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New insights into the expression and functions of the Kaposi's sarcoma-associated herpesvirus long noncoding PAN RNA

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Abstract

The Kaposi's sarcoma-associated herpesvirus (KSHV) is a clinically relevant pathogen associated with several human diseases that primarily affect immunocompromised individuals. KSHV encodes a noncoding polyadenylated nuclear (PAN) RNA that is essential for viral propagation and viral gene expression. PAN RNA is the most abundant viral transcript produced during lytic replication. The accumulation of PAN RNA depends on high levels of transcription driven by the Rta protein, a KSHV transcription factor necessary and sufficient for latent-to-lytic phase transition. In addition, KSHV uses several posttranscriptional mechanisms to stabilize PAN RNA. A cis-acting element, called the ENE, prevents PAN RNA decay by forming a triple helix with its poly(A) tail. The viral ORF57 and the cellular PABPC1 proteins further contribute to PAN RNA stability during lytic phase. PAN RNA functions are only beginning to be uncovered, but PAN RNA has been proposed to control gene expression by several different mechanisms. PAN RNA associates with the KSHV genome and may regulate gene expression by recruiting chromatin-modifying factors. Moreover, PAN RNA binds the viral latency-associated nuclear antigen (LANA) protein and decreases its repressive activity by sequestering it from the viral genome. Surprisingly, PAN RNA was found to associate with translating ribosomes, so this noncoding RNA may be additionally used to produce viral peptides. In this review, I highlight the mechanisms of PAN RNA accumulation and describe recent insights into potential functions of PAN RNA.

1. INTRODUCTION

Herpesviruses infect a wide range of organisms from invertebrates to mammals. Due to millions of years of virus-host co-evolution, herpesviruses generally have low pathogenicity and narrow host range, but they can cause significant disease in humans and present problems for agriculture and aquaculture (Davison, 2002; Davison et al., 2008; McGeoch, 2005; van Beurden and Engelsma, 2012). Mammalian herpesviruses are classified into three subfamilies alpha, beta, and gamma that diverged in a mammalian ancestor ~200 million years ago. Herpesviruses have large (~120–240 kb) double-stranded nuclear genomes, and

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they use the host cell machinery for transcription and RNA processing. As a result, herpesvirus mRNAs resemble those of the host in that they are RNA polymerase II (Pol II)-transcribed, capped, polyadenylated transcripts, but most herpesvirus genes contain no introns. Given the recent appreciation of the widespread expression of noncoding RNAs (ncRNAs), it is not surprising that herpesviruses also encode ncRNAs. Herpesviruses utilize a wide variety of cellular RNA biogenesis pathways for ncRNA maturation. Specific herpesviruses produce ncRNAs that are stable introns, RNA polymerase III (Pol III) transcripts, long polyadenylated RNAs, nonpolyadenylated Pol II transcripts, antisense RNAs, and tRNA-like RNAs. In addition to these ncRNAs, miRNAs are found in all mammalian herpesviruses (Swaminathan, 2008; Tycowski et al., 2015; Zhu et al., 2013). In most of these cases, little is understood about the functions of herpesviral ncRNAs, but their unique structures and the current literature suggest a broad variety of roles for ncRNAs in herpesvirus replication. Here, I review the literature on one unique herpesvirus ncRNA, the polyadenylated nuclear (PAN) RNA encoded by the Kaposi's sarcoma-associated herpesvirus (KSHV). I further recommend to two recent reviews that provide additional perspectives on this viral RNA (Campbell et al., 2014b; Rossetto and Pari, 2014).

KSHV is a human gammaherpesvirus initially identified based on its close association with Kaposi's sarcoma, a common AIDS-associated malignancy (Chang et al., 1994). KSHV was additionally associated with the lymphoproliferative disorders primary effusion lymphoma (PEL), a subset of multicentric Castleman's disease (MCD), and KSHV inflammatory cytokine syndrome (KICS) (Dittmer and Damania, 2013; Du et al., 2007; Ganem, 2006; Greene et al., 2007). Similar to other herpesviruses, the KSHV life cycle includes a latent phase in which the viral DNA is maintained in infected host cells as a circular episome. During latency, no viral replication occurs and only a few viral genes are expressed. However, upon reactivation to the lytic phase, a sophisticated cascade of gene expression leads to the production of infectious virions. PAN RNA accumulates to high levels during the lytic phase of KSHV infection.

2. THE DISCOVERY OF PAN RNA

PAN RNA (also called nut-1 or T1.1) was identified and characterized independently by the laboratories of Drs. Ganem and Miller (Staskus et al., 1997; Sun et al., 1996; Zhong and Ganem, 1997; Zhong et al., 1996). PAN RNA was found in KSHV-infected PEL cell lines as well as in tissue derived from a Kaposi's sarcoma patient lesion. Characterization of the RNA showed that, as the name implies, PAN RNA is a polyadenylated transcript that accumulates in the nucleus of lytically infected cells. PAN RNA is 1077 nucleotides (nt), transcribed by Pol II, and its promoter has typical Pol II promoter elements. Several pieces of data led to its assignment as a noncoding transcript. First, the only open reading frames are short (<65 amino acids) and these have suboptimal translation initiation sequences. Second, PAN RNA is primarily, if not exclusively, nuclear as assessed by both fractionation and in situ hybridization. Third, while PAN RNA can be found in higher molecular weight complexes, the RNA does not co-sediment with polysomes. Together, these data strongly support a nuclear noncoding function for PAN RNA.

