DISSERTATION

SINDBIS VIRUS USURPS THE CELLULAR HUR PROTEIN TO STABILIZE ITS TRANSCRIPTS AND PROMOTE INFECTIONS OF MAMMALIAN AND MOSQUITO CELLS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctorate of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2010

COLORADO STATE UNIVERSITY

July 14th, 2010

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KEVIN J. SOKOLOSKI ENTITLED THE MOSQUITO AND MAMMALIAN HUR PROTEINS REGULATE SINDBIS VIRUS RNA STABILITY AND VIRAL TITER IN TISSUE CULTURE CELLS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

Sindbis virus usurps the cellular HuR protein to stabilize its transcripts and promote infections of mammalian and mosquito cells

Members of the genus *Alphavirus* are recognized as significant human pathogens. Infection of vertebrate hosts often results in febrile illness and occasionally severe encephalitis. The archetypical alphavirus is Sindbis virus, which we have utilized in these studies. The genomic and subgenomic RNAs of Sindbis virus strongly resemble cellular mRNAs as they are capped at their 5' ends and polyadenylated at their 3' termini. These features allow the viral RNAs to act like cellular mRNAs and make them prime substrates for the cellular mRNA decay machinery.

Sindbis virus RNAs are indeed subject to degradation by the cellular mRNA decay machinery in cell culture models of infection. Nevertheless, they decay by a mechanism that is different from the majority of cellular mRNAs as the decay of Sindbis virus transcripts is predominantly deadenylation-independent. As cellular mRNAs are often regulated by elements present in their 3' untranslated regions (UTR), we hypothesized that these viral 3'UTR elements were functioning similarly to cellular mRNA stability elements resulting in the enhancement of viral infection.

The primary goal of the research described in this dissertation was to characterize in mechanistic detail how the Sindbis virus 3'UTR represses deadenylation. To this end we used both cell free extracts and tissue culture systems to assay the effects of the viral 3'UTR on transcript stability. Interestingly, multiple elements were found to be independently repressing deadenylation in mosquito cytoplasmic extracts. Further examination revealed that a major stability determinant was the U-rich element (URE) observed in the 3'UTR of many alphaviruses. The ability to repress deadenylation in our cell free extract system was similarly observed with the UREs of Venezuelan equine, eastern equine, western equine and Semliki Forest viruses. Taken together, these data strongly assert that the repression of deadenylation via the URE is evolutionarily conserved. Prior to this study, the URE had no ascribed function. The repression of deadenylation imparted by the URE correlated with the binding of a cellular 38kDa factor. This 38kDa factor was determined to be the cellular HuR protein. Both the human and mosquito HuR proteins were found to bind with high affinity to the Sindbis virus 3'UTR. Reduction of cellular HuR protein levels using RNAi resulted in an increase in the rate of viral RNA decay. Furthermore, a significant decrease in the titer of progeny virus was observed. A similar effect on viral titer was observed when the predominant HuR binding site, the URE, was deleted from the viral 3'UTR.

Taken together these observations identify a novel *Alphavirus* / host interface that significantly impacts viral biology. Furthermore these studies have confirmed our hypothesis that the members of genus *Alphavirus* have indeed evolved RNA stability elements that resemble cellular mRNA stability elements for the purpose of

enhancing viral infection. Furthermore these studies identify a potential therapeutic anti-viral target - the cellular HuR protein.

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Acknowledgments

The work contained herein is the summation of my academic career here at Colorado State University. My success is undoubtedly as much a creature of the efforts of others as much as my own. No matter how much ink I scatter upon this page my debt will likely never be repaid.

The last five years of my life have included many milestones. First and foremost I married the love of my life and welcomed my beautiful daughter into this world. These are without a doubt the proudest moments of my life. I whole heartedly thank them both for their understanding of the late nights that, unfortunately, more often than not turned into early mornings. Without their sacrifices I would not be sitting here writing these words.

Without the understanding and hard work of my advisors this endeavor would never have reached the stage that it is in now. Through both difficulty and adversity my mentors have stood behind me, educating and supporting me to become who I am today. Furthermore, I would like to acknowledge and applaud the efforts of my committee who have undoubtedly shaped my understanding and appreciation of science.

I would like to extend my gratitude to the many friends whose company I have enjoyed during my time in the lab. It is certain that without them the constant drone of machinery would have brought about absolute madness many years ago. I count myself as lucky, having had the opportunity to grow as a scientist alongside some of the finest people I have ever met. I will always remember the walks with friends in

times of joy and those of sorrow. It is these moments, where standing side-by-side; I learned the true merits of friendship. I cannot express how grateful I am for their help, even for the removal of dried gels and the sharing of time points so that I would have the opportunity to limit the time away from my family.

Nevertheless, this endeavor has not been without its consequences. While I am thankful for the friendships forged, I am also mournful for those lost during this endeavor. Unfortunately, my understanding of the reasons as to why are often apparent too late. Moreover, I have learned the hard way that the there is far more to life than molecules that are too small to see.

I would like to end with the five life lessons that I learned during my time in the lab: 1) If it is a weekend and you need a specific reagent, you will have exactly half the amount that you require, 2) If something cannot be explained, it likely involves the Lsm complex, 3) Always take time to enjoy art, especially when in a new city, 4) You can save yourself a year of work by working an extra hour a day (but remember if the experiment doesn't work you haven't gained any time but still get the extra headache) and 5) Bats (and the viruses they carry) are coming to get all of us.

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Introduction

Arboviral diseases are increasingly being recognized as a global public health concern. The enlarged geographical distribution of competent vector mosquitoes is aiding the spread of arboviruses into naïve populations resulting in epidemics. Members of the genus Alphavirus are responsible for some of the largest outbreaks of arboviral disease ever recorded (Calisher, 1994; Ligon, 2006). While the majority of infections result in febrile illness, serious encephalitis may develop leading to death. Mortality rates associated with epidemics of eastern equine encephalitis virus may be as high as 30% (Feemster, 1957). Currently treatment for Alphavirus disease is limited, often consisting solely of supportive care. Examination of Alphavirus-host interactions will likely yield innovative targets for the development of treatment options. One relatively under explored area of host-virus interactions is the interface between viral RNAs and the cellular mRNA decay machinery. The goal of this project was to understand how alphavirus transcripts successfully avoid degradation in order to promote a productive infection. Understanding how Alphaviruses interact with the cellular mRNA decay machinery may give fundamental insights into not only virus biology but may illuminate potential strategies to effectively treat severe incidences of arboviral disease.

Cellular mRNA Decay

The cellular mRNA decay machinery is a robust system capable of selectively removing unwanted or aberrant transcripts from the host

transcriptome. Indeed, as much as 50% of host cell regulation of gene expression occurs at the level of post-transcriptional control rather than through modulation of transcription rates (Garcia-Martinez et al., 2004; Cheadle et al., 2005b; Cheadle et al., 2005a).

Virtually all cellular mRNAs are cotranscriptionally capped at the 5' end with a $^{7\text{-me}}$GpppG cap and polyadenylated at the 3' end (Hagler and Shuman, 1992, Sheiness and Darnell, 1973). These features impart stability to the mRNAs and influence the efficiency of their translation (Garneau et al., 2007). Many proteins are also deposited onto the nascent transcript during transcription. For instance, the deposition of the exon junction complex (EJC), a large molecular weight complex deposited upstream of the exon-exon junction (Le et al., 2000) as a result of splicing, identifies the transcript as mature. The proteins associated with a given mRNA form the messenver ribonucleoprotein (mRNP). The composition of the mRNP directs the fate of the mRNA and serves to act as a liaison between the RNA and cellular processes. As described below, components of these mRNPs directly influence the decay of a given mRNA.

The decay of an mRNA may be the result of one of many cellular enzymatic activities. The predominant mechanism of mRNA decay involves the deadenylation of the transcript followed by exonucleolytic degradation. Additionally, the transcript may be slated for decay by endonuclease-mediated cleavage. Furthermore, the selection of a transcript as a decay substrate may be initiated as a result of the cellular RNA quality control machinery. These pathways, and their regulation, are described below.

Deadenylation: Removal of the Poly(A) Tail

For the majority of cellular mRNAs the primary and rate limiting step of degradation is deadenylation (Brewer and Ross, 1988; Shyu et al., 1991; Wilson and Treisman, 1988). As depicted in Fig. 1, deadenylation is the process by which the 3' poly(A) tail is removed from a transcript. Removal of the poly(A) tail results in translational silencing (Gallie, 1991; Huarte et al., 1992) and additionally serves to expose the 3' end of the transcript to exonuclease-mediated decay (Allmang et al., 1999). Furthermore, the removal of the poly(A) tail enhances the decapping of the RNA substrate, a phenomenon termed deadenylation-dependent decapping (Beelman et al., 1996; Hatfield et al., 1996). Within the cell there are multiple deadenylases with different functions, four of which have been characterized to date – namely CCR4, CAF1, PAN2 and poly(A)-specific ribonucleases (PARN). In addition to the above well-characterized deadenylases, there are a variety of less studied proteins that likely possess deadenylase activity. One of these, Nocturnin, which bears significant homology to CCR4, is impacted by the circadian rhythm (Baggs and Green, 2003).

