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The many roles of the regulatory protein ICP27 during herpes simplex virus infection

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TABLE OF CONTENTS

1. ABSTRACT

Herpes simplex virus type 1 (HSV-1) protein ICP27 is a multifunctional regulator of gene expression that assumes different roles during the course of infection. Early in infection, ICP27 mediates the inhibition of cellular splicing, whereas, later it helps to recruit cellular RNA polymerase II (RNAP II) to viral replication sites and to facilitate viral RNA export. ICP27 has also been shown to stimulate translation of viral transcripts. ICP27 performs its activities by interacting with RNA and with an assortment of proteins. ICP27 binds viral RNAs in its role as an export adaptor. An ever increasing number of cellular proteins have been shown to interact with ICP27, including splicing factors, export proteins and RNAP II. A number of protein motifs within ICP27 have been predicted based upon sequence comparisons; however, detailed structural information is not yet available. Although much has been learned about the mechanisms by which ICP27 performs its roles, relatively little is known about how its activities are regulated. The roles and activities of ICP27 are the subject of this review.

2. INTRODUCTION

ICP27 is an HSV-1 immediate-early (IE) or alpha protein that is required for viral replication (1). It is a 512-amino-acid phosphoprotein that performs a number of functions during productive infection including contributing to the shut off of host protein synthesis (2), stimulating viral early gene expression and DNA synthesis (3,4), and inducing viral late gene expression (5,6). ICP27 has also been implicated in the repression of host gene transcription after infection (7), in blocking the cell cycle in the G1 phase (8) in the prevention of apoptosis (9,10), in the activation of stress kinases JNK and p38 (11) and in the nuclear sequestration of foci containing the stress protein Hsc70 (12). In early studies on the regulatory functions of ICP27, it was demonstrated that ICP27 was both a positive and negative regulator of the expression of different target genes in transfection studies (13,14). Subsequent studies demonstrated that the activation and repression of target gene expression by ICP27 occurred post-transcriptionally at the level of RNA processing (15), including splicing (16) and polyadenylation (17). More recent studies have

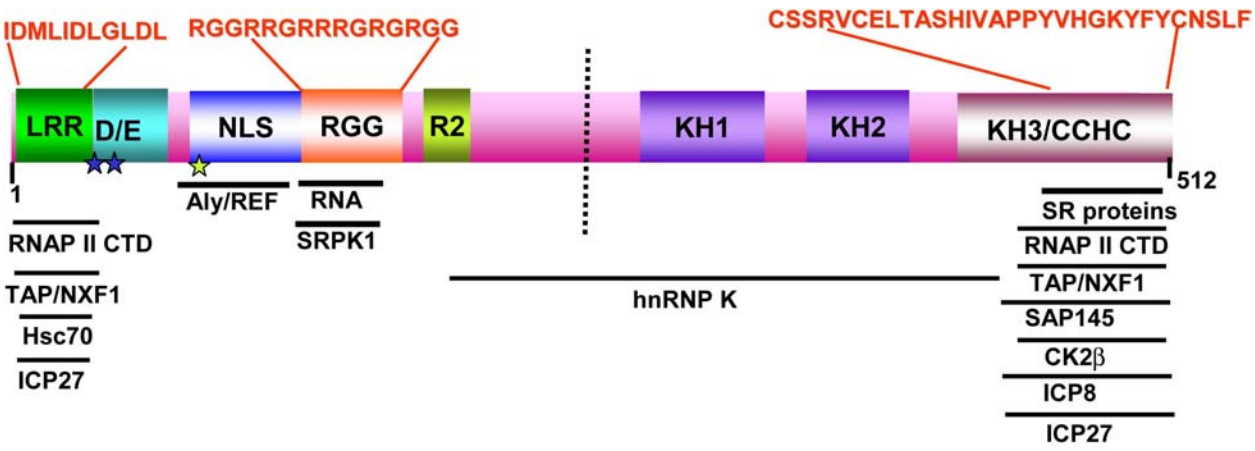

Figure 1. Schematic depiction of ICP27 showing structural motifs based on sequence homology to other proteins. The motifs include: leucine-rich NES-like sequence (LRR); acidic region (D/E); nuclear localization signal (NLS); RGG-box RNA binding motif (RGG); arginine-rich R2 region (R2); KH1, KH2 and KH3 domains, and a zinc-finger-like domain (CCHC).The stars mark phosphorylation sites. The amino acid sequence of selected motifs is shown as are the regions of ICP27 shown to interact with cellular and viral proteins and with RNA.

uncovered additional post-transcriptional activities for ICP27 including facilitating viral RNA export (18) and stimulating translation of viral RNAs to which it is bound (19-21). ICP27 also acts at the transcriptional level in that it has been shown to be required for the stimulation of viral late gene transcription (6).

In probing the mechanisms by which ICP27 acts, it was found that ICP27 binds RNA *in vivo* and *in vitro* (18,22,23), and it also interacts with a large number of proteins, both viral and cellular (24-33). The regions of ICP27 required for its RNA binding and protein interactions are shown in Figure 1. ICP27's various activities appear to be regulated temporally throughout the course of infection. It has not yet been elucidated how ICP27 switches among its various roles, although it is post-translationally modified by phosphorylation (34) and arginine methylation (22), (Figure 1) and both modifications have been demonstrated to affect protein-protein interactions (35,36).

ICP27 is one of two essential HSV-1 IE proteins and unlike the transcriptional activator ICP4, which functions in transcription initiation, ICP27 is multifunctional. It appears to be intimately associated with cellular and viral RNA metabolism from transcription and processing through export to the cytoplasm and translation. Determining the mechanisms of its actions and how its diverse functions are coordinately regulated is important for understanding the dynamics of HSV-1 lytic infection and viral take-over of the host cell. Interestingly, ICP27 is the only HSV-1 IE protein that has homologues in all of the human herpesviruses and throughout the herpesvirus family. When studies have been performed on these homologues, functions similar to those described for ICP27 have been found. For example, Epstein-Barr virus (EBV) SM protein (also called EB2 and Mta) has been shown to shuttle between the nucleus and cytoplasm; to mediate the cytoplasmic accumulation of EBV gene transcripts; to enhance processing of an EBV transcript; to affect RNA splicing, and to associate with a splicing factor (37-41). Similarly, ORF 57 of herpesvirus saimiri functions post-transcriptionally and interacts with a splicing factor (42-44). Other ICP27 homologues have also been shown to function post-transcriptionally, including UL69 of human cytomegalovirus (HCMV) (45,46) and ORF57 of Kaposi's sarcoma herpes virus (KSHV) (47). Further, SM, UL69 and ORF57 have all been shown to interact with cellular RNA export proteins, similarly to ICP27, and to facilitate viral RNA export (45,48-52). The activities and required domains of these homologs have only begun to be defined. In contrast, much is known of the activities of ICP27. In this review, the mechanisms by which ICP27 performs its various roles will be detailed along with recent insights into how its various activities may be regulated.

3. POST-TRANSCRIPTIONAL ACTIVITIES OF ICP27

3.1. Pre-mRNA splicing inhibition

Infection with some viruses can alter cellular mRNA processing to favor viral gene expression. During HSV-1 infection, host cell splicing is inhibited and ICP27 mediates this inhibition (16,24,30,53,54). This results in decreased levels of cytoplasmic cellular spliced mRNAs, and thus contributes to the shut off of host protein synthesis (2,55). This is not disadvantageous for HSV-1 gene expression because most HSV-1 transcripts are intronless (56). Because spliced mRNAs are more efficiently exported than intronless transcripts (57), splicing inhibition also results in the alteration of cellular splicing-dependent export pathways. RNA export will be described in detail in the next section.

