

A Kaposi's sarcoma virus RNA element that increases the nuclear abundance of intronless transcripts

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The Kaposi's sarcoma-associated herpesvirus produces a 1077 nucleotide noncoding, polyadenylated, exclusively nuclear RNA called PAN that is highly expressed in lytically infected cells. We report that PAN contains a novel post-transcriptional element essential for its abundant accumulation. The element, PAN-ENE (PAN RNA expression and nuclear retention element), increases the efficiency of 3'-end formation *in vivo* and is sufficient to enhance RNA abundance from an otherwise inefficiently expressed intronless β -globin construct. The PAN-ENE does not concomitantly increase the production of encoded protein. Rather, it retains the unspliced β -globin mRNA in the nucleus. Tethering of export factors can override the nuclear retention of the PAN-ENE, supporting a mechanism whereby the PAN-ENE blocks assembly of an export-competent mRNP. The activities of the PAN-ENE are specific to intronless constructs, since inserting the PAN-ENE into a spliced β -globin construct has no effect on mRNA abundance and does not affect localization. This is the first characterization of a *cis*-acting element that increases RNA abundance of intronless transcripts but inhibits assembly of an export-competent mRNP.

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Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV; HHV-8) is the causative agent of Kaposi's sarcoma (KS; reviewed in Dourmishev *et al*, 2003; Moore and Chang, 2003; Verma and Robertson, 2003). KS is the most common AIDS-associated cancer and is endemic to populations in sub-Saharan Africa and the Mediterranean. KSHV is also associated with the lymphoproliferative disorders primary effusion lymphoma (PEL) and multicentric Castleman's disease. KSHV is a member of the gammaherpesvirus family, whose life cycle includes both latent and lytic phases. In infected individuals, latent KSHV infection is observed in B cells, while KS tumors

are comprised predominantly of latently infected endothelial cells with a subset undergoing lytic replication.

When latently infected PEL cells are reactivated, the most abundant lytic phase transcript is a 1077-nucleotide (nt) polyadenylated nuclear RNA (also called PAN; T1.1; *nut1*; Sun *et al*, 1996; Zhong *et al*, 1996). PAN comprises as much as 80% of the polyadenylated RNA in these lytically infected cells (Sun *et al*, 1996; Song *et al*, 2001). Like most messenger RNAs, PAN is transcribed by RNA polymerase II and acquires a 5' 7-methylguanosine cap and poly-A tail. However, unlike most mRNAs, PAN is exclusively nuclear and is not spliced (Sun *et al*, 1996; Zhong *et al*, 1996). Moreover, even though the 5' portion of the transcript overlaps with the viral protein-coding gene *K7* (Wang *et al*, 2002), the PAN RNA itself is apparently noncoding. No protein partners for PAN have been identified, but a subpopulation (<5%) of PAN RNA associates indirectly with Sm proteins, consistent with its observed enrichment in nuclear speckles (Sun *et al*, 1996; Zhong and Ganem, 1997).

While nuclear polyadenylated RNAs have been described (Bendena *et al*, 1989; Liu *et al*, 1997; Andersen and Panning, 2003), polyadenylation of transcripts is usually coupled to export from the nucleus (Eckner *et al*, 1991; Huang and Carmichael, 1996; Custodio *et al*, 1999; Brodsky and Silver, 2000; Hilleren *et al*, 2001; Jensen *et al*, 2001; Dower and Rosbash, 2002; Hammell *et al*, 2002; Libri *et al*, 2002; Dower *et al*, 2004). In both yeast and mammalian systems, mutations in *cis*- or *trans*-acting 3'-end formation factors, or formation of 3' ends by a mechanism independent of the normal 3'-end formation machinery leads to nuclear retention of transcripts. Consistent with a role for 3'-end formation in the release of RNA from a chromatin-bound state, unprocessed transcripts accumulate at the site of transcription, and polyadenylation is not required when mRNA transcripts are injected directly into the nucleus of *Xenopus* oocytes (e.g., see Saavedra *et al*, 1997). In some cases, proper 3'-end formation and subsequent release from transcription sites appear to be sufficient for export (Dower and Rosbash, 2002; McCracken *et al*, 2002; Dower *et al*, 2004). In yeast, 3'-end formation has been implicated in the recruitment of export factors to the mRNA (Hector *et al*, 2002; Lei and Silver, 2002).

Intronless cDNA constructs are often expressed at much lower levels than their intron-containing counterparts, an effect that has been attributed to nearly every stage of mRNA and protein biogenesis, including transcription, processing, RNA stability, export, translation, localization and even protein stability (for a review, see Le Hir *et al*, 2003). Accordingly, many naturally occurring intronless genes contain *cis*-acting elements that enhance gene expression both in their natural context and when transferred to intronless reporter genes. Transcripts from the hepatitis B virus (HBV) and the woodchuck hepatitis virus (WHV) contain *cis*-acting post-transcriptional regulatory elements (HBV-PRE, WPRE) that increase both the RNA accumulation and protein

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expression from intronless genes (Huang and Liang, 1993; Huang and Yen, 1995; Donello *et al.*, 1998). Functionally similar sequences have been found in the herpes simplex virus thymidine kinase gene (*HSV-TK-PPE*; Liu and Mertz, 1995) and in the mouse histone *H2a* gene (Huang and Carmichael, 1997). The HIV *Rev* responsive element (RRE) and the Mason Pfizer monkey virus constitutive transport element (CTE) recruit viral and cellular export factors that allow the cytoplasmic accumulation of unspliced RNA transcripts (Cullen, 2003).

Since KSHV PAN RNA is expressed at very high levels, but is neither spliced nor exported to the cytoplasm, we examined it for the presence of *cis*-acting elements important for nuclear RNA accumulation. We define a 79-nt element that is both necessary for the high abundance of PAN RNA and sufficient to increase transcript levels from an intronless β -globin construct. The element functions post-transcriptionally, most likely at the level of 3'-end processing. In contrast to all previously characterized *cis*-acting enhancers of intronless protein-coding gene expression, this element retains the intronless transcripts in the nucleus, an effect that can be overcome by splicing or by tethering of export factors to the transcript.

Results

A 115-nt region is necessary for high levels of PAN RNA

To identify *cis*-acting sequences that contribute to the nuclear accumulation of PAN, we co-transfected HEK293 cells with a plasmid containing the entire PAN transcribed region, ~1 kb of upstream promoter sequence, and 40 bp of downstream sequence (Figure 1A), along with a second plasmid encoding the viral transactivator Rta, necessary for PAN transcription (Song *et al.*, 2001; Chang *et al.*, 2002). Northern analysis revealed a prominent band corresponding to wild-type

(WT) PAN RNA (Figure 1B, lane 1), whereas no PAN RNA was observed in the absence of Rta (data not shown).

Analysis of constructs that delete approximately 300 nt of the PAN transcribed region and overlap one another by 70 nt (Figure 1A) showed a dramatic reduction in PAN RNA abundance for the 3'-most deletion (Figure 1B, lane 5). The $\Delta 1$ construct also exhibited downregulation, but this effect was more variable. Therefore, PAN $\Delta 4$ was chosen for further characterization. The deleted sequences in $\Delta 4$ lie 50 nt upstream of the cleavage and polyadenylation hexanucleotide, AAUAAA.

The first 70 nt of the $\Delta 4$ region are part of the overlapping $\Delta 3$ deletion, which showed no decrease in PAN RNA levels. Sequential deletion of 57 or 58 nt corresponding to the remainder of $\Delta 4$ revealed that $\Delta 4c$ and $\Delta 4d$ had the greatest effect on the steady-state levels of PAN RNA (Figure 1B). Since our subsequent analyses argue that this 115 nt region also acts to retain intronless transcripts in the nucleus (see below), we call it the PAN-ENE for RNA expression and nuclear retention element.