I. The CCR4-NOT Complex

The CCR4-NOT complex, which consists of CCR4 and CAF1 in addition to NOT1-NOT5, exhibits poly(A)-specific nuclease activity in both yeast, where it is the predominant deadenylase (Yamashita et al., 2005; Tucker et al., 2001),

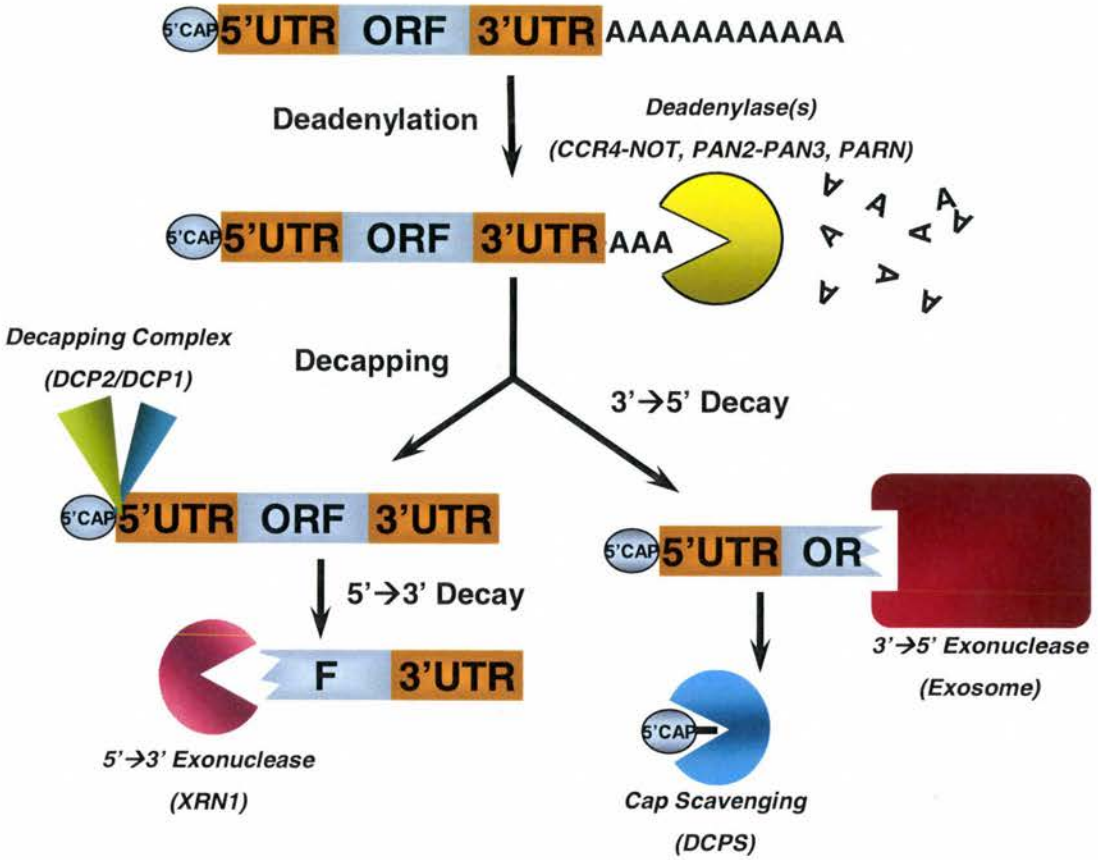


Figure 1- The Deadenylation-Dependent Pathways of mRNA Decay. Cellular mRNAs predominantly undergo deadenylation-dependent mRNA decay. Degradation is initiated via removal of the 3' poly(A) tail by way of one (or more) of the cellular deadenylases. Following removal of the poly(A) tail the body of the mRNA may be degraded in either a 5'→3' or 3'→5' direction. Transcripts may be degraded in the 3'→5' direction by the exosome with the remaining 5' capped small RNA degraded by DCPS. Alternatively, instead of decaying in a 3'→5' manner, the 5' cap structure may be removed via the decapping activity of the DCP2/DCP1 complex. In this pathway the remaining body of the transcript is eliminated in the 5'→3' direction via XRN1.

and in metazoans (Albert et al., 2000; Schwede et al., 2008; Temme et al., 2004; Temme et al., 2010). The CCR4-NOT complex components in yeast that possess deadenylase activity are Ccr4p and Caf1p (Tucker et al., 2001). In yeast, Ccr4p appears to be the predominant deadenylase (Ohn et al., 2007), but in *Drosophila melanogaster* CAF1 appears to be the primary deadenylase as demonstrated using RNAi of CCR4-NOT components (Temme et al., 2010). In humans the CCR4-NOT complex is diverse in composition and function (Lau et al., 2009), as several complexes exhibit deadenylase activity. To date, at least 4 distinct CCR4-NOT complexes have been observed in humans with each complex associating with known members of RNA metabolism (Lau et al., 2009). CCR4-NOT activity is regulated at the deadenylation substrate level as the activity of the CCR4-NOT complex can be inhibited by the presence of Poly(A) Binding Protein (Pab1p in yeast, or PABPC1 in mammals) (Tucker et al., 2002), or activated by PABPC1 depending on the presence of activating factors (Funakoshi et al., 2007). Interactions between the CCR4-NOT complex and histone deacetylases have also been reported (Laribee et al., 2007), underscoring their role in transcriptional regulation.

II. The PAN2-PAN3 Complex

In yeast the PAN2-PAN3 complex deadenylates target mRNAs in a Pabp1-dependent manner (Brown et al., 1996). Pan2p is the enzymatic component of the PAN2-PAN3 complex in yeast (Boeck et al., 1996). This particular deadenylase complex has been observed "trimming" the poly(A) tails of

reporter constructs, after which the deadenylation of the remaining poly(A) tail is likely performed by another deadenylase (Yamashita et al., 2005,Tucker et al., 2001). Additionally the PAN2-PAN3 complex is involved in remodeling the poly(A) tail after export from the nucleus (Brown et al., 1996). In trypanosomes, RNAi of Pan2 reveals that several transcripts may be regulated directly by the PAN2-PAN3 complex (Schwede et al., 2009).

III. PARN

Poly(A)-specific ribonuclease (PARN) acts as a multimer (Martinez et al., 2000) to deadenylate RNA substrates in a cap-dependent manner in mammals (Wu et al., 2009; Martinez et al., 2001; Martinez et al., 2000; Nilsson et al., 2007; Gao et al., 2000; Dehlin et al., 2000). The presence of the nuclear cap binding complex as well as eIF4G inhibits the activity of PARN (Balatsos et al., 2006; Gao et al., 2000). PARN has also been shown to regulate the expression of mRNAs in *Xenopus laevis* oocytes in conjunction with cytoplasmic polyadenylation (Copeland and Wormington, 2001; Kim and Richter, 2006). PARN is the predominant deadenylase in cell free mRNA decay systems (Ford and Wilusz, 1999; Opyrchal et al., 2005; Sokoloski et al., 2008a; Sokoloski et al., 2008b). Curiously, there are no clear homologs of PARN in either *Saccharomyces cerevisiae* or *Drosophila melanogaster*, but PARN homologs have been identified in most other metazoan species including mosquitoes (Opyrchal et al., 2005). PARN activity is likely regulated by the association of RNA binding proteins with the transcript as exhibited by the recruitment of PARN

to transcripts interacting with the instability factors KH-type splicing regulatory proteins (KSRP), CUG binding protein 1 (CUGBP1) and tristetraproline (TTP) (Moraes et al., 2006; Gherzi et al., 2004; Lai et al., 2003).

Exonuclease Meditated Degradation of Unadenylated mRNAs

Deadenylated mRNAs are translationally silenced and are prime substrates for exonucleolytic degradation. Following deadenylation the body of the mRNA is degraded either in the 3'→5' direction via the exosome or in a 5'→3' direction via the XRN1 exoribonuclease (Garneau et al., 2007). The end result of either of these pathways is usually the complete degradation of the body of the transcript. These processes are highly regulated and rapid, as decay intermediates are difficult to observe.