In studies to address the mechanism of splicing inhibition by ICP27, it was shown that ICP27 expression during viral infection caused a redistribution of splicing factors (54,58). Further, ICP27 co-immunoprecipitated with

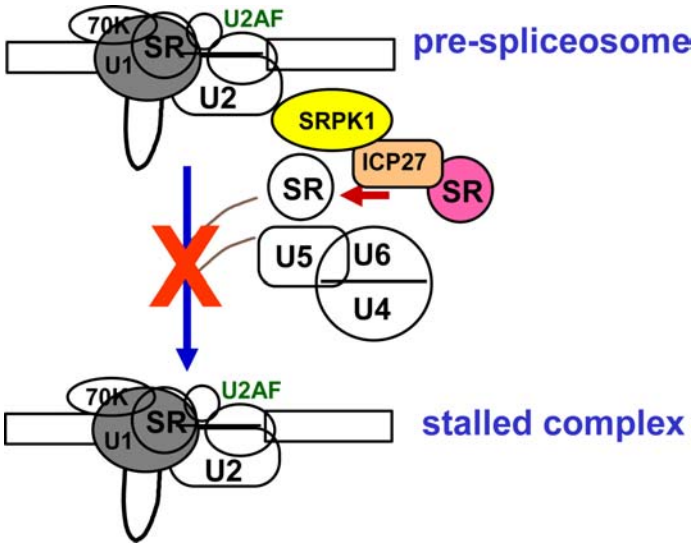

Figure 2. Model of splicing inhibition by ICP27. ICP27 interacts with SRPK1 and recruits it to the nucleus. ICP27 also interacts with SR splicing proteins. The recruitment of SRPK1 to the nucleus results in aberrant phosphorylation of SR proteins, which affects their ability to participate in spliceosome assembly. Spliceosome complex formation stalls at complex A stage before the entry of the tri-snRNP U5-U4/U6 (30)

anti-Sm antisera and altered the phosphorylation of two co-precipitating splicing proteins (59). ICP27 was also found to interact with splicing protein SAP145 in a yeast two hybrid analysis (24). These findings suggested that ICP27 mediates inhibition by interacting with splicing factors. Subsequently, we reported that ICP27 interacted with splicing factor SRp20 in a yeast two-hybrid screen (30). SRp20 is a member of a highly conserved family of serine-arginine-rich essential splicing factors termed SR proteins with roles in both constitutive and alternative splicing (60). Furthermore, SR proteins have been shown to be required for spliceosome assembly (61). Co-immunoprecipitation and co-localization studies showed that ICP27 also interacts with other members of the SR protein family and the C-terminus of ICP27 was required for these interactions (Figure 1).

Interestingly, ICP27 expression resulted in hypophosphorylation of SR proteins (30). An intermediate level of phosphorylation is required for SR proteins to perform their roles in splicing (61-63) and consequently, it was found that spliceosome complex formation is stalled in wild type HSV-1 infected cells at the pre-spliceosomal complex A stage before the first transesterification reaction (24,30,53). Furthermore, ICP27 was shown to interact with SR protein kinase 1 (SRPK1), a member of a family of conserved kinases that are highly specific for arginine/serine dipeptides (64,65). Interaction with ICP27 resulted in a compartmental redistribution of SRPK1 *in vivo*, in that this predominantly cytoplasmic kinase was largely relocalized to the nucleus (30). The RGG box RNA binding motif at residues 138 to 152 in the ICP27 polypeptide was required to be intact for its interaction with SRPK1 (Figure 1). Further, the interaction of ICP27 with SRPK1 altered the activity of the kinase *in vitro*. SR protein substrates purified from HeLa cells or expressed as recombinant proteins in bacteria were less phosphorylated in kinase assays in the presence of ICP27 (30). Infection with a viral mutant in which the RGG box was deleted did not show hypophosphorylation of SRp20, indicating that the interaction of ICP27 with SRPK1 is necessary for the decreased phosphorylation of SR proteins during HSV-1 infection (Dai-Ju and Sandri-Goldin, unpublished results). These findings indicate that pre-mRNA splicing inhibition by ICP27 results from its interaction with SRPK1, thus triggering aberrant SR protein phosphorylation (Figure 2).

3.2. The role of ICP27 in 3' end formation

In vitro studies looking at 3' end formation, showed that some viral polyadenylation sites, present primarily on late transcripts, were utilized inefficiently by cellular polyadenylation factors, and these sites were operationally defined as weak (66). ICP27 added to the extracts stimulated the use of these sites (15,17). Further analysis, using *in vitro* polyadenylation assays, suggested that ICP27 helped to recruit the cellular cleavage stimulation factor, Cst64, which is required for poly (A) site recognition, to "weak" HSV-1 poly (A) sites (67). However, a study investigating differential polyadenylation of two transcripts that arise from the HSV-1 UL24 gene by the use of two different poly (A) sites, one weak and one strong, found that the accumulation of transcripts during infection from the weak poly (A) site did not require ICP27 (5). In addition, another study using various constructs of the viral late glycoprotein C gene, stably integrated into transfected cells, showed that the stimulatory effect of ICP27 occurred post-transcriptionally but was independent of the inserted polyadenylation site (68). Thus, the role of ICP27 in enhancing the use of weak poly (A) sites during viral infection has not been firmly established.

In higher eukaryotes, it has been shown that 3' processing is coupled to the elongating RNA polymerase II complex (69,70). Proteins in the cleavage and

polyadenylation complex and in the cleavage stimulation complex bind to the C-terminal domain of RNAP II so that there will be a local concentration of these factors at sites on the nascent RNA where their action is needed. For example, Cst64 binds to the C-terminal domain (CTD) of RNAP II (71). As will be described in Section 4.1, ICP27 was shown to bind to the CTD of RNAP II (29). However, it has not been shown whether or not ICP27 remains bound to the CTD during elongation. If this were the case, ICP27 might be able to recruit cleavage and polyadenylation factors to viral polyadenylation sites. To determine if ICP27 remains bound to the CTD during elongation would require analysis using chromatin immunoprecipitation (ChIP) experiments. This has not yet been reported. It also has not been determined if ICP27 associates with polyadenylation factors *in vivo*. The studies showing that ICP27 recruited Cst64 were performed in nuclear extracts *in vitro* (67). Co-immunoprecipitation experiments or analysis of ICP27-protein complexes would be needed to determine if ICP27 associates with polyadenylation complex proteins during infection. Thus, confirming the proposed role for ICP27 in stimulation of polyadenylation of late transcripts will require further studies.

3.3. ICP27 and the export of viral mRNA

The export of eukaryotic mRNAs from the nucleus to the cytoplasm involves an elaborate machine that is highly conserved from yeast to humans. Eukaryotic pre-mRNAs are processed after synthesis in the nucleus by capping at the 5' end, cleavage and polyadenylation to form the 3' end, and splicing to remove intervening sequences. Following processing, mRNAs must be exported through the nuclear pore complex to the cytoplasm for translation, which requires recognition by export factors to direct the mRNAs to nuclear export receptors for translocation through the nuclear pore complex (72-74). TAP/NXF1, in conjunction with its heterodimeric partner, p15/NXT is the major nuclear export receptor for mRNAs in metazoans (75-80). The yeast homologue of TAP/NXF1, termed Mex67p, has been shown to function as the mRNA export receptor in yeast (81-84). TAP/NXF1 has been shown to shuttle between the nucleus and cytoplasm, cross-link to poly (A^+) RNA, localize at the nuclear pores and interact directly with nucleoporins (75,78,82,83,85). Further, overexpression of TAP/NXF1 stimulates mRNA export (75,85) and knock down of TAP/NXF1 by RNA interference blocked nuclear export of poly (A+) RNA indicating a direct role in mRNA export (86,87).