Given the current understanding of the number of long ncRNAs (lncRNAs) in the nucleus of mammalian cells (Cech and Steitz, 2014; Rinn and Chang, 2012; Ulitsky and Bartel, 2013), the identification of a herpesviral noncoding polyadenylated nuclear RNA does not seem particularly novel. However, it is worth noting that in 1996, relatively few nuclear polyadenylated RNAs had been characterized (e.g. XIST, H19) (Lee and Jaenisch, 1997; Leighton et al., 1996). Moreover, other gammaherpesviruses were known at that time to encode abundant nuclear RNAs, but these were quite different from PAN RNA. The Epstein-Barr virus (EBV) EBERs are small RNA Pol III-transcribed latent phase RNAs (Rosa et al., 1981). The New World monkey pathogen, herpesvirus saimiri (HVS), encodes HSURs, which are non-polyadenylated Pol II-transcribed RNAs with trimethylguanosine caps similar to spliceosomal small nuclear RNAs (snRNAs) (Lee et al., 1988; Murthy et al., 1986). However, unlike HSURs and snRNAs, PAN RNA is not trimethylguanosine capped nor does it directly associate with the Sm complex (Sun et al., 1996; Zhong and Ganem, 1997). Thus, the description of a lytic phase nuclear, polyadenylated RNA that otherwise resembles an mRNA was quite unexpected.

3. HOW DOES PAN RNA ACCUMULATE TO HIGH LEVELS IN LYTIC PHASE CELLS?

PAN RNA is by far the most abundant RNA produced by KSHV during lytic phase. Its expression levels have been estimated to be as high as 5×10^5 copies per cell. Comparatively, the highly expressed housekeeping GAPDH mRNA, is estimated to be only $\sim 10^3$ copies per cell and the abundant U2 snRNA involved in splicing of major class introns is $\sim 5 \times 10^5$. In fact, PAN RNA constitutes as much as 80% of the polyadenylated RNA in a lytically reactivated cell. Granted, this percentage is in the context of lytic infection during which KSHV efficiently degrades the majority host mRNAs through its host shut-off activity (Glaunsinger and Ganem, 2006). Nonetheless, the abundance of PAN RNA is quite remarkable when compared with other polyadenylated RNAs. To achieve such high levels of PAN RNA, the virus uses a robust transcriptional induction coupled with multiple mechanisms of stabilization of the RNA after induction.

3.1 Transcriptional regulation of PAN RNA

PAN RNA is a delayed early transcript that is strongly induced by the KSHV immediate early transcription factor Rta (ORF50) (Lukac, 2012; Miller et al., 2007; Staudt and Dittmer, 2007). Rta is both necessary and sufficient to reactivate latently infected KSHV, and it is the primary driver of latent-to-lytic reactivation in cells. Rta uses at least two modes of transcription activation. Rta directly binds to viral gene promoters and activates their transcription. Alternatively, Rta activates viral gene transcription by interacting with the cellular RBP-J κ protein (Chang et al., 2005; Liang et al., 2002; Lukac, 2012). PAN RNA promoter falls into the former class as it is directly bound by Rta at an Rta-response element (RRE) approximately 45–75 bp upstream of the PAN RNA transcription start site (TSS) (Figure 1A, yellow) (Chang et al., 2002; Song et al., 2001; 2002). In fact, sequence specificity required for direct binding by Rta was in large part defined using the PAN RNA promoter. In transfection experiments with PAN RNA driven by its own promoter, expression is undetectable unless Rta expression constructs are co-transfected. However,

low levels of PAN RNA have been observed in Rta-null viruses (Rossetto et al., 2013), suggesting that basal transcription or Rta-independent viral transcription programs can induce low levels of PAN RNA.

Additional cellular and viral factors are involved in the regulation of PAN RNA transcription. The PAN RNA promoter is largely RBP-J κ independent, but an RBP-J κ binding site is present upstream in the promoter that provides an additional ~2-fold boost in reporter assays in the presence of Rta (Figure 1A, pink) (Liang et al., 2002). In addition, binding sites for the cellular transcription factors YY1 and Sp1 were identified in the PAN RNA promoter and both YY1 and Sp1 bind to the promoter (Chang et al., 2005). However, the function of YY1 and Sp1 in PAN RNA transcription remains unknown. Interestingly, the KSHV posttranscriptional activator ORF57 (Mta; described below), has been implicated in transcriptional regulation of PAN RNA (Palmeri et al., 2007). While this observation was initially surprising, examples of the coupling of posttranscriptional events in gene expression with transcription is significantly more common than previously appreciated (Schwartz and Ast, 2010), so this potential contribution should not be overlooked. Together, these data suggest that Rta is the primary driver of PAN RNA transcription but other factors contribute to high levels of PAN RNA transcription.

3.2 Posttranscriptional regulation of PAN RNA

The steady-state levels of an RNA are dictated by both synthesis and decay rates, so in order to reach high levels of nuclear accumulation PAN RNA must be substantially unaffected by nuclear decay. In eukaryotes, nuclear RNAs are subject to RNA quality control (QC) pathways that degrade misprocessed (pre-)mRNAs, RNAs that are inefficiently exported, or RNAs that do not form a proper ribonucleoprotein particle (RNP) (Doma and Parker, 2007; Fasken and Corbett, 2009; Schmid and Jensen, 2008). However, the mechanisms that promote decay or protect transcripts from nuclear RNA QC in mammals remain poorly characterized. One contributing factor is the presence of an intron (and/or the changes in mRNP composition that occur upon splicing), which protects RNAs directly or indirectly from nuclear RNA QC pathways (Bresson and Conrad, 2013; Conrad et al., 2006; Lei et al., 2011; Stubbs et al., 2012; Wang et al., 2007). PAN RNA resembles an mRNA in that it has a cap, a poly(A) tail and it is transcribed by Pol II, but because it is intronless and nuclear, it mimics an aberrant RNA that should be subject to nuclear decay. Presumably for this reason, PAN RNA evolved multiple potentially redundant posttranscriptional strategies that protect it from decay. These mechanisms use cis-regulatory elements within PAN RNA as well as trans-acting KSHV and host factors. Moreover, investigations of its nuclear accumulation have led to insights into host cell nuclear RNA decay pathways that extend beyond the scope of virology into the fundamentals of human cell molecular biology.