I. 3'→5' Decay

The mediator of 3'→5' exonucleolytic degradation is the exosome, a high molecular weight complex that possesses nuclease activity. The best studied eukaryotic exosome is that of *S. cerevisiae* where the core exosome is composed of 10 subunits (Houseley et al., 2006). Many of the subunits contain RNase PH domains, which although required in yeast, lack catalytic activity (Dziembowski et al., 2007). A single subunit of the yeast exosome, Rrp44p (also known as Dis3p), has both exonuclease and endonuclease activity (Lebreton et al., 2008; Schaeffer et al., 2009). Mutations in one, but not both, of the nuclease activities of Rrp44p are tolerated in yeast (Schaeffer et al., 2009). Enzymatic

activity has also been observed with Rrp6p, a component of the nuclear exosome (van Hoof et al., 2000). The function of Rrp6p is vital to the maturation of the 5.8 S rRNA (Briggs et al., 1998; van Hoof et al., 2000). Activity of the exosome is regulated by the superkiller (SKI) complex (Brown et al., 2000; Araki et al., 2001). The SKI complex inhibits translation of poly(A)-minus mRNAs in yeast (Benard et al., 1999). Similarly the mammalian exosome is a large complex with apparently synonymous functions to the yeast complex (Allmang et al., 1999). In the mammalian exosome the RNase PH domain-containing subunits augment the binding of the exosome to AU-rich sequences (Anderson et al., 2006). Degradation of the body of the mRNA by the exosome results in the formation of a 5' $^{7\text{-me}}$GpppG moiety. The 5' $^{7\text{-me}}$GpppG fragment is recycled by the cap-scavenger enzyme DCPS (Liu et al., 2002).

II. 5'→3' Decay

Alternatively to the 3'→5' pathway described above the transcript may be degraded via the 5'→3' decay pathway. In this pathway the 5' $^{7\text{-me}}$Gpp is removed via a decapping event following deadenylation. This exposes the 5' end of the mRNA to exonucleolytic decay, by the generation of a 5' monophosphate (Beelman et al., 1996; Hatfield et al., 1996). In yeast, removal of the poly(A) tail allows the Lsm1p-7p complex to associate with the 3' end of the mRNA (Tharun et al., 2000, Chowdhury and Tharun, 2009; Chowdhury and Tharun, 2008; Chowdhury et al., 2007; Tharun and Parker, 2001). This complex is required in yeast for decapping (Tharun et al., 2000; Tharun and Parker, 2001), however its

function in mammals has yet to be characterized (Ingelfinger et al., 2002). The removal of the 5' cap is mediated by the DCP1/DCP2 complex, of which DCP2 contains the decapping activity (Wang et al., 2002; van Dijk et al., 2002a) and also appears to direct some degree of substrate specificity via sequence preference (Cohen et al., 2005). A third componentin yeast, Dhh1p, a DEAD-box helicase is required for decapping (Coller et al., 2001). Binding of Dhh1p is regulated by Stm1p in yeast (Balagopal and Parker, 2009) leading to an enhancement of decapping. The removal of the 5' cap structure is enhanced by many factors in both yeast and higher organisms. Examples of these factors are human enhancer of decapping large subunit (HEDLS), the enhancer of decapping (EDC) proteins and Pat1p (Fenger-Gron et al., 2005, Pilkington and Parker, 2008; Harigaya et al., 2010). Removal of the 5' cap leaves a 5' monophosphate which is a prime substrate for XRN1, the 5'→3' exonuclease which degrades the remainder of the mRNA (Stoecklin et al., 2006; Hatfield et al., 1996).

Endonuclease Mediated RNA Decay

RNA decay may alternatively initiate via an endonuclease-mediated cleavage event. As outlined below the cleavage of a transcript is more often than not an irreversible process. As a result these events are highly controlled and transcripts are often targeted in a highly specific manner. Regardless of how or why a target is cleaved, it exposes the cleavage products to both 5'→3' and 3'→5' decay.

I. The RNA Interference (RNAi) Pathway

The RNAi pathway utilizes small antisense RNAs to direct cleavage of specific RNAs. In the RNAi response, dsRNA is detected and cleaved by Dicer to yield small 21-mer dsRNA fragments (Hammond, 2005). These fragments are then incorporated into the RISC complex and one strand is retained via an argonaute (AGO) protein (Hock and Meister, 2008; Liu et al., 2004). This complex in turn binds to complementary ssRNAs and slices them via the RNase H-like activity of the PIWI domain of the AGO protein (Hock and Meister, 2008). The generation of dsRNA is a common feature of many RNA viruses, leading to the hypothesis that RNAi acts as an antiviral response in invertebrates where an interferon response is lacking (Keene et al., 2004; Myles et al., 2008; Zambon et al., 2006). Indeed RNAi has been successfully used to target viral infection in insects (Cirimotich et al., 2009; Franz et al., 2006) and examples of viral encoded inhibitors of RNAi (for instance, Flock House virus' B2 protein) have been demonstrated (Berry et al., 2006).

II. Non-small RNA Mediated Endonucleases

Endonucleolytic cleavage of a transcript may be initiated by a variety of proteins not linked with RNAi. Examples of this include polysomal ribonuclease 1 (PMR1), erythroid-enriched endonuclease (ErEN) and RNase L. PMR is unique amongst the endonucleases in that it targets transcripts that are associated with polysomes (Pastori et al., 1991; Chernokalskaya et al., 1998). Cleavage by the

ErEN endonuclease in contrast is inhibited by the presence of PABPC1 *in vitro* (Rodgers et al., 2002; Wang and Kiledjian, 2000) suggesting that the primary targets of ErEN are not being actively translated. RNase L is activated by increased levels of 2'-5'-oligoadenylate following activation of the dsRNA response (for instance during viral infection). Activation of RNase L (typically as result of an interferon response) results in the nonspecific cleavage of RNAs at UU and UA motifs leading to translational arrest via cleavage of the ribosomal RNA (Carroll et al., 1996; Silverman et al., 1983).

The RNA Quality Control Pathways

An additional role the cellular mRNA decay machinery plays is that of quality control. The accumulation and expression of aberrant transcripts is undoubtedly a waste of energy for the cell and expression of dysfunctional proteins may be detrimental. The expression of dominant negative or gain-of-function mutations is an obvious area of concern. It is presumably for these reasons that cells have evolved mechanisms to actively monitor the transcriptome. The major surveillance and quality control mechanisms are described below.

I. Nonsense Mediated Decay (NMD)

The removal of transcripts containing premature termination codons (PTCs) from the host transcriptome is achieved via nonsense mediated decay (NMD). NMD recognizes mRNAs containing a PTC by sensing either the

presence of an EJC after the PTC (Le and Seraphin, 2008) and / or the distance between the PTC and the 3' end (presumably due to the poly(A) tail) (Buhler et al., 2006, Amrani et al., 2004). Either way, detection of the PTC leads to the formation of the SMG-SURF complex on the target RNA. Arguably the most important protein within the NMD pathway is UPF1, an RNA helicase with RNA-dependent ATPase activity (Bhattacharya et al., 2000; Czaplinski et al., 1995). Currently the precise events leading to the association of UPF1 with the stalled ribosome are unclear. Nevertheless the interaction of UPF1 with the prematurely terminating ribosome results in the association of UPF2 and UPF3 in a "licensing" step (Serin et al., 2001; Weng et al., 1996; He et al., 1997). The formation of these interactions leads to phosphorylation of UPF1 in metazoans (Denning et al., 2001; Kashima et al., 2006). Phosphorylation of UPF1 appears to be dependent on the presence of UPF2 and UPF3 (Kashima et al., 2006). This phosphorylation event in turn leads to the degradation of the bound transcript. The degradation of the PTC-containing mRNA can occur by several mechanisms. The NMD substrate may be degraded via an endonuclease cleavage mediated by SMG6 (Huntzinger et al., 2008; Eberle et al., 2009), and the fragments eliminated via the exonucleolytic pathways described above (Cao and Parker, 2003). SMG5 and SMG7 may activate the deadenylation-dependent decay of the NMD substrate (Couttet and Grange, 2004; Lejeune et al., 2003; Chen and Shyu, 2003). Alternatively, in yeast, degradation of the NMD substrate can be initiated by removal of the 5' cap independent of deadenylation (Cao and Parker, 2003; Muhlrad and Parker, 1994).

II. No-Go Decay (NGD)

Cellular transcripts with strong secondary structures that impede movement of the ribosome during translation are substrates for no-go decay (NGD) (Doma and Parker, 2006). Currently the NGD pathway is poorly understood. Endonucleolytic cleavage of NGD-substrates in yeast is mediated via Dom34p and Hbs1p (Passos et al., 2009; Doma and Parker, 2006). Both Dom34p and Hbs1p exhibit similarities to the translation termination factors eRF1 and eRF3, suggesting a sensory role for the proteins in the detection of NGD substrates (Davis and Engebrecht, 1998; Inagaki et al., 2003).