Nuclear export of metazoan mRNA has been closely linked to splicing (57). The basis of this connection was originally thought to reside in a protein complex that is deposited on pre-mRNAs undergoing splicing at a specific position just upstream of exon junctions (88-90). This Exon Junction Complex or EJC consists of at least eight proteins, which have been shown to function in splicing, nuclear export and mRNA surveillance (91,92). One of these proteins, termed Aly/REF, was initially found to be a component of the EJC and to interact directly with TAP/NXF1 (93). Aly/REF is recruited to pre-mRNA sites near exon junctions (88,89) by a DEAD-box helicase termed UAP56, which functions in spliceosome assembly (94) and which also appears to have role in mRNA export (95). Thus, it was generally accepted that one or more proteins in the EJC mark the mRNA for export through the TAP/NXF1 pathway (96). More recent studies analyzing the composition of the EJC have found that the EJC is composed of a stable core of four proteins that serve as a scaffold for other factors to associate (97) and that Aly/REF and UAP56 are minor and unstable components of the EJC. Instead, the linkage of mRNA export and splicing mediated by Aly/REF now appears to occur through another export complex termed TREX for transcription-export (98), because it was first defined in yeast, where it was shown that mRNA export was directly linked to transcription through the TREX complex (99). In humans, the TREX complex has been shown to associate with spliced mRNA, but not with unspliced pre-mRNA *in vitro* (98). Transcription was not required for this recruitment. Further, the human TREX complex colocalizes with splicing factors in nuclear speckle domains *in vivo*, suggesting that recruitment of the human TREX complex to spliced mRNA is not directly coupled to transcription, but is instead coupled to transcription indirectly through splicing (98). The TREX complex is recruited to a region near the 5' end of mRNA, with the TREX component Aly/REF bound closest to the 5' cap (100). Both TREX recruitment and mRNA export require the cap, and these roles for the cap are splicing dependent. CBP80, which is bound to the cap, associates efficiently with TREX, and Aly/REF mediates this interaction (100). These findings indicate that the CBP80-Aly/REF interaction results in recruitment of TREX to the 5' end of mRNA, where it functions in mRNA export such that the RNA would be exported in a 5' to 3' direction through the nuclear pore (Figure 3).

Most cellular mRNAs are multiply spliced, whereas the majority of HSV-1 transcripts are intronless. Further, ICP27 inhibits cellular pre-mRNA splicing at early times of infection. This presents a problem for HSV-1 because of the coupling of export to splicing (57), regardless of whether this occurs through the EJC or the TREX complex recruited to the 5' cap. Following infection with HSV-1, the cell is presented with thousands of viral intronless transcripts that would not compete efficiently with cellular transcripts for export. Thus, ICP27 shifts the balance by shutting down the cellular splicing machinery (16), so that cellular pre-mRNAs are not processed and exported (2,30). ICP27 interacts with SR proteins and other spliceosomal components (24,30), and thus encounters Aly/REF, which associates with splicing factors (25,27). We have reported that ICP27 co-immunoprecipitates and colocalizes with Aly/REF in HSV-1 infected cells, and that ICP27 recruits Aly/REF from spliceosomes to sites of HSV-1 transcription (27,28) (Figure 3). Interestingly, SM, the EBV homologue of ICP27, and ORF57, the KSHV homologue, have also been found to interact with Aly/REF (49,51), whereas UL69, the HCMV homologue, interacts with UAP56, which recruits Aly/REF to spliced RNA (45). ICP27 binds to HSV-1 intronless transcripts through its RGG box RNA binding motif (18) and the RNA-ICP27-Aly/REF complex binds to TAP/NFX1 (25,27,28). Thus, HSV-1 transcripts are exported through the nuclear pore

