Herpesvirus saimiri ORF57: a post-transcriptional regulatory protein

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TABLE OF CONTENTS

1. ABSTRACT

Herpesvirus saimiri (HVS) is the prototype gamma-2 herpesvirus and is a useful model to study the basic mechanisms of lytic replication in this herpesvirus subfamily. This review focuses upon the role of an essential lytic protein, ORF57, which is functionally conserved in all classes of herpesviruses. ORF57 is a multidomain, multifunctional protein responsible for both activation and repression of viral gene expression at a post-transcriptional level. ORF57-mediated repression of gene expression is determined by mRNA processing signals, in particular the presence of an intron within the target gene. This may also be linked to the ability of ORF57 to redistribute SC-35 and U2 splicing factors into specific nuclear domains. ORF57 also plays a pivotal role in transactivating viral gene expression by specifically mediating the nuclear export of HVS intronless transcripts. ORF57 has the ability to shuttle between the nucleus and the cytoplasm, bind viral RNA and recruit cellular nuclear export proteins, such as hTREX components and TAP, onto the viral mRNA. This enables the efficient nuclear export and cytoplasmic accumulation of virus intronless mRNA.

2. HERPESVIRUS SAIMIRI : THE PROTOTYPE GAMMA-2 HERPESVIRUS

Herpesvirus saimiri (HVS) is the prototype gamma-2 herpesvirus, or rhadinovirus. HVS was originally isolated from its natural host, the squirrel monkey, *Saimiri sciureus*, in which it causes a persistent asymptomatic infection (1). The genome of HVS (strain A11) consists of a unique internal low G+C content DNA segment (L-DNA) of approximately 110 kbp, which is flanked by a variable number of 1444 bp high G+C content tandem repetitions (H-DNA) (2). Analysis indicates it shares significant homology with the herpesviruses: Epstein-Barr virus (EBV), bovine herpesvirus 4, Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8) and murine gammaherpesvirus 68 (MHV68) (2-7). The genomes of EBV, KSHV, MHV68 and HVS have been shown to be generally colinear, in that homologous sequences are found in approximately equivalent locations and in the same relative orientation. However, conserved gene blocks are separated by unique genes respective to each virus (2-6). These unique genes usually encode proteins with homology to cellular proteins and are thought to play a role in
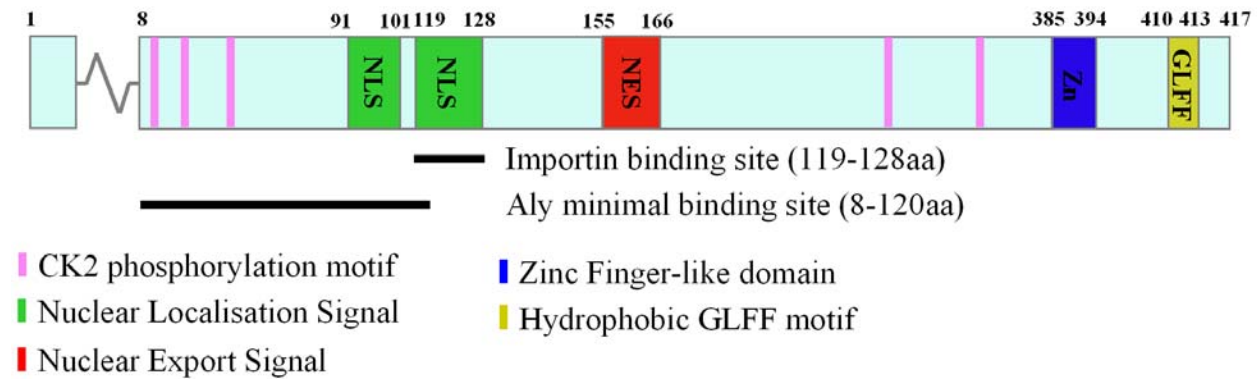
Figure 1. Schematic representation of the multidomain HVS ORF57 protein. The NLSs are required to interact with importin-α allowing nuclear import and are also required for nucleolar targetting. The leucine-rich NES and Aly binding domain are needed for nucleo-cytoplasmic shuttling of ORF57. Both the zinc finger-like domain and the GLFF motif are required for transactivation of viral genes, however only the zinc finger-like region is essential for transrepression. The precise role of CK2 phosphorylation in ORF57 is unknown but probably has a role in gene activation.

transformation, immune evasion and long term persistence of the viral episome. Cellular homologues encoded by HVS include U RNA homologues (HSURs), anti-apoptotic proteins (ORF16/vBcl2 and ORF 71/vFLIP), a cyclin D homologue (ORF72/vCyclin), complement control inhibitory proteins (ORF4/CCPH and ORF15/vCD59), nucleotide metabolism enzymes (ORF2/DHFR and ORF70/TS), a viral superantigen (ORF14/vSag) and cytokine homologues (ORF13/vIL-17and ORF74/vGCR) (2).