III. Non-Stop Decay (NSD)

The non-stop decay (NSD) pathway removes mRNAs that lack a stop codon. Current models of NSD include the assembly of the SKI complex adjacent to the stalled ribosome (van Hoof et al., 2002b) or degradation of the NSD substrate via the 5'→3' decay pathway (Inada and Aiba, 2005). As stated earlier, the SKI complex is associated with exosome-mediated decay in yeast (van Hoof et al., 2000; Brown et al., 2000). In yeast, assembly of this complex leads to the release of the stalled ribosome, presumably due to Ski7p given its similarity to eRF3 (van Hoof et al., 2002; Benard et al., 1999), and activation of deadenylation and subsequent 3'→5' degradation of the transcript (Frischmeyer et al., 2002; van Hoof et al., 2002b). Nevertheless, in the absence of Ski7p, the component responsible for the SKI complex-ribosome interaction, degradation of the NSD

substrate occurs in a 5' manner (Inada and Aiba, 2005). The ability of the NSD machinery to shunt NSD substrates into either exonucleolytic pathway highlights the importance of removing aberrant mRNAs from the host transcriptome.

Subcellular Localizations of mRNA Decay

The subcellular site of mRNA decay has been a widely discussed topic for a number of years. Using fluorescent microscopy, foci containing components of mRNA metabolism have been observed in the nucleus and cytoplasm (Sheth and Parker 2003). Both Processing Bodies (P-Bodies) and Stress Granules (SGs) are prime examples of cytoplasmic foci that are involved in determination of the fate of mRNAs.

I. The Processing Body

Current models propose that many mRNA decay events occur in processing bodies (P-bodies) (Parker and Sheth, 2007; Coller and Parker, 2005). Many RNA decay factors, including the 5'→3' exoribonuclease XRN1, the cytoplasmic Lsm complex, UPF1 and components of the RNAi decay machinery were found to localize in these foci (Swisher and Parker, 2010; Eulalio et al., 2007; Zheng et al., 2008; Brogna et al., 2008). However P-bodies are not required for decay as dissolution of the P-body by RNAi-mediated reduction of GW182 (a scaffolding protein) results in disappearance of the cytoplasmic foci without apparent loss of decay activities associated with P-body constituents (Eulalio et al., 2007; Stalder and Muhlemann, 2009). Moreover, in yeast, mRNA

decay can occur on polysomal mRNAs. The decay associated with polysomal mRNAs is initiated via decapping allowing the mRNA to finish ongoing translation (Hu et al., 2009; Hu et al., 2010). This further implies that P-bodies are not the only locations where mRNA decay may occur, as ribosome components are not associated with P-bodies. Over expression of exosome components has revealed an additional cytoplasmic site perhaps involved in mRNA decay. These foci, termed exosome granules, contain PARN as well as components of 3'→5' decay pathway (Lin et al., 2007).

II. The Stress Granule

In contrast to P-bodies, Stress Granules (SGs) do not appear to contain active decay enzymes (Anderson and Kedersha, 2008). Instead SGs are associated with translationally stalled mRNAs and their regulatory factors (Kedersha et al., 2000; Kedersha et al., 1999; Piecyk et al., 2000). Notable components of SGs include the regulatory RNA binding proteins TIA-1 and TIAR, which have been shown to shunt bound mRNAs to the SG (Izquierdo, 2006; Kedersha et al., 2000; Lopez, et al., 2005; Zhang et al., 2005; Kedersha et al., 1999; Piecyk et al., 2000). Current models put forth that stress granules are cytoplasmic sites of triage, where the fate of the transcript is decided. The evidence for this is observation that RNA regulatory factors are observed associating with mRNAs in stress granules (Brennan et al., 2000; Lai et al., 2003). Association of these factors is believed to lead to decay of the mRNA in the closely associated P-body (Anderson and Kedersha, 2008).

Regulation of mRNA decay

Cellular mRNA decay rates are often regulated by sequences within the 3'UTR of mRNAs. Notable examples of 3'UTR elements are the AU-rich elements (AREs), GU-rich elements (GREs), pyrimidine-rich elements and the 3' stem loop of histone mRNAs. Sequences within the open reading frames of mRNAs have also been noted as determinants of stability. These regulatory elements, through RNA-binding proteins, modulate both the manner and rate by which the transcript is decayed.

I. The AU-Rich Element (ARE)

AREs are perhaps the best studied examples of 3'UTR elements regulating mRNA decay. Between 5-8% of human mRNAs contain an ARE (Bakheet et al., 2006; Khabar et al., 2005; Raghavan et al., 2004). ARE regulatory elements are capable of acting as either instability or stability determinants depending on the RNA-binding proteins associated with the ARE. Initially AREs were defined by the presence of the AUUUA pentamer, a sequence associated with rapid decay of mRNAs (Chen and Shyu, 1995; Chen et al., 1995). However the AUUUA pentamer, in itself, is not a determinant of instability (Lagnado et al., 1994). The context of the AUUUA sequence modulates function. For instance, while the AUUUA pentamer by itself is insufficient to activate decay, a nonamer motif UUAUUUAUU is sufficient to activate RNA decay (Zubiaga et al., 1995). AREs were initially subdivided into three categories based on sequence (Chen and Shyu, 1995), and later further

refined (Wilusz et al., 2001). Class I AREs contain one or more copies of the AUUUA pentamer within a U-rich tract. Class II AREs contain 2 or more of the AUUUA pentamer in an overlapping arrangement. The final type of ARE, Class III, are predominantly U-Rich tracts that lack the canonical AUUUA pentamer. Important examples of cellular transcripts containing AREs include those that encode cytokines and growth factors. The ability of AREs to act as a stabilizer or destabilizer, depending on the repertoire of bound proteins, allows for a rapid change in protein expression as a result of stimuli (Bakheet et al., 2006; Khabar et al., 2005; Raghavan et al., 2004).

II. GU-Rich Elements

Bioinformatic analyses of activated T-cells revealed a number of transcripts that were regulated but lacked a discernable ARE (Raghavan et al., 2004). Further examination of these transcripts revealed a G/U rich sequence, with an 11-mer (UGUUUGUUUGU) termed the GRE (Vlasova et al., 2008). These sequences, similar to the ARE, modulate mRNA decay through interaction with RNA-binding proteins. CUGBP1, as well as HuR, appear to be major GRE-interacting partners (Rattenbacher et al., 2010; Lee et al., 2010). As stated above, CUGBP1 interacts with PARN leading to the enhancement of deadenylation (Moraes et al., 2006).

III. Pyrimidine Rich Elements (PREs)

Pyrimidine Rich Elements (PREs) have also been shown to affect mRNA turnover. In contrast to the ARE regulatory element, the PRE is associated with a net stabilizing effect (Irwin et al.,1997; Lee et al., 2010). Examples of transcripts that contain PREs are the α-globin and β-globin mRNAs (Yu and Russell, 2001; Kiledjian et al., 1995). Similar to AREs, the functions of PREs are controlled largely by the association of *trans*-acting factors. Examples of these factors are the poly(C) binding proteins (PCBPs) and the polypyrimidine tract binding protein (PTB), which stabilize mRNAs upon binding (Wang et al., 1999; Kiledjian et al., 1999; Irwin et al., 1997; Pautz et al., 2006).

IV. The Histone 3' Stem Loop

The 3' stem loop of histone mRNAs is associated with stabilization. Histone mRNAs, unlike the majority of cellular mRNAs, lack a poly(A) tail. Since the poly(A) tail aids in translation and protects the transcript from exonucleolytic decay an alternative element is required to fill the absence of the poly(A) tail. In histone mRNAs this element is the 3' stem loop (Dominski et al., 1995; Gallie et al., 1996). This stem loop protects the 3' end of the non-polyadenylated histone transcripts via the interaction of stem loop binding protein (SLBP). Furthermore this feature aids in the translation of the histone mRNAs (Williams et al., 1994). SLBP binding and stabilization of histone mRNAs is cell-cycle dependent, as the abundance of SLBP varies during the cell cycle and is significantly decreased

during G1 (Lanzotti et al., 2004; Zheng et al., 2003). Taken together these indicate an important role for SLBP in the regulation of histone expression.