Cleavage and polyadenylation signals are necessary for PAN-ENE activity

The steady-state level of an RNA is dictated by its rates of synthesis and decay. RNA synthesis includes both transcription and processing of the nascent transcript to generate a mature RNA. To evaluate a role for the PAN-ENE in 3'-end processing, RNase protection assays (RPA) were performed and the ratio of transcripts that are correctly cleaved to those that read through the PAN cleavage site determined. Consistent with data from the Northern blots (Figure 1B), the RPAs show a substantial decrease in the levels of $\Delta 4$ cleaved RNA relative to WT (Figure 2B). There is an approximately 10-fold decrease in the cleaved-to-read-through ratio for the PAN $\Delta 4$ transcripts compared to WT (corresponding to 20 and 74% of the detected transcripts cleaved for $\Delta 4$ and WT, respectively). This observation supports a role for the PAN-ENE in increasing the efficiency of 3'-end formation, although any alteration in the stability of the processed $\Delta 4$ transcript might also lead to an apparent change in the cleaved-to-read-through ratio. However, half-life determinations (see below, Figure 3) demonstrate no significant difference between the WT and $\Delta 4$ cleaved transcripts.

To verify that the PAN-ENE acts through the normal 3'-end formation machinery, we replaced the PAN cleavage and polyadenylation signals, including the AAUAAA and the downstream GU-rich sequences, with a hammerhead ribozyme (Figure 2C; Eckner *et al.*, 1991; Libri *et al.*, 2002; Nott *et al.*, 2003). Our rationale was that this substitution, which generates a 3'-end through a distinct mechanism, should greatly diminish the difference between the WT and the $\Delta 4$ RNA levels. This is indeed the case, as the relative abundance of the WT and the $\Delta 4$ transcripts decreased from approximately 25-fold to about two-fold. The results in Figure 2 therefore support a role for the PAN-ENE in the cleavage and/or polyadenylation of PAN RNA.

The PAN-ENE does not affect rates of transcription or decay of PAN RNA

To test the possibility that the PAN-ENE plays other roles in RNA metabolism, we examined the relative rates of transcription and degradation of the PAN WT and $\Delta 4$ transcripts.

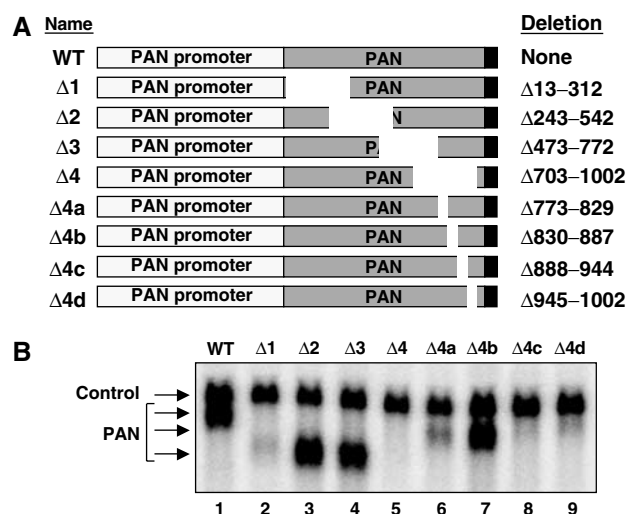


Figure 1 Sequences near the 3'-end of PAN are necessary for high levels of PAN RNA. (A) Schematic diagram of the PAN RNA constructs. Numbering refers to the PAN start site (+1) as defined by Zhong *et al.* (1996). (B) Northern blot analysis of the constructs listed in (A). The control is mRNA signal from a co-transfected β -globin construct. High-molecular-weight smears representing read-through transcripts were cut off for presentation.

