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Viral miRNA Targeting of Bicistronic and Polycistronic Transcripts

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Abstract

Successful viral infection entails a choreographic regulation of viral gene expression program. Kaposi's sarcoma-associated herpesvirus (KSHV) encodes numerous miRNAs that regulate viral life cycle. However, few viral targets have been identified due to the lack of information on KSHV 3' untranslated regions (3'UTRs). Recent genome-wide mapping of KSHV transcripts and 3'UTRs has revealed abundant bicistronic and polycistronic transcripts. The extended 3'UTRs of the 5' proximal genes of bicistronic and polycistronic transcripts offer additional regulatory targets. Indeed, a genome-wide screening of KSHV 3'UTRs has identified several bicistronic and polycistronic transcripts as the novel targets of viral miRNAs. Together, these works have expanded our knowledge of the unique features of KSHV gene regulation program and provided valuable resources for the research community.

Introduction

Mechanisms of regulation of gene expression at the RNA level

Viruses have evolved a plethora of mechanisms to regulate the expression of their genes. Examples include transcriptional regulation, RNA import and export, RNA stability, alternative mRNA splicing, modifications of 5' untranslated region (UTR), alternative polyadenylation, mRNA editing and non-canonical translation mechanisms [1–3]. While viral genomes are usually compact, a number of viruses produce bicistronic and polycistronic transcripts as a mechanism to maximize their coding capacities [1,2].

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The processing of the 3' mRNA ends and 3'UTRs are subjected to extensive regulation in eukaryotic cells [1,2]. Alternative polyadenylation produces a subset of transcripts with different coding potentials and/or different lengths of 3'UTRs [4]. 3'UTRs, defined as the portion of an mRNA transcript extending from the stop codon to the polyadenylated tail, often harbor regulatory sequences. Therefore, the length of a 3'UTR can influence not only the stability but also the localization, transport, and translational properties of the mRNA [4–6]. Indeed, the amount of protein generated by an mRNA depends on its 3'UTR length [7*, 8*]. A general shortening in 3'UTR length is associated with increased proliferation, dedifferentiation, and diseases such as cancer [7*, 8*, 9, 10*, 11, 12]. In general, transcripts with longer 3'UTRs are more prone to negative regulation [7*, 8*]. For examples, in active proliferating cells such as activated CD4+ T lymphocytes, cancer cells and developmental embryo of zebrafish, alternative polyadenylation is extensive and transcripts with shorter 3'UTRs usually produce higher levels of proteins in part due to the loss of miRNA-mediated repression [7*, 8*, 13]. Nevertheless, if the 3'UTR is too short, it could render the transcript unstable [13]. In addition to regulating alternative polyadenylation, longer lengths of poly(A)-tails are coupled with increased translational efficiencies [14*, 15*, 16]. Thus, the extended 3'UTRs of bicistronic and polycistronic transcripts offer additional regulation at both transcriptional and post-transcriptional levels. Similarly, for viruses, the 3'UTRs of the 5' proximal ORFs in bicistronic and polycistronic transcripts comprise the 5' distal ORFs. As a result, viral genes encoded by bicistronic and polycistronic transcripts containing the same polyadenylation signal often harbor different lengths of 3'UTRs, thus providing an additional mechanism for regulating the expression of viral genes.

The expression of bicistronic and polycistronic transcripts also creates a conundrum for the viruses. Because the translation machinery does not usually engage the start codons at internal positions within an mRNA, an eukaryotic transcript generally encodes only one functional polypeptide [17]. However, many viruses subvert the translational constraints of the host and their mRNAs do not conform to the one protein per mRNA paradigm [1,2]. Viral mechanisms of translational programming include internal ribosome entry, ribosome shunting, leaky scanning, frameshifting, control of termination and reinitiation, and functional recoding [2].

The discovery of cellular and viral microRNAs (miRNAs) has expanded the repertoire of mechanisms of gene regulation. miRNAs are short (21–23 nt), single-stranded noncoding RNAs, which are processed from 70–80 nt precursor miRNAs (pre-miRNAs) hairpins by RNase III-like enzymes Drosha and Dicer [18]. One strand of the hairpin duplex is loaded into an Argonaute family protein (AGO) to form the core of RNA-induced silencing complexes (RISC) [18]. miRNAs bind to partially complementary sequences in the 3'UTRs of target transcripts to inhibit their translation and/or induce their degradation [19,20]. It is estimated that over 30% of transcripts are subjected to miRNA regulation in humans [21]. Cellular miRNAs regulate almost every cellular gene-regulatory pathway, including cellular differentiation, innate immunity, apoptosis and oncogenic transformation [18,19]. The first viral miRNA was discovered in human B cells latently infected by the gammaherpesvirus Epstein-Barr virus in 2004 [22*]. To date, most known viral miRNAs are encoded by herpesviruses [23]. These miRNAs are implicated in the regulation of viral life cycle and the development of their associated diseases [23,24].