3.2.1 The PAN RNA ENE—The most mechanistically well-defined mode of posttranscriptional regulation of PAN RNA is the stabilization driven by a 79-nt element near its 3' end called the ENE (Figure 1A, green). The ENE was defined by deletion analysis that showed it is necessary for PAN RNA accumulation in transfected cells (Conrad and Steitz, 2005). When the ENE was placed into an intronless β -globin reporter, it dramatically increased the nuclear levels of the reporter mRNA, but had little or no effect on its

efficiently exported spliced counterpart (Conrad and Steitz, 2005). The ENE lost activity in reporters in which the conventional poly(A) signal was replaced with a hammerhead ribozyme suggesting it enhanced 3'-end formation efficiency or otherwise required the presence of a poly(A) tail. In this study, RNA stability was unaffected by the ENE, but it is essential to note that this analysis was performed with the general transcription inhibitor Actinomycin D (see below). At that time, splicing was known to enhance polyadenylation and export, but PAN RNA was not subject to RNA export (Lu and Cullen, 2003; Nott et al., 2003; Proudfoot et al., 2002). Therefore, the simplest interpretation of the data was that the ENE promoted intronless RNA expression by enhancing 3' end formation. Since both PAN RNA and the ENE-containing intronless β -globin reporter accumulated in the nucleus, the ENE was further proposed to have nuclear retention function. Thus, the element was dubbed the ENE, for expression and nuclear retention element. However, studies described below convincingly showed that the nuclear accumulation of ENE-containing RNAs is driven by inhibition of nuclear decay, not retention, so this nomenclature is misleading.

Subsequent studies showed that the ENE is a potent cis-acting inhibitor of nuclear RNA decay. A transcriptional pulse-chase assay demonstrated that PAN RNA lacking the ENE is considerably less stable in cells than wild-type PAN RNA (Conrad et al., 2006). Interestingly, observations from our lab show that treatment of cells with Actinomycin D or other general transcription inhibitors leads to the stabilization of a number of nuclear RNAs (S. Bresson and N. Conrad, unpublished observations), potentially explaining why the initial experiments used to characterize the ENE showed no effects on PAN RNA half-life (Conrad and Steitz, 2005). Moreover, the ENE inhibits deadenylation and decay in nuclear extracts (Conrad et al., 2006). Together, these observations strongly support a stabilizing function for the ENE. The predicted secondary structure of the ENE revealed a stem structure interrupted by a U-rich loop (Figure 2A) leading to the proposal that the ENE interacts in cis with the poly(A) tail to protect the RNA from unknown nuclear RNA decay factors. Indeed, the ENE interacts with the poly(A) tail in vitro, and it binds the poly(A) tail in extracts from transfected cells (Conrad et al., 2006; Mitton-Fry et al., 2010). Extensive mutational analysis further validated the model by showing that the ENE stem and U-rich loop are essential for binding to the poly(A) tail and for increased RNA stability (Conrad et al., 2007). These observations neatly explained the requirement for a poly(A) tail for ENE activity (Conrad and Steitz, 2005) and demonstrated that the ENE interacts in cis with the PAN RNA poly(A) tail to protect it from decay.

In principle, the ENE could interact with the poly(A) tail by forming canonical base pairing between the poly(A) tail and the U-rich loop, similar to box H/ACA snoRNAs (Weinstein and Steitz, 1999). Surprisingly, the co-crystal structure of the core ENE with an oligo(A)₉ revealed that the ENE forms a triple helix structure (Figure 2B, 2C) (Mitton-Fry et al., 2010). The triple helix is formed by a five-adenosine stretch (A5–A9, orange) that forms Hoogsteen base pairs with one strand of the U-rich loop and traditional Watson-Crick base pairs with the other strand (Figure 2D) (Conrad, 2013). The additional hydrogen bonds stabilize the structure and reduce the “breathing” that may occur if the structure was exclusively A:U Watson-Crick base-pairing. Importantly, the 3'-end of the oligo(A) tail is buried in the structure. The top and bottom view of the ENE-oligo(A)₉ structure and the examination of the 3'-most adenosine (A9) show that the tail is effectively clamped inside of

the ENE and hidden from the outside, particularly at the 3' end (Figure 2C, left inset; Figure 2E). In addition to the triple helix, interactions between the oligo(A)₉ and the ENE extend into the stem where three adenosines (A2–A4, yellow) interact with the G:C base pairs of the bottom stem (green) through A-minor motifs as predicted by mutational analysis (Figure 2C, right inset) (Conrad et al., 2007; Doherty et al., 2001; Mitton-Fry et al., 2010; Nissen et al., 2001). Thus, the in vitro, cell-based, and the structural data all converge to validate the model that the ENE binds the poly(A) tail and sequesters the 3'-end of the RNA from nuclear RNA decay factors.

Once the ENE structure-function relationship was discovered, the results allowed the identification of new viral ENE-like elements. Most importantly for this review, a bioinformatic search identified a predicted ENE in the rhesus rhadinovirus (RRV) and equine herpesvirus-2 (EHV-2) (Tycowski et al., 2012). Both of these viruses are in the same subcategory of gammaherpesvirus as KSHV, the γ_2 -herpesviruses (McGeoch, 2005), and the presence of an ENE-like element suggested that these viruses encode PAN RNA homologs. Moreover, the putative transcripts were found at syntenic positions on the viral genomes. Indeed, the presence of a nuclear polyadenylated lytic phase transcript was verified in an RRV-infected B-cell line (Tycowski et al., 2012). Thus, while sequence comparisons alone were not sufficient to identify these viral lncRNAs, the conservation of ENE-like elements revealed the presence of likely PAN RNA homologs in other γ_2 -herpesviruses. Selective pressure for PAN RNA conservation further suggests an important function(s) for PAN RNA. Interestingly, ENE-like elements were also identified in polydnaviruses, mimivirus, and picorna-like dicistrovirus (Tycowski et al., 2012) demonstrating that the mechanism evolved multiple times independently.