2.2 HVS Life cycles

Like all herpesviruses, HVS has two distinct forms of infection, lytic replication and latent persistence. Throughout latent infection the HVS genome is maintained within a dividing cell population as a high copy number circularised episome, without chromosome integration. The persistence of the HVS genome is mediated by the latently-expressed ORF73 protein, which tethers the HVS genome to host mitotic chromosomes via an interaction with the chromosomal-associated protein, MeCP2 (8-10).

Gene expression during lytic replication is sequentially regulated and occurs in three main temporal phases: immediate-early (IE), delayed-early (DE) and late (L) (19). The IE genes do not require cellular protein synthesis and in HVS two transcriptional activating proteins are produced. The first is encoded by the ORF50 gene, and is known as the replication and transcription activator (Rta). HVS ORF50 produces two transcripts, the first, ORF50a, is spliced containing a single intron which is detected at early times during the productive cycle. The second, ORF50b, is expressed later and is produced from a promoter within the second exon (11). The ORF50 gene products are well conserved among all gamma-2 herpesviruses (12-14), and play a pivotal role in regulating the latent-lytic switch in gamma-2 herpesviruses (15-19). HVS ORF50a functions as sequence specific transactivator, activating transcription following direct interactions with specific response elements (REs) within HVS promoters

(20-24). Once bound HVS ORF50a recruits and interacts with the TATA Binding Protein (TBP) (25), suggesting that it recruits components of the TFIID complex, thereby allowing transcription initiation by RNA polymerase II.

The second HVS IE gene, ORF57, encodes a multifunctional protein. This review considers the current understanding of the many functions performed by the HVS ORF57 protein. In particular, it discusses how this multidomain, multifunctional protein facilitates both transactivation and repression of HVS gene expression at a post-transcriptional level.

3. HVS ORF57 : A CONSERVED MULTIDOMAIN, MULTIFUNCTIONAL PROTEIN

HVS ORF57 is a 52 kDa protein homologous to gene products identified in all classes of herpesviruses, including the EBV Mta protein transactivator encoded by BMLF1, ORF57 of KSHV, ICP27 of herpes simplex virus (HSV-1), ORF4 of varicella-voster virus and UL69 in human cytomegalovirus (2, 5, 26-30).

HVS ORF57 is comprised of several domains, which are functionally conserved between these viral homologues (Figure 1). The amino-terminus of ORF57 contains an RNA-binding region (31), two nuclear localisation signals (NLSs) (32, 33) and a leucine-rich nuclear export signal (NES) (31), which is essential for nucleo-cytoplasmic shuttling (20, 34-36). The carboxy-terminus includes a zinc finger-like domain and a hydrophobic GLFF consensus sequence, both of which are involved in the regulation of gene expression (35). ORF57 has also been shown to interact with a number of cellular proteins, primarily involved in nuclear import and export pathways. Figure 1 highlights domains within ORF57 which are responsible for the interactions with importin alpha (33) and the nuclear export factor, Aly (37). In addition, ORF57 contains numerous putative phosphorylation motifs, including several potential casein-
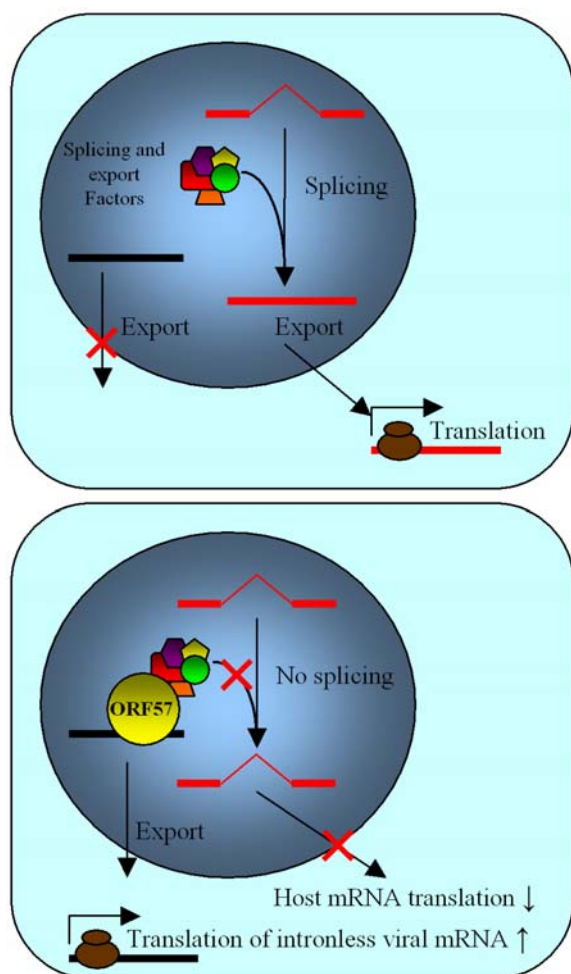
Figure 2. HVS ORF57 can activate and repress gene expression at a post-transcriptional level. Schematic representation of ORF57's role in the activation of intronless transcripts and repression of intron-containing genes. In the absence of ORF57 viral intronless transcripts (black) are retained in the nucleus. However, ORF57 recruits cellular mRNA export factors to these intronless viral transcripts enabling efficient nuclear export. In contrast, ORF57 redistributes essential splicing factors into discrete nuclear domains, that in turn with the sequestration of nuclear export factors by ORF57, reduces the nuclear export and translation of intron-containing genes (red).