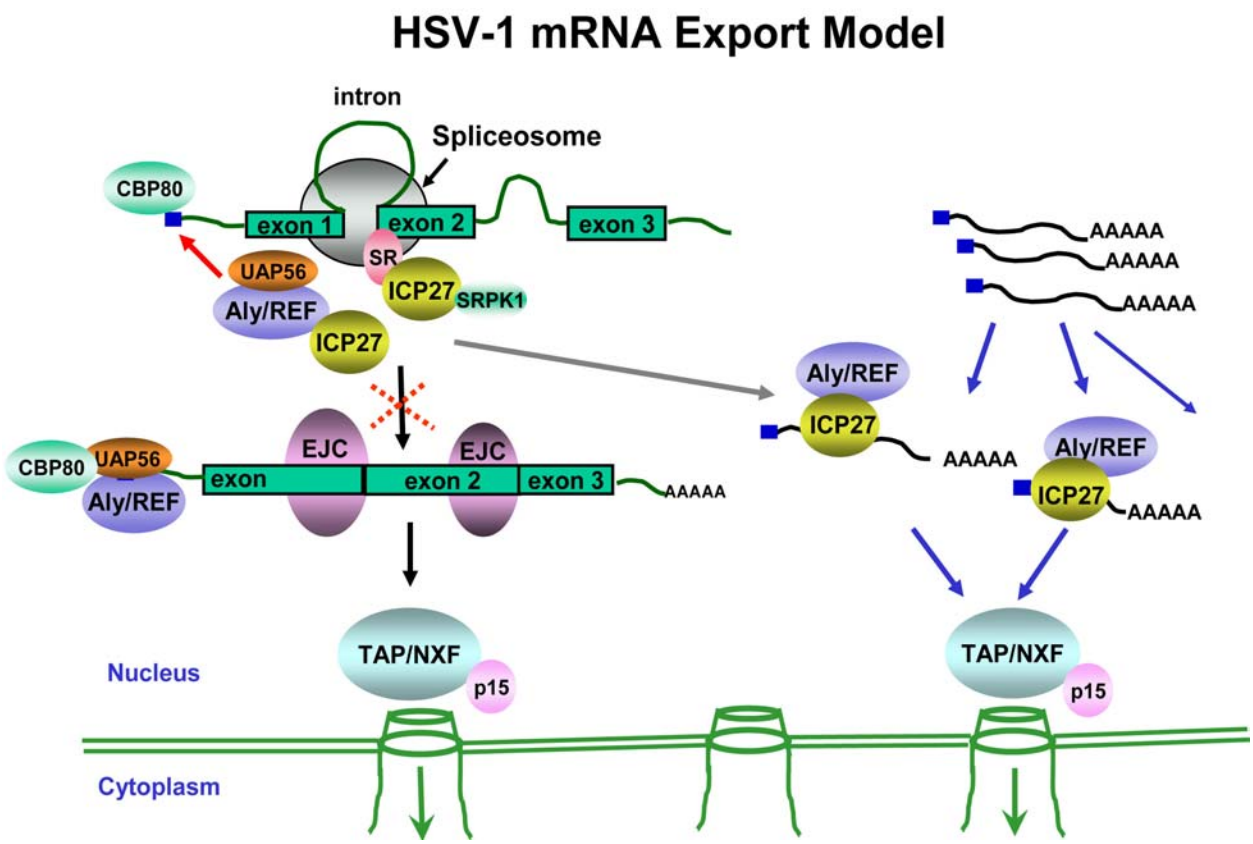

Figure 3. Model of HSV-1 RNA export. Export of metazoan mRNA is closely linked to splicing. A complex of proteins, termed the EJC, is deposited on the spliced RNA at a position just upstream of exon junctions. Aly/REF, one of the components of this complex is recruited to exon junctions by UAP56, which appears to play a role in splicing and export. It has recently been shown that Aly/REF is also recruited by UAP56 to the 5' cap of the mRNA, where it interacts with CBP80 and, as part of the TREX complex, marks the 5' end of the mRNA for export (98). Aly/REF interacts directly with TAP/NXF1 and the RNP complex is exported to the cytoplasm. In HSV-1 infected cells, ICP27 inhibits host cell splicing by interacting with SR splicing factors and an SR specific kinase, SRPK1, which results in inappropriate phosphorylation of SR proteins. Splicing complex formation is stalled before the first catalytic step and export of incompletely spliced RNA is blocked. ICP27 interacts with Aly/REF and recruits it to sites of HSV-1 transcription. ICP27 binds viral mRNAs and the ICP27-Aly/REF-RNA complex is directed to TAP/NXF1. ICP27 interacts directly with TAP/NXF1 and viral RNP complex is exported to the cytoplasm.

complex. It has not yet been determined to which region of the RNA ICP27 binds or if Aly/REF associates with CBP80 at the 5' cap in viral mRNAs.

Two regions of ICP27 have been shown to be required to be intact for interaction with TAP/NXF1 (28), which are the leucine-rich N-terminus and the C-terminus, which encodes a predicted KH domain and a zinc-finger-like motif (Figure 1). Mutations in either the N-terminus or the C-terminus confine ICP27 to the nucleus (18,27,28,101). To determine the fate of viral mRNA in infections with ICP27 mutants that are defective in export, a combination *in vitro* export assay and *in situ* hybridization assay was developed (Johnson, L. A., and Sandri-Goldin, R.M., unpublished results). These studies showed that at 8 hours after infection with wild type HSV-1 greater than 90% of ICP27 protein had left the nucleus by 30 minutes after the start of the *in vitro* export assay. There was a concomitant decrease in nuclear poly (A+) RNA. In contrast, in a mutant with a deletion of the leucine-rich N-terminus, which cannot interact with TAP/NXF, ICP27 was confined to the nucleus during the course of the assay, and so was poly (A+) RNA. In assays with an ICP27 mutant with a deletion of the RGG box RNA binding motif, ICP27 was exported as efficiently as wild type ICP27 because the RGG box resides outside the regions required for interaction with TAP/NXF. However, poly (A+) RNA mostly remained in the nucleus, reaffirming that ICP27 RNA binding is required for proficient viral RNA export (Johnson and Sandri-Goldin, unpublished results). These findings were further supported by microarray analysis on nuclear and cytoplasmic RNA fractions from cells infected with wild type HSV-1 or with ICP27 mutants. The microarrays consisted of DNA oligonucleotide probes representing the complete repertoire of HSV-1 transcripts (102). The interaction of ICP27 with TAP/NXF1 was required for efficient export of viral transcripts because HSV-1 RNAs were retained in the nucleus of cells infected with ICP27 mutants in the TAP/NXF interaction region. Further, ICP27 must bind viral transcripts for them to be

exported efficiently because there was a nuclear retention seen in the samples from infections with the RGG box deletion mutant (Johnson and Sandri-Goldin, unpublished results). Thus, HSV-1 mRNA export requires binding by ICP27 and occurs through the TAP/NXF1 pathway via ICP27 as an export adaptor (Figure 3).

3.4. A Role for ICP27 in translation

Proteomic studies involving immunoprecipitation of ICP27 and mass spectrometric identification of coprecipitated proteins showed an association of ICP27 with the cellular translation initiation factors poly A binding protein (PABP), eukaryotic initiation factor 3 (eIF3), and eukaryotic initiation factor 4G (eIF4G) (19). Co-immunoprecipitation studies and *in vitro* binding studies confirmed these associations *in vivo* and *in vitro*. These results suggest that ICP27 may play a role in stimulating translation of viral mRNAs. Support for this proposal was provided by Ellison *et al.* (20), who examined how ICP27 influences the expression of the HSV-1 protein VP16. Deletion of ICP27 reduced the levels of VP16 mRNA without altering its nuclear export or stability; however, the translational yield of the VP16 mRNA produced in the absence of ICP27 was reduced up to 80-fold relative to that for wild-type infection, suggesting a defect in translation. In the absence of ICP27, the majority of cytoplasmic VP16 mRNA was not associated with actively translating polyribosomes but instead co-sedimented with 40S ribosomal subunits, indicating that the translational defect is likely at the level of initiation. These effects were mRNA specific, because polyribosomal analysis of two cellular transcripts indicated that ICP27 was not required for efficient translation of these mRNAs (20). More recently, the effects of ICP27 on the accumulation, nuclear export, and translation of HSV-1 late mRNAs encoding VP16, ICP5, and gD were examined (103). Fontaine-Rodriguez and Knipe (103) confirmed that ICP27 stimulates translation of VP16, and further showed that translation of another HSV-1 late protein, ICP5, was also stimulated. Translation levels of both VP16 and ICP5 mRNA were reduced during infections with the ICP27 null virus mutant d27-1, and with ICP27 C-terminal deletion mutants n406 and n504. In contrast, translation of another late mRNA encoding gD was not affected by ICP27. These findings demonstrated that ICP27 functions to increase the translation levels of a subset of HSV-1 late genes, and that this function requires the C-terminus of ICP27. In another study, using a tethered function assay, Larralde *et al.* (21) showed a cytoplasmic activity for ICP27 in directly enhancing mRNA translation *in vivo* in the absence of other viral factors. These investigators also showed that ICP27 was associated with polyribosomes. Therefore, ICP27 does appear to have a role in translation of viral transcripts, although the precise nature of that role has yet to be fully elucidated.

A feasible model for the proposed role of ICP27 in translation is that it binds to nascent transcripts in its role as an RNA export factor, and ICP27 may remain bound to viral transcripts upon translocation through the nuclear pore complex. Subsequent interaction with translation initiation factors may result in recruiting these factors to viral mRNAs to facilitate translation. A similar model has been proposed for the cellular protein SRp20, which has been shown to export a subset of cellular intronless RNAs (104,105) and to associate with translation initiation factors and polysomes (106). However, it has not been established how or when export factors are removed from the RNA undergoing translation.

4. TRANSCRIPTIONAL STIMULATION OF HSV-1 EXPRESSION BY ICP27

4.1. ICP27 associates with RNA polymerase II

ICP27 has also been shown to stimulate expression of some early genes and transcription of some late viral genes and the accumulation of early and late gene products is severely reduced in ICP27 viral mutant infections (3,6,107-109). Furthermore, ICP27 has been shown to associate with cellular RNA polymerase II (RNAP II) holoenzyme, and this interaction was found to be independent of DNA and RNA (110). A number of cellular proteins that are involved in RNA processing, including 5' capping, splicing, and 3' cleavage and polyadenylation, have been found to bind to the C-terminal domain (CTD) of RNAP II, which acts a platform to bring these factors to sites on the nascent transcript where their action is needed (111,112). Therefore, we asked if ICP27 interacted directly with RNAP II CTD, in accord with its post-transcriptional activities. *In vitro* binding assays demonstrated that ICP27 does interact with the CTD, and both the N-terminus and C-terminus of ICP27 were required to be intact for this interaction to occur. In virus infected cells, ICP27 N-terminal and C-terminal mutants did not coimmunoprecipitate or colocalize with RNAP II (29) .

