critical role in the establishment and/or maintenance of KSHV latent infection *in vivo* and, hence, in KSHV-induced oncogenesis.**

RNA interference | viral pathogenesis | viral latency

**M**icroRNAs (miRNAs) are a class of ≈22-nt-long noncoding RNAs found in all metazoan eukaryotes, and >200 different miRNAs have so far been identified in humans (reviewed in ref. 1). miRNAs are initially transcribed as part of one arm of an ≈80-nt-long RNA stem-loop that in turn forms part of a longer transcript called a primary miRNA (pri-miRNA) (2). The first step in miRNA processing is mediated by the nuclear RNase III enzyme Droscha, which cleaves pri-miRNA stem-loops to release the ≈60-nt long pre-miRNA hairpin intermediate (3). After export to the cytoplasm, pre-miRNAs are further processed by the RNase III enzyme Dicer to give an ≈22-bp miRNA duplex (4, 5). One strand of this duplex is then incorporated into the RNA-induced silencing complex (RISC), where it acts to guide RISC to mRNAs bearing complementary sequences (6–8). RISC binding to mRNA can inhibit gene expression by one of two mechanisms. If the mRNA bears an essentially perfectly complementary sequence, binding induces cleavage by the RISC component Ago2, leading to mRNA degradation (9–11). However, if the mRNA contains an imperfectly complementary target, RISC binding may instead induce translational inhibition (11–13). Unlike mRNA cleavage, which can be mediated by a single RISC, translation inhibition is highly cooperative and may require binding by several RISCs, potentially bearing different miRNAs, to be effective (14).

Successful viral replication depends on the ability of the infecting virus to hijack the biosynthetic machinery of target cells while simultaneously inactivating innate host cell defense mechanisms, such as the IFN pathway or apoptosis induction. Although many viruses encode proteins that specifically inactivate host cell defense factors, the small size of viral genomes limits the utility of this approach. The small size of miRNA stem-loop precursors could offer an attractive alternative way for these

intracellular pathogens to turn off specific host genes. However, because miRNAs act exclusively on mRNAs, they cannot exert a marked phenotypic effect until the preexisting encoded protein has decayed. Because many lytic viral life cycles are very short, miRNAs may therefore not represent a useful tool for lytic viruses to modify the host cell environment. In contrast, in the case of viruses that routinely establish latent infections as part of their life cycle, miRNAs may represent an effective way to reshape host cell gene expression to their benefit.

Among virus families, one stands out as using latent infection as an almost invariant component of their life cycle, i.e., the herpesviruses. Moreover, an analysis of one human family member, Epstein–Barr virus (EBV), has recently identified five viral miRNAs and shown that these are expressed in latently EBV infected cells (15). Here, we extend these earlier data by demonstrating that a second, distantly related pathogenic human herpesvirus, termed Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8, encodes at least 11 previously undiscovered miRNAs that are all expressed in latently KSHV infected cells. The expression of an array of these viral miRNAs in KSHV-infected cells suggests that down-regulation of host cell mRNAs by miRNA-mediated RNA interference may represent a critical step in the establishment and/or maintenance of latent infections by KSHV.

## Methods

**Cell Culture and RNA Preparation.** BC-1, BCBL-1 and BJAB cells were maintained in RPMI medium 1640 supplemented with 20% FCS. Where necessary, TPA (Sigma, final concentration 25 ng/ml) was added for 48 h before RNA preparation. Total cellular RNA was prepared by using Trizol reagent (Invitrogen).

**Cloning of Small RNAs.** The cloning of small RNAs from BC-1 cells was conducted as described by Lau *et al.* (16) using 750 μg of BC-1 total RNA. Classification of small RNA cDNA sequences was based on cDNA sequence analysis in the GenBank database ([www.ncbi.nlm.nih.gov/GenBank/index](http://www.ncbi.nlm.nih.gov/GenBank/index)), the miRNA registry database ([www.sanger.ac.uk/Software/Rfam/mirna](http://www.sanger.ac.uk/Software/Rfam/mirna)), the rRNA database ([www.psb.ugent.be/rRNA/blastrrna.html](http://www.psb.ugent.be/rRNA/blastrrna.html)), and human tRNA database (<http://rna.wustl.edu/GtRDB/Hs/Hs-seqs.html>).