The study of viral molecular biology does not solely elucidate viral mechanisms, but it additionally leads to insights about host cell molecular mechanisms. Studies of the PAN RNA ENE led to two important discoveries regarding host cell nuclear ncRNA metabolism. First, the realization that PAN RNA used a 3'-end triple helix to stabilize nuclear RNAs led to the discovery of similar elements in host RNAs. Two nuclear ncRNAs, MALAT1 and MEN β use triple helices for stabilization (Brown et al., 2014; 2012; Conrad, 2013; Wilusz et al., 2012). Second, the mechanism of the ENE suggested that the element inhibited a poly(A) tail-dependent nuclear RNA decay pathway, but the pathway remained unknown until recently. PAN RNA lacking the ENE was used to characterize a poly(A) tail-dependent nuclear RNA decay pathway in which the poly(A) binding protein, PABPN1, stimulates hyperadenylation by the canonical poly(A) polymerases, PAP α and PAP γ , and this targets the degradation of the hyperadenylated RNAs by the nuclear exosome (Bresson and Conrad, 2013). In addition, RNA-seq studies on cells depleted of PABPN1 showed that PABPN1 is involved in the decay of a large number of nuclear ncRNA transcripts (Beaulieu et al., 2012) and inhibition of PAP hyperadenylation depletion has similar global effects (Bresson et al., unpublished studies). Thus, studies of the ENE led to the characterization of a host RNA QC pathway that degrades a considerable number of nuclear RNAs.

3.2.2. The KSHV ORF57 protein enhances PAN RNA stability—A second mode of posttranscriptional enhancement of PAN RNA levels involves the KSHV ORF57 protein, also called Mta. ORF57 is essential for virus replication and is conserved among

herpesviruses, but no host homologs are known (Conrad, 2009; Han et al., 2007; Han and Swaminathan, 2006; Majerciak et al., 2007; Majerciak and Zheng, 2009; Sandri-Goldin, 2008; Sergeant et al., 2008; Toth and Stamminger, 2008). This family of proteins is best characterized for their potential roles in mRNA export, but early studies of ORF57 demonstrated that co-expression of ORF57 with PAN RNA increased the nuclear accumulation of PAN RNA (Kirshner et al., 2000; Nekorchuk et al., 2007). Moreover, upon deletion of ORF57 from KSHV, PAN RNA levels are reduced during viral infection (Han and Swaminathan, 2006; Majerciak et al., 2007). Thus ORF57 enhances PAN RNA levels independent of RNA export. A role for ORF57 in nuclear RNA stability was first formally demonstrated in transfected cells using an unstable ENE-lacking PAN RNA (Sahin et al., 2010) and a subsequent study showed that wild-type PAN RNA is also stabilized by ORF57 (Massimelli et al., 2011). Several lines of evidence show that ORF57 binds PAN RNA (Table 1). The 3'-most ~300 nt of PAN RNA are necessary for ORF57 activity but deletion of this element can be complemented by direct tethering of ORF57 to the RNA (Sahin et al., 2010), thereby demonstrating that recruitment of ORF57 to the RNA is essential for its stabilization activity. Additional studies refined the element, called the ORF57-responsive element (ORE) or Mta-responsive element (MRE) to the 5'-most 79 nt of PAN RNA (Massimelli et al., 2011; Sei and Conrad, 2011) (Figure 1A, 1B). The ORE is predicted to form three stem loop structures and specific bases in the second loop (Figure 1B, blue) are particularly important for binding and ORF57 responsiveness. While the ORE appears to be the principal driver of ORF57 binding to PAN RNA, other regions also bind to ORF57 in transfected and in lytically reactivated cells (Kang et al., 2011; Sei et al., 2015).

It remains unknown how ORF57 association with PAN RNA protects the transcript from cellular decay, but the cytoplasmic poly(A)-binding protein, PABPC1 (described below) and ALYREF have been implicated in its mechanism (Li et al., 2012; Massimelli et al., 2011; 2013; Stubbs et al., 2012). ALYREF is a component of the nuclear mRNA export machinery that binds to ORF57 (Boyne et al., 2008; Majerciak et al., 2006; Malik et al., 2004; Nekorchuk et al., 2007; Reed and Cheng, 2005). ALYREF binds to PAN RNA in an ORF57-dependent fashion, and ALYREF is sufficient to upregulate PAN RNA levels when it is artificially tethered to PAN RNA in the absence of ORF57 (Stubbs et al., 2012). Moreover, complementation of an ORF57 deletion virus with an ORF57 mutant that cannot interact with ALYREF decreases PAN RNA accumulation during lytic reactivation (Li et al., 2012). These data support the model that ALYREF is an effector of ORF57-mediated stabilization, but additional studies are required to further test this idea. The cellular decay pathway(s) inhibited by ORF57 are unknown, but because ORF57 is more active on ENE-lacking constructs, it seems likely that it serves to inhibit poly(A)-tail dependent decay by PABPN1. Even so, ORF57 appears to inhibit the decay of both hypo- and hyperadenylated PAN RNA, suggesting more than one mechanism of stabilization (Sahin et al., 2010). Further studies are necessary to understand the host decay pathways and ORF57-mediated mechanisms that protect PAN RNA from decay.