Some cellular RNA processing factors that associate with RNAP II CTD interact predominantly with a specific phosphorylated form of the CTD, whereas, others do not show a marked preference when binding (113-116). The RNAP II CTD in all eukaryotes is highly conserved and consists of tandem repeats of a heptapeptide, YSPTSPS, which is repeated 52 times in humans. The CTD is reversibly phosphorylated on serine-2 and serine-5 in the heptapeptide repeat (117). Unphosphorylated RNAP II is recruited to the promoter, where a pre-initiation complex forms. At initiation of transcription, serine-5 is phosphorylated primarily by cdk7, which is associated with general transcription factor TFIIH (115,117). Thus, RNAP II found at the promoters of genes is mostly phosphorylated on serine-5 (118). This form is also referred to as hypophosphorylated or RNAP IIA. During elongation, another kinase P-TEFb, which consists of the cyclin-dependent kinase cdk9 and one of several cyclin T isoforms, phosphorylates serine-2 (119). The elongating form of RNAP II is hyperphosphorylated and is called the RNAP IIO form, which has a slower migration (120). RNA processing factors involved in cleavage and polyadenylation associate with the phospho-serine 2 form of the CTD or RNAP IIO, and this form of the CTD is required for proper 3' end formation (111). In HSV-1 infected cells, an intermediately phosphorylated form has been found beginning around 5 hours after infection (121).

HSV-1 protein ICP22 and kinase UL13 have been shown to mediate the phosphorylation of RNAP II resulting in the RNAP IIi form (122,123). Further, it has been shown that ICP22 interacts with cdk9 in a complex and that the HSV-1 kinase Us3 may also be involved in the intermediate phosphorylation of RNAP II (124). The exact nature of the modifications in phosphorylation and role of the RNAP IIi form during viral transcription have not been illuminated.

4.2. ICP27 recruits RNA polymerase II to viral replication sites

During HSV-1 infection, RNAP II is redistributed to sites of viral transcription (121). We looked at the distribution of RNAP II in wild type and ICP27 mutant virus infected cells to determine if the interaction of ICP27 with RNAP II led to this redistribution. To determine if ICP27 associates with the RNAP II initiating or elongating transcription complex, we performed colocalization studies using monoclonal antibodies that are specific for different phosphorylated forms of the RNAP II CTD. ICP27 was seen to colocalize with RNAP II stained by an antibody that recognizes unphosphorylated RNAP II, which is the form that is recruited to promoters, and the phosphoserine-5 form found in the initiation complex. This colocalization was also seen in staining with an antibody that recognizes an epitope in the N-terminus of RNAP II, thus all forms, phosphorylated and unphosphorylated are recognized (29). We were particularly interested in determining if ICP27 colocalized with the hyperphosphorylated phosphoserine-2 form of RNAP II, which is found predominantly in elongating transcription complexes, and with which factors required for 3' end formation have been found to associate (113,116,125). Antibody H5 has been reported to specifically recognize serine-2 phosphorylation in the CTD (126). At early times after infection with wild type HSV-1, H5 staining was seen to colocalize with ICP27, suggesting that ICP27 binds to the CTD of RNAP II in a phosphorylation independent manner because it associates with both phosphorylated and unphosphorylated forms (29). This was an important finding because this indicates that ICP27 does not just associate with the elongating CTD to use it as a platform to reach RNA processing sites, but instead that ICP27 could also play a role in the recruitment of unphosphorylated RNAP II to HSV-1 transcription sites, and namely to HSV-1 promoters. This would suggest a transcriptional role for ICP27.

Next, we looked at viral transcription-replication compartments, which can be identified by antibody staining of the HSV-1 transcriptional activator ICP4. In wild type infected cells, RNAP II was redistributed into ICP4-containing viral transcription-replication compartments (29). Redistribution was also seen in cells infected with ICP27 mutants that lie outside of the N-terminal and C-terminal regions of ICP27, which are required for interaction with RNAP II. In contrast, in cells infected with ICP27 mutants bearing lesions in the N-terminus and C-terminus, RNAP II was diffusely distributed throughout the nucleus, and ICP4-containing transcription-replication compartments were poorly formed (29). These data suggest that ICP27 is required for the efficient recruitment of RNAP II to viral transcription sites, and that formation of transcription-replication compartments requires RNAP II redistribution. Thus, stimulation of transcription of viral genes by ICP27 may stem from its interaction with RNAP II CTD and its role in recruiting RNAP II to viral replication sites.

A model for the transcriptional stimulation of HSV-1 early and late gene expression by ICP27 is as follows. During viral infection, ICP27 interacts with the CTD of RNAP II, which acts as a platform for RNA processing factors. ICP27 recruits unphosphorylated RNAP II to sites of HSV-1 transcription and transcription initiation on viral genes becomes highly active. ICP27 mutants that cannot interact with RNAP II show reductions in viral transcription and this may result from the inability of these mutants to recruit sufficient amounts of RNAP II to viral transcription-replication sites, which in turn do not assemble into full-fledged replication compartments.

4.3. Elongating RNAP II is degraded during HSV-1 infection

An unexpected finding in our studies on the association of ICP27 and RNAP II was that the RNAP IIO form, which is the elongating form of RNAP II and which is phosphorylated primarily on serine-2 in the CTD, becomes degraded at late times after HSV-1 infection (29). Western blot analysis with antibodies specific for unphosphorylated, serine-5 phosphorylated and serine-2 phosphorylated CTD showed that it was the serine-2 form specifically that was degraded. Proteasome inhibitors prevented the degradation, and ubiquitinated forms of RNAP II were found during late times after infection, indicating the degradation occurred through ubiquitination and the proteasome pathway. Viral transcription was required for this degradation to occur because inhibition of viral transcription by addition of actinomycin D prevented the decrease in serine-2 CTD levels. Further, in infections with ICP27 mutants that fail to recruit RNAP II to viral replication sites, viral transcription is greatly reduced, and RNAP II serine-2 levels were decreased to a lesser extent and the degradation occurred much later in infection. We conclude that the RNAP II degradation occurs because of highly active viral transcription (29).