**RT-PCR and Northern Blot Analyses.** RT-PCR and Northern blot analyses were performed as described (17) except that the annealing temperature during the PCR amplification was set at 50°C and 30 PCR cycles were performed for all KSHV sequences. To remain in the linear range of the assay, only 25 cycles were run for the cellular *GAPDH* mRNA. Thirty micrograms of

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Abbreviations: miRNA, microRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; EBV, Epstein–Barr virus; KSHV, Kaposi's sarcoma-associated herpesvirus; PEL, primary effusion lymphoma.

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**Table 1. Composition of the small RNA cDNA library obtained from BC-1 cells**

Type	Number of clones	Percentage
KSHV miRNAs	167	18
EBV miRNAs	222	23
Cellular miRNAs	168	18
Noncoding RNA fragments	182	19
mRNA fragments*	177	18
Not annotated*	28	3
Not matched	15	2
Total number sequenced	959	100

\*It is possible that there are cellular miRNAs in these classes that were not identified in this study.

total RNA per sample was used in each Northern blot. The specific probe used for each miRNA was the full-length antisense DNA oligonucleotide. The probe used for the U6 Northern analysis was 5'-CGTTCCAATTTTAGTATATGTGCTGC-CGAAGCGA-3'. The primers used for RT-PCR are listed in *Supporting Text*, which is published as supporting information on the PNAS web site.

## Results

Although KSHV is primarily known as the etiologic agent of Kaposi's sarcoma, KSHV is also associated with two B-cell derived cancers termed primary effusion lymphoma (PEL) and multicentric Castleman's disease (18–21). Several B cell lines derived from PEL patients have been described; we selected one of these, BC-1, for analysis (22). BC-1 was chosen because latent KSHV infection of these cells is stable under normal growth conditions, with only very few cells ( $\leq 2\%$ ) undergoing spontaneous activation of lytic replication (23), and because the KSHV genome resident in BC-1 cells has been fully sequenced. Of note, BC-1, like many other PEL B cell lines, is also latently coinfecting with EBV (23).

We size selected and cDNA cloned 18- to 24-nt-long RNAs from BC-1 cells (16) and sequenced 959 different cDNA clones. As shown in Table 1,  $\approx 18\%$  of the short RNAs obtained were derived from KSHV, and a further  $\approx 23\%$  were of EBV origin. Cellular miRNAs accounted for  $\approx 18\%$  of the short RNA clones obtained, and this grouping included 34 different miRNAs, several of which have been reported only in rodents (Table 3, which is published as supporting information on the PNAS web site). The remaining cDNAs, representing  $\approx 41\%$  of the total,

represent breakdown products of cellular noncoding RNAs, mRNAs, or cellular transcripts of unknown origin.

Analysis of the KSHV sequences identified 14 distinct candidate viral miRNAs, with the most prevalent, named miR-K7, being cloned 51 times (Table 2). Criteria for validation of the authenticity of candidate miRNAs have been established (24). In addition to identification in a library of size fractionated RNA (Table 2), these include detection of the  $\approx 22$ -nt RNA by Northern analysis. Moreover, as noted above, a defining characteristic of authentic miRNA is that they are derived by processing of an  $\approx 80$ -nt precursor RNA hairpin.

Analysis of the KSHV sequence context of the 14 candidate miRNAs shown in Table 2 revealed that all sequences derived from computer-predicted RNA hairpins of the expected  $\approx 80$ -nt size (Fig. 1). As predicted based on previous analyses of human pri-miRNAs (16, 25, 26), the mature KSHV miRNAs all derived from one arm of the RNA hairpin and the precursor stem extended significantly beyond the mature miRNA sequence. This extension is required for efficient processing of the pri-miRNA precursor by Drosha (3, 25).

As noted above, miRNA processing gives rise to an  $\approx 20$ -bp miRNA duplex bearing 2-nt 3' overhangs. One strand of this duplex is then incorporated into RISC while the second is degraded (1). It has been proposed that selection of the incorporated strand depends on the relative stability of the base pairing around the 5' nucleotide of each strand, with the strand containing the less tightly base paired 5' end being selected for incorporation (27, 28). However, if the difference in base pairing is small, then each strand may be incorporated into RISC. Moreover, the nonincorporated strand, termed the star strand, may persist long enough to be captured during cloning, albeit at low frequency. Inspection of the predicted KSHV pri-miRNA hairpins (Fig. 1) shows that four of these, i.e., miR-K4, miR-K6, miR-K8, and miR-K9, give rise to two mature miRNAs. Following convention, the miRNA derived from the 5' arm of the stem-loop precursor is designated by a "5p" suffix, whereas the miRNA derived from the 3' arm is designated by "3p" (Table 2). Inspection of Table 2 shows that three of the four KSHV miRNAs, which derive from the RNA strand complementary to a more common KSHV miRNA, were only recovered once (miR-K6-5p and miR-K8-5p) or twice (miR-K9-3p). These three KSHV RNA may therefore represent the star strand of a miRNA duplex intermediate. However, miR-K4-3p was recovered six times, which suggests that it may be an authentic miRNA (see below).