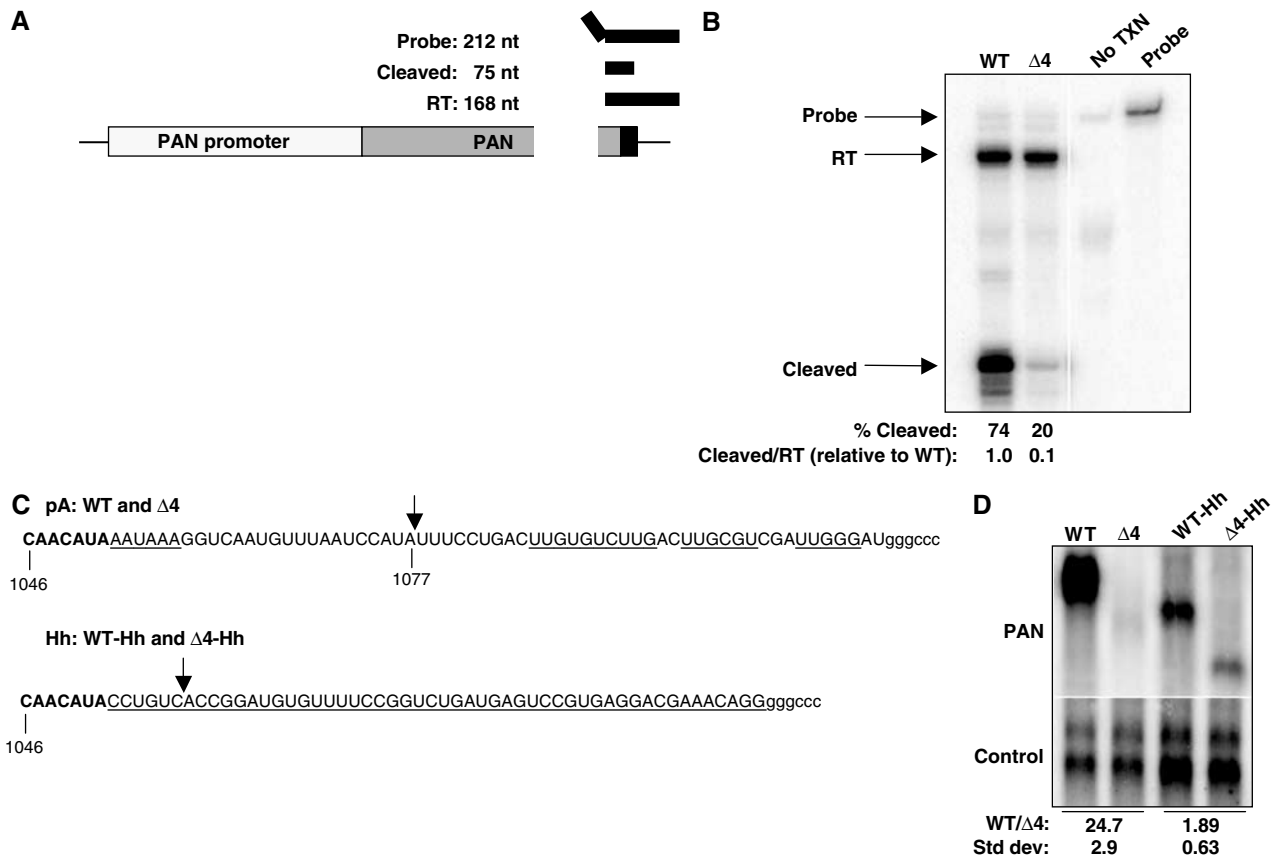


Figure 2 PAN-ENE likely affects 3'-end formation. (A) Schematic diagram of the RPA. Probe complementarity does not extend into the $\Delta 4$ deletion, so that the same-sized fragments are produced for all constructs tested. (B) Results from an RPA with RNA from cells transfected with the WT or $\Delta 4$ construct, or untransfected (no TXN) cells. The Probe lane shows 1% of the total. The values under each lane are percent cleavage and the ratio of the cleaved to read-through signals normalized to WT. The latter values are shown with standard deviations in Figure 4B. (C) RNA sequence of the 3'-end formation elements of the polyadenylated (pA) and hammerhead ribozyme (Hh) constructs. The sequence shown begins with PAN nt 1046, with common nucleotides depicted in bold face and cleavage sites indicated by arrows. Important *cis*-acting sequences are underlined: the polyadenylation hexanucleotide, downstream GU-rich regions, and the entire hammerhead ribozyme. Vector sequence is in lowercase letters. (D) Northern blot of RNAs from cells transfected with the indicated constructs. Quantitation was performed by normalization to a control probe that hybridizes to the co-transfected Rta mRNA. The ratios are the normalized WT PAN to $\Delta 4$ signals, with the standard deviation from four independent experiments.

Nuclear run-on experiments were performed to verify that the decrease in steady-state levels of $\Delta 4$ does not occur at the stage of RNA transcription. Such analyses measure the RNA polymerase density on a gene and are interpreted as a direct readout of transcriptional frequency.

Cells were transfected with the PAN WT or $\Delta 4$ constructs, the Rta expression plasmid, and a β -globin construct to control for transfection efficiency and RNA recovery (Figure 3A and B). To assess the background signal of the PAN probe, nuclei from cells in which the Rta plasmid was replaced with empty vector were examined. The results of multiple experiments demonstrated no significant difference between the polymerase density on the WT and $\Delta 4$ genes. Coupled with the observation that the PAN-ENE acts in an orientation-specific manner (see below), these results establish that the element affects a post-transcriptional step in gene expression.

The relative rates of decay of the PAN WT and $\Delta 4$ transcripts were examined using the transcription inhibitor actinomycin D (Figure 3C and D). The cleaved WT and $\Delta 4$ transcripts exhibited half-lives of over 24 h, whereas those of the $\Delta 4$ and WT read-through products were less than 6 h. Although in both cases the $\Delta 4$ RNAs were slightly less stable

than WT, the lack of a major difference between WT and $\Delta 4$ decay rates suggests that the ENE does not act by stabilizing the cleaved transcript. When taken with the results presented in Figure 2, these data strongly support the conclusion that the PAN-ENE acts as a *cis*-acting enhancer of 3'-end formation.

PAN-ENE activity localizes to a 79-nt element

To delineate the boundaries of the PAN-ENE, we performed RNase protection analyses (see Figure 2) on 12–14 nt deletions of the PAN-ENE (Figure 4A). The results confirm Northern blot analyses (Figure 1) in that the $\Delta 4c$ and $\Delta 4d$ deletions more dramatically affect PAN RNA levels than the $\Delta 4a$ and $\Delta 4b$ deletions, even though the cleaved to uncleaved ratios for $\Delta 4a$ and $\Delta 4b$ were somewhat lower than WT (Figure 4B). Although deletion of the 3'-most 12 nt of the PAN-ENE ($\Delta 4d5$) was never tested because of cloning difficulties, the adjacent upstream sequences ($\Delta 4d3$ and $\Delta 4d4$) were dispensable for activity. Likewise, two 25 nt deletions that span the 50 nt between the PAN-ENE and the cleavage and polyadenylation signal had no effect on steady-state levels of PAN RNA (data not shown). We conclude that the

core of the PAN-ENE resides within the 79 nt from +888 to +966 (-87 to -165 relative to the AAUAAA), but that upstream sequences may enhance the activity of the element.

Inspection of the core PAN-ENE sequence indicates that it can be divided into three separate domains (Figure 4C). The first and last domains are G/U- and U-rich, respectively, while the internal domain has only minimal U content, with sporadic CA or AC dinucleotides. These sequence features may be biologically relevant since U- and GU-rich upstream and downstream elements are known binding sites for essential cleavage and polyadenylation factors (Gilmartin *et al.*, 1995; Takagaki and Manley, 1997). Similarly, repeats of CA dinucleotides are bound by hnRNP L, which contributes to

the expression of the intronless *HSV-TK* gene (Liu and Mertz, 1995).

PAN-ENE is sufficient to increase the RNA, but not protein expression of a heterologous intronless gene

The dependence on introns for high levels of gene expression has been particularly well characterized in the case of human β -globin mRNA (Collis *et al.*, 1990; Liu and Mertz, 1996; Lu and Cullen, 2003). To ask whether the PAN-ENE increases the abundance of a heterologous transcript, we constructed PAN-ENE fusions with β -globin reporters. Beginning with an intronless β -globin construct, we inserted into the 3'UTR either the 300 nt PAN fragment that is deleted in $\Delta 4$ ($\beta\Delta 1,2$ -PF4) or one ($\beta\Delta 1,2$ -79F), two ($\beta\Delta 1,2$ -79Fx2), three ($\beta\Delta 1,2$ -79Fx3), or five ($\beta\Delta 1,2$ -79Fx5) copies of the core 79 nt PAN-ENE (Figure 5A). As controls, one to four copies of the core PAN-ENE were inserted in the reverse orientation ($\beta\Delta 1,2$ -79R, $\Delta\beta\Delta 1,2$ -79Rx2, $\beta\Delta 1,2$ -79Rx3, $\beta\Delta 1,2$ -79Rx4).

Consistent with previous reports, Northern analyses showed a dramatic decrease in RNA levels produced from the intronless β -globin reporter construct (Figure 5B and C). The PAN-ENE restored mRNA levels by about 11- or five-fold for the 300 nt $\Delta 4$ fragment or the core 79-nt PAN-ENE, respectively. The regions deleted in the $\Delta 1$ - $\Delta 3$, $\Delta 4a$, and $\Delta 4b$ constructs (Figure 1) were also tested, but did not increase RNA levels of intronless β -globin (data not shown). These results confirm the conclusion of the deletion experiments (Figure 4) that the core PAN-ENE is critical for activity, while the upstream flanking region increases its efficiency.

The insertion of multiple copies of the core PAN-ENE had even more dramatic effects on β -globin mRNA levels (Figure 5B, lanes 6-8, and 5C). Compared to a single copy of the PAN-ENE, two or three inserts enhanced three- or four-fold, respectively, no additional RNA expression boost was observed with five copies. Strikingly, with three copies of the PAN-ENE, the steady-state level of the intronless β -globin mRNA approached that of spliced β -globin transcripts. We conclude that the PAN-ENE is both necessary and sufficient for high-level RNA production from two different intronless genes, *PAN* and *β -globin*.