In yeast and mammalian cells, it has been reported that the elongating form of RNAP II can be ubiquitinated and degraded under conditions in which the transcription elongation complex becomes stalled (127,128). This can occur because of DNA damage or because of piling up of transcription complexes in highly active regions. We propose the following model to explain how and why RNAP IIO gets degraded during HSV-1 infection. HSV-1 transcription is very robust, especially after the initiation of DNA replication, which starts at about 5 hours after infection. Once DNA synthesis commences, all classes of viral genes, IE, early and late are transcribed to high levels. Many HSV-1 genes comprise co-linear transcript families that have different start sites but the same 3' polyadenylation site or the same 5' start site but

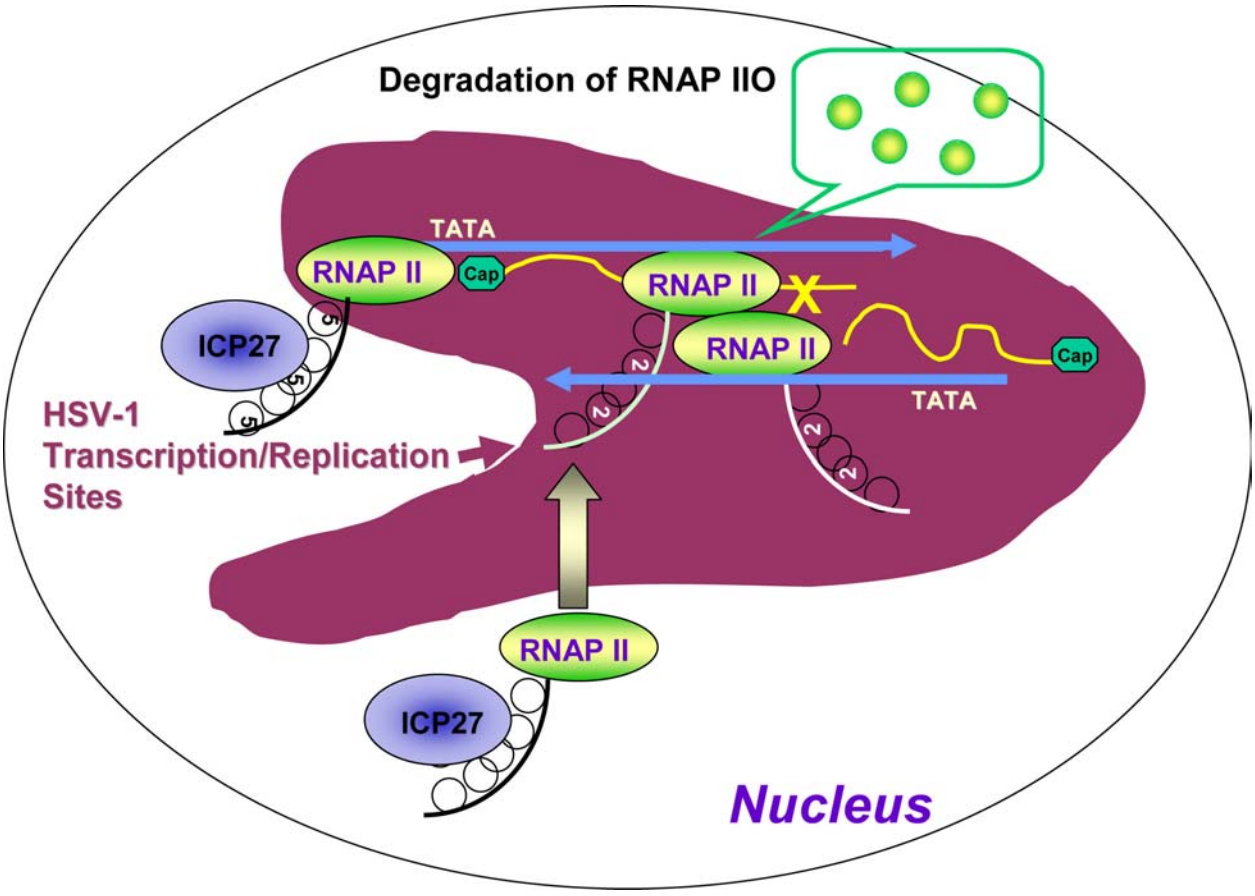

Figure 4. RNAP II is degraded during HSV-1 infection. ICP27 interacts with the CTD of RNAP II and helps to recruit RNAP II to sites of viral transcription and replication, resulting in robust viral transcription. In highly transcriptionally active regions of the genome, elongating RNAP II complexes may collide if transcription is occurring on both strands at the same time, or may pile up if the lead complex is slower than complex that follow. The stalled complex becomes ubiquitinated and undergoes proteasomal degradation to clear the path for approaching RNAP II complexes.

different polyadenlyation sites (56). Intergenic regions are short so that the 3' end of some genes is adjacent to the promoter region of others. Finally, HSV-1 transcription occurs on both strands of the viral genome and genes are not clustered according to kinetic class. Elongating RNAP II complexes could pile-up in multi-transcript regions especially if transcription complexes were moving at different rates or transcription complexes on opposite strands could collide resulting in stalling. Removal of these stalled complexes by proteasomal degradation would clear the path for other approaching complexes to proceed as has been demonstrated for stalled complexes in yeast and mammals (127-130) (Figure 4).

5. HSC70 NUCLEAR FOCUS FORMATION REQUIRES ICP27

The chaperone protein Hsc70 is part of a large group of proteins that assist in the folding and unfolding of proteins and in the assembly and disassembly of macromolecular structures (131-134). In addition to protein folding, it has also been demonstrated that Hsc70 and an interacting co-chaperone U-box protein termed CHIP (C-terminus of Hsc70-interacting protein), target aberrant forms of CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) protein for proteasomal degradation by promoting their ubiquitination (135). Burch and Weller (136) reported that in herpes simplex virus 1 (HSV-1) infected cells, Hsc70 was sequestered in nuclear foci that also contained components of the 26S proteasome and ubiquitin-conjugated proteins. In subsequent studies, these authors also found that Hsp90 and Hsp40 were redistributed to these sites, which they termed VICE-domains, for virus-induced-chaperone-enriched (137). The formation of these domains or foci at the periphery of viral replication compartments suggests that they may function to rid virus-infected nuclei of misfolded and ubiquitinated proteins through proteasomal degradation.

As described in the preceding section, we reported that during HSV-1 infection when viral transcription is robust, cellular RNAP II, and specifically the serine-2 phosphorylated form of the CTD found in elongating transcription complexes (113,117,138), undergoes ubiquitination and proteasomal degradation (29). Based upon the reported composition of the Hsc70 nuclear

foci or VICE domains, we asked whether the formation of these discrete nuclear structures at the periphery of viral replication compartments was related to active viral transcription and RNAP II degradation. We found that ICP27 also interacts directly with Hsc70 and that ICP27 is required for the sequestration and formation of Hsc70 foci (12). Both the N- and C-terminus of ICP27 are required for the interaction. During infection with N- and C-terminal ICP27 mutants that cannot interact with Hsc70, nuclear foci do not form. Further, N- and C-terminal mutants also cannot interact with RNAP II, resulting in low levels of viral transcription, poor formation of viral transcription-replication compartments and delayed and decreased degradation of RNAP II (29). These results indicate that there is a correlation between the formation of Hsc70 nuclear foci and RNAP II degradation during highly active viral transcription. Further evidence for this correlation was the observation that addition of proteasome inhibitors during wild type HSV-1 infection prevented the formation of Hsc70 nuclear foci, suggesting that the foci form, at least in part, to provide components of the proteasome machinery for the degradation of RNAP II during robust viral transcription. Expression of a dominant negative Hsc70 mutant, which has been shown to interfere with endogenous Hsc70 activity (139,140), prevented the formation of Hsc70 nuclear foci and reduced RNAP II degradation. This in turn had adverse effects on viral replication (12). These results lead us to postulate that Hsc70 foci serve an important role during viral infection, which includes aiding in the ubiquitination and proteasomal degradation of stalled RNAP II complexes on viral genomes. Thus, HSV-1 appears to have evolved a mechanism to insure efficient viral transcription by clearing stalled elongation complexes. We postulate that these foci either serve as sites where misfolded or ubiquitinated proteins are targeted and are consequently degraded, or more likely, that these foci serve as reservoirs or storage sites for the proteasomal degradation machinery, much like splicing speckles are thought to serve as storage sites for mRNA processing factors. The localization of the foci at the periphery of viral transcription-replication compartments would provide an efficient access to stalled transcription complexes, which would then be ubiquitinated and degraded.