**Table 2. KSHV miRNA sequences and their genomic location**

miRNA	Sequences 5' to 3'	Hits	Length, nt	KSHV sequence coordinates
miR-K1	AUUACAGGAAACUGGGUGUAAGC	5	22	121911–121890
miR-K2	AACUGUAGUCCGGUCGAUCUG	3	22	121750–121729
miR-K3	UCACAUUCUGAGGACGGCAGCGA	11	23	121608–121586
miR-K4-5p	AGCUAAACCGCAGUACUCUAG(G)	14	21–22	121476–121455
miR-K4-3p	UAGAAUACUGAGGCCUAGCUGA	6	22	121438–121417
miR-K5	UAGGAUGCCUGGAACUUGCCGG	11	22	121287–121265
miR-K6-5p	CCAGCAGCACCUGAAUCCUACGG	1	22	120817–120796
miR-K6-3p	UGAUGUUUUCGGGUGUUGAG	35	22	120786–120765
miR-K7	UGAUCCAUUGUUGCGCGCU(CA)	51	21–23	120380–120358
miR-K8-5p	CUCCUCACUAAACGCCCGC	1	20	120006–119987
miR-K8-3p	CUAGGCGGACUGAGAGAG(CA)	15	19–21	119967–119947
miR-K9-5p	ACCCAGCUGCGUAAACCCCGCU	9	22	119359–119338
miR-K9-3p	CUGGGUUAUCGCAGCUGCGUA	2	21	119325–119305
miR-K10	UAGUGUUGUCCCCCGAGUGGC	1	22	117991–117970

Sequence variation in recovered KSHV miRNAs is indicated by parentheses surrounding the variable nucleotides.

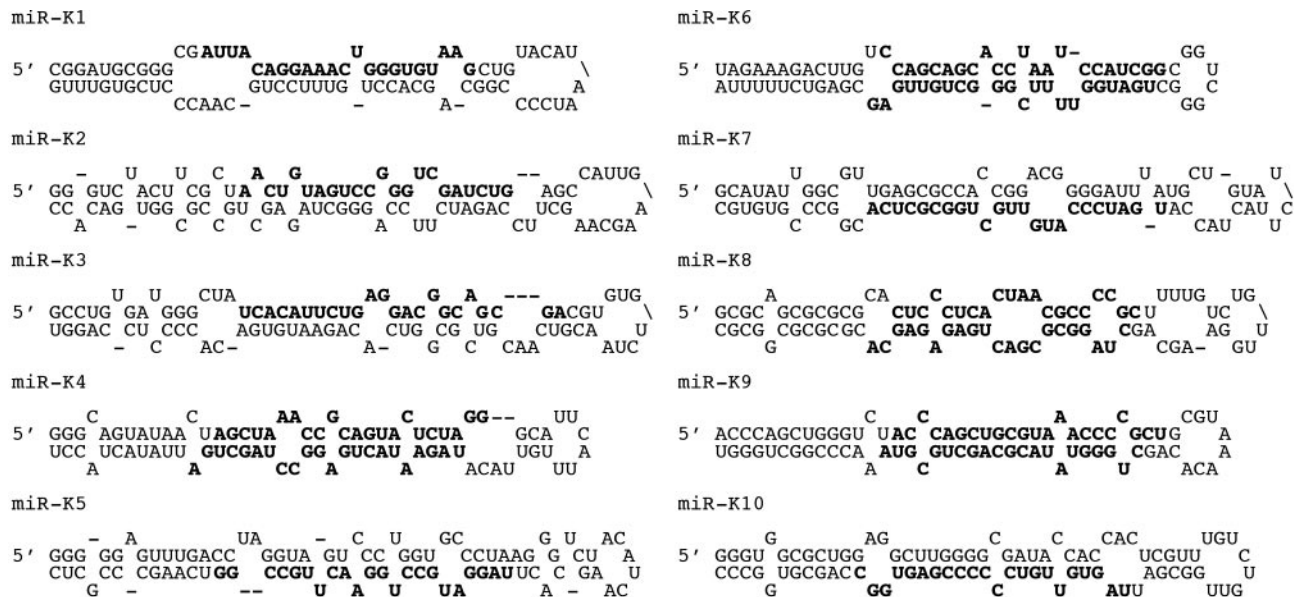


Fig. 1. Predicted stem-loop structures of KSHV pri-miRNAs. These structural predictions were derived by using MFOLD (46). Mature miRNA sequences are highlighted.

