Uncoupling of hTREX demonstrates that UAP56 and hTHO-complex recruitment onto herpesvirus saimiri intronless transcripts is required for replication

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Herpesvirus saimiri (HVS) ORF57 nucleocytoplasmic shuttle protein binds viral RNA and interacts with the cellular nuclear export adaptor protein, Aly, to access the TAP-mediated nuclear export pathway. This enables the efficient nuclear export of HVS intronless mRNAs. Herein, we extend these studies and demonstrate that ORF57 recruits several members of hTREX, namely Aly, UAP56 and hTHO-complex proteins, onto the viral mRNAs to assemble an export-competent ribonucleoprotein particle. Moreover, using a transdominant form of Aly which inhibits UAP56 and hTHO-complex association with viral intronless mRNA, we show that complete hTREX recruitment is required for efficient HVS mRNA nuclear export and replication.

In contrast with cellular genes, the majority of the herpesvirus lytically expressed genes lack introns. Herpesviruses replicate in the host cell nucleus and therefore require their intronless mRNAs to be efficiently exported from the nucleus to allow translation. To undergo efficient nuclear export, cellular mRNAs undergo various post-transcriptional processing events, including capping, splicing and polyadenylation (Bentley, 2005; Erkmann & Kutay, 2004; Vargas et al., 2005). It is evident that splicing is particularly important for mRNA nuclear export (Luo & Reed, 1999). The distinct multi-protein complexes hTREX and the exon–exon junction complex are both recruited to mRNA in a cap- and splicing-dependent manner. hTREX, which comprises Aly/REF, UAP56 and the multi-protein hTHO-complex, is pivotal in directing nuclear export of mRNA (Cheng et al., 2006; Masuda et al., 2005). Once bound to mRNA, Aly stimulates recruitment of the mRNA export factor, TAP (Le Hir et al., 2001). TAP interacts with p15 (Fribourg et al., 2001) and nucleoporins, providing the connection between the ribonucleoprotein particle (RNP) and the nuclear pore (Bachi et al., 2000; Grant et al., 2002; Huang & Steitz, 2001).

To facilitate efficient viral intronless mRNA export, herpesviruses encode a conserved protein which has an essential role in post-transcriptional mechanisms (Bello et al., 1999; Boyne et al., 2008a; Boyne & Whitehouse, 2006a; Sandri-Goldin, 2004; Smith et al., 2005; Swaminathan, 2005). Herpesvirus saimiri (HVS) ORF57 binds viral mRNA and shuttles between the nucleus and cytoplasm, promoting nuclear export of viral intronless mRNAs (Boyne et al., 2008a; Boyne & Whitehouse, 2006b; Colgan et al., 2009; Cooper et al., 1999; Goodwin et al., 1999, 2000; Goodwin & Whitehouse, 2001; Whitehouse et al., 1998). We have demonstrated that ORF57 interacts with Aly, providing the connection between the viral RNP and the nuclear export factor, TAP. Moreover, disruption of the TAP-mediated nuclear export pathway had a profound effect on HVS replication (Williams et al., 2005). This suggests that the ORF57–Aly–TAP interaction is required for the efficient nuclear export of intronless transcripts.

Herein, we demonstrate that ORF57 associates with other core components of hTREX forming an export competent viral RNP. Moreover, we show that hTREX recruitment onto the viral intronless mRNA is required for efficient HVS replication.

hTREX contains Aly, UAP56, the multi-protein hTHO-complex (hTho1, hTho2, fSAP79, fSAP35 and fSAP24) and Tex1. We have previously demonstrated that, during an HVS infection, ORF57 co-localizes with hTREX proteins in the nucleolus (Boyne & Whitehouse, 2006b). Therefore, we assessed whether ORF57 interacts directly with hTREX proteins. Aly-, UAP56- and hTho1-myc fusion proteins were produced by PCR amplifying and cloning their respective coding regions into pcDNA3.1myc-His-B (Invitrogen), producing pAly-myc, pUAP56-myc and pHTho1-myc. Transfection of each myc construct into
293T cells resulted in expression of the full-length myc-tagged proteins (data not shown). To assess whether ORF57 interacts with hTREX components, co-immunoprecipitations were performed; 293T cells were co-transfected with each hTREX-myc construct in the presence of pGFP or pORF57GFP. Thirty-six hours post-transfection, cell lysates were incubated with a GFP-specific antibody (Clontech) and the immunocomplex was captured using protein-A agarose. Control lysates were incubated with protein-A agarose beads alone. hTREX proteins were then detected by immunoblotting using a 1:2000 dilution of myc-specific antibody (Sigma). Results show that ORF57 interacts with hTREX core components (Fig. 1a).