kinase-2 phosphorylation sites (38). Therefore phosphorylation is probably an important step in the regulation of ORF57's many functions.

4. HVS ORF57 MODULATES GENE EXPRESSION AT A POST-TRANSCRIPTIONAL LEVEL

The HVS ORF57 protein is an essential multifunctional protein, responsible for both repression and transactivation of viral gene expression at a post-transcriptional level (16, 31, 34) (Figure 2). This is highlighted by initial studies investigating ORF57's effect on the HVS intron-containing ORF50a gene. The HVS

ORF50 gene encodes two transcripts, the first, ORF50a, contains a single intron and is detected at early times during the productive cycle, whereas the second, ORF50b, is intronless and is expressed later from a promoter within the second exon (11, 20). Experiments have shown that ORF57 specifically represses expression of the intron-containing ORF50a gene, in contrast to the intronless ORF50b gene. This reduction of ORF50a expression by ORF57 has been measured by a reduction in the downstream activation of a variety of HVS ORF50 responsive promoters (34).

4.1 ORF57 represses viral gene expression

The repressive effect of HVS ORF57 on ORF50a gene expression is believed to be linked to the presence of an intron within the ORF50a coding region. This intron-related repression was further investigated using transient expression studies. A range of HVS promoters for the genes encoding DNA polymerase, thymidine kinase, major capsid protein and glycoprotein B were inserted upstream of the Chloramphenicol Acetyl Transferase (CAT) and Beta-Galactosidase (β-Gal) coding regions, and reporter based assays performed to assess the transactivation capabilities of ORF57. It was determined from these results that ORF57 transactivated the promoter-β-Gal constructs to a much greater extent than the promoter-CAT constructs (34). This suggested that ORF57 functions as a reporter gene dependent regulator, as the promoter sequences in the CAT and β-Gal constructs were identical. However, upon further examination, the promoter-CAT constructs contained the small t-antigen intron and SV40 poly(A) region, in contrast, the β-Gal constructs only contained the SV40 poly(A) signal. Therefore, to determine if ORF57-mediated transactivation was dependent on the reporter gene or on the presence of the small t antigen intron, the mRNA processing signals of the CAT and β-Gal expression vectors were swapped, in that, the CAT constructs only contained the SV40 poly(A) region and the β-Gal constructs contained the small t-antigen intron and SV40 poly(A) signal. Results demonstrated that ORF57 regulation was independent of the target reporter gene and is determined by the mRNA processing signals (34). Thus, repression of gene expression by ORF57 is dependent on the presence of the small t-antigen intron in the target gene sequence. These results further support the suggestion that ORF57–mediated repression of the ORF50a gene is due to the presence of its intron. At present, it is unknown whether ORF57 also affects the expression of cellular intron-containing genes and host cell splicing in general. Interestingly, the majority of HVS transcripts are intronless, therefore ORF57 may decrease the processing of cellular spliced transcripts thereby contributing to host protein synthesis shut-off.