6. ICP27 DIRECTS MANY ACTIVITIES DURING INFECTION

How does ICP27 orchestrate so many activities? Although a definitive answer cannot be given to the question posed, the likely explanation is through a changing series of protein-protein interactions. ICP27 interacts with many proteins, both viral and cellular (Figure 1). These include an interaction with itself to form multimers (31), with viral proteins ICP4 (33) and ICP8 (32,141), and with cellular proteins including: splicing proteins SRp20 and other SR proteins (30), SAP145 (24) and SRPK1 (30); RNA export proteins Aly/REF (25,27,28) and TAP/NXF1 (28); RNA polymerase II (29,110); Hsc70 (12); hnRNP K (142); CK2 (26,142); and translation factors PABP, eIF3 and eIF4G (19). ICP27 is also involved in re-localizing a number of the proteins with which it interacts including SR proteins (30,54), SRPK1 (30), Aly/REF (28), RNAP II (29), Hsc70 (12) and CK2 (26). Thus, in some ways ICP27 serves as a viral chaperone protein. Cellular chaperone proteins assist in protein folding and in protein holding, which aids in assembly of protein complexes by targeting proteins to the complex (143). ICP27 is probably not involved in protein folding, but in protein holding. That is, it binds to proteins to transfer them to sites where their action is required to benefit viral replication. Studies to chart the dynamics of the interactions of ICP27 throughout the course of infection using live cell imaging, while still underway in my lab, have shown that ICP27 is predominantly nuclear at 3 hours after infection and is associated with splicing factors in coalesced splicing speckles. Association of ICP27 with Aly/REF moving into structures that resemble viral replication compartments can be visualized beginning at 5 hours (Li and Sandri-Goldin, unpublished results). Further studies will probe the dynamics of these interactions throughout infection.

7. REGULATION OF ICP27'S ACTIVITIES THROUGHOUT INFECTION

7.1. Post-translation modifications: arginine methylation

Protein arginine methylation by type I methyltransferases, which add one or two methyl groups to arginine, has been shown to affect a number of eukaryotic processes including protein transport, transcription and cell signaling (35). ICP27 was shown to be methylated *in vivo* and the arginine-glycine-rich RGG box motif, which is required for binding to RNA (18,22), was shown to be one of the targets for methylation (22). Because ICP27 is multifunctional and it appears to alter its activities throughout the course of infection, we postulate that arginine methylation could modify its affinity for some of its interacting partners and thus act as a switching control. We probed the arginine methylation of ICP27 by MALDI-TOF Mass Spectrometry, and identified three arginines within the RGG box motif (Figure 1), R138, R148 and R150, which were methylated in ICP27 purified from wild type HSV-1 infected cells (Souki, Gershon and Sandri-Goldin, unpublished results). These residues were differentially methylated in the nucleus and cytoplasm. Viral point mutants were engineered in which the three arginine residues within the RGG-box were mutated to lysine. Recombinant viral mutants were constructed bearing single, double and triple mutations. Compared to wild-type HSV-1, growth of mutant R150K, and of the double mutant R138, 150K was reduced about one log, and plaque size was smaller, whereas, growth of R148K was similar to wild-type. Immunofluorescence staining of ICP27 during infection with mutants R150K, R138, 150K, and R138,148,150K demonstrated that ICP27 rapidly shuttled to the cytoplasm at early times after infection. Similar results were seen under hypomethylation conditions during wild type infection, when the general methyltransferase inhibitor adenosine dialdehyde was added (Souki and Sandri-Goldin, unpublished results). Further, the R150K and R138K, as well as the double mutant were unable to interact with SRPK1 or to recruit it to the nucleus. Arginine methylation has been shown to

affect both nucleocytoplasmic transport and protein-protein interactions of RNA binding proteins (144). In these studies we found that arginine methylation appears to affect ICP27's export activity and to regulate the interaction of ICP27 with SRPK1. In ongoing studies, we are seeking to determine which other ICP27 protein-protein interactions may be affected or modulated by arginine methylation and whether arginine methylation has any effect on ICP27 RNA binding *in vivo*.

7.2. Post-translational modifications: phosphorylation

Another post-translational modification that has been demonstrated to regulate protein function is phosphorylation. ICP27 has been demonstrated to be phosphorylated on serine residues during infection (34). Phospho-peptide mapping of ICP27 purified from HSV-1 infected cells showed that there are two serine residues at positions 16 and 18 within the leucine-rich N-terminal region required for export that are phosphorylated by CK2, and a serine at position 114 within the nuclear localization signal (NLS) that is phosphorylated by PKA (34). In transient expression studies, mutation of the serine at 114 to alanine diminished import of ICP27 to the nucleus (34), suggesting that phosphorylation by PKA may regulate the ability of ICP27 to interact with importins. To further study the role of phosphorylation on the activities of ICP27, we constructed serine to alanine and serine to glutamic acid substitution mutations at each of the known sites (Rojas and Sandri-Goldin, unpublished results). Single, double and triple recombinant viral mutants were constructed and characterized. In one step growth curves, viral yields were reduced 2 to 3 logs compared wild type HSV-1 for all of the mutants. This indicates that phosphorylation of ICP27 is important for its function. Further, immunofluorescence studies showed that the localization of ICP27 was predominantly nuclear even at late times after infection, suggesting that phosphorylation has a role in regulating ICP27 shuttling. In addition, viral replication/transcription compartment formation was greatly delayed, in that compartments were not fully formed even by 8 hours after infection compared to WT HSV-1 (Rojas and Sandri-Goldin, unpublished results). These data suggest that phosphorylation of ICP27 is an important regulatory mechanism that may modulate its ability to function in its various roles during viral infection.

8. THE DOMAINS REQUIRED FOR THE MULTIPLE INTERACTIONS OF ICP27

The regions of ICP27 that are involved in its interactions with proteins and RNA have been mapped by the analysis of mutants, and have been broadly mapped to protein motifs in both the N-terminal and C-terminal halves of the molecule (Figure 1). Although several protein motifs have been identified based upon sequence comparisons, no structural information on ICP27 has been published to date. The predicted protein structural motifs in the interacting regions of ICP27 were based upon sequence comparisons with proteins whose structures have been determined. While this can help to determine protein motifs, it will really be necessary to perform structural analysis on ICP27 to determine if the predicted motifs do indeed form in the global folding of the protein.

8.1. RNA binding by ICP27 confers some structural rigidity

The N-terminus of ICP27 is largely hydrophilic and contains a leucine-rich region required for export (LRR), an acidic region composed of several aspartic and glutamic acids (D/E), a nuclear localization signal (NLS), and two arginine-rich regions, R1 and R2 (Figure 1). The R1 region contains an RGG box RNA binding motif, which is required for binding to viral RNA (18,22). To investigate the structure of the N-terminus of ICP27 NMR studies were performed. The N-terminal 160 amino acid peptide of ICP27, which includes the RGG box, was expressed in *E. coli* and was found to be highly soluble (Corbin-Lickfett, Cocco and Sandri-Goldin, unpublished results). Two dimensional NMR analyses revealed that the ICP27 N-terminus is mostly unfolded but the addition of generic structured nucleic acid substrates to the N-terminus conferred some rigidity to its structure (Corbin-Lickfett, Cocco and Sandri-Goldin, unpublished results).