We next performed Northern analyses to confirm that the KSHV miRNAs listed in Table 2 are actually expressed in KSHV-infected cells. For this purpose, we derived RNA from BC-1 cells as well as from the cell lines BCBL-1 and BJAB. BCBL-1 cells are a second, independent PEL-derived cell line infected with KSHV, but these cells differ from BC-1 in that they are not coinfecting with EBV. Moreover, up to 5% of BCBL-1 cells spontaneously enter lytic KSHV replication under normal culture conditions (29, 30). BJAB is a B cell line that is not infected by KSHV or EBV and that here serves as a negative control. In addition to BC-1 and BCBL-1 cells cultured under normal conditions, we also analyzed BC-1 and BCBL-1 cells that had been treated with TPA for 48 h. TPA treatment induces lytic KSHV replication in a significant percentage of both BC-1 and BCBL-1 cells (23, 30), and this treatment was therefore designed to see whether lytic replication activated expression of any KSHV miRNA.

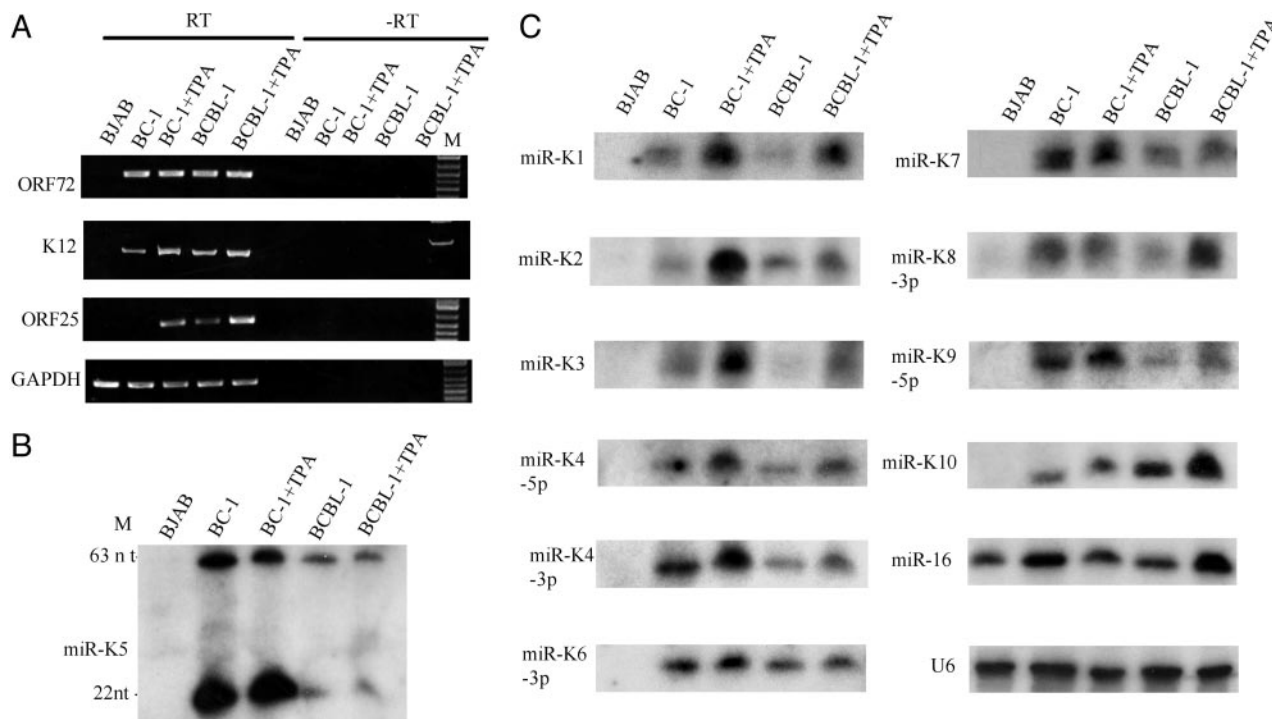
To confirm that TPA treatment was indeed inducing lytic KSHV replication, we used immunofluorescence to quantitate the number of BC-1 and BCBL-1 cells expressing the ORF59 protein, a KSHV early lytic protein, before and after TPA treatment (Fig. 4, which is published as supporting information on the PNAS web site). These data revealed that  $\approx 0.9\%$  of BC-1 cells expressed ORF59 before TPA treatment, whereas  $\approx 20\%$  expressed ORF59 after treatment. Similarly,  $\approx 4.3\%$  of BCBL-1 cells expressed ORF59 before TPA treatment and  $\approx 29\%$  after treatment. Analogous results were also obtained by quantitative competitive RT-PCR using primers specific for the *ORF50* gene, encoding the major activator of KSHV lytic replication (Fig. 4B). Therefore, although TPA treatment indeed induces a substantial increase in the level of lytic KSHV replication in both BCBL-1 and BC-1 cells, the majority of cells remain latently infected even after TPA treatment.