3.2.3. PABPC1 modulates PAN RNA stability—The cytoplasmic poly(A)-binding protein has also been implicated in PAN RNA stability, but by two different mechanisms. PABPC1 shuttles rapidly between the nucleus and cytoplasm and interacts with nuclear and

cytoplasmic RNAs, but at steady-state PABPC1 is predominantly cytoplasmic (Afonina et al., 1998; Hosoda et al., 2006). During the course of KSHV lytic reactivation, PABPC1 relocalizes and becomes primarily nuclear due to the activity of the KSHV host cell shut-off protein SOX (Lee and Glaunsinger, 2009). PABPC1 co-purifies with PAN RNA from lytically reactivated cells, supporting its interaction with PAN RNA during viral infection (Table 1) (Borah et al., 2011). Transient expression of SOX is sufficient for PABPC1 relocalization in uninfected cells and this further induces PAN RNA accumulation in a largely poly(A)-tail dependent fashion. In contrast, expression of a SOX mutant that does not induce PABPC1 relocalization does not increase the expression of PAN RNA. Expression of a nuclear-retained version of PABPC1 is sufficient to increase PAN RNA levels further supporting the idea that PABPC1 is the effector of SOX-driven PAN RNA upregulation (Borah et al., 2011). Upon overexpression, a significant portion of PABPC1 accumulates in the nucleus leads to dramatic increases in ENE-lacking PAN RNA half-life as assessed by a transcription pulse-chase experiments (S.M. Bresson and N.K. Conrad, unpublished observations). Taken together, these data support the model that SOX-dependent nuclear relocalization of PABPC1 contributes to PAN RNA accumulation during lytic infection by binding to its poly(A) tail and inhibiting poly(A) tail-dependent decay.

Interestingly, PABPC1 was also pulled down from cellular extract by a biotinylated MRE element but not a size-matched control (Massimelli et al., 2011; 2013) and PABPC1 binds to ORF57 in vitro (Massimelli et al., 2013). The same study reported that PABPC1 knockdown increases the accumulation of PAN RNA, suggesting a negative role for PABPC1 in PAN RNA accumulation. The authors proposed that in the absence of ORF57, PABPC1 suppresses PAN RNA accumulation by a PABPC1-MRE interaction but the presence of ORF57 alleviates the suppression (Majerciak and Zheng, 2015; Massimelli et al., 2013). Thus, distinct approaches suggest that nuclear PABPC1 stabilizes PAN RNA (Borah et al., 2011) or suppress PAN RNA in the absence of ORF57 (Massimelli et al., 2013).

In summary, several different mechanisms are responsible for the high levels of nuclear PAN RNA. PAN RNA promoter is a strong, Rta-dependent promoter that is highly transcribed upon lytic reactivation. At least three posttranscriptional mechanisms appear to contribute to PAN RNA stability: the ENE is a cis-acting enhancer of RNA stability, ORF57 increases PAN RNA stability, and PABPC1 relocalization binds to the poly(A) tail to protect it from decay. The extent to which each of these three posttranscriptional mechanisms contributes to PAN RNA stability in the context of lytic infection will be difficult to discern. Each mechanism can function independently of the other in transfected cells, so it seems likely that they act redundantly to stabilize PAN RNA during lytic infection. If these pathways are indeed redundant, PAN RNA will only be completely destabilized by deletion of the ENE, knockout of ORF57, and inactivation of PABPC1, which will be difficult to establish empirically. Consistent with this idea, PAN RNA levels are only ~1.5–2.5-fold lower in ORF57-deleted virus (Verma et al., 2015; Majerciak et al., 2007) suggesting that PABPC1 overexpression and/or the ENE compensate for ORF57 deletion in the context of viral infection. In contrast, no studies using a virus specifically deleted for the ENE have been reported, so its effects on steady-state levels during viral replication have not been verified. Alternatively, these pathways may not be redundant in the context of lytic infection. For example, the ENE may support stability at one stage in

either viral infection cycle or RNP biogenesis, while ORF57 stabilizes PAN RNA during other stages of virus life cycle or RNP biogenesis. In addition, distinct PAN RNA RNPs could potentially use different methods for stability. Thus, the potentially redundant or independent roles of the ENE, PABPC1, and ORF57 in PAN RNA stability remain to be explored in the context of viral lytic infection.

4. FUNCTIONS OF PAN RNA

The remarkable abundance of PAN RNA during lytic infection and the multiple mechanisms used by the virus to promote its accumulation suggest that high levels of PAN RNA are necessary for its function. Of course, the central question has yet to be addressed: What is the function of PAN RNA? A complete understanding of PAN RNA function and molecular mechanisms remains a long way off, but several recent publications hint at potential functions for PAN RNA in viral gene expression, chromatin remodeling, control of latent-to-lytic transition, and even as a protein coding RNA.

4.1 PAN RNA is essential for viral replication

The study of PAN RNA functions has proceeded slowly due to technical obstacles in manipulating KSHV infection systems. PEL cell lines harboring latently infected KSHV can be induced to undergo lytic reactivation, but the difficulty in transfecting these cells limits manipulation of viral or host cell gene products. In addition, conventional RNAi approaches are significantly less robust for nuclear RNAs than cytoplasmic mRNAs. These limitations were overcome by introducing antisense oligonucleotides that knockdown PAN RNA with endogenous RNase H activity by nucleofection (Borah et al., 2011; Ideue et al., 2009). Upon PAN RNA knockdown, viral gene expression and viral titers were significantly diminished (Borah et al., 2011; Campbell et al., 2014a). Generation of a true null virus has been complicated by the fact that, until recently (Brulois et al., 2012), the only manipulable infectious clone was a bacmid clone containing the entire KSHV genome, BAC36 (Zhou et al., 2002). Unfortunately for PAN RNA studies, BAC36 bacmid clone has an ~9 kb bp duplication including the PAN RNA gene (Yakushko et al., 2011), so attempts to delete the PAN RNA gene were unsuccessful. Once the duplication was reported, Rossetto and Pari implemented an ambitious two-step procedure to generate a PAN RNA null virus in a BAC36 derivative (Rossetto and Pari, 2012). To do so, they initially generated a bacmid that removed the duplicated sequences from BAC36 to create a new wildtype bacmid (BAC36CR). Subsequently, they deleted the 3' 634 nt of PAN RNA from BAC36CR to create BAC36CR PAN. Consistent with the knockdown results, deletion of PAN RNA gene compromised viral gene expression and production of infectious virus. Importantly, both the knockdown and knockout strategies controlled for the complicating effects of the K7 ORF that partially overlaps with PAN RNA (Figure 1A, orange). Together, these studies established that PAN RNA is essential for production of infectious KSHV virions.