Repression of intron-containing genes maybe linked to observations showing that HVS infection results in the redistribution of SC-35 and U2 splicing factors in HVS-infected cell nuclei (39). Furthermore, the redistributed SC-35 protein colocalised with ORF57 and expression of ORF57 alone is sufficient to cause the redistribution of the spliceosome components (39). These results suggest that the mechanism by which ORF57

downregulates intron-containing genes involves the redistribution of the spliceosome complex. The exact mechanism of ORF57-mediated repression is unknown, however this reorganisation of spliceosome components may disrupt the efficiency of the splicing process or the stability of the intron-containing genes leading to poor RNA processing and subsequent translation. However, ORF57-mediated splicing inhibition may involve mechanisms more complex than simply redistributing spliceosome components. For example, HSV-1 ICP27 is believed to mediate splicing inhibition by interacting with multiple splicing factors and altering their phosphorylation status (40, 41). Although, the exact mechanism of how ORF57-mediated repression occurs and how this relates to the interaction with the spliceosome components is yet to be fully elucidated, it is believed to involve the carboxy-terminal region of the protein. In particular, a zinc finger-like domain, which is conserved in all ORF57 homologues, is essential for repression (35). Similarly, it is required for the redistribution and intense nuclear spotting of the SC-35 spliceosome component. However, the exact role of the zinc finger-like domain in the repressive properties of ORF57 is unknown. It is probably required for the self interaction of ORF57, enabling it to function as a multimer. Alternatively it may be required for specific interactions with cellular proteins which in turn are required for ORF57's repressive properties.

4.2 ORF57 is required for efficient nuclear export of viral mRNAs

Transient transfection studies have shown that HVS ORF57 can activate reporter gene expression from a range of heterologous and viral promoter-reporter constructs, with mRNA processing signals which do not contain the small t antigen intron (20, 34, 35). Moreover, northern blot analysis demonstrated that the increase in reporter activity did not correlate with a similar increase in reporter RNA levels in the presence of ORF57 (34). This suggested that ORF57 acts at a post-transcriptional level to modulate gene expression. Deletion analysis of the ORF57 carboxy-terminus demonstrated that two domains are essential for its post-transcriptional activation (35). The zinc finger-like domain is required for transactivation as well as the repressive properties of ORF57, suggesting multimerisation or specific protein-protein interactions with cellular proteins are required for the transactivation properties. In addition, mutations within a conserved hydrophobic GLFF domain result in a distinct ORF57 subcellular localisation (35). Analysis demonstrated that a proportion of the GLFF mutant protein was present in the cytoplasm, this subcellular localisation has never been observed with the wild type ORF57 protein or any other mutants tested to date. At present the implication of this result is yet to be elucidated. It is possible however, that these carboxy-terminal hydrophobic residues are involved in protein-protein interactions with a host cell nuclear protein which retains ORF57 in the nucleus. Alternatively, this hydrophobic domain is itself a novel nuclear localisation or retention signal.

Several possible mechanisms have been investigated to determine how ORF57 activates gene expression at a post-transcriptional. It could affect the processing, transport or translational efficiency of viral mRNAs. To further investigate these possibilities, it was initially determined whether ORF57 has the ability to bind viral mRNA using northwestern analysis. Recombinant ORF57 protein was expressed and purified as an amino-terminal GST fusion protein, and the filter bound protein incubated with RNA probes specific for HVS intronless transcripts or a cellular transcript. Results demonstrated that ORF57 has the ability to specifically bind the viral intronless mRNA transcripts, but did not interact with the control cellular RNA (31). The ability of ORF57 to bind intronless viral mRNA has also been confirmed using RNA-immunoprecipitation assays (32). At present, the RNA binding domain within HVS ORF57 has not been fully delineated. RNA binding by the ORF57 homologue HSV-1 ICP27 is mediated by an arginine and glycine-rich sequence resembling a RGG box motif, a putative RNA binding determinant found in a number of cellular nuclear proteins involved in mRNA and rRNA metabolism (42). ORF57 does not encode an RGG box motif, but contains a relatively arginine-rich amino-terminus. RNA binding analysis of an ORF57 amino terminus-GST fusion product suggested that this arginine-rich region is likely to mediate RNA binding (31). However, it cannot be excluded that the carboxy-terminal portion of the ORF57 protein may also include RNA binding determinants. Therefore, further analysis is required to identify the functional domain(s) contained within ORF57 which are responsible for RNA binding.

Another intriguing question is the mechanism by which ORF57 specifically recognises intronless viral mRNAs. At present, experiments are being undertaken using systematic evolution of ligands by exponential enrichment (SELEX) approaches (43, 44), to identify RNA sequences which preferentially interact with the ORF57 protein. Preliminary analysis of these sequences reveals a bias for G/U rich sequences (Colgan and Whitehouse, unpublished observations). Two-dimensional structural predictions of these sequences yielded large, low complexity loops due to insufficient base-pairing. This therefore suggests that the important features of these ORF57-responsive RNA species lies in their sequence rather than their structure.