To confirm that the BC-1 cells used in this analysis were indeed expressing latent KSHV gene products under normal culture conditions, we performed an RT-PCR analysis of RNA preparations derived from BC-1, as well as from the BCBL-1 and BJAB cells, using primers specific for a latently expressed KSHV mRNA (*ORF72*, encoding v-cyclin), a gene expressed in latently infected cells that is activated during lytic infection (K12), and a gene characteristic of lytic infection (*ORF25*, which encodes

the major KSHV capsid protein) (31–33). As shown in Fig. 2A, noninduced BC-1 cells expressed *ORF72* mRNA and a low level of K12 transcripts, but *ORF25* mRNA was not detected, consistent with latent KSHV infection. Treatment of BC-1 cells with TPA increased expression of K12 and induced *ORF25* mRNA expression, consistent with induction of lytic KSHV replication. BCBL-1 cells, in contrast, expressed not only *ORF72* and K12, but also a low level of *ORF25* mRNA, consistent with the tendency of BCBL-1 to enter lytic replication spontaneously (29). However, TPA induction clearly enhanced the expression of K12 and *ORF25*, as expected. The control cell line BJAB was negative for all KSHV transcripts, but expressed a comparable level of the cellular *GAPDH* mRNA (Fig. 2A).

As noted above, miRNA processing proceeds through an  $\approx 60$ -nt pre-miRNA intermediate before giving rise to the mature  $\approx 22$ -nt miRNA (1). As shown in the Northern analysis presented in Fig. 2B, both the  $\approx 62$ -nt miR-K5 pre-miRNA and the 22-nt mature miR-K5 were readily detectable in both latently infected and TPA-treated BC-1 cells, with little or no activation by TPA. In BCBL-1 cells, the  $\approx 62$ -nt pre-miR-K5 RNA was readily detectable, but mature miR-K5 was expressed at only low levels. Finally, no miR-K5-specific signal was detectable in BJAB cells (Fig. 2B).

In Fig. 2C, we present a more complete Northern analysis of the expression of the mature KSHV miRNAs listed in Table 2. As may be readily observed,  $\approx 22$ -nt RNAs that were specifically recognized by the KSHV miRNA-specific probes were detected for all of the miRNAs analyzed in the KSHV-infected cell lines BC-1 and BCBL-1, but not in the control BJAB cells. Of note, both miR-K4-5p and miR-K4-3p, derived from opposite strands of a single pri-miRNA hairpin (Fig. 1) were detected. To save space, only the mature KSHV miRNA signals are shown, although the predicted  $\approx 70$ -nt pre-miRNA precursors were also detected in most cases. Doublet bands were detected for miR-K7 and miR-K8-3p, consistent with the recovery of more than one size of mature miR-K7 and miR-K8-3p documented in Table 2. For the majority of the KSHV miRNAs, there was no evidence of enhanced expression after TPA treatment, but some TPA induction ( $\leq 2$  fold) was observed for miR-K1, miR-K2, and miR-K3. As a loading control, we also performed a Northern analysis for the constitutively expressed snRNA U6 and for the