Therefore, we sought to identify native HSV-1 sequences that interact with the ICP27 N-terminus with high affinity. Although a previous study, using a yeast three-hybrid approach, identified 31 HSV-1 sense RNAs 35 to 225 nucleotides in length, which interacted with preferred specificity for ICP27 (23), these RNAs mapped to 28 different open reading frames. Thus, it would have been difficult to determine high affinity binding sequences from this library. Instead, we used a series of 30 nucleotide sequences within the glycoprotein C (gC) gene. ICP27 was shown previously to bind to gC RNA in UV cross linking experiments (18). EMSA experiments were performed with bacterially expressed ICP27 N-terminal peptide from amino acids 1 through 160. In all, 19 gC sequences were tested. For 7 of the gC sequences tested, the ICP27 N-terminus was able to strongly shift in EMSA assays. There were also sequences within the pool of 30mers tested that had intermediate shifts (7/19) and several that did not shift at all (5/19) (Corbin-Lickfett, Cocco and Sandri-Goldin, unpublished results). The 30mer sequences that strongly shifted are rich in C residues, suggesting that the ICP27 RGG box may be binding to novel sequence motifs, different from what has been previously described for another RGG box protein, which was shown to bind to G-quartets (145). The data from NMR analysis of nucleic acid-bound and unbound ICP27 N-terminus indicates that it forms a highly flexible, dynamic structure, but also that it has a compact structure, not unfolded or in a random coil state but in a state that is referred to as molten globular (Corbin-Lickfett, Cocco and Sandri-Goldin, unpublished results). Studies are underway to determine the solution structure of other important ICP27 domains and eventually of the entire protein.

8.2. Intramolecular interaction of the N- and C-termini of ICP27

An interesting dichotomy in the structure of ICP27 is that for several of its protein-protein interactions, mutation of either the N-terminal region or the C-terminal

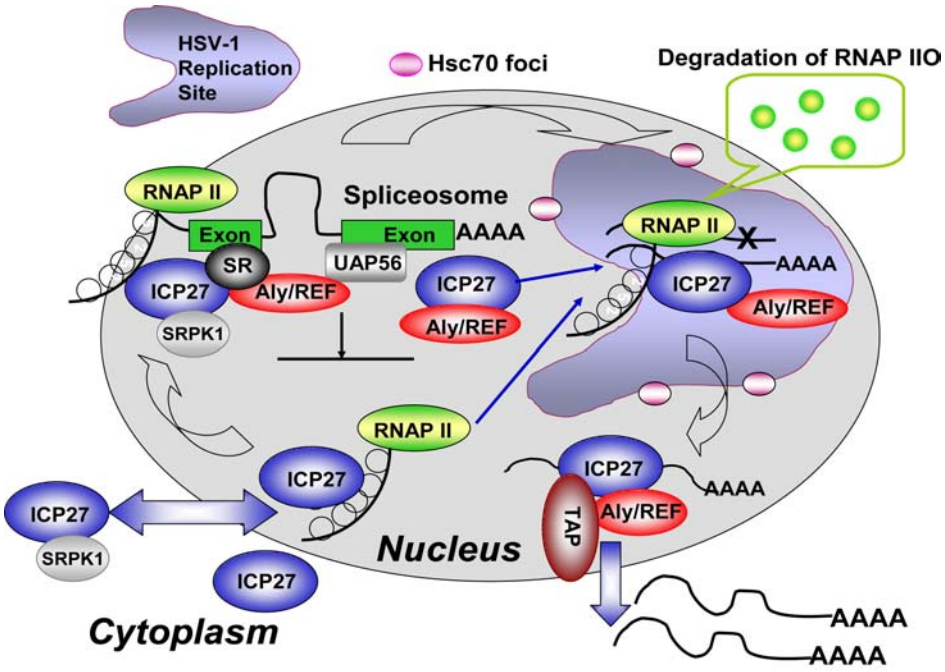

Figure 5. Model of the multifunctional activities of ICP27. ICP27 affects several cellular processes to facilitate viral replication. ICP27 interacts with a number of cellular proteins, some of which it recruits to new locations as depicted. ICP27 also binds viral RNA to direct it to the cytoplasm through the TAP/NXF1 pathway. ICP27 stimulates HSV-1 transcription by recruiting RNAP II to viral replication sites, through its interaction with RNAP II CTD. A novel consequence of highly active viral transcription is the degradation of RNAP II, which may be part of the reason that chaperone proteins, including Hsc70, are sequestered into foci that lie at the periphery of viral replication compartments. ICP27 helps to direct Hsc70 to these foci, which also contain components of the 26S proteasome and the ubiquitination machinery (136).

region reduces or abrogates the interaction. This has been shown for the interaction of ICP27 with itself to form multimers (31), as well as for the interaction with RNAP II CTD (29), TAP/NXF1 (28), CK2 (142) and Hsc70 (12). These findings led us to suggest that ICP27 may undergo intramolecular head-to-tail interactions. That is, proper folding, resulting in an intramolecular interaction between the N- and C-terminal regions of ICP27, may be necessary for the association of ICP27 with certain viral or host cell proteins. To determine if this occurs, Bi-molecular Fluorescence Complementation or BiFC (146) was performed. BiFC is performed by fusing fragments of a fluorescent protein, which have been split in half, to two proteins thought to interact. If the two proteins interact and come into contact, the fragments of the fluorescent protein are able to fold properly and form a bimolecular fluorescence complex that restores fluorescence. In our experiments, the two fragments of yellow fluorescent protein (YFP) were tagged to the N- and C-termini of ICP27 to determine if ICP27 undergoes an intramolecular interaction (Hernandez and Sandri-Goldin, unpublished results). To rule out an intermolecular interaction, each fragment of YFP was singly tagged to different molecules of ICP27 and co-expressed. BiFC analysis was performed in transient transfection assays using live cells and fluorescence from each of the fusion proteins was monitored at different times after HSV-1 infection. Upon expression of the intramolecular YFP/ICP27 fusion protein, YFP fluorescence was restored and the intermolecular YFP/ICP27 fusion proteins showed no fluorescence. An ICP27 mutant, with two cysteine to serine substitutions in the C-terminus, also failed to restore fluorescence, further demonstrating that the C-terminus of ICP27 must be intact for proper folding and intramolecular interaction (Hernandez and Sandri-Goldin, unpublished results). Thus, ICP27 folds to mediate an interaction between its N- and C-terminus.

9. PERSPECTIVES

ICP27 is a key player in HSV-1 infection. ICP27 is multifunctional and has been implicated in all aspects of gene expression from transcription through RNA processing and export to translation. Key questions remain including how ICP27 switches among its various activities during the course of infection; how its export and import activities may be controlled by post-translational modifications; how ICP27 recognizes viral transcripts by determining the RNA recognition sequence/structure, and how ICP27 "escorts" interacting proteins to sites that favor viral gene expression. Future studies will be focused on charting the dynamics of ICP27's intermolecular interactions. In the model of HSV-1 gene expression showing the known functional activities and interactions of ICP27 (Figure 5), it is currently unclear which of these activities and interactions are essential for HSV-1 infection. Nor is it clear which activities attributed to ICP27 are actually directly mediated by ICP27 and which are

mediated indirectly, for example by products whose expression requires ICP27. Structural information on ICP27 will be required to design targeted mutations that will disrupt specific interactions without disrupting the structural integrity of the protein. It will be necessary to dissect each of these interactions and activities to determine their overall importance to viral infection.

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