4.2 PAN RNA associates with host and viral chromatin

Xist is the most extensively studied abundant polyadenylated lncRNA. Xist is essential for the chromatin modifications that drive inactivation of one copy of the X-chromosome during mammalian sex chromosome dosage compensation. Similar epigenetic functions have been

ascribed to other nuclear lncRNAs (Lee, 2012; Marchese and Huarte, 2014; Yang et al., 2014). Several different studies suggest that PAN RNA may be playing a similar role during KSHV lytic reactivation (Rossetto et al., 2013; Rossetto and Pari, 2012; 2011). Both PAN RNA knockdown and knockout approaches demonstrated that loss of PAN RNA compromised viral gene expression (Borah et al., 2011; Campbell et al., 2014a; Rossetto and Pari, 2012). Herpesvirus gene expression patterns are highly regulated such that activation of downstream genes depends on preceding events in viral life cycle. Therefore, changes in gene expression upon PAN RNA depletion cannot be interpreted to be definitive proof of a direct effect of PAN RNA regulation of KSHV genes. To test for direct roles for PAN RNA in KSHV and host gene expression, Pari and colleagues used chromatin isolation by RNA purification (ChIRP) (Chu et al., 2012) to show that PAN RNA associates with KSHV and cellular gene promoters (Rossetto et al., 2013). Nearly, 35 KSHV promoters were identified representing an appreciable fraction of the KSHV transcription units. Host promoters associating with PAN RNA represent a large variety of functions including gene expression, immune response, cell death and development. These observations lend support to the hypothesis that PAN RNA associates with promoters and directly modulates transcription.

The mechanisms of regulation of transcription by PAN RNA remain unknown, but several possibilities have been suggested. In one model, PAN RNA recruits cellular factors that remove repressive chromatin modifications and/or promote activating modifications. In cells infected with a PAN-deletion bacmid, the repressive H3K27 trimethylation of the Rta promoter is higher than in wild-type infections (Rossetto and Pari, 2012). In addition, the demethylases JMJD3 and UTX associated with the Rta promoter in wild-type but not PAN RNA deleted viral strains and ChIRP assays showed an interaction between PAN RNA and the Rta promoter (Rossetto et al., 2013; Rossetto and Pari, 2012). A formaldehyde-based crosslinking RNA immunoprecipitation assay suggests an interaction between PAN RNA and JMJD3 and UTX (Table 1). Interestingly, the MLL2 protein also bound PAN RNA in this assay. MLL2 is a methyltransferase that provides transcriptionally activating histone methylation patterns, leading to the speculation that PAN RNA activates transcription by promoting the removal of repressive methylations and addition of activating chromatin marks (Rossetto and Pari, 2012). PAN RNA additionally crosslinks SUZ12 and EZH2 (Table 1) (Rossetto et al., 2013), which promote repressive chromatin marks. Thus, Pari and colleagues proposed that PAN RNA may be responsible for both positive and negative epigenetic regulation of host and viral gene transcription (Figure 3).

An affinity pull-down with in vitro transcribed PAN RNA identified the host interferon-regulatory factor 4 (IRF4) protein as a potential PAN RNA binding protein and this interaction was also observed in a formaldehyde-based crosslinking assay (Table 1) (Rossetto and Pari, 2011). Expression of PAN RNA reduces the activity of an IRF4-responsive promoter in a luciferase assay, suggesting that PAN RNA negatively regulates IRF4 activity (Figure 3). In addition, expression of PAN RNA in uninfected cells decreases the expression of several immune responsive genes. Thus, PAN RNA may function to mute the host-mediated immune responses during KSHV infection (Rossetto and Pari, 2011). Interestingly PAN RNA expression is sufficient to increase the proliferation rate of several cell types, suggesting that PAN RNA positively regulates genes involved in cell proliferation (Rossetto et al., 2013).

4.3 PAN RNA is engaged with translating ribosomes

As described above, multiple lines of evidence support the conclusion that PAN RNA is a nuclear noncoding RNA. Surprisingly, translational profiling experiments (Ingolia, 2014) showed that several small ORFs near the 5' end of PAN RNA are engaged with active ribosomes during KSHV lytic reactivation (Arias et al., 2014) (Figure 1A, purple). Importantly, the engagement of the putative PAN RNA ORFs with ribosomes terminates at in-frame stop codons, which has proven to be a strong predictor of bona fide translation as opposed to noise in the assay (Guttman et al., 2013). As expected from its predominantly nuclear localization, the fraction of PAN RNA used for translation is small. However, given its high abundance, the export of a small fraction of PAN RNA yields a biologically relevant quantity of cytoplasmic "mRNA". At $\sim 5 \times 10^5$ copies per cell, if as little as 0.5% of PAN RNA escapes the nucleus, 2.5×10^3 cytoplasmic PAN RNA molecules represent more transcripts than many abundant cellular mRNAs. Due to the technical limitations of fractionation and FISH approaches, even the most robust experimental data cannot definitively rule out that a small fraction PAN RNA is cytoplasmic. In fact, under certain conditions, PAN RNA has been observed in the cytoplasm (Massimelli et al., 2011). Therefore, the amount of protein produced by PAN RNA could well be biologically relevant despite the primarily nuclear localization of PAN RNA. Importantly, these data do not exclude nuclear noncoding functions for PAN RNA. It seems highly unlikely that KSHV would generate vast amounts of nuclear PAN RNA if its only function is to serve as an otherwise conventional mRNA. Thus, in addition to its noncoding function(s), PAN RNA may be employed as an mRNA to produce viral peptides (Arias et al., 2014). However, it should be noted that the presence of these PAN RNA-derived peptides has not been confirmed and their possible functions are unknown.