**Fig. 2.** Analysis of KSHV miRNA expression. (A) RT-PCR analysis performed using RNA samples derived from KSHV-infected BC-1 and BCBL-1 cells cultured under normal conditions or in the presence of TPA. ORF72 (*v-cyclin*) is a latent KSHV gene, and K12 is a latent gene that is activated during lytic replication, whereas ORF25 is strictly lytic. KSHV-negative BJAB cells served as a control. (B) Northern analysis of miR-K5 pre- and mature miRNA expression using a DNA probe perfectly complementary to mature miR-K5 (Table 2). The RNA samples used were the same as analyzed in A. Synthetic RNA markers (M) of 63 and 22 nt in length were run in parallel. (C) Similar to B, except that DNA probes complementary to the indicated viral miRNAs, a cellular miRNA (miR-16), or cellular U6 snRNA, were used.

cellular miRNA miR-16. As shown in Fig. 2C, the signal obtained for both of these small cellular RNAs was consistent across all five RNA samples tested. The fact that TPA treatment, which increases the number of BC-1 cells entering lytic KSHV replication by  $\approx 20$ -fold (Fig. 4), has little or no effect on KSHV miRNA expression (Fig. 2) argues strongly against the hypothesis that the KSHV miRNAs observed in uninduced BC-1 and BCBL-1 cells originate exclusively from the small percentage of cells undergoing spontaneous lytic KSHV replication.

An interesting question is the average level of expression of these viral miRNAs on a per cell basis. To determine this level, we purchased synthetic forms of the KSHV miR-K7 and miR-K6-3p miRNAs and then performed a Northern analysis using BC-1 RNA and BJAB RNA to which a standard curve of the synthetic KSHV miRNAs had been added. As shown in Fig. 5, which is published as supporting information on the PNAS web site, this analysis revealed that latently KSHV infected BC-1 cells express an average of  $\approx 2,200$  copies of miR-K6-3p and  $\approx 800$  copies of miR-K7 per cell. These results are comparable to expression data for four cellular miRNAs obtained in HeLa cells, which detected between  $\approx 700$  and  $\approx 10,000$  copies per cell (34).

The KSHV DNA genome exists in latently infected cells in the form of an unintegrated episome, and previous work using BC-1 and BCBL-1 cells has reported that these cell lines contain an average of  $\approx 59$  and  $\approx 70$  copies of the KSHV genome, respectively (35). These numbers likely represent an upper limit for latently infected cells, because the KSHV genome copy number increases substantially during lytic replication. To confirm that these earlier numbers are representative of the KSHV genome copy number present in the cells analyzed here, we performed a quantitative competitive PCR analysis using DNA derived from uninduced BC-1 and BCBL-1 cells and primers specific for the viral *ORF25* gene. These data (Fig. 6, which is published as

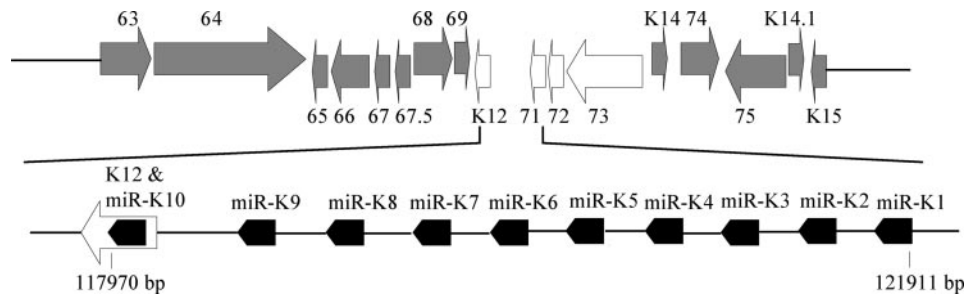
supporting information on the PNAS web site) revealed that each BC-1 cell on average contained  $\approx 83$  copies, and each BCBL-1 cell contained  $\approx 74$  copies, of the KSHV genome. Therefore, in the case of BC-1, we can calculate that each episomal KSHV genome on average gives rise to  $\approx 27$  copies of miR-K6-3p and  $\approx 10$  copies of miR-K7. Because miRNAs are thought to be fairly stable (1), this finding suggests that the promoter(s) responsible for KSHV miRNA transcription are likely to be, at most, modestly active.

## Discussion

KSHV was initially discovered in AIDS-associated Kaposi's sarcoma (19) and is now known to play a key role in all forms of this disease (36). In addition, KSHV is specifically associated with two forms of B cell lymphoma (20, 21). Although KSHV infection is fairly innocuous in immunocompetent individuals, KSHV can be a serious opportunistic pathogen in immunocompromised patients and is the most common cause of cancer in human immunodeficiency disease type 1 (HIV-1)-infected patients. As a result, Kaposi's sarcoma has become the most commonly reported cancer in parts of Africa (37) and is likely to emerge as a serious problem in other areas where HIV-1 infection is increasing.