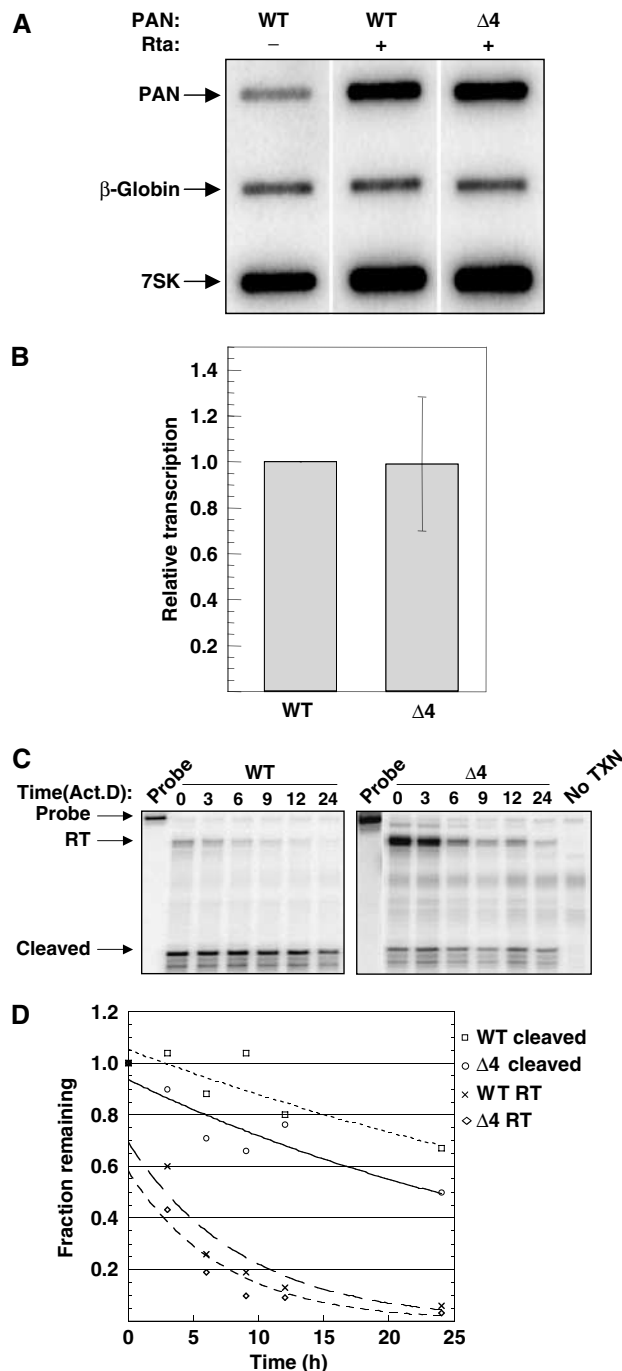


Figure 3 PAN-ENE does not alter transcription rate or transcript half-life. (A) A representative slot blot from a nuclear run-on experiment with nuclei isolated from cells transiently transfected with PAN WT or $\Delta 4$, and β -globin constructs. In the left-most panel, Rta was omitted to assess the background of the PAN probe. A probe for the cellular RNA polymerase III-transcribed 7SK RNA was also included. The probes are full-length RNA transcripts complementary to PAN, β -globin, or 7SK. (B) Relative transcription levels of the PAN WT and $\Delta 4$ constructs are graphed with the WT value set at 1.0. The data represent the average of three independent experiments with standard deviations shown. (C) RNase protection analysis of RNAs from cells transiently transfected with the PAN WT (left panel) or $\Delta 4$ (right panel) constructs. Actinomycin D was added 18-24 h post-transfection and cells were harvested at the indicated times. The probe used in the $\Delta 4$ panel had five-fold higher specific activity than the WT probe. The probe lanes show ~1% of the total. Intervening samples between the $\Delta 4$ probe and subsequent lanes were omitted for clarity. No TXN shows RNase protection of RNA from untransfected cells. (D) Graphical representation of the decay experiments; each point is the average of three or four independent experiments. \square : WT cleaved; \circ : $\Delta 4$ cleaved; \times : WT RT; \diamond : $\Delta 4$ RT.

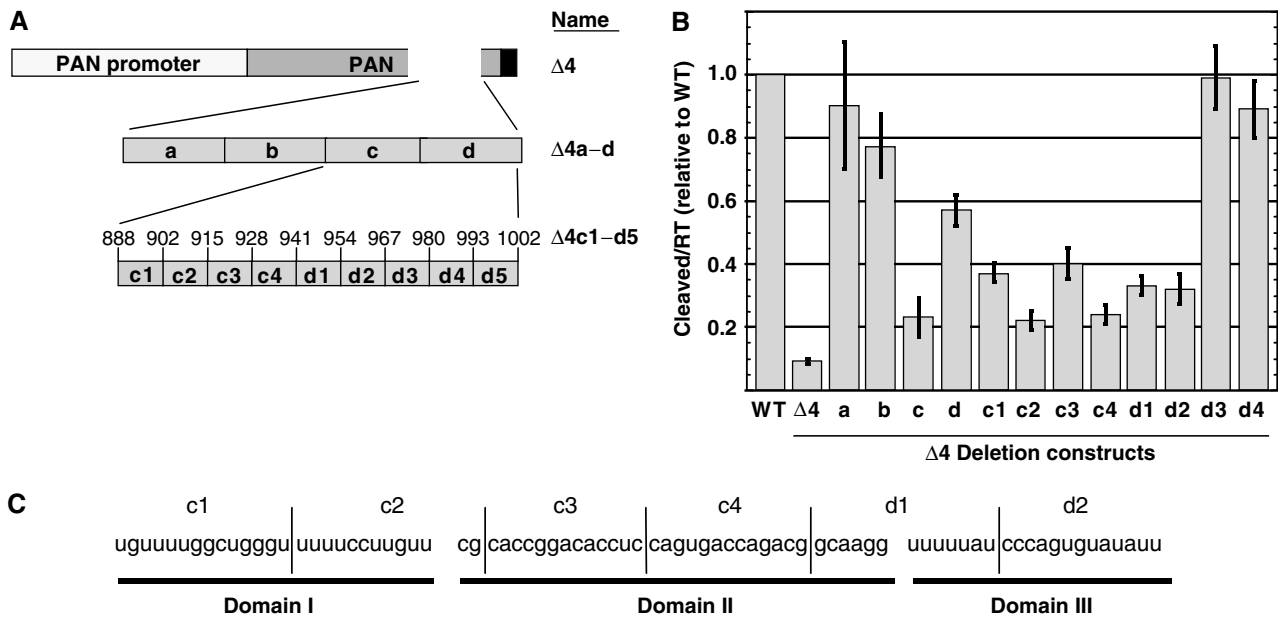


Figure 4 Definition of the 79-nt PAN-ENE core element. (A) Schematic showing the $\Delta 4$ and the $\Delta 4$ subdeletion constructs, with boxed areas representing deletions. The $\Delta 4a-d$ constructs contain 57–58 nt deletions (Figure 1), while the $\Delta 4c1-d5$ constructs contain 12–14 nt deletions. (B) Quantitation of data from RPAs with the $\Delta 4$ deletion series. RPAs (Figure 2) were quantitated by setting the cleaved-to-read-through ratio of WT transcripts to 1.0 for each independent experiment; each deletion construct was compared to the WT value. The data are the average of three independent experiments, with the error bars indicating standard deviation. (C) The sequence of the 79 nt core PAN-ENE. Vertical lines demarcate the $\Delta 4c1-d2$ regions. Horizontal lines separate the three sequence domains of the element.