V. Open Reading Frame Regulatory Elements

Finally, elements within the open reading frames (ORFs) of transcripts regulate the stability of the transcript. Notable examples of transcripts with ORF stability elements are c-fos, c-myc, IFNβ, *Drosophila* Hsp83 and HIS3 in yeast (Semotok et al., 2008; Wellington et al., 1993; Chen et al., 1992; Herrick and Jacobson, 1992; Paste et al., 2003). The best characterized examples of these elements are those found in c-fos and c-myc. In c-fos this ORF element, termed major protein coding-region determinant of instability (mCRD) requires the ribosome in order to induce rapid deadenylation of reporter constructs, implying a role of translation in regulating stability (Chen et al., 1992; Schiavi et al., 1994; Wellington et al., 1993). Current models infer that translation of the mCRD region leads to assembly of the RNA-binding protein UNR on the mCRD. UNR binding, through an unknown mechanism, but likely involving PABP leads to recruitment of the CCR4 deadenylase and rapid deadenylation of the transcript (Schiavi et al., 1994; Shyu et al., 1991). For c-myc the regulation of stability via elements in exons 2 and 3 is also dependent on translation (Pistoi et al., 1996; Yeilding and Lee, 1997; Yeilding et al., 1996). Current models involve a mechanism reminiscent of NGD in which decay is induced by pausing of the ribosome at rare codons (Yeilding and Lee, 1997). The *Drosophila* Hsp83 mRNA is regulated via the binding of SMAUG to multiple elements within the coding region (Semotok et

al., 2008). Previously an interaction between SMAUG and the CCR4-NOT complex has been described, leading to a model involving the rapid deadenylation of SMAUG bound transcripts during development (Semotok et al., 2005).

RNA Binding Proteins and the Regulation of mRNA Decay

As stated above, the functions of stability determinants, such as the ARE, are highly dependent on the proteins associated with the element. A number of factors bind the regulatory elements described above. These are often classified as either destabilizing or stabilizing factors, however the effects of RNA-binding proteins are highly dependent on their context (Barreau et al., 2005).

I. Destabilizing Factors

A large number of proteins interact with AREs and influence the fate of the bound transcript (Zhang et al., 2002a). For instance the RNA-binding proteins (RBPs) TTP, KSRP, AUF1 and CUGBP1 exhibit a net destabilizing effect (Fenger-Gron et al., 2005; Lykke-Andersen and Wagner, 2005; Lai et al., 2003; Moraes et al., 2006; Lal et al., 2004; Blaxall et al., 2002). In particular, the binding of KSRP activates the deadenylation of an mRNA by PARN (Chou et al., 2006; Gherzi et al., 2004). Similarly, CUGBP1 interacts with the PARN deadenylase to enhance the rate of decay (Moraes et al., 2006). In contrast, the binding of TIA-1 and TIAR to AREs results in translational silencing (Kedersha et al., 1999; Piecyk et al., 2000) and the relocalization of ARE-containing transcripts in response to

cell stress (Zhang et al., 2005; Kedersha et al., 1999). Binding of the zinc-finger antiviral protein (ZAP) decreases the translation of bound RNAs. ZAP expression confers resistance to several RNA virus families; notable examples include *Retroviridae* and *Togaviridae* (Bick et al., 2003). ZAP interacts with the exosome leading to degradation of target RNAs (Guo et al., 2007).

II. HuR, a Potent RNA Stability Factor

HuR is a member of the embryonic lethal abnormal vision (ELAV) superfamily and the Hu family of proteins. In humans there are four Hu family members each with different tissue expression patterns. HuC and HuD are expressed solely in neurons, whereas HuB is expressed in both neurons and gonads (Dalmau et al., 1992). HuB, HuC and HuD play a role in neuronal development and function (Kasashima et al., 1999; Pascale et al., 2004; Quattrone et al., 2001). In contrast HuR (also known as HuA) is expressed in a wide range of cell types (Ma et al., 1996; Good, 1995). The function of HuR is diverse, and HuR is a major regulator of post-transcriptional control (Fan and Steitz, 1998b; Peng et al., 1998). In Drosophila the Hu protein homolog ELAV is strictly neuronal and plays an essential role in development (Yao and White, 1994; Yao et al., 1993). Homology searches for ELAV superfamily members in *Aedes aegypti* mosquitoes identify at least 4 potential homologs, as indicated by a high degree of shared identity, (NCBI Protein REFSEQ#s XP_001658986.1, XP_001659335.1, XP_001657938.1, XP_001660922.1) implying a more diverse

range of functions for the Hu proteins in mosquitoes than *Drosophila* spp. An alignment of these sequences is presented in Appendix A.

i. HuR Organization

The ELAV superfamily members all exhibit similar protein organization, as shown in Fig. 2. For instance, HuR consists of 3 RNA Recognition Motifs (RRMs) and a Hinge region. RRM1 and RRM2 bind to AREs (Chen et al., 2002). HuR RRM3 interacts with poly(A) sequences (Ma et al., 1997) and serves as an oligomerization domain (Kasashima et al., 2002). Crystallographic structures of HuR have illustrated the RRM-RNA interaction using small RNAs (Iyaguchi et al., 2009). Additionally, examination of RRM1 using crystallography reveals a potential redox sensing mechanism in response to the cellular environment (Benoit et al., 2010). The Hinge region contains the HuR nucleocytoplasmic shuttling (HNS) sequence which regulates the subcellular localization of HuR. The HNS exhibits similarity to the M9 sequence of hnRNP A1 (Fan and Steitz, 1998a). As shown in Fig. 2, post-translational modifications are observed throughout the HuR protein. The functions of these modifications are described below.

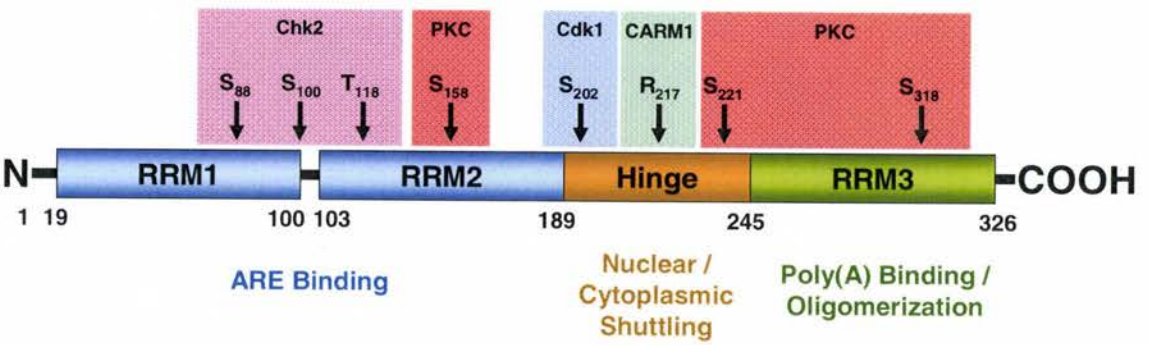


Figure 2- Domain Structure of the Mammalian HuR Protein. The cellular HuR protein consists of 3 RNA Recognition Motifs (RRMs) and a Hinge region. The amino acid position of each element is noted below. The first two RRMs have been connected with the binding of AU-rich elements (ARE) and are characteristic of the ELAV superfamily. RRM3 mediates interaction of HuR with poly(A) regions of RNAs and has been implicated in the assembly of HuR oligomers. The Hinge region is involved in the nuclear / cytoplasmic shuttling of HuR in response to cellular stimuli. Sites of post-translational modifications and the responsible kinases (Chk2, PKC and Cdk1) / methylases (CARM1) are indicated above.

ii. HuR Binds Many mRNAs

As determined by HuR immunoprecipitation and microarray analysis of the co-immunoprecipitated RNAs, HuR is believed to interact with as much as 15% of the cellular transcriptome (Lopez de Silanes et al., 2004). Bioinformatic examination of HuR associated transcripts reveals an interaction motif that is primarily U-rich (Lopez de Silanes et al., 2004; Meisner et al., 2004). Transcript specific studies of HuR binding have identified HuR as an interaction partner with all classes of ARE. Cellular mRNAs that are regulated by HuR include cytokines and growth factors (Yeap et al., 2002; Wang et al., 2000a; Nabors et al., 2001; Lopez de Silanes et al., 2004; Levy et al., 1998; Lal et al., 2004; Katsanou et al., 2005; Fan and Steitz, 1998b; Chen et al., 2002; Atasoy et al., 2003). Taken together these observations lead to the conclusion that HuR is a major nexus in the regulation of gene expression at the level of RNA stability.

iii. RNA Stabilization via HuR Binding

A principal role of HuR is to stabilize transcripts upon binding. Over expression of HuR was originally recognized to stabilize ARE-containing transcripts in tissue culture cells (Fan and Steitz, 1998b). Using *in vitro* systems, a stabilization of the body of ARE-containing substrates indicated a role for HuR in the repression of 3'→5' exonuclease mediated decay (Ford et al., 1999). HuD decreases the deadenylation of the GADp-43 mRNA upon association (Beckel-Mitchener et al., 2002). More recently stabilization of transcripts prior to deadenylation was observed in relation to HuR binding of the casein mRNA