Although lytic infection by KSHV has been proposed to contribute to KSHV-associated pathogenesis (38), latently infected cells are likely to play a critical role in all KSHV-induced tumors. Therefore, understanding how KSHV is able to establish and maintain a latent infection and disrupt the normal regulation of cell proliferation is important to understanding KSHV pathogenesis and potentially for the future development of drugs that disrupt KSHV replication *in vivo*.

Latent infection by KSHV involves the expression of only a few of the 88 known KSHV ORFs. Specifically, all latently KSHV-infected cells, including the PEL-derived cell line BC-1, express



**Fig. 3.** Genomic location of the KSHV miRNAs. (Upper) A schematic of the 3' end of the KSHV DNA genome with known ORFs, their orientation and expression pattern indicated. The KSHV miRNAs identified in this report are arrayed between ORF71 and K12, in noncoding sequences, with the exception of miR-K10, which overlaps the K12 ORF. White boxes, KSHV genes expressed during latent infection; gray boxes, KSHV genes expressed only during lytic infection; black boxes, KSHV pri-miRNA stem-loop precursors. The genomic orientation of each gene or miRNA is indicated by the arrow at the end of each box.

the KSHV genes v-FLIP (ORF71), v-cyclin (ORF72), and LANA-1 (ORF73) (31–33). Although these three genes are not induced by activation of lytic infection, a small number of additional KSHV genes, e.g., K12/Kaposin and vIRF2, express low levels of mRNA in latently infected cells but are activated during lytic infection (32, 33). Interestingly, the three genes most characteristic of latent KSHV infection, i.e., ORFs 71, 72, and 73, are located adjacent to one another in the KSHV genome (39). These three ORFs are separated from the K12 gene, which is expressed at low levels during latency, by an  $\approx 4$  kb KSHV sequence that lacks any significant ORFs, representing the largest coding gap within the unique region of the KSHV genome (39) (Fig. 3). Remarkably, 9 of the 10 KSHV miRNAs identified in this analysis are located within this coding gap, whereas the 10th, miR-K10, is found within the adjacent ORF for the K12 protein (Fig. 3). Moreover, all 10 KSHV miRNAs are oriented in the same genomic direction (Fig. 3).

There are two obvious questions raised by our discovery of multiple virally encoded miRNAs within KSHV infected cells: (i) How are these viral miRNAs expressed? (ii) What do these viral miRNAs do? Analysis of cellular miRNA transcription has shown that all miRNAs analyzed so far are transcribed by RNA polymerase II and that pri-miRNAs are capped, polyadenylated transcripts structurally analogous to pre-mRNAs (17, 40). One pri-miRNA may contain one or a cluster of several pre-miRNA hairpins that are individually excised by Drosha during the first step in miRNA processing. Pre-miRNA stem-loops are generally found either within the exons of noncoding mRNAs or within the introns of coding or noncoding mRNAs.

The fact that all 10 KSHV miRNA stem-loops are located in one short segment in the  $\approx 141$ -kb KSHV genome, and are all oriented in the same direction, suggests that all 10 miRNAs may derive from a single pri-miRNA transcript. A candidate pre-mRNA that could serve as the KSHV pri-miRNA has been described by Li *et al.* (41), who reported a K12 mRNA that initiated at position 123,842, i.e., near the C-terminus of ORF73, and was polyadenylated at position 117,432, i.e., 3' to the K12 ORF (Fig. 3). Importantly, this mRNA contains a single intron extending from 123,594 to 118,779 that would encompass all of the KSHV pre-miRNA stem-loops except miR-K10 (Fig. 3 and Table 2). Although the hypothesis that nine of the KSHV miRNAs are processed out of an intron present in a K12 mRNA is attractive, it remains possible that one or more KSHV miRNAs are instead, or also, derived from an exonic location within a noncoding RNA(s). Indeed, the finding that miR-K1, miR-K2, and miR-K3 expression is enhanced  $\approx 2$ -fold by TPA treatment, whereas the remaining KSHV miRNAs are essentially unaffected (Fig. 2), seems difficult to reconcile with the idea that they all derive from the same pri-miRNA precursor. Finally, we note that the miR-K10 stem-loop is currently unique in being located within a known coding ORF (Fig. 3). Because

K12 is certainly expressed in KSHV infected cells, we can only conclude that miR-K10 processing is inefficient and that the bulk of the K12 mRNA is exported to the cytoplasm before nuclear Drosha cleavage. The low level of recovery of miR-K10 as a cDNA clone (Table 2) may be consistent with this hypothesis, although miR-K10 was certainly detectable by Northern analysis (Fig. 2).