Since the PAN-ENE is derived from a nontranslated RNA, we evaluated its effects on translational output of the β -globin-PAN-ENE constructs. We analyzed protein levels by Western blot (Figure 5D) and compared them to a titration of protein produced from $\beta\Delta 1$ (lanes 1–4). Unfortunately, the limited linear range of Western blotting procedures provides only a rough approximation of relative protein expression (lanes 1–3). Even so, the data demonstrate that the protein levels do not mirror the mRNA levels. While each PAN-ENE fusion (lanes 6–10) showed an increase in protein expression compared to intronless β -globin (lane 5) or the reverse orientation controls (lanes 11–14), none of these constructs produced protein at levels similar to that of the spliced β -globin RNA. Since the translational yields of intronless transcripts are known to be lower than those of spliced messages (Lu and Cullen, 2003; Nott *et al.*, 2004), it is more appropriate to compare the protein production of $\beta\Delta 1,2$ and its derivatives. Surprisingly, the relative protein yield from these constructs peaks with only two copies of the PAN-ENE (lane 8), even though the message levels are highest with three or five copies (lanes 9 and 10). Thus, translational yields from β -globin-PAN-ENE mRNA transcripts are lower than those of either spliced or unspliced messages possessing the same coding potential.

Intronless β -globin-PAN-ENE mRNA is retained in the nucleus

Since PAN is an exclusively nuclear transcript, we reasoned that the poor translational efficiency of the β -globin-PAN-ENE mRNA might result from its retention in the nucleus. The nucleocytoplasmic distribution of β -globin mRNA was therefore examined by fluorescence *in situ* hybridization (Figure 6). The spliced β -globin messages were predomi-

nantly cytoplasmic (panels A–D) as were the unspliced $\beta\Delta 1,2$ (panels E and F) and the reverse orientation control $\beta\Delta 1,2-79R_{x4}$ (panels I and J) transcripts. Strikingly, the $\beta\Delta 1,2-79F_{x5}$ constructs showed predominantly nucleoplasmic staining with exclusion of the nucleolus. Decreasing the number of PAN-ENE inserts roughly correlates with a decrease in the ratio of nuclear to cytoplasmic signal (data not shown). These results suggest that the PAN-ENE is not only important for the high PAN RNA levels, but also can serve as a nuclear retention element for unspliced β -globin mRNA.

Direct tethering of export factors can overcome the ENE-induced nuclear retention

In a simplified model of mRNA export, the mRNA is first bound by an adaptor protein, which recruits the export receptor responsible for carrying the mRNP to the nuclear pore and cytoplasm (Figure 7A). It is possible that the ENE blocks export of PAN and β -globin messages at any of these stages of export. To distinguish among these possibilities, we artificially tethered known export factors (for reviews, see Dimaano and Ullman, 2004; Erkmann and Kutay, 2004), including the adaptor REF2-1, the receptor TAP, and UAP56, a general export factor also involved in splicing, to β -globin-ENE reporter constructs. By analyzing which, if any, of these factors bypasses ENE-mediated retention *in vivo*, we can identify the stage of export blocked by the ENE.

Six binding sites for the bacteriophage MS2-coat protein were inserted into the coding region or 3'UTR of $\beta\Delta 1,2-79F_{x5}$ to create M- $\beta\Delta 1,2-79F_{x5}$ and $\beta\Delta 1,2-M-79F_{x5}$, respectively (Figure 7B; Lykke-Andersen *et al.*, 2000). We co-expressed these constructs with MS2-coat protein fusions of export factors and examined the nucleocytoplasmic distribution of β -globin RNA by *in situ* hybridization (Figure 7C). As

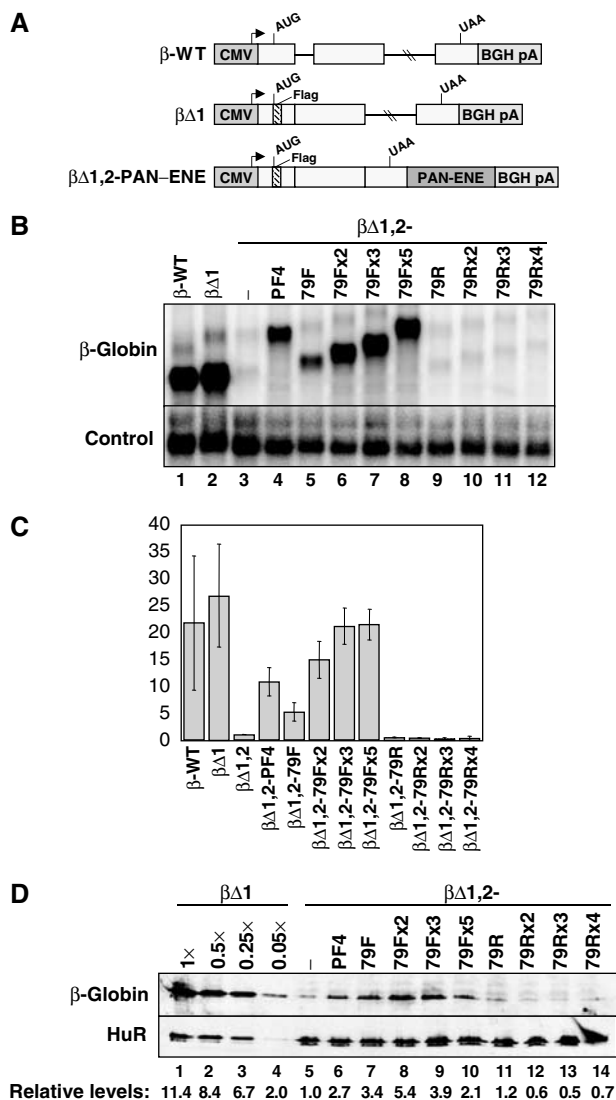


Figure 5 PAN-ENE rescues RNA levels, but not translation of a heterologous intronless transcript. (A) Schematic representation of the β -globin constructs. The vector sequence includes the CMV promoter and bovine growth hormone polyadenylation signals (BGH pA). The light gray boxes and lines represent the β -globin exons and introns, respectively. The hatched box depicts an N-terminal Flag tag and the dark gray box (PAN-ENE) shows the site of insertion of the tested PAN fragments in forward or reverse orientation. The start (AUG) and stop (UAA) codons are also shown. (B) Northern blot analysis of the β -globin-PAN chimeric RNAs. The control is the same blot hybridized to a probe for the co-transfected Rta construct. Similar results were observed in the absence of Rta. (C) Quantitation of the results of Northern blot analysis. The β -globin signals, normalized to the control probe to correct for loading and transfection efficiency, are graphed. $\beta\Delta 1,2$ signal was arbitrarily set at 1.0, with other signals referenced to that signal. The relative levels shown are the averages for three or four independent experiments, with error bars representing standard deviation. (D) Western blot of the β -globin protein produced from the intronless β -globin constructs. Total protein from approximately 2×10^5 cells was loaded in each lane, except lanes 2, 3, and 4 in which serial dilutions of the $\beta\Delta 1$ sample were loaded. The bottom panel shows the same membrane after stripping and re-probing with an antibody to HuR. Relative β -globin signals shown below are the average of two independent experiments.

expected, β -globin reporters containing the ENE were predominantly nuclear in the absence of MS2-fusions (data not shown) or in the presence of MS2-coat protein alone (panels