Initial studies to investigate how ORF57 mediates transactivation demonstrated that it did not affect the levels of RNA, therefore analysis was performed to assess whether ORF57 affected the nuclear-cytoplasmic transport of viral mRNAs. To this end, total, nuclear and cytoplasmic RNA was isolated separately from cells transfected with plasmids expression either glycoprotein B or capsid late intronless mRNAs in the absence and presence of ORF57. Northern blot analysis was then performed in order to determine the effect of ORF57 on the subcellular localisation of a viral late mRNAs. The results of the northern blot analysis showed that ORF57 did not affect the quantities of viral transcripts but is required for the efficient cytoplasmic accumulation of glycoprotein B and capsid transcripts (31). This suggests that ORF57 affects viral mRNA transport and is required for the efficient nuclear export of viral intronless transcripts (Figure 2).
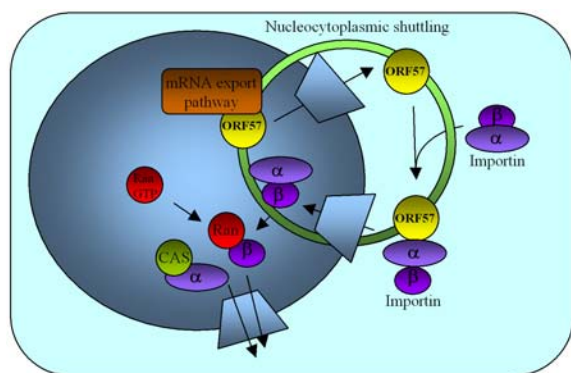
Figure 3. HVS ORF57 enters the nucleus via an interaction with the cellular importin pathway. Schematic representation of importin-mediated nuclear import of ORF57. ORF57 interacts directly with importin α via ORF57's NLSs. Importin α then forms a heterodimeric complex with importin β allowing passage through the nuclear pore. Once in the nucleus, binding of Ran-GTP to importin β causes dissociation of the import complex. Once released, importin subunits are then recycled to the cytoplasm.
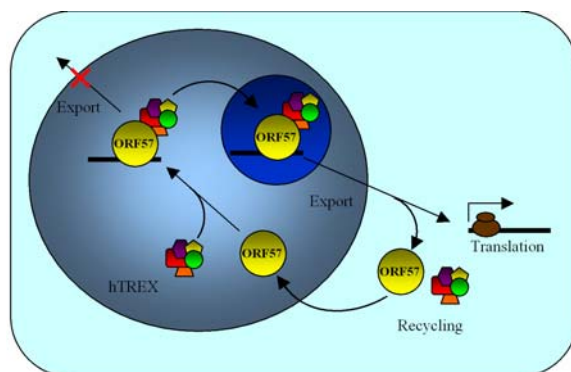

Figure 4. HVS ORF57 recruits the nuclear export factor TAP, via an interaction with hTREX, to export viral mRNA. Schematic representation of ORF57-mediated nuclear export of intronless viral mRNA. ORF57 associates with viral intronless mRNA, then recruits Aly and other components of the hTREX complex. This viral mRNP then trafficks through the nucleolus. Aly then recruits the nuclear export factor, TAP and p15. This complex then docks at the nuclear-pore complex (NPC) and translocates into the cytoplasm. Viral mRNA is then efficiently translated. The export factors are then recycled into the nucleus.

The observations that HVS ORF57 binds viral mRNA and enhances nuclear export of intronless viral mRNA suggested that ORF57 may function by shuttling between the nucleus and the cytoplasm. In order to determine whether ORF57 shuttles, an interspecies heterokaryon assay was utilised (31, 42, 45, 46). Monkey Cos-7 cells were transiently transfected with ORF57, then plated with mouse 3T3 cells in medium containing cycloheximide. The cells were then fused using polyethylene glycol and incubated for 1 hour in the presence of cycloheximide. Cells were then fixed, incubated with Hoescht dye and ORF57 localisation observed using an ORF57-specific antibody. Hoechst dye allowed the differentiation between monkey and mouse cell nuclei to identify interspecies heterokaryons. Monkey cells stained diffusely throughout their nuclei, whereas mouse cell nuclei stained with a distinctive speckled pattern. Analysis of the interspecies heterokaryons demonstrated that ORF57 was expressed in both monkey and mouse cell nuclei (31, 37). These results indicate that ORF57 has the ability to shuttle between the nucleus and cytoplasm.