miRNAs and bicistronic and polycistronic transcripts of Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV, a gammaherpesvirus, possesses a ~165-kb genome of double stranded DNA and encodes close to 90 genes and 12 pre-miRNAs, which generate 25 mature miRNAs and several variants carrying an additional 5' adenosine (A) in relation to their respective prototypic miRNAs [25–30]. Infection by KSHV is associated with the development of Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease, malignancies commonly found in immunocompromised patients [31]. Like all herpesviruses, the life cycle of KSHV consists of latent and lytic replication phases. Only a limited number of KSHV genes are expressed during latency while most of viral lytic genes are expressed during lytic replication [31]. Complex mechanisms regulate the expression of KSHV genes during latent and lytic replication phases, both of which are required for the development of KSHV-related malignancies [31].

KSHV miRNAs are constitutively expressed during latency and their expressions are maintained during lytic replication [27,28]. This mode of expression implicates their important roles in viral life cycle and KSHV-induced malignancies. Specifically, the expression of KSHV miRNAs is regulated in the same mode as those of viral latent genes residing in the latent locus, which is controlled by both constitutively active latent and inducible lytic promoters [32]. Interestingly, pre-miR-K10 and -K12 are resided in the 3'UTR of Kaposin B. Drosha regulates Kaposin B expression through processing of the pre-miRNA *cis* elements [33]. The expression of Drosha is downregulated during KSHV lytic replication resulting in decreased *cis*-regulation of Kaposin B transcripts and pre-miR-K10 and -K12 [33]. It is possible that reduced Drosha expression could also affect the processing of other pre-miRNAs at the late stage of KSHV replication.

KSHV miRNAs have been shown to regulate cell cycle, apoptosis, inflammation, angiogenesis, cellular transformation and immune evasion by targeting cellular genes in addition to viral life cycle [24,34–53]. Several KSHV miRNAs regulate viral replication by directly targeting lytic genes [54–56,57*]. Ribonomics approaches including high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (HITS-CLIP) and photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) have been used to search for the targets of KSHV miRNAs [26*,57*,58*]. These studies have identified many targets of KSHV miRNAs. However, most of them are cellular targets and only a few of them are viral targets [24,59].

Previous studies have shown that a number of KSHV genomic loci encode for bicistronic and polycistronic transcripts. ORF50/K8/K8.1 is a cluster gene locus in which all three genes share a common poly(A) signal but use alternative promoters and alternative RNA splicing to express each gene [60–62]. ORF71/72/73 is another cluster gene locus, which transcribes three alternative transcripts with polyadenylation from the same poly(A) signal [63]. The translation of one of the 5' distal gene ORF71 (vFLIP) is mediated by an internal ribosomal entry mechanism [64–66]. Hypoxia-induced polycistronic transcripts ORF34/35/36/37 encode two short upstream ORFs, uORF35.1 and uORF35.2, within the 5' leader sequence of the ORF35–36 bicistronic transcript [67–69]. Both uORFs function as

negative regulators of ORF35 while the second one allows the translation of the downstream ORF36 gene by a termination-reinitiation mechanism [67,68].

Recently, several groups have taken genome-wide approaches to map the KSHV transcripts and 3'UTRs [70^{••},71^{••},72^{••},73^{••}]. These studies have revealed numerous novel bicistronic and polycistronic transcripts in several KSHV genomic loci. In a genome-wide screening of 3'UTRs, a number of novel targets of KSHV miRNAs including several bicistronic and polycistronic transcripts were identified [73^{••}]. These studies have expanded our knowledge of the unique features of KSHV gene regulation program. In the following sections, we review and discuss the identification and mapping of the novel KSHV transcripts and 3'UTRs with emphasis on bicistronic and polycistronic transcripts that are targeted by KSHV miRNAs.