A previous report documenting the expression of five viral miRNAs in EBV-infected cells (15), together with this current report documenting the expression of at least 11 miRNAs in KSHV-infected cells, suggests that miRNAs may play a critical role in the establishment and/or maintenance of latent infections initiated by these, and possibly other, herpesviruses. The fairly high level of expression of the KSHV miRNAs, i.e.,  $\approx 2,200$  copies of miR-K6-3p and  $\approx 800$  copies of miR-K7 per BC-1 cell (Fig. 5), and the observation that KSHV and EBV each account for a third or more of the miRNAs detected in BC-1 cells (Table 1), is certainly consistent with this hypothesis. Although we have not observed any significant sequence similarity between the 14 KSHV miRNAs described here (Table 2) and the five previously reported EBV miRNAs, it is certainly possible that one or more of these viral miRNAs targets the same cellular mRNA.

Animal miRNAs generally target imperfectly complementary target sites located in the 3' UTR of mRNAs and then induce translational arrest (1). This process is highly cooperative, and it has been proposed that this cooperativity has evolved to allow the coordinate posttranscriptional regulation of gene expression by different miRNAs acting in concert (14). Nevertheless, vertebrate miRNAs are fully capable of inducing mRNA cleavage when they encounter a perfectly complementary mRNA target (10, 11), and this form of RNA interference is indeed the norm for plant miRNAs (1).

Although human miRNAs and their mRNA targets have likely coevolved to permit one miRNA to coordinately target multiple mRNAs, this seems unlikely to be true for the cellular targets of viral miRNAs. Therefore, we hypothesize that viral miRNAs may, in fact, function to induce the degradation of one or a small number of host mRNAs. Nevertheless, it is also possible that the KSHV miRNAs do function as true miRNAs that, perhaps acting in concert with each other or with cellular miRNAs, block the translation of specific host mRNAs. Finally, it is possible that one or more of the KSHV miRNAs are actually designed to regulate viral mRNA expression, as has indeed been proposed for one of the EBV miRNAs (15).

Based on these three possible scenarios, we have used computational methods to perform searches for human and KSHV mRNA that contain potential targets for each of the KSHV miRNAs listed in Table 2. Cellular mRNAs that contain potential targets highly complementary to a KSHV miRNA are listed in Table 4, which is published as supporting information on the PNAS web site, cellular mRNAs that contain potential partially

complementary targets within the 3' UTR are listed in Table 5, which is published as supporting information on the PNAS web site, and cellular mRNAs that contain potential partially complementary targets within the coding region are listed in Table 6, which is published as supporting information on the PNAS web site. Finally, KSHV mRNAs that contain potential targets partially complementary to a KSHV miRNA are listed in Table 7, which is published as supporting information on the PNAS web site. We did not detect any highly complementary targets for KSHV miRNAs within the coding regions of the KSHV genome.

Thus far, it has proven very difficult to identify authentic mRNA targets for vertebrate miRNAs. Indeed, only one vertebrate target, the HOX8 mRNA cleaved by miR-196, has been confirmed (42), although many others have been proposed based on computer analyses (1, 43). Because we have not yet analyzed the expression level of candidate target mRNAs or proteins in the presence or absence of specific KSHV miRNAs, the validity of the potential mRNA targets listed in Tables 4–7 is unclear. This is particularly true for the partially complementary targets listed in Tables 5–7, because genuine miRNA regulatory sites frequently do not display a statistically significant level of complementarity (43). With this caveat in mind, we note that

Tables 4–6 include a number of interesting candidate target genes whose products are involved in a range of cellular activities, including apoptosis and signaling. A number of B cell-specific genes were also detected as potential targets in this computational analysis, including the mRNAs encoding SWAP-70, ETV5, and BLR1, which have been reported to be repressed in KSHV latently infected cells based on microarray analysis (44, 45). Although the identification of the mRNA targets for the KSHV miRNAs described in this manuscript is clearly a critical goal, the fact that these KSHV miRNAs are expressed at readily detectable levels in latently KSHV infected cells (Fig. 2) and are fully conserved in all KSHV genome sequences present in the database argues that they are likely to play a key role in KSHV replication and pathogenesis.

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