4.4 PAN RNA regulates LANA

Due to the high abundance of PAN RNA in lytically reactivated cells, an attractive model proposes that PAN RNA titrates cellular or viral proteins that would otherwise serve to negatively regulate viral lytic phase. Direct evidence supporting this idea has recently been reported (Campbell et al., 2014a). The KSHV latency associated nuclear antigen (LANA) protein is one of a few viral proteins expressed during latency. LANA is essential for establishment and maintenance of viral latency, and it represses lytic gene promoters (Ballestas and Kaye, 2011; Lieberman, 2013; Verma et al., 2007). Many of these functions of LANA rely on its ability to bind at several loci on the KSHV genome. In a sense, LANA and Rta are opposing factors, with LANA driving latency and Rta driving lytic reactivation. Therefore, production of KSHV virions requires LANA activity to be suppressed while Rta activity is activated.

Campbell et al. (2014) reported that PAN RNA binds to LANA early in the lytic reactivation cycle to drive lytic replication by suppressing LANA DNA-binding (Campbell et al., 2014a). Recombinant LANA directly binds to PAN RNA in vitro and formaldehyde crosslinking assays further support an interaction between LANA and PAN RNA during viral lytic phase (Table 1). Interactions between LANA and PAN RNA map primarily to the N-terminal 70 amino acids of LANA. Importantly, the 3' end of PAN RNA (nt 600–1062) binds LANA in vitro, whereas the 5' end does not, supporting a specific interaction between

the 3'-end of PAN RNA and LANA. LANA binding to DNA is necessary for its activity and LANA is removed from viral DNA during lytic reactivation. Several pieces of evidence support the model that PAN RNA titrates LANA away from viral DNA to reduce its activity. First, PAN RNA abrogates LANA binding to histone H3 in a dose-dependent fashion in vitro. In contrast, PAN RNA does not affect interactions between H3 and cellular proteins EHMT2 or KDM5A. Second, nucleofection of PAN RNA decreases the association of LANA with the viral genome as assessed by chromatin immunoprecipitation (ChIP). Third, knockdown of PAN RNA in lytically reactivated cells increases LANA association with viral DNA at several regions of the genome. Together, these data support the model that PAN RNA interacts with LANA and prevents it from re-associating with the KSHV genome (Figure 3).

These studies imply a feed-forward mechanism in which Rta expression initiates the lytic transcriptional program, including PAN RNA. In the early phases of the lytic program, LANA may still be sufficiently active to re-establish or maintain latency. However, if Rta activity is strong, PAN RNA levels rise and titrate LANA activity, further shifting the balance towards lytic reactivation. Thus, full lytic reactivation is only realized when lytic transcription program has reached a point in which Rta is active and PAN RNA inactivates LANA. Another compelling aspect of this model is that PAN RNA inactivation of LANA is presumably fully reversible because PAN RNA does not degrade LANA. Should the full lytic program not be achieved, PAN RNA levels will plateau and the RNA will slowly degrade. LANA's nuclear binding equilibrium will then shift back to favor its association with the viral genome. At least in cultured cells, KSHV enters latency as its default mode (Speck and Ganem, 2010), so the virus has evolved robust mechanisms to ensure that lytic replication only occurs in the appropriate cellular environment. In principle, this feed-forward mechanism could contribute to this regulation by reversibly regulating LANA in a fashion that relies on robust Rta activity. That is, lytic phase occurs only when Rta is active enough to drive PAN RNA levels to high enough titers to overcome LANA repression.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Studies of PAN RNA accumulation have led to significant insights into the function and mechanisms of viral proteins ORF57 and Rta. In addition, studies of PAN RNA led to the discovery of an unanticipated mechanism of stabilization of nuclear RNAs by triple helix formation that is used beyond the herpesviruses. Moreover, investigation of the decay of PAN RNA lacking the triple helix forming ENE aided significantly in the characterization of an unknown nuclear RNA decay pathway. Given these precedents, it seems likely that studies of PAN RNA will continue to elucidate questions relevant to general molecular biology and KSHV gene expression. For example, it will be interesting to determine the cis- and trans-acting factor(s) responsible for PAN RNA nuclear retention. Furthermore, it is of interest to understand whether the multiple factors that stabilize PAN RNA are truly redundant or if the virus uses each of them at different times in infection or RNA biogenesis. Rta is not absolutely required for PAN RNA transcription (Rossetto et al., 2013), so understanding the mechanisms driving PAN RNA synthesis in the absence of Rta will lead to greater understanding of alternate transcription programs employed by KSHV. Thus,

further understanding of the mechanisms promoting PAN RNA accumulation will lead to greater insights into host and viral gene expression.

Functional analysis of PAN RNA is in its early stages, but it has become clear that PAN RNA is essential for viral gene expression and viral replication. Despite the fact that the field has only recently begun to develop the tools to functionally study PAN RNA, there have been five distinct mechanisms ascribed to PAN RNA (Figure 3). Whether PAN RNA performs each of these functions during viral replication remains to be explored, so caution is warranted in assigning these proposed functions to PAN RNA. Varying amounts of data support each of these mechanisms, but for now, each should be considered a hypothetical model for PAN RNA function, not an established mechanism. Even with this caveat in mind, the idea that PAN RNA is a multifunctional abundant viral lncRNA is compelling. For viral proteins, multifunctionality appears to be the rule, rather than the exception, so it follows that a viral lncRNA would also be multifunctional. In addition to canonical lytic reactivation, PAN RNA is also expressed in Rta-independent transcription programs and perhaps even in some uninduced cells (Rossetto et al., 2013). Moreover, PAN RNA is abundant in KSHV virions (Bechtel et al., 2005; Rossetto et al., 2013), so it is present in newly infected cells prior to viral transcription. Therefore, PAN RNA is found in a variety of cellular contexts, and it is reasonable to speculate that it plays different roles in each of these distinct environments. Given its abundance, PAN RNA could associate with different proteins and form multiple distinct RNPs with distinct functions. Thus, PAN RNA may indeed prove to be a dynamic multifunctional viral lncRNA.