Identification of 3'UTRs of KSHV genes

The KSHV genome was sequenced from the lymphoid cell line BC-1 in 1996 [25] and its transcripts were subsequently mapped [74]. However, until the recent reports, only ~30% of KSHV genes have been experimentally determined for their 3'UTRs. Recently four laboratories reported genome-wide mapping and screening of KSHV transcripts [70^{••},71^{••},72^{••},73^{••}]. Using a modified polyadenylation-sequencing (PA-seq) protocol, Majerciak et al. performed high-throughput RNA deep sequencing (RNAseq) in three KSHV-positive B-cell lines (JSC-1, BCBL-1 and TREx BCBL-1) and identified 67 active RNA poly(A) sites for the expression of ~90 KSHV genes and a few antisense transcripts. The PA-seq analysis identified numerous regions of the KSHV genome with multiple genes sharing common poly(A) sites, indicating that a large fraction of poly(A) sites is likely shared by cluster genes such as bicistronic or polycistronic transcripts. Among the mapped poly(A) sites, only one-third is used for the expression of single genes [70^{••}]. McClure et al. also applied RNAseq analysis in TREx-RTA BCBL-1 cells and identified 82 poly(A) sites [71^{••}]. Using 3' rapid amplification of cDNA ends (3'RACE) analysis and tiled microarray analysis, they confirmed 71 KSHV 3'UTRs [71^{••}]. By performing RNAseq and ribosome profiling in induced and non-induced iSLK-219 cells, Arias et al. provided a refined and expanded annotation of KSHV transcriptome including transcription start sites, poly(A) sites, splice junctions, and initiation/termination codons of known and new viral features in the genomic space [72^{••}]. Finally, we carried out a genome-wide 3'RACE analysis and identified 83 3'UTRs of KSHV genes in non-induced and TPA-induced BCBL-1 cells [73^{••}]. Of the 83 3'UTRs, 34 (41%) were from viral genes with bicistronic and polycistronic transcripts [73^{••}]. While these studies generally identified most of the same poly(A) sites, some reported more sites than the others had, which could be due to the variations of methodology. Based on the data sets from these four studies, a number of emerging concepts of transcriptional regulation controlling the expression of KSHV genes during viral latent and lytic infection have arisen. Although using different cell lines and viral strains, all four studies have shown that, similar to mammalian transcripts, KSHV RNA polyadenylation employs two major poly(A) signals (PAS), AAUAAA (69%–83%) and AUUAAA (9%–17%). However, KSHV transcripts have fewer PAS variants than human transcripts have. Compared to human 3'UTRs, the lengths of KSHV 3'UTRs are shorter [73^{••},75]. A large number of KSHV genes are expressed as bicistronic or polycistronic transcripts with the 5' proximal genes

possessing relatively long 3'UTRs that contain the downstream gene(s). These extended 3'UTRs could be subjected to regulation of protein and RNAs including viral and cellular miRNAs (Table 1). These results reveal numerous unique features and complex regulatory mechanisms of KSHV transcriptome.

Bicistronic and polycistronic transcripts targeted by KSHV miRNAs

To identify viral targets of KSHV miRNAs, we performed a genome-wide screening with the KSHV 3'UTR reporters for their responsiveness to KSHV miRNAs [73**]. We identified 28 3'UTRs that were responsive to KSHV miRNAs, 12 of which were 3'UTRs from viral genes with bicistronic and polycistronic transcripts (Table 1 and Figure 1), including two pairs which were identified in previous studies (miR-K7-5p:ORF50 and miR-K9*:ORF50) [54,56]. Multiple 3'UTRs from the ORF31-33 locus or the ORF71-73 locus were regulated by the same miRNA, respectively (Figure 1) [73**]. ORF30-33 locus encodes lytic genes ORF30, ORF31, ORF32 and ORF33. These genes are associated with the late stage of viral replication in murine gammaherpesvirus 68 [76–78]. Specifically, ORF33, a tegument protein, is involved in virion maturation [79,80]. All of the 3'UTR reporters from the ORF30-33 locus were repressed by miR-K3-5p except the ORF30 3'UTR, suggesting that miR-K3-5p might play an important role in the late stage of viral replication [73**]. Since ORF30 shares most of the 3'UTR regions with ORF31, it remains to be determined if the ORF31 coding sequence in the ORF30 3'UTR region could block miR-K3-5p repression of this 3'UTR. Three essential latent genes, vFLIP, vCyclin and LANA, that regulate KSHV latency and promote cell growth and survival, are encoded by the ORF71-73 locus [81]. Mutagenesis analyses of 3'UTR reporters revealed that ORF71-73 transcripts were targeted by miR-K10a-3p and miR-K10b-3p as well as their variants through different target sites [73**]. Although vFLIP, vCyclin and LANA have diverse functions during viral latency, their excessive expression may cause unwanted stress and thus affect cell proliferation, or even be detrimental to the cells [82]. In this scenario, viral miR-K10a-3p and miR-K10b-3p as well as their variants may play a fine-tuning role in regulating the expression of KSHV latent genes. These results suggest that the bicistronic and polycistronic transcripts that share the same poly(A) site and some transcripts with 3'UTR harboring the same miRNA targeting sites may frequently display similar regulation patterns. McClure et al. introduced chimeric 3'UTR luciferase reporters into TReX-BCBL-1 cells to study the regulatory activity of KSHV 3'UTRs [71**]. They found that 52% and 13% of KSHV 3'UTRs conveyed negative and positive regulation, respectively. Interestingly, a similar regulation mode was observed in KSHV-negative BJAB cells and HEK 293 cells, indicating that cellular rather than virus-derived *trans* factors are likely involved in most of the regulation conveyed by viral 3'UTRs in this system [71**]. Thus, besides viral factors/miRNAs, cellular factors/miRNAs may also target the 3'UTRs to regulate the expression of KSHV genes. In fact, our recent study has demonstrated that hsa-miR-1258 inhibits KSHV lytic replication by directly targeting RTA 3'UTRs [83]. It is thus anticipated that additional cellular factors/miRNAs that regulate the expression of KSHV genes by targeting their 3'UTRs would be identified and functionally delineated in the future studies. In this regard, results of these recent studies have provided valuable resources for the research community [70**,71**,72**,73**].