Furthermore, co-immunoprecipitation assays were performed using pGFP- or pORF57GFP-transfected cell lysates, precipitating with fSAP79-, fSAP24- and Tex1-specific antibodies. ORF57 was then detected by immunoblotting using a 1:1000 dilution of GFP-specific antibody (Supplementary Fig. S1, available in JGV Online). These combined results show that ORF57 interacts with several hTREX components.

To address possible overexpression artefacts from these transfection studies, we next assessed whether ORF57 interacted with hTREX components during an HVS lytic infection. Moreover, to assess whether these interactions were direct or due to RNA bridging, experiments were also performed in the absence or presence of RNaseA. Owl monkey kidney (OMK) cells remained uninfected or were infected with HVS-A11 at an m.o.i. of 1. After 24 h, the cell lysates remained untreated or were incubated for 30 min at 37 °C with 20 μg RNaseA ml⁻¹. Supplementary Fig. S2 (available in JGV Online) confirms that RNaseA treatment was successful, as previously described (Carlile et al., 1998). The untreated and treated cell lysates were then used in co-immunoprecipitations, as described above, using an ORF57-specific antibody. Immunoblotting was then performed using fSAP79- and hTho1-specific antibodies. UAP56 and Aly could not be utilized in this analysis, as both the ORF57 and hTREX antibodies were of polyclonal origin, and cross-reactivity of the immunoglobulins masked the UAP56 and Aly immunoprecipitations. However, results revealed that ORF57 interacts with fSAP79 and hTho1 during lytic replication and that these interactions were RNA-independent (Fig. 1b).

To determine which hTREX protein interacted directly with HVS ORF57, radio-labelled ORF57 was generated by in vitro coupled transcription/translation (ITT), as previously described (Williams et al., 2005), and used in GST pull-down experiments with GST-Aly, GST-UAP56 and GST-hTho1 fusion proteins. Analysis showed that, in contrast with UAP56 and hTho1, ORF57 only bound directly to GST-Aly (Fig. 1c).

hTREX proteins are recruited to mRNAs in a splicing-dependent manner; therefore, we next determined whether hTREX proteins associated with intronless HVS transcripts by using an ORF57-dependent mechanism, using RNA

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**Fig. 1.** HVS ORF57 interacts with hTREX components. (a) 293T cells were transfected with either pGFP (i) or pORF57GFP (ii) and hTREX-myc constructs. Immunoprecipitations were performed using beads alone (control) or a GFP-specific antibody and immunoblots were performed using a myc-specific antibody. Total cell lysate from pGFP or pORF57GFP-transfected cells served as positive controls (Input). (b) Immunoprecipitations were performed on mock-infected (i) or HVS-infected (ii) or RNaseA-treated HVS-infected (iii) OMK cell lysates, using beads alone or an ORF57-specific antibody. Immunoblots were performed using fSAP79- and hTho1-specific antibodies. OMK cell lysate served as a positive control (Input). (c) Recombinant GST, GST-Aly, GST-UAP56, GST-hTho1 bound to beads were separated by SDS-PAGE and proteins were visualized by Coomassie staining (i). Bound recombinant GST-fusion proteins were incubated with 35S-Met-labelled ORF57 produced by ITT (ii). Bound proteins were separated by SDS-PAGE and the dried gel was exposed to autoradiograph film for 16 h.
immunoprecipitations (RNA-IPs) (Boyne & Whitehouse, 2006b). 293T cells transfected with pORF47, expressing HVS intronless mRNA, in the absence or presence of pGFP or pORF57GFP were UV-irradiated to cross-link protein and RNA. Protein-A agarose was coupled to CBP80-, Aly-, UAP56- and hTho1-specific antibodies and incubated with each cell lysate. After washing, RNA was eluted and RT-PCR was performed to identify any positive interactions. Precipitations were performed with the CBP80-specific antibody to demonstrate that intronless mRNAs could be precipitated using this technique. RNA-IPs using cell extracts transfected with pORF47/pGFP failed to show an interaction between Aly, UAP56 or hTho1 and the ORF47 mRNA (Fig. 2a). In contrast, extracts transfected with both pORF47 and pORF57GFP displayed a clear interaction between Aly, UAP56 and hTho1 and the intronless mRNA (Fig. 2a). Moreover, this analysis was repeated with cells expressing a second HVS intronless mRNA, gB (Williams et al., 2005), and similar results were observed (Fig. 2b). These data show that ORF57 is required for the recruitment of hTREX proteins onto viral intronless mRNA.