This analysis demonstrates that ORF57 has the ability to bind viral RNA, shuttle between the nucleus and cytoplasm and is required for efficient cytoplasmic accumulation of virus mRNA. This suggests that ORF57 is a nucleo-cytoplasmic shuttle protein that plays a pivotal role in mediating the nuclear export of HVS intronless transcripts.

5. HVS ORF57 INTERACTS WITH NUCLEAR IMPORT AND EXPORT FACTORS

An intriguing question regarding the functioning of virus-encoded nucleo-cytoplasmic shuttle proteins is the mechanism they utilise to be transported through the nuclear pore complex. Macromolecule trafficking into and out of the nucleus is mediated by soluble transport receptors (47). These receptors bind specific proteins or RNA cargoes and interact with nuclear pore proteins which subsequently allow the translocation of the receptor cargo through the nuclear pore complex (NPC). Analysis has shown that ORF57 interacts with both nuclear import and nuclear export factors enabling it to shuttle in and out of the nucleus (Figure 3 and 4).

5.1 ORF57 interactions with Importin α isoforms 1 and 5

The most widely characterised nuclear transport pathway mediates the nuclear import of proteins that contain a classical nuclear localisation signal (NLS) (47). These basic, generally lysine-rich NLSs serve as recognition sites for an NLS adaptor protein, termed importin α or karyopherin α (48), which forms a heterodimeric complex with importin β or karyopherin β. Importin β functions as a transport receptor molecule binding to the nuclear pore complex via a direct interaction with specific nucleoporins (49, 50). Once in the nucleus, binding of Ran-GTP to importin β causes dissociation of the import complex (51, 52). Upon release from the cargo, importin subunits are then recycled to the cytoplasm. Importin β is recycled rapidly, whereas the export of importin α is mediated by the nuclear export factor, CAS, which binds importin α preferentially in the presence of Ran-GTP (53-55). In the cytoplasm the conversion of Ran-GTP to Ran-GDP, facilitated by RanGAP, triggers the dissolution of the export complex (56-58), allowing participation in additional rounds of nuclear import (Figure 3).

A yeast 2-hybrid screen using ORF57 as bait, demonstrated that ORF57 interacts with importin α

isoforms 1 and 5. Sequence analysis has revealed that importin α is composed of three distinct domains (50, 59). A basic amino-terminal importin β-binding domain, a large central domain composed of eight to ten Armadillo (arm) repeats and an acidic carboxy-terminus which mediates an interaction with the nuclear export factor, CAS (53-55). Co-crystallisation studies of importin α and a monopartite NLS has identified two possible binding sites (60, 61). The major site which lies between the first and fourth arm repeats and the minor site located between arm repeats four and eight. At both sites, the NLS binds in an extended antiparallel conformation, via trytophan and asparagine residues (60, 61). Moreover, the bipartite nucleoplasmin NLS simultaneously binds to both major and minor sites (60, 61). From deletion analysis studies of both ORF57 and importin α, it is inferred that ORF57 contains a monopartite NLS which specifically binds importin α via the minor repeats between the fourth and eighth arm repeats (33).

5.2 ORF57 utilises the TAP-mediated nuclear export pathway

In order for ORF57 to exit the nucleus, it must also interact with soluble exit transport receptors. Sequence analysis of the HVS ORF57 protein identified a leucine-rich region, homologous to nuclear export signals (NESs) found in many nucleo-cytoplasmic shuttle proteins. Results demonstrated that fusion of the ORF57 NES to a heterologous GFP protein resulted in its nuclear export, supporting the hypothesis that the leucine-rich sequence can function as a NES (31). CRM1 (chromosomal region maintenance 1) or Exportin 1, has been identified as a nuclear export receptor for proteins encoding a leucine-rich NES, in a process that also requires the GTP bound form of Ran (62, 63). This suggests that exportin 1 is the bridging protein for the interactions of NES containing proteins and the nuclear pore complex. However, a direct interaction between ORF57 and CRM-1 has never been demonstrated. Moreover, interspecies heterokaryon assays demonstrated that ORF57 could still shuttle between the nucleus and cytoplasm in the presence of the CRM-1 specific inhibitor, Leptomycin B. These data suggest that ORF57 primarily shuttles between the nucleus and cytoplasm via a CRM-1 independent pathway. The question therefore remains regarding the function of the ORF57 leucine-rich NES, perhaps it allows the use of a CRM-1 dependent RNA export pathways when the major CRM-1 independent pathway is compromised.