Conclusion and outlook

Recent advancements in new molecular biology technologies have permitted reexamination of the genomic architecture and gene expression networks of KSHV. Multiple genome-wide technologies including modified PA-seq, RNAseq, Ribo-seq and 3'RACE analyses have been adapted and used to map the transcriptome and identify the 3'UTRs of KSHV genes, resulting in the considerable expansion of our knowledge of the genomic features of KSHV. While preliminary characterizations of the 3'UTRs of KSHV genes have been achieved, further investigations promise to demonstrate how they are regulated to orchestrate the coordinated expressions of KSHV genes during the viral life cycle. A better understanding of the gene regulation networks could not only provide insights into the molecular basis of KSHV life cycle but also help elucidate the pathogenesis of KSHV-induced malignancies.

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Highlights

- Genome-wide mapping has delineated the features of KSHV transcriptome and 3'UTRs.
- Over 30% of KSHV transcripts are bicistronic and polycistronic transcripts.
- Viral miRNAs regulate viral life cycle.
- KSHV bicistronic and polycistronic transcripts are frequent targets of viral miRNAs.

Figure 1.

KSHV 3'UTRs and targets of KSHV miRNAs. Genes are labeled in blue with arrowheads indicating the transcription orientation. Intergenic regions are labeled in gray. 3'UTRs of viral transcripts are indicated as purple lines. Transcripts targeted by KSHV miRNAs are marked with the respective miRNAs in red with bicistronic and polycistronic transcripts marked with '*'. miR-K3-5p targeting sites in the ORF30-33 cluster are marked with yellow rectangles while miR-K10a/b-3p targeting sites in the ORF71-73 cluster are marked with red rectangles. Adapted from [73**].

Table 1

Bicistronic and polycistronic transcripts targeted by KSHV miRNAs

Name of 3'UTRs	# of 3'UTRs	Target or not	miRNA	Reference(s)
ORF-K1	2	Y	miR-K1/K3	[73**]
ORF9	1	N		
ORF10	1	Y	miR-K10	[73**]
ORF70	1	N		
ORF2	1	Y	miR-K10	[73**]
ORF-K4.2	1	N		
ORF21	1	N		
ORF24	1	N		
ORF25	1	Y	miR-K8	[73**]
ORF26	1	N		
ORF30	1	N		
ORF31	1	Y	miR-K3	[73**]
ORF32	1	Y	miR-K3	[73**]
ORF34	1	Y	miR-K1	[73**]
ORF35	1	N		
ORF36	1	N		
ORF40	2	N		
ORF43	1	Y	miR-K4	[73**]
ORF48	1	N		
ORF47	1	N		
ORF46	1	N		
ORF50	3	Y	miR-K7/K9	[54,56,73**]
ORF56	1	N		
ORF59	1	Y	miR-K11	[73**]
ORF60	1	N		
ORF61	1	N		

Name of 3'UTRs	# of 3'UTRs	Target or not	miRNA	Reference(s)
ORF67	1	N		
ORF66	1	N		
ORF72	1	Y	miR-K10	[73**]
ORF73	1	Y	miR-K10	[26*, 73**]
ORFK4	1	N		