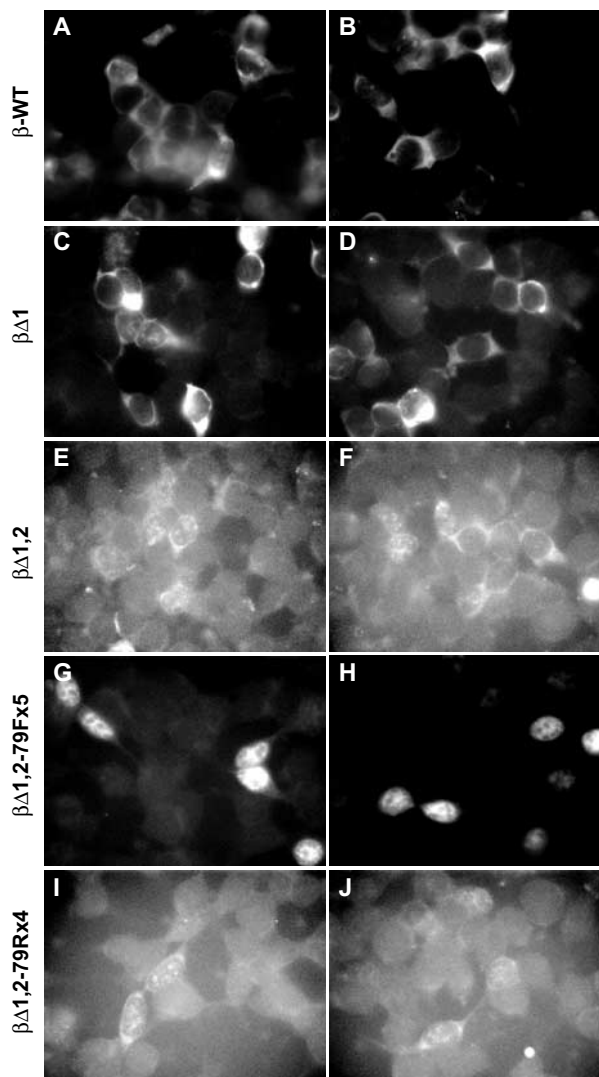


Figure 6 Multiple copies of the PAN-ENE cause accumulation of the intronless β -globin mRNA in the nucleus. Fluorescence *in situ* hybridization of HEK293 cells transfected with the indicated β -globin reporter constructs. Two panels are shown for each construct to illustrate the heterogeneity observed in both signal strength and localization. Since only a subset of the cells are transfected, only a fraction of the cells in each panel show hybridization signal. Signal-to-noise ratios are lower with the $\beta\Delta 1,2$ and $\beta\Delta 1,2$ -79Rx4 constructs (panels E, F, I, and J), due to their relatively low levels of mRNA.

a and b). In contrast, MS2-TAP co-expression yielded clear accumulation in the cytoplasm (panels c and d). MS2-UAP56 and MS2-REF2-1 also generated predominantly cytoplasmic signal, but nuclear staining often remained (panels e, f, g and h). MS2 fusions with other mammalian REF-family proteins, REF1-I (also known as Aly) and REF1-II (Stutz *et al*, 2000), were also tested, but their expression led to truncation of the reporter constructs, likely due to cryptic splicing (data not shown). No increase in cytoplasmic signal was observed when fusions to SRm160, RNPS1, or hnRNP A1 were co-expressed with the reporter constructs (data not shown). Since direct binding of export factors to ENE-containing transcripts leads to efficient export, we conclude that the ENE acts to inhibit recruitment of these factors to the mRNP in cultured cells.

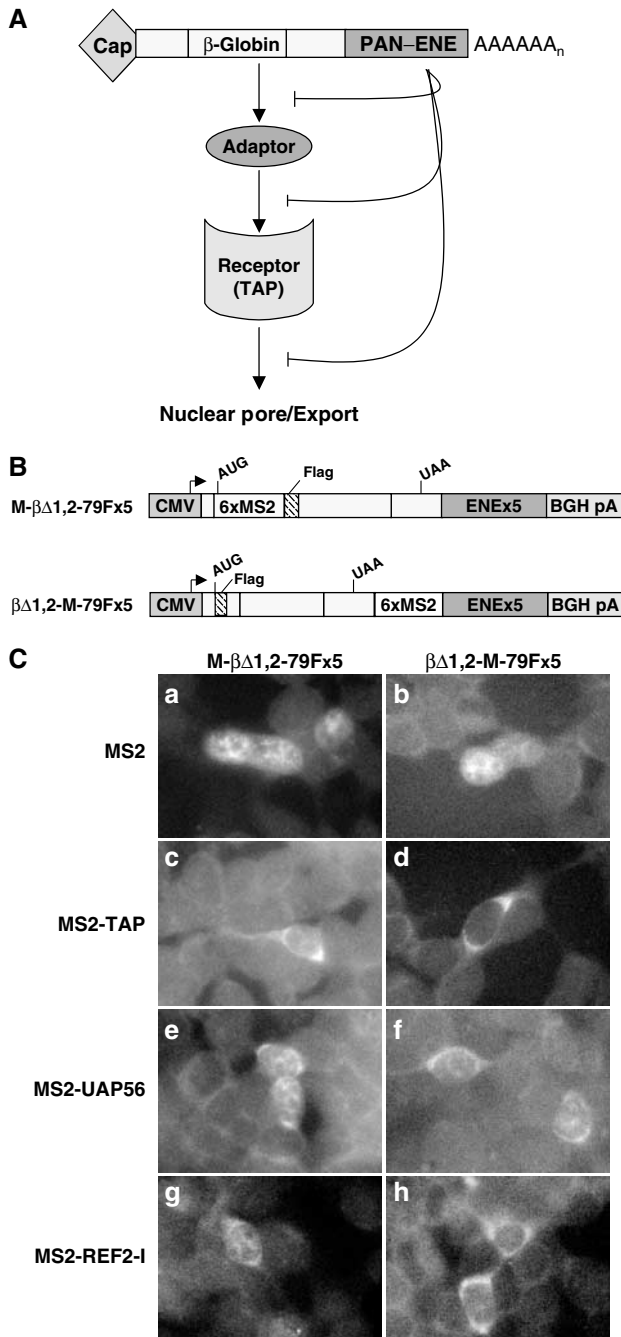


Figure 7 Tethering of export factors leads to cytoplasmic accumulation of β-globin-ENE mRNA. (A) Schematic of export-competent mRNP formation. (B) Schematic of ENE-containing reporter constructs with MS2-binding sites. (C) *In situ* hybridization of β-globin reporter constructs M-βΔ1,2-79Fx5 (a, c, e, g) or βΔ1,2-M-79Fx5 (b, d, f, h) co-transfected with MS2-coat protein alone (a, b) or MS2-coat protein fused to TAP (c, d), UAP56 (e, f), or REF2-I (g, h).

Splicing reverses the nuclear retention of the β-globin-PAN-ENE mRNA

Splicing has been reported to promote the efficient export of mRNA by recruitment of export factors (reviewed in Reed, 2003). Furthermore, the splicing-associated export factor UAP56 is capable of exporting ENE-containing transcripts (Figure 7C). We therefore investigated whether nuclear retention by the PAN-ENE is specific to unspliced transcripts.

We restored the second intron of β-globin to the intronless constructs containing three or five copies of the PAN-ENE in the forward orientation and three or four copies of the reverse PAN-ENE to generate βΔ1-79Fx3, βΔ1-79Fx5, βΔ1-79Rx3, and βΔ1-79Rx4, respectively. Transfected cells were analyzed for β-globin RNA and protein levels, as well as for β-globin RNA localization.

Figure 8 shows that both the RNA and protein levels produced by these spliced constructs were not altered by the presence of the PAN-ENE. *In situ* hybridization showed that, in contrast to the βΔ1,2-79Fx5 transcripts, the βΔ1-79Fx5 transcripts were predominantly cytoplasmic (Figure 7C). Thus, splicing can surmount the retention conferred by the PAN-ENE. Both the forward- and reverse-orientation PAN-ENE constructs exhibited slightly more nuclear staining than βΔ1. This may be due to the increased level of pre-mRNA transcripts generated from these constructs (data not shown). Alternatively, it is formally possible that an increasing length of the 3'UTR decreases the export efficiency of β-globin RNA.