HVS infection of OMK cells leads to production of infectious virions and we therefore utilized this property to investigate whether complete hTREX-complex recruitment is required for HVS replication. We have previously shown that ORF57 can form a ternary complex with Aly and TAP (Williams et al., 2005); however, by expressing a transdominant form of Aly, we can assess whether this ternary complex is sufficient for mRNA export or whether UAP56 and the hTHO-complex are required. pAlyΔC-myc has 20 residues deleted from the C-terminus of Aly, which disrupts the interaction between Aly and UAP56 (Luo et al., 2001). However, it still retains its ability to interact with ORF57 and TAP. Therefore, as ORF57 only interacts directly with Aly, overexpression of pAlyΔC-myc inhibits UAP56 and hTHO-complex recruitment, as UAP56 bridges the interaction between Aly and the hTHO-complex (Masuda et al., 2005). It should be noted that, although unlikely, AlyΔC may affect this complex by another unidentified mechanism; moreover, short interfering RNA techniques cannot be used in these types of assays, due to toxicity issues.

Confirmation that AlyΔC-myc interacts with ORF57 and TAP differently from UAP56 was obtained using GST-pulldown analysis. ORF57, UAP56 and TAP were expressed and bound to GST-affinity beads as previously described (Williams et al., 2005). The protein-bound beads were then incubated with either pmyc-, pAly-myc- or pAlyΔC-myc-transfected cell lysates and pulldown analysis was performed. Immunoblotting using a myc-specific antibody demonstrated that Aly-myc interacted with ORF57, TAP and UAP56, suggesting that it bridges the interaction between these proteins. In contrast, AlyΔC-myc retains the ability to interact with ORF57 and TAP but is unable to associate with UAP56 (Fig. 3a). These results suggest that, as ORF57 only interacts directly with Aly, AlyΔC-myc is an ideal mutant to inhibit the recruitment of UAP56 and hTHO-complex to the viral intronless mRNA.

One caveat to this experiment is that expression of pAlyΔC-myc may also act in a dominant-negative capacity to inhibit spliced mRNA nuclear export (Luo et al., 2001). Therefore, it was important to allow expression of the spliced ORF57 protein prior to accumulation of pAlyΔC-myc. To this end, transient transfection of pAlyΔC-myc was performed 8 h after HVS infection and ORF57 protein levels were assessed 24 h post-infection. Results show that comparable amounts of ORF57 were expressed in untransfected and pmyc-, pAly-myc- and pAlyΔC-myc-transfected cell lysates (Fig. 3b, i). Moreover, immunoblotting was performed at 48 h post-infection, confirming expression of Aly-myc and AlyΔC-myc (Fig. 3b, ii).

To determine whether AlyΔC-myc inhibited the recruitment of UAP56 and the hTHO-complex onto intronless RNAs, RNA-IPs were performed on HVS-infected OMK cells (m.o.i. of 1), which were transfected 8 h later with 4 μg pmyc, pAly-myc or pAlyΔC-myc. A GFP transfection control indicated a transfection efficiency of approximately 40–50%. Similar results were observed (Fig. 2), in which recruitment of hTREX components onto the viral RNA was observed in the presence of ORF57. However, RNA-IPs using cell extracts expressing AlyΔC-myc showed a decrease in association of UAP56 and hTho1 to ORF47 mRNA (Fig. 3c), suggesting that AlyΔC-myc inhibits the recruitment of UAP56 and the hTHO-complex to intronless mRNAs. Importantly, RNA-IPs performed using a TAP-specific antibody showed that TAP is still recruited to the intronless viral mRNA, irrespective of Aly status. RNA-IPs using an ORF57-specific antibody produced ORF47

![Fig. 2. HVS ORF57 recruits hTREX to intronless viral mRNA. 293T cells were transfected with (a) pORF47 and (b) pGFP in the presence of either pGFP or pORF57GFP and incubated for 24 h. Following UV cross-linking, RNA-IPs were performed using the antibodies indicated (No Ab, beads alone). Total RNA extracted from mock-transfected and ORF47/gB-transfected cells served as controls (Input).](http://vir.sgmjournals.org)
RT-PCR products of a similar intensity, suggesting that ORF57 was not a limiting factor in this assay (Fig. 3c).