In summary, PAN RNA is an abundant KSHV transcript that is essential for viral replication. While it may code for some peptides, it primarily serves as a viral lncRNA that is important for regulation of gene expression. KSHV uses several factors to ensure the high levels of expression of PAN RNA during lytic phase, and dissection of those mechanisms has revealed functions extending beyond PAN RNA to other viral genes and to host gene expression. Functions of PAN RNA in transcription regulation, peptide synthesis and latent-to-lytic transition are beginning to be uncovered, but significantly more work needs to be done to understand the mechanisms of this RNA and its important roles in the viral life cycle. Future studies of PAN RNA are sure to uncover exciting novel molecular mechanisms involved in KSHV and host cell gene expression.

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Highlights

- PAN RNA is an unusually abundant viral lncRNA
- I review posttranscriptional and transcriptional mechanisms that are essential for PAN RNA
- I review new insights into multiple potential functions for PAN RNA

Figure 1. Cis-acting regulatory elements of PAN RNA

(A) PAN RNA gene with several features highlighted including the Rta-responsive element (yellow), overlapping K7 ORF (orange), putative peptide coding regions (purple), the ORE/MRE (blue), the ENE (green), and an upstream RBP-J κ site (pink). The diagram is approximately to scale, but the broken lines point out a region where the scale is disrupted. (B) Sequence and predicted secondary structure of the core ORE element. Mutational analysis supports that the nucleotides shown in blue bind directly to ORF57 (Massimelli et al., 2011; Sei and Conrad, 2011).

Figure 2. Structure of the PAN ENE

(A) The predicted secondary structure of the ENE as determined by M-fold (Conrad et al., 2006; Zuker, 2003). (B) Schematic of the core ENE and an oligo(A)₉ that were used for structure determination. The color scheme is used in all structures: green, upper/lower stem; orange, A5–A9 which form the triple helix; purple, U-rich loop that form either side of the triple helix; yellow, A2–A4 which form A-minor interactions with the bottom stem. (C) Multiple views of the ENE-oligo(A)₉ structure with the ENE in surface view and oligo(A)₉ in stick view. The inset on the left is a close-up of A9 and the inset on the right is a close-up of the A-minor interactions. The grey residues are the GAAA tetraloop at the top of the structure and the bulged AU 3' to the U-stretch on the right as drawn in (B). A1 makes no contacts with the ENE and is not shown. (D) Base-pairing in the A9 triple helix. H-bonds are shown with dashed lines. Nitrogen atoms are blue, oxygen atoms are red and hydrogen atoms are white. The Hoogsteen contacts occur in the major groove formed by conventional Watson-Crick base pairing. The same hydrogen bonding pattern is observed for A5–A9. (E) Views of the ENE structure from the top and bottom of the helix. Only A2 and part of A3 protrude from the main stem structure. ENE structures were derived from PDB ID: 3P22.12 (Mitton-Fry et al., 2010).

Figure 3. A multifunctional regulator of KSHV gene expression?

Cartoon summarizing the five distinct mechanisms proposed for PAN RNA function. See text for details.

Table 1

Potential PAN RNA binding proteins

Protein	Method*	Potential function	Publication(s)
HnRNP C	CLIP(UV), NP	Nuclear retention, RNA chaperone	Conrad, 2008; Borah et al., 2011
ORF57	CLIP(UV), RIP, PD, HITS-CLIP, PD-MS, LT	Stabilizes PAN RNA by binding an element (ORE/MRE) in its 5' end	Sahin et al., 2010; Kang et al., 2011; Massimelli et al., 2011; Sei and Conrad, 2011; Rossetto and Pari, 2011; Massimelli et al., 2012; Sei et al., 2015
PABPC1	NP	Stabilizes PAN RNA by binding its poly(A) tail	Borah et al., 2011
PABPC1	PD	Decreases PAN RNA levels	Massimelli et al., 2011; Massimelli et al., 2012
ALYREF	CLIP(UV)	ORF57 co-factor to increase PAN RNA stability	Stubbs et al., 2012
UTX	CLIP(F)	Activates transcription by removing repressive H3K27Me3	Rossetto and Pari, 2012
JMJD3	CLIP(F)	Activates transcription by removing repressive H3K27Me3	Rossetto and Pari, 2012
MLL2	CLIP(F)	Activates transcription by promoting H3K4 histone methylation	Rossetto and Pari, 2012
IRF4	CLIP(F) PD-MS	Represses IRF4-driven transcription	Rossetto and Pari, 2011
SUZ12	CLIP(F)	Represses transcription by promoting H3K27 methylation	Rossetto et al., 2013
EZH2	CLIP(F)	Represses transcription by promoting H3K27 methylation	Rossetto et al., 2013
LANA	CLIP(F) IVB	Titrate LANA function to promote lytic replication	Campbell et al., 2014a
ORF59	PD-MS CLIP(F)	DNA targeting	Rossetto and Pari, 2011
H2A	PD-MS CLIP(F)	Unknown	Rossetto and Pari, 2011
RPA70	PD-MS CLIP(F)	Unknown	Rossetto and Pari, 2011

* Abbreviations: CLIP, crosslinking RNA immunoprecipitation with ultraviolet light (UV) or formaldehyde (F) as the crosslinker. UV strictly crosslinks direct RNA-protein interactions whereas formaldehyde crosslinks RNA-protein and protein-protein interactions. Thus, UV CLIP reveals direct RNA-protein interactions but omit proteins in a complex that do not directly interact with RNA. In contrast, formaldehyde captures more peripherally associating proteins, but tends to have higher background (Conrad, 2008; Niranjana Kumari et al., 2002). NP, native purification; RIP, RNA immunoprecipitation without crosslinking; PD, pull-down with immobilized RNA; HITS-CLIP, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (Licatalosi et al., 2008); LT, label transfer; PD-MS, RNA pulldown followed by mass spectrometry. Additional proteins were identified by this method but not further validated (Rossetto and Pari, 2011); IVB, in vitro binding with recombinant proteins.