Recent analysis has therefore been directed towards ORF57's interaction with other nuclear export factors. Additional yeast 2-hybrid screening identified a direct interaction between ORF57 and the nuclear export protein, Aly. Aly is a component of the multi-protein human transcription and export (hTREX) complex, which also contains the proteins; UAP56 (an RNA-helicase) and the hTHO-complex (hTho1, hTho2, fSAP79, fSAP35 and fSAP24) (64). Initially, Aly was thought to be a component of another multi-protein complex, the exon-exon junction complex (EJC), which is deposited 20-24 nucleotides upstream of the exon-exon boundary, as a consequence of splicing. However, recent analysis has shown that Aly and UAP56 are in fact associated exclusively with hTREX and not with the EJC (65, 66). In contrast to being associated at the exon-exon junction, Aly and hTREX are recruited exclusively to the 5' end of the first exon (67). This recruitment occurs in a cap and splicing dependent manner and is mediated via a direct interaction between Aly and the cap-binding protein, CBP80. The mechanism for hTREX recruitment also provides an elegant explanation for the directionality observed in mRNA export in the Balbiani rings of *Chironomas tentans* (68), as 5' recruitment of hTREX grants 5'-polarity to the messenger ribonucleoprotein (mRNP) complex. Once bound to mRNA Aly stimulates recruitment of the cellular mRNA export factor, TAP. TAP is a multidomain protein comprising an amino-terminal Aly binding domain, central domain which interacts with p15 (69) and nucleoporins (70) and a carboxy-terminal domain which also binds nucleoporins (71, 72). Therefore, the TAP/p15 heterodimer provides the connection between the ribonucleoprotein particle (mRNP) and the nuclear pore allowing export from the nucleus to the cytoplasm. As expected, depletion of TAP leads to a block in mRNA export in a number of model systems (73-75).

The interaction of ORF57 and Aly has been confirmed using both GST-pulldowns and co-immunoprecipitations. In addition, ORF57 also colocalises and interacts with other hTREX components, namely UAP56, hTho1 and fSAP79 (Colgan, Boyne and Whitehouse, unpublished observations). Moreover, as Aly is thought to provide the connection between the mRNP and the nuclear export factor TAP, GST pulldowns were also performed to determine whether a ternary complex could form between ORF57, Aly and TAP. A very weak interaction was observed between ORF57 and TAP directly, but this was greatly enhanced when supplemented with purified Aly protein (37). This ternary interaction was only moderately reduced with ribonuclease treatment, suggesting this protein complex formation is RNA independent. Together, these data indicate that Aly, TAP and ORF57 can form a ternary complex and that the interaction of TAP with ORF57 is enhanced and stabilised by Aly.

Delineation of the interacting domains of ORF57 and Aly demonstrated that the amino-terminus of ORF57 is required to interact with Aly. This region has been narrowed down to amino acids 8-120, although a stronger interaction is seen with amino acids 8-251, suggesting sequences between amino acids 121-251 either contribute directly to the interaction with Aly or to the folding of the amino-terminal domain. In contrast, ORF57 interacts with both the amino and carboxy-terminal domains of Aly with equal affinity. These observations are interesting as the amino and carboxy-terminal domains of Aly are also important for Aly's interaction with RNA and TAP (76-78). Therefore, the interaction domains for ORF57 and TAP may directly overlap within the Aly amino and carboxy-terminal domains. Alternatively, ORF57 and TAP might interact with either domain of Aly in such a way that in ternary complexes, ORF57 is associated with the amino-terminus and TAP with the carboxy-terminus or *vice versa*.

These data suggest that HVS ORF57-mediated nuclear export of intronless HVS mRNA occurs through the nuclear export factor TAP-mediated pathway (Figure 4).

To confirm whether TAP is specifically required for HVS mRNA export, total, nuclear and cytoplasmic RNA was isolated separately from cells transfected with plasmids expressing glycoprotein B mRNA in the absence and presence of ORF57 and a transdominant mutant of TAP. Results demonstrated that when TAP activity is compromised in the cell, the nuclear export of glycoprotein B mRNA is significantly reduced. These data suggest that the ORF57-TAP interaction is required for the efficient nuclear export of late viral transcripts and that viral transcripts use the TAP-mediated cellular mRNA export pathway. In addition, experiments were also performed to assess whether inhibition of viral mRNA export by the dominant negative form of TAP might block HVS replication. Results showed that over expression of the transdominant TAP reduced virus titres by approximately 100 fold demonstrating that disruption of the cellular mRNA export pathway has a profound effect on HVS replication.