(Nagaoka et al., 2006). Furthermore, knockdown of HuR by RNAi reduces the stability of target RNAs (for example Gantt et al., 2006). The precise mechanism of stabilization via HuR is unclear. To date, three models have been put forth for the stabilization of mRNAs by HuR. First, HuR binding to regulatory elements in mRNAs may lead to a reorganization or displacement of previously bound destabilizing RNA-binding factors (Linker et al., 2005; Lal et al., 2004; Blaxall et al., 2002). Similarly, HuR binding may compete with miRNAs thereby preventing their regulation of target mRNAs (Bhattacharyya et al., 2006). The second model involves the exclusion of other RNA-binding proteins from regulatory elements. Evidence in support of this particular model rests with the oligomerization domain of the Hu proteins, RRM3. HuR forms oligomers on the TNFα mRNA in a cooperative fashion (Fialcowitz-White et al., 2007). Deletion of RRM3 from both HuR and HuD abrogates the stabilizing effects of these proteins (Fan and Steitz, 1998b; Anderson et al., 2000). Indeed RRM3 is vital for ARE-binding of HuR (Fan and Steitz, 1998b). It is possible that oligomerization of HuR results in exclusion of other ARE-binding proteins, thereby inhibiting their regulation of the fate of the mRNA. The third and final model for HuR stabilization involves the interaction of HuR with other RNA-binding proteins. Using the stabilization of the casein mRNA as an example, HuR interacts with PABP (Nagaoka et al., 2006). It is possible that this interaction acts to stabilize the PABP:poly(A) interaction, resulting in repression of deadenylation. In any of the above models the binding of HuR is implied to affect other factors regulating the stability of the target mRNA as currently no evidence exists for a direct HuR:decay enzyme

interaction. The above models are not mutually exclusive, and it is likely that a combination of all three is leading to the stabilization of HuR-bound transcripts.

iv. Translational Enhancement via HuR Binding

An additional consequence of HuR binding is the enhancement of translation. Cellular mRNAs bound to HuR have an increased association with polysomes, an event associated with enhanced translation (Gantt et al., 2006; Kawai et al., 2006; Lu et al., 2009; Nguyen et al., 2009; Perlewitz et al., 2010). As an example, the GLUT1 mRNA in the presence of HuR is highly associated with polysomes. Knockdown of HuR using RNAi results in decreased association with polysomes and a decrease in GLUT1 protein (Gantt et al., 2006). Similar observations have been made during male germ cell development with the Brd2 and GCNF mRNAs (Nguyen et al., 2009). The mechanism(s) behind these observations are currently unclear. The leading hypothesis is that HuR, presumably through the interaction of other factors (for instance PABP), is acting to enhance the reinitiation event leading to polysome formation.

v. Hu Family Proteins May Direct Alternative Splicing

Hu family members also influence the splicing pattern of several transcripts in the nucleus. Notable examples of cellular transcripts include Ikaros, NF1 and calcitonin. Binding of the Hu proteins near splice junctions results in the differential inclusion of exons. The inclusion of alternative exons occasionally leads to alternative polyadenylation by the incorporation of other polyadenylation

signals into the transcript (Zhu et al., 2008; Zhu et al., 2007; Zhu et al., 2006; Hahm et al., 1994; Zhou et al., 2007).

vi. Enhancement of Nuclear Export of mRNAs via HuR Binding

HuR, via the HNS, is predominantly nuclear in quiescent cells (Fan and Steitz, 1998a) but translocates to the cytoplasm following various stimuli. Examination of the mechanisms of HuR shuttling from the nucleus to the cytoplasm reveals a high degree of redundancy as multiple export and import pathways act on HuR (Rebane et al., 2004). HuR translocation from the nucleus to the cytoplasm also impacts mRNA localization. An example is the export of C/EBPβ mRNAs from the nucleus following differentiation of adipocytes. The C/EBPβ mRNA has no introns, necessitating an alternative mode of transport from the nucleus to the cytoplasm as splicing deposits a molecular tag, the EJC, which identifies the transcript as a properly formed mRNA. Following drug-induced differentiation the C/EBPβ transcript moves from the nucleus to the cytoplasm where immunoprecipitation reveals a HuR:RNA interaction (Gantt et al., 2005). Prior to the induction of differentiation no interaction between the C/EBPβ mRNA and HuR was observed. Knockdown of HuR using siRNAs resulted in decreased C/EBPβ expression as a result of decreased export of the mRNA (Gantt et al., 2005).

vii. The Localization of HuR is Highly Regulated

The subcellular localization of the Hu proteins greatly influences their function (Ma et al., 1996; Keene, 1999; Atasoy et al., 1998; Antic and Keene, 1997). As described above HuR is predominantly nuclear, but may shuttle to the cytoplasm. The shuttling of HuR is due to interactions with protein ligands. Coimmunoprecipitation revealed protein:protein interactions between HuR and SETα, SETβ, pp32 and APRIL (Gantt et al., 2005; Gallouzi et al., 2001). Three of these factors, SETα / β and pp32 are known inhibitors of protein phosphatase 2A (PP2A) implying complex regulation at the level of phosphorylation (Gantt et al., 2005; Gallouzi et al., 2001). These shuttling events are regulated by several signaling pathways. HuR is a target for regulation by the mitogen-activated protein kinases (MAPKs), MAPK activated protein kinase (MK2), AMP-activated kinase (AMPK), the cell-cycle checkpoint kinase 2 (Chk2), members of the protein kinase C (PKC) family, cyclin dependent kinase 1 (Cdk1) and the CARM1 methylase (Ming et al., 2001; Winzen et al., 1999; Winzen et al., 2004; Subbaramaiah et al., 2003; Tran et al., 2003; Wang et al., 2004; Wang et al., 2002; Wang et al., 2003; Abdelmohsen et al., 2007; Doller et al., 2008b; Doller et al., 2007; Doller et al., 2008a).

a. MAPK and MK2

MAPK and MK2 are kinases associated with inflammation. Activation of the MAPK and MK2 kinases correlates with an increase in the stabilization of mRNAs encoding key inflammatory proteins, such as tumor necrosis factor

(TNF), IL-8, IL-4, cyclooxgenase-2 (COX-2) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (Dean et al., 2001; Winzen et al., 1999; Winzen et al., 2004; Jin et al., 2007; Lasa et al., 2000; Fan and Steitz, 1998a; Lin et al., 2006). Both MAPK and MK2 are believed to induce the cytoplasmic localization of HuR via a downstream methylase, as niether MAPK or MK2 phosphorylates HuR directly and phosphorylated HuR is not detected (Lin et al., 2006).

b. AMPK

In contrast to MAPK and MK2, AMPK activation leads to the nuclear retention of HuR and destabilization of cytoplasmic targets (Wang et al., 2004; Wang et al., 2002). Cellular AMPK acts as a sensor of metabolic stress (Wang et al., 2003) and is activated by depletion of cellular ATP reserves. Inhibition of AMPK increases the cytoplasmic abundance of HuR thereby stabilizing the cyclin B1, cyclin A and p21 transcripts (Wang et al., 2004; Wang et al., 2002). Nuclear sequestration of HuR in response to AMPK activation is mediated by post-translational modification of Importin α, a nuclear import cohort of the cytoplasmic HuR protein (Wang et al., 2003; Martinez-Chantar et al., 2006).

c. Chk2

Chk2-mediated phosphorylation of HuR reduces binding to the human SIRT1 mRNA (Abdelmohsen et al., 2007). Curiously, phosphorylation of HuR by Chk2 leads to decreased SIRT1 mRNA stability. At first this seems contradictory

to the role of HuR as a stability factor, however it is far more likely that combinatorial regulation is directing the stability of SIRT1 mRNA (Abdelmohsen et al., 2007). The stability (and instability) of HuR-bound mRNAs is dependent on the context of the HuR protein. Several models were postulated in Wilusz and Wilusz (2007) as to how HuR regulation may lead to simultaneous stabilization and destabilization on different mRNAs- 1) The bound HuR protein may have altered binding preference as a result of post-translational modification, 2) The binding of HuR may be enhanced by accessory factors, 3) HuR may be bound in such a manner that the post-translational modifying enzyme may not have access to vital residues and 4) HuR may enhance the binding of miRNAs to the HuR-bound transcript by exposing the docking sites for the small RNAs (Kim et al., 2009)

d. PKC

The PKC family of serine/threonine protein kinases are regulators of HuR. PKCα and PKCδ directly phosphorylate HuR in the hinge region leading to cytoplasmic localization of HuR (Doller et al., 2007; Doller et al., 2008a). Furthermore, post-translational modification of HuR by the PKCs has been postulated to influence mRNA binding as PKC phosphorylated serine residues in both RRM2 and RMM3 (Doller et al., 2008b). These events are presumed to inhibit the binding of HuR to ARE and poly(A) sequences, similar to the first model described in Wilusz and Wilusz (2007).