Discussion

We have identified a *cis*-acting element important for the nuclear accumulation of KSHV PAN RNA. The PAN-ENE exhibits two activities *in vivo*: it increases the steady-state levels of intronless transcripts and confers their nuclear retention. Our deletion analysis suggests a minimal length for the PAN-ENE of approximately 79 nt; we have not tested whether the entire 79 nt core PAN-ENE sequence is essential for nuclear retention activity. Since the tethering of export factors overcame the nuclear localization of the β-globin-ENE RNAs (Figure 7), mechanistically the ENE acts at an early stage of mRNP formation. Splicing also generates cytoplasmic β-globin-ENE transcripts, demonstrating that retention is recessive to the effects of splicing on export. Previously characterized *cis*-acting enhancers of intronless gene expression have been reported to promote export or to be export neutral (Huang and Liang, 1993; Huang and Yen, 1995; Liu and Mertz, 1995; Huang and Carmichael, 1997; Popa *et al*, 2002). Thus, the PAN-ENE is unique in its nuclear retention activity. The PAN-ENE is also distinct from canonical cleavage and polyadenylation upstream sequence elements (USEs; e.g., see Brackenridge *et al*, 1997; Natalizio *et al*, 2002) in that USEs do not compensate for lack of introns (Gruss *et al*, 1979) and function in 3'-end formation of intron-containing genes (Moreira *et al*, 1995; Brackenridge *et al*, 1997).

Enhancement of 3'-end formation independent of export

The PAN-ENE is necessary as well as sufficient to elevate the levels of both PAN and β-globin transcripts (Figures 1 and 5). Our data argue that it does so by increasing the efficiency of 3'-end processing. The ratio of cleaved-to-read-through transcripts is greatly diminished when the PAN-ENE is deleted. It was somewhat surprising that this change is primarily due to a decrease in the Δ4 cleaved transcript levels and not due to an increase in Δ4 read-through transcripts (Figure 2B), so we further tested the hypothesis that the ENE enhances 3'-end formation. Generating a 3'-end in a fashion independent of the normal 3'-end formation machinery greatly diminished

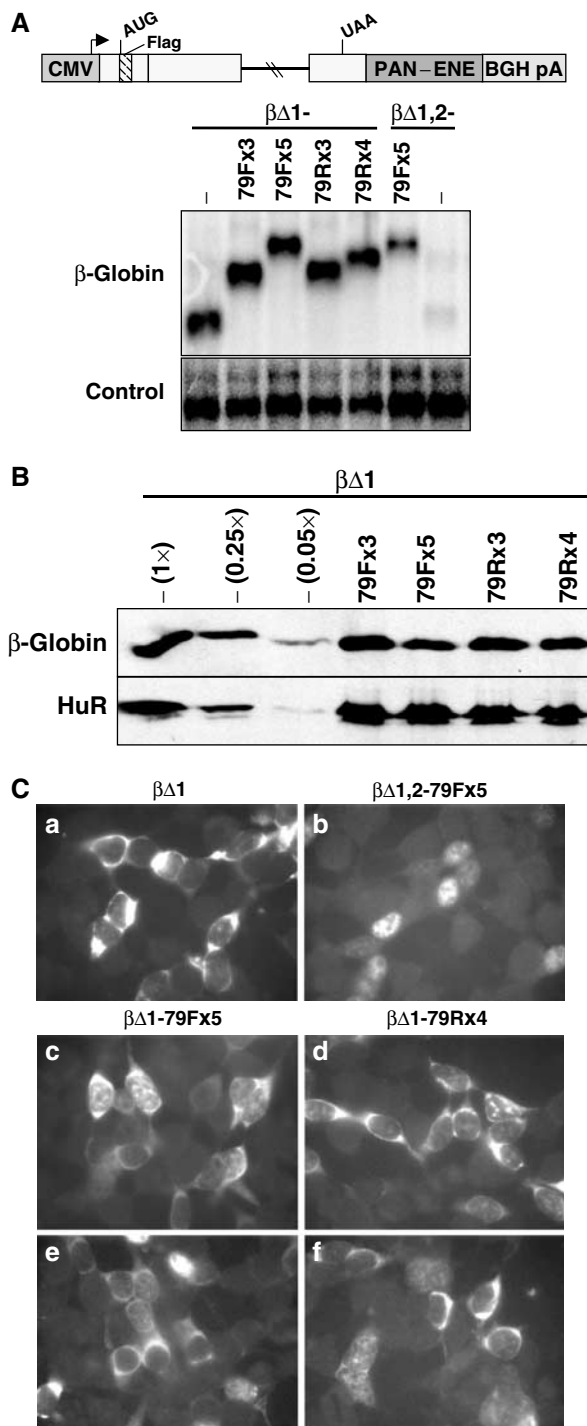


Figure 8 Splicing is dominant over PAN-ENE-mediated retention. Schematic diagram: Northern (A), Western (B), and *in situ* hybridization (C) analyses of intron-containing β -globin-PAN-ENE constructs. Analyses were performed as in Figures 5B, D, and 6. Panels c and e are independent fields showing β 1-79Fx5; d and f show β 1-79Rx4.

the difference in RNA levels of WT and Δ 4 (Figure 2D). Neither the half-lives of the cleaved WT and Δ 4 transcripts nor their transcription rates differ significantly (Figure 3). These results are consistent with the data of others demonstrating that the increased abundance from intron-containing genes compared to the same mRNAs generated from genes

lacking introns is due to increased 3'-end formation efficiency (Lu and Cullen, 2003; Nott *et al.*, 2003). Taken together, these data strongly argue that the ENE enhances the 3'-end formation of intronless transcripts. The lack of any observable increase in Δ 4 read-through RNAs is therefore likely due to their inherent instability. To date, attempts to stabilize the PAN read-through transcripts using downstream cleavage and polyadenylation signals (e.g., see Natalizio *et al.*, 2002) have failed (data not shown).

While it seems likely that the PAN-ENE acts to enhance 3'-end formation, our results are also consistent with a model in which the PAN-ENE inhibits a polyadenylation-dependent pre-steady-state RNA decay pathway (i.e., one in which decay occurs too quickly to be observed with steady-state analyses). At first glance, this model seems somewhat far-fetched, but recent studies linking the nuclear exosome with transcription and polyadenylation argue that this hypothesis should not be overlooked (Vasudevan and Peltz, 2003).

Consistent with the idea that 3'-end formation promotes release of the transcript from the DNA, our data suggest that the retention promoted by the PAN-ENE occurs subsequent to chromatin release. The nuclear accumulation of the β -globin-PAN-ENE mRNA is diffusely nucleoplasmic and not punctate, indicating that the transcripts are not restricted to sites of transcription (Figure 6). Furthermore, in nucleocytoplasmic fractionation experiments under conditions where unprocessed read-through transcripts are \sim 90% nuclear, a significant percentage of both the β -globin-PAN-ENE and PAN polyadenylated RNAs leak into the cytoplasmic fraction (data not shown). We believe that our *in situ* hybridization (Figures 6–8) and not the fractionation results represent the *bona fide* nucleocytoplasmic distribution of these transcripts for several reasons. First, multiple oligonucleotide probes that hybridize to different regions in the PAN or β -globin transcripts show *in situ* hybridization results identical to previously published work (Sun *et al.*, 1996) and the present data. Second, the cytoplasmic signals are clearly not being washed away in the *in situ* protocol, since cytoplasmic signal can be observed even with poorly expressed transcripts (Figure 6, panels E, F, I and J). Finally, the distribution of PAN in the fractionation experiments closely resembles that of U6 snRNA, an exclusively nuclear transcript (data not shown). We conclude that the ENE promotes 3'-end formation and concomitant release from chromatin, even though export is inhibited.