6. HVS ORF57 TRAFFICKS THROUGH THE NUCLEOLUS

Analysis of the subcellular localisation of ORF57 during a HVS infection demonstrated that at times of intronless mRNA export a proportion of ORF57 is localised to the nucleolus (32). Detailed deletion and mutational analysis of the ORF57 protein identified two distinct NLSs positioned towards the amino-terminus of the protein which are both required for nucleolar targeting (32). Moreover, a loss of either NLS was sufficient to prevent ORF57 localising to the nucleolus, however the nuclear localisation of ORF57 remained unperturbed, confirming both NLSs are functional. For many years the exclusive role of the nucleolus was thought to be the site of ribosomal RNAs transcription, processing and assembly into the ribosome subunits (79). Recent studies however, suggest it has additional non-classical roles in many aspects of cell biology including cell cycle regulation, viral replication, tumourigenesis and cellular stress responses (80-82). This plurifunctional nature of the nucleolus has been highlighted by extensive proteomic analysis of human nucleoli (83, 84). To date, the nucleolar proteome database archives 728 nucleolar proteins and functional classification of these proteins reinforces the multiple roles of the nucleolus (85). Interestingly, an increasing number of key proteins from both RNA and DNA viruses have been shown to localise to the nucleolus. These include proteins encoded by viruses such as coronaviruses, influenza, HIV-1, adenoviruses and herpesviruses (86). Therefore, virus-nucleolar interactions are likely to have important implications in the life cycle of many viruses. However at present, the precise functional role of these virus-nucleolar co-localisations has not been determined.

Interestingly, double indirect immunofluorescence labelling of HVS-infected cells with ORF57 and the nuclear export hTREX antibodies revealed a drastic alteration in each of the mRNA export proteins. In contrast to the speckled pattern of localisation seen in uninfected cells, the hTREX components were almost exclusively redistributed to the nucleolus, where they co-localised with ORF57 (32). This finding is the first time that components of hTREX have been shown to be re-localised to the nucleolus and indicates a possible role for the nucleolus in HVS mRNA nuclear export (Figure 4). To test this hypothesis, mRNA was isolated from nuclear and cytoplasmic fractions of cells transfected glycoprotein B mRNA expression vector in the presence of wild type ORF57 or a mutant ORF57 construct which was unable to traffick to the nucleolus. In the presence of wild type ORF57 there was a shift in glycoprotein B mRNA localisation from the nuclear fraction to the cytoplasmic fraction, symptomatic of ORF57-mediated viral mRNA nuclear export. In contrast, in the presence of the nucleolar excluded mutant ORF57, glycoprotein B mRNA is retained in the nuclear pool, indicative of a failure in ORF57-mediated viral mRNA nuclear export. Confirmation of this observation was shown by restoring the ability of the mutant ORF57 protein to traffick to the nucleolus using a heterologous nucleolar localisation signal. This retargeting to the nucleolus in turn rescued the mutant's ability to mediate nuclear export of viral intronless mRNA (32). At present the role of the nucleolus in HVS ORF57-mediated nuclear export remains unknown. However, presumably, there is an advantage for the herpesvirus to co-localise its mRNA export protein and co-factors inside the nucleolus. A number of different RNAs are known to be processed during nucleolar trafficking and it is possible that the herpesvirus mRNA is being modified in some fashion. Alternatively, nucleolar trafficking of the HVS mRNP could allow the virus to avoid surveillance mechanisms that may otherwise degrade foreign viral intronless mRNAs prior to translation.

7. SUMMARY

HVS ORF57 encodes an IE multifunction protein which is conserved in all herpesviruses. It is responsible for both repression and transactivation of viral gene expression at a post-transcriptional level. Repression of gene expression is independent of the target reporter gene and is determined by the mRNA processing signals, in particular the presence of an intron within the target gene sequence. Moreover, repression of intron-containing genes maybe linked in part to observations showing ORF57 results in the redistribution of SC-35 and U2 splicing factors into discrete nuclear speckles. In contrast, transactivation of gene expression is mediated at the mRNA nuclear export level. ORF57 associates specifically with viral mRNA and triggers the recruitment of Aly, and other TREX components to form an export competent viral mRNP, which, in turn, leads to the recruitment of TAP and subsequent mRNA export. There are however, many intriguing questions yet to be addressed, in particular whether ORF57 has specific viral mRNA binding sites and where these might reside in the mRNA. It is interesting to note that ORF57 homologues have recently been shown to synergistically enhance the transactivation capabilities of other viral transcriptional proteins (87, 88) and also directly

enhance mRNA translation by associating with polyribosomes (89), indicating a function in translation. This suggests that ORF57 may act on several levels to regulate viral gene expression. Further experiments are now required to determine whether ORF57 functions as a sequence specific mRNA binding protein and whether ORF57 has additional roles in mRNA processing.

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