e. Cdk1

Increased nuclear localization of HuR can also be a consequence of Cdk1-mediated phosphorylation (Abdelmohsen et al., 2008). This interaction has been identified as an important regulator of cell division, as HuR retention in the nucleus is associated with decreased anti-apoptotic effects (Kim and Gorospe, 2008; Kim et al., 2008a). Mutants of HuR in which Cdk1-targeted serine residues have been altered are associated with increased cell proliferation and a cancer phenotype (Kim et al., 2008b).

f. CARM1

The CARM1 methylase (also known as PRMT4) methylates arginine residues in the hinge region of HuR (Li et al., 2002). Similarly to phosphorylation by PKC α/δ HuR methylation enhances the cytoplasmic accumulation of HuR (Li et al., 2002). This is presumed to act by inhibiting the association of protein ligands to HuR, such as the karyopherins, preventing import to the nucleus. Currently, no clear biochemical difference between HuR and methyl-HuR has been observed. Thus the HuR relocalization event may be sufficient to explain the biological phenomenon associated with CARM1 activation.

viii. The Biological Impacts of HuR

As described above HuR regulates gene expression at multiple levels, most notably RNA stability and translation. HuR is a highly regulated protein as

exhibited by the number of signaling pathways leading to post-translational modification of HuR. It is through the combined activity of these pathways, acting as effectors of cellular stimuli, that HuR function is determined. HuR regulates transcripts at the level of RNA stability in response to many types of cell stress. As described below, over expression of HuR correlates with tumorigenesis, conversely senescence is associated with reduced HuR levels. Furthermore the HuR protein is essential for viability, as exhibited in recent tamoxifen-inducible HuR knockout mouse models (Ghosh et al., 2009).

Multiple forms of stress, including oxidative, heat shock, UV irradiation and hypoxic stress are known to impact HuR. A unifying principle in all of these responses is the relocalization of HuR from the nucleus to the cytoplasm (Abdelmohsen et al., 2008; Tran et al., 2003; von and Gallouzi, 2010; Amadio et al., 2008; Li et al., 2008; Jeyaraj et al., 2005; Song et al., 2005; Wang et al., 2000b; Gallouzi et al., 2000; Gallouzi et al., 2001). Oxidative treatment of tissue culture cells induces activation of MK2, resulting in the post-translational modification of HuR. HuR translocates from the nucleus to the cytoplasm where it stabilizes transcripts involved in the host response to oxidative stress such as urokinase plasminogen activator (uPA), urokinase plasminogen activator receptor (uPAR), and γ-glutamylcysteine synthetase heavy subunit (γ-GCSh) (Tran et al., 2003; Song et al., 2005). Heat shock induces protein:protein interactions between HuR, pp32 and APRIL resulting in CRM1-mediated export of HuR from the nucleus (Abdelmohsen et al., 2009; Gallouzi et al., 2000; Gallouzi et al., 2001). Moderate heat shock induces degradation of HuR via

ubiquitination, leading to destabilization of HuR targets and decreased cell survival (Abdelmohsen et al., 2009). UV irradiation enhances the translation of p53 and inhibits cell proliferation, via HuR (Mazan-Mamczarz et al., 2003). Interestingly not all responses to oxidative stress mediated by HuR result in stabilization. HuR is phosphorylated by Chk2 in response to oxidative stress. Chk2 activation leads to release of the SIRT1 mRNA from HuR thereby destabilizing the transcripts and decreasing cell longevity (Abdelmohsen et al., 2007; Wilusz and Wilusz, 2007). The above examples indicate the widespread roles of HuR in response to stress.

Over expression of HuR is associated with cancer and is therefore a potential indicator of prognosis (Barbisan et al., 2009; Guo and Hartley, 2006; Mazan-Mamczarz et al., 2008; Denkert et al., 2006; Denkert et al., 2004b; Denkert et al., 2004a). As stated above, mRNA targets of HuR include factors involved in cell cycle regulation, such as several cyclins and p53. Excess HuR is postulated to lead to rampant cell division resulting in cancer phenotype (Mazan-Mamczarz et al., 2008). Cell senescence, in tissue culture models, is associated with a decrease in HuR abundance (Wang et al., 2001; Yi et al., 2010). In contradiction to this observation are *in vivo* studies examining tissue biopsies from a wide range of human subjects which noted no significant change in the level of HuR expression with respect to age (Masuda et al., 2009).

The evidence above indicates that HuR is an important node regulating gene expression. The conservation of HuR further stresses its role as an essential regulator in biological systems. The sheer number of putative HuR

targets (Lopez de Silanes et al., 2004) coupled with the complex regulation (by the activity of several signaling pathways) of HuR underscores how deeply integrated HuR is in cellular function.

Virus / Host mRNA Decay Machinery Interactions

As described below, interfaces between viral transcripts and the cellular mRNA decay machinery have been observed. Generally, these interfaces benefit the virus by either enhancing the efficiency of viral processes or by evading cellular persecution. Otherwise recognition of the viral RNA by the host mRNA decay machinery could result in interference of viral function. The simplest strategy that viruses employ to evade the host decay machinery is to hide activators or substrates from detection. Alternatively, some viruses have evolved to utilize aspects of the host RNA decay machinery to their advantage. Moreover some viruses have evolved to encode their own RNA decay factors leading to regulation of the host transcriptome. The goal of this section is to provide an overview of known interactions between viruses and cellular RNA decay processes

I. Viral Evasion of Host RNA Decay

Viral evasion of the host RNA decay machinery is a common strategy amongst both DNA and RNA viruses and is likely to be required for a productive infection (Sokoloski et al., 2006). One way that evasion is achieved is by simple sequestration of the viral RNAs to prevent access by the decay machinery. For

example Reoviruses replicate their RNAs within nucleocapsid structures to prevent the recognition of the dsRNA genome by the host cell (Edelmann et al., 2004). Other viruses may rely upon the formation of membranous compartments to segregate their dsRNA formed during replication from the host defense mechanisms (Uchil and Satchidanandam, 2003; Kopek et al., 2007). Flock House virus encodes the B2 (FHVB2) protein which coats the dsRNA preventing its recognition by the host cell (Li et al., 2002). Furthermore FHVB2 interacts with the PAZ domain of Dicer, indicating that the repression of the dsRNA response by B2 is multifaceted (Singh et al., 2009). Additionally many plant viruses encode similar factors that bind to dsRNAs such as the p19 protein of the Tombusviruses (Scholthof, 2006; Chapman et al., 2004). The above strategies reduce the host response by limiting the interaction of dsRNA and the subsequent activation of downstream effectors. Alternatively, evasion may be mediated by the nature of the viral genome itself rather than concealment of viral RNAs. As an example, the genome of interferon resistant Hepatitis C Virus (HCV) has UU and UA dinucleotide motifs underrepresented, thereby reducing the ability of RNase L to cleave the viral genome (Han and Barton, 2002).

DNA viruses interact more with the quality control aspects of the RNA decay machinery. By their nature DNA viruses transcribe their RNAs in the nucleus, thus requiring them to develop mechanisms for export and evasion of nuclear RNA surveillance pathways. For instance the pre-mRNA processing enhancer (PPE) of the Herpes Simplex thymidine kinase mRNA serves to stabilize the transcript and enhance its export from the nucleus (Guang and

Mertz, 2005). Similarly the post-transcriptional regulatory Element of Hepatitis B virus aids in mRNA export from the nucleus (Heise et al., 2006). Retroviruses must export the unspliced viral genomic RNAs from the nucleus. As unspliced mRNAs are usually degraded, this has led to the evolution of elements such as exhibited in Rous Sarcoma Virus (RSV) (Weil and Beemon, 2006). The RSV Stability Element (RSE) consists of a pair of stem-loop structures (Weil et al., 2009). The RSV RSE allows viral RNAs to evade the NMD machinery by preventing recognition of the viral transcripts as targets. The precise mechanism of stabilization is unclear. The current model involves the "shortening" of the distance between the termination codon and the poly(A) tail. This in effect would prevent recognition of the viral RNA as an NMD target. Similar mechanisms have been noted using reporter systems whereby shortening the distance between the termination codon and the poly(A) tail has improved RNA stability (Eberle et al., 2008).