While the PAN-ENE is sufficient for nuclear retention of β -globin transcripts, the PAN-ENE is likely redundant with other *cis*-acting nuclear retention elements in the PAN transcript. Although insertion of one PAN-ENE detectably shifts the nucleocytoplasmic distribution of the intronless β -globin transcript to the nucleus (data not shown), multiple copies of the PAN-ENE are required for a predominantly nuclear signal (Figure 6). Furthermore, no cytoplasmic accumulation of the PAN Δ 4 transcript is seen by *in situ* hybridization (data not shown).

Splicing and deposition of export factors are dominant to PAN-ENE-mediated nuclear retention

Restoration of the second intron of β -globin into the β -globin-ENE construct increased the efficiency of export (Figure 8C), consistent with previous data showing that splicing and export are linked (Reed, 2003). Mechanistically, the simplest

model is that splicing leads to the deposition of export factors on the transcript. However, a direct role for splicing in export remains controversial (Dimaano and Ullman, 2004; Erkmann and Kutay, 2004). Alternative mechanisms include the possibility that splicing remodels the mRNP, passively allowing export factors to bind. Indeed, if export and 3'-end formation are normally directly coupled, the ENE may promote 3'-end formation in a fashion that is uncoupled from export. Since splicing and 3'-end formation are linked (Le Hir *et al.*, 2003), restoration of the second intron of β -globin may override the ENE pathway and promote the use of the export-coupled pathway. Finally, it is possible that the intron or the spliceosome interferes with ENE function directly by occluding ENE-binding proteins or by altering RNA secondary structure.

The observation that direct tethering of export factors TAP, UAP56, and REF2-1 is sufficient for export of the β -globin-ENE mRNAs (Figure 7) provides insight into the mechanism of PAN-ENE-induced nuclear retention. While TAP lies downstream in the export pathway, both UAP56 and REF act at an upstream step (Luo *et al.*, 2001; Rodrigues *et al.*, 2001; Custodio *et al.*, 2004) and are recruited to processed mRNPs at the site of transcription (Custodio *et al.*, 2004). Our data indicate that the PAN-ENE does not interfere with any of these proteins' activities, since tethering of any one is sufficient to overcome the export block (Figure 7). Therefore, the PAN-ENE must alter mRNP assembly, likely by inhibiting or bypassing export factor recruitment at the site of transcription.

How does the presence of the ENE alter export factor recruitment by an intronless β -globin transcript? One possibility is that the ENE forms a secondary structure that masks the site(s) responsible for binding of export factors. Structured RNAs can be refractory to REF binding (Masuyama *et al.*, 2004), so it is possible that addition of a structured element to the β -globin message may decrease its export efficiency. However, we think this is unlikely since the ENE reverse complement, which has predicted structural features similar to the ENE (data not shown; Zuker, 2003), does not induce nuclear retention (Figure 6), nor does the addition of six structured MS2-binding sites (data not shown). Moreover, the PAN-ENE also functions when placed in the 3'UTR of the firefly luciferase transcript (data not shown), demonstrating that the ENE does not simply mask specific export-factor binding sites in the β -globin transcript.

One of many alternative models speculates that enhanced 3'-end processing of the β -globin-ENE RNA releases it from the transcription site too quickly for an export-competent mRNP to be assembled. In a β -globin transcript lacking an intron or the ENE, decreased processing efficiency would increase the dwell time at the site of transcription and therefore provide a greater opportunity for export factors to bind in the absence of splicing. A similar model has been proposed in yeast, where decreased elongation efficiency leads to more efficient mRNP assembly (Jensen *et al.*, 2004). An assumption inherent in this model is that co-transcriptional assembly is obligatory to form an export-competent mRNP and that once the RNA is released from the site of transcription recruitment of export factors is significantly less likely. One prediction of this model is that the 3'-end formation and processing activities of the ENE are causally linked and therefore

inseparable. We are currently testing this prediction by mutagenesis of the ENE. These and further analyses will provide experimental data necessary to support or reject this hypothesis.

Does PAN-ENE function in KSHV infection?

Without knowing the function of the PAN RNA, it is difficult to assess the activity of the PAN-ENE with respect to KSHV biology. Since PAN is so abundantly expressed in the nuclei of lytic phase cells, both its quantity and localization seem likely to be important for PAN function. High levels of PAN may titrate important cellular factors that would otherwise inhibit viral production. Such cellular factors may be important not only for PAN maturation and localization, but may also become unavailable to cellular messages because of their sequestration by PAN. Further dissection of the PAN-ENE and its presumed cellular co-factors will lead to insights into mRNA retention and export, 3'-end formation, and KSHV biology.

Materials and methods

Cell culture and transfection

HEK293 cells were grown in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 2mM L-glutamine and 1 \times penicillin streptomycin solution (Sigma). Transfections were performed with TransIT-293 (Mirus Corporation) according to the manufacturer's protocol.

Plasmid constructs, Northern blotting, and RPAs

Plasmid construction, Northern blotting, and RPAs were performed using standard techniques; the details are given in Supplementary data.

Nuclear run-on assays

Nuclear run-on experiments were modified from Ashe *et al.* (1997); details of the procedure are given in Supplementary data. The signals were quantitated using the ImageQuant software (Molecular Dynamics). First, a background value was subtracted from the volume for each slot based on the general background signal on the filter. Then, PAN signal was normalized to that of β -globin, as a control for transfection efficiency and RNA recovery. Since the PAN background was relatively high in the samples that had not been transfected with Rta, the normalized 'Rta negative' value was subtracted from that of the samples that had been transfected with Rta. This value for the PAN WT-transfected constructs was then normalized to 1.0, and the $\Delta 4$ values are given relative to that of WT. Experiments were also performed in which the β -globin construct was not co-transfected. In these cases, the signals were first normalized to the 7SK signal instead of β -globin. This correction step made virtually no difference in the final values because, in contrast to the PAN signal, the β -globin probe showed very low background.

Western blotting and protein extracts

Total protein was recovered from the Trizol organic phase according to the manufacturer's protocol or obtained by direct lysis of cells in 1 \times loading buffer (187.5mM Tris-HCl (pH 6.8), 2.1% SDS, 10% glycerol, 140mM β -mercaptoethanol, 0.05% bromophenol blue), followed by brief sonication. Both protein isolation protocols yielded identical results. Antibodies for Western blotting were diluted to 15 μ g/ml (anti-globin) or 1:30000 (anti-HuR) in TTBS (100mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% Tween-20) with 10% non-fat dry milk. Electrophoresis, transfer, and Western blotting were performed by general techniques, and proteins were detected using Western Lightning (Perkin-Elmer, Inc.) reagents. Quantitation was performed using Quantity One software (Bio-Rad) on scanned autoradiographs. Since the HuR signal was clearly out of the linear range, β -globin levels were not normalized to HuR signal.

In situ hybridization

In situ hybridization was performed based on Kendirgi *et al* (2003); the details are outlined in the Supplementary data. A cocktail of three digoxigenin-labeled 39-mer oligodeoxynucleotides that hybridize to the 5', central, and 3' portions of the β -globin coding sequence were hybridized to fixed cells and detected with anti-digoxigenin-Rhodamine Fab fragments (Roche). While the probe mixture showed the best signal-to-noise ratio, each probe was also tested individually and the results were the same as those presented in Figures 6–8.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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