To test whether the lack of UAP56 and hTHO-complex recruitment to intronless mRNAs prevented nuclear export, an mRNA export assay was performed as previously described (Williams et al., 2005). Northern blotting was used to detect ORF47 mRNA in the nuclear or cytoplasmic fraction of cells transfected with pORF47 in the presence of pORF57/pAly-myc or pORF57/pAlyΔC-myc. Little ORF47 mRNA was detected in the cytoplasmic RNA fraction of cells transfected with pORF47 alone, whereas cells co-transfected with pORF47/pORF57/pAly-myc displayed a clear shift of ORF47 mRNA into the cytoplasmic fraction, indicative of ORF57-mediated mRNA nuclear export. However, upon co-transfection with pAlyΔC-myc, the majority of ORF47 mRNA was retained in the nuclear pool at levels similar to those seen for ORF47 alone, symptomatic of a failure in ORF57-mediated mRNA nuclear export (Fig. 3d). These results suggest that UAP56 and hTHO-complex recruitment are required for efficient nuclear export of intronless transcripts.

To assess the effect of inhibiting UAP56 and hTHO-complex recruitment onto intronless mRNAs on HVS replication, OMK cells were infected and transfected as above. Cells were then incubated for 5 days until destruction of the cell sheet. The supernatants were then harvested and the viral titres were measured by plaque assay. Results demonstrate that similar titres were produced from pmyc and pAly-myc pre-transfected cells, showing that expression of these constructs had little effect on HVS replication. In contrast, the virus titre from pAlyΔC-myc-transfected cells was greatly reduced (Fig. 3e). Complete inhibition of virus replication was not achieved due to the low transfection efficiency of OMK cells. These
results demonstrate that disruption of the hTREX complex onto intronless transcripts can have a profound effect on HVS replication.

These data suggest that recruitment of the complete hTREX complex by ORF57 is required for HVS replication. The use of the transdominant Aly mutant allows the formation of a minimal ternary complex on the viral intronless mRNA comprising ORF57–Aly–TAP, as identified by RNA-IPs. However, we suggest that this complex is not sufficient for efficient virus replication, and thus UAP56 and the hTHO-complex have an unidentified, yet essential, role in mRNA nuclear export of HVS intronless transcripts. Moreover, these interactions may be conserved within herpesviruses, as hCMV UL69 also interacts with UAP56 (Lischka et al., 2006). This is of interest, as RNA interference studies in several models show variation in the requirement of hTREX components for mRNA export, suggesting redundancy in lower eukaryotic systems for certain TREX components. Aly has been shown to be non-essential for mRNA export (Gatfield & Izaurralde, 2002; MacMorris et al., 2003). In contrast, UAP56 is required for bulk mRNA nuclear export (Gatfield et al., 2001; MacMorris et al., 2003). At present, the specific role of the hTHO-complex is not fully elucidated; however, yeast strains carrying mutations of the THO-complex have several phenotypes, including a rapid degradation of a subset of RNAs and the accumulation of mRNAs in post-transcriptional site-associated foci (Assenholt et al., 2008; Rehwinkel et al., 2004).

These observations and other data suggest that the recruitment of hTREX to intronless viral mRNAs, forming an export-competent RNP, may be conserved within herpesviruses. We have recently demonstrated that KSHV ORF57 forms a ribonucleoprotein particle containing hTREX (Boyne et al., 2008b). Whether similar complexes are recruited by the ORF57 homologues in other herpesvirus subfamilies, such as HSV-1 ICP27 and hCMV UL69, is unknown. However, it has been shown previously that these proteins do interact with specific hTREX components, such as Aly and UAP56 (Chen et al., 2002; Koffa et al., 2001; Lischka et al., 2006). Therefore, it will be of interest to determine the specific role of the complete hTREX complex in the life cycle of these herpesviruses.

In summary, we propose that HVS ORF57 recognizes and binds the intronless viral transcripts and recruits hTREX, leading to the assembly of an export-competent viral intronless RNP. Moreover, the recruitment of hTREX is essential for efficient viral mRNA nuclear export and HVS replication.

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