It should be noted however that not all of the evasions of DNA viruses and the RNA decay machinery involve the quality control machinery. A notable example is the PAN-ENE element found in the nuclear PAN RNA encoded by Kaposi's Sarcoma Herpes Virus (KSHV). The nuclear accumulation of the PAN RNA is dependent on the base pairing of the ENE element with the 3' poly(A) tail (Conrad et al., 2006). The KSHV ORF57 protein binds to the PAN RNA in the nucleus resulting in increased RNA stability (Sahin et al., 2010). Currently the purpose and functions of these events are unclear; nevertheless they are believed to preserve the RNA from the nuclear decay machinery.

II. Viral Subversion of Host Factors

Many viruses have evolved to utilize aspects of the RNA decay machinery to direct, and in some cases enhance, viral replication. An example of this phenomenon is the binding of PCBP to the 5' cloverleaf structure of the Poliovirus Internal Ribosome Entry Site (IRES) (Murray et al., 2001). This interaction, along with the covalently attached VPg protein, serves to protect the 5' end of the uncapped poliovirus genome. Additionally some viruses have evolved interdependency on factors of the RNA decay machinery. An example of this is the role of the cellular Lsm1-7 complex in the replication of Brome Mosaic virus where the LSM complex directs a change from translation to replication. This event is believed to be mediated by removal of the 5' cap from the Brome Mosaic virus transcripts, resulting in the inhibition of translation (Mas et al., 2006; Galao et al., 2010). Furthermore, some viruses have evolved to utilize the regulatory factors described above to enhance viral infection. For instance an interaction between Hepatitis C Virus (HCV) and the cellular HuR protein has been shown to impact viral infection (Spangberg et al., 2000).

III. Viral Encoded Protein Effectors of Decay

It is also not uncommon for viruses to activate the decay of cellular transcripts by encoding their own decay factors. The KSHV shutoff and exonuclease protein (SOX, ORF37) induces aberrant polyadenylation and nuclear retention of PABPC1 (Glaunsinger et al., 2005; Lee and Glaunsinger,

2009; Covarrubias et al., 2009). Additionally, Poxviruses encode two factors with Nudix motifs, similar to that found in DCP2, that have observable decapping activities (Parrish and Moss, 2006; Parrish et al., 2007). The orthomyxoviruses, bunyaviruses and arenaviruses exhibit cap-snatching activity, thereby destabilizing cellular mRNAs via a decapping-like activity (Rao et al., 2003). The severe acute respiratory syndrome (SARS) coronavirus induces the degradation of cellular transcripts and alter translation in infected cells (Kamitani et al., 2006; Kamitani et al., 2009; Narayanan et al., 2008; Tohya et al., 2009). Poliovirus encodes the 2A protease that cleaves many cellular factors, such as PABPC1, inhibiting the translation of host mRNAs and enhancing viral translation (Joachims et al., 1999). Thus, we see that viruses are not only evading and utilizing aspects of the cellular mRNA decay machinery to their advantage but they are encoding their own proteins that modulate the host environment altering host RNA function.

The findings above indicate a wide breadth of interactions between viruses and the host RNA decay machinery. Nevertheless the precise impact of RNA decay on a viral infection is poorly understood. Given the extent of interaction between the decay machinery and viruses one can easily hypothesize that viral transcripts must be substrates for the cellular mRNA decay machinery. Examination of many RNA viruses, for instance the alphaviruses, reveals a high degree of similarity between the viral RNAs and cellular mRNAs in both function and organization. For example, both cellular mRNAs and the alphavirus genomic and subgenomic RNAs are 5' capped and 3' polyadenylated. Given this similarity

we hypothesize that, like cellular mRNAs, viral transcripts will possess regulatory elements that modulate their interactions with the host decay machinery. Here we propose to examine the relationship between the Alphaviruses and the host RNA decay machinery in an effort to characterize the impact of RNA turnover on viral infection. Understanding the interactions between the Alphavirus RNAs and the host decay machinery will increase not only our fundamental understanding of viral biology but may also give insight into novel therapeutic targets.

An Overview of Alphavirus Biology

The genus *Alphavirus*, of the family *Togaviridae*, is a group of diverse positive-sense RNA viruses. In addition to being divided by serogroup, the members of genus *Alphavirus* may be divided spatially. Geographically the members of genus *Alphavirus* may be classified as Old World Alphaviruses, those that evolved in the eastern hemisphere; and the New World Alphaviruses, those observed in the western hemisphere (Luers et al., 2005; Powers et al., 2001). Through phylogenetic analysis it is believed that the alphaviruses originated in the western hemisphere and emerged into the eastern hemisphere as indicated by the characterization of recombinant alphaviruses (Powers et al., 2001). The vast majority of the members of the genus are arboviruses; viruses which are spread by arthropod vectors. Members of the family are known to cause significant illness in children and the elderly. This dissertation focuses primarily on the alphaviruses that utilize a mosquito vector – in particular Sindbis virus (SinV), the archetypical member of the *Alphavirus* genus.

In the sylvatic environment the replication of SinV is maintained through an enzootic transmission cycle between the mosquito vector and avian reservoirs and, to a lesser extent, small rodents (Griffin, 2001). In the enzootic cycle the specific vector mosquito species typically varies between the alphaviruses primarily consisting of *Culex* spp. The epizootic life cycle of the alphaviruses often involves a change in both the reservoir host and vector mosquito species. Notably the mosquito species widely linked with urban replication cycles are the *Aedes albopictus* (Asian Tiger) mosquito, the *Aedes aegypti* mosquito and, to a lesser extent, members of the genus *Culex* (Griffin, 2001).

Infection of the vector mosquito begins with the intake of a blood meal from a viremic reservoir host. The period of time from the infectious bloodmeal to infectivity is termed the extrinsic incubation period, and is ~3-14 days (Scott and Weaver, 1989). Upon entry into the midgut the virus initiates replication within the posterior midgut epithelium (Jackson et al., 1993). Vector competence is, at least in part, determined by the efficiency of viral interaction with the midgut epithelia (Scott and Weaver, 1989; Houk et al., 1990). Viral escape from the midgut tissues results in dissemination of the virus into the hemolymph and fat body where replication allows the virus to reach the titers required for salivary gland infection (Dubrulle et al., 2009; Jackson et al., 1993; McLintock, 1978). Infection of the mosquito salivary glands is required for efficient transmission of the Alphaviruses (Jackson et al., 1993). Infection of a mosquito results in a persistent infection that will continue throughout the life of the mosquito (Mims et al., 1966). There is some evidence of alphaviruses being passed vertically through

mosquito generations to non-hematophagous males (Fulhorst et al., 1994; Lindsay et al., 1993). Furthermore, the over wintering of infected ova has been reported (Lindsay et al., 1993).

Replication of the virus within the mammalian host initiates at the local site of mosquito feeding. Upon feeding the mosquito releases a bolus of salivary gland proteins and the infectious alphavirus into the injection site (Turell et al., 1995; Griffin, 2001). Components of the salivary emission modulate the local immune response towards a more permissive environment (Wasserman et al., 2004; Wanasen et al., 2004). Locally the alphaviruses initiate their replication in the muscle tissues and Langerhan's cells prior to producing the primary viremia. Viral dissemination further continues via spread to regional lymph nodes and then to secondary target organs. It should be noted that the potential for transmission to a mosquito from an infected human is quite limited, as the titer of the viremia noted for the majority of alphavirus infections (including SinV) in the human host rarely reaches levels required for lateral transmission (Griffin, 2001). Certain alphavirus infections (such as those with eastern equine encephalitis) may result in encephalitis. In these instances it is likely that infection of the brain itself is mediated via infection of the olfactory neurons, as demonstrated in mouse models (Charles et al., 1995; Ryzhikov et al., 1995).

The pathology associated observed with alphavirus infection can be categorized into two groups: the encephalitides and the non-encephalitides. Infection with the non-encephalitide alphaviruses results in limited pathology. Notably in the majority of human cases febrile illness is typically reported

alongside moderate to severe polyarthralgia (depending on the particular alphavirus); Sindbis virus is a member of this group. With the other group of alphaviruses encephalitis may develop, and typically this pathology is associated more often with western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV) (in equines) and eastern equine encephalitis (EEEV) viruses. In those individuals who develop encephalitis, there is a chance of developing severe neurological sequelae as a result of viral infection. In other mammalian hosts (such as equines) the infection may be far more severe exhibiting a higher degree of mortality.

In mammalian cell culture models the cytopathology is far more pronounced than in mosquito cells. Focal degeneration and pyknosis of the infected cell nuclei is readily apparent in infected 293T (human embryonic kidney), BHK-21 (baby hamster kidney) and Vero (African green monkey kidney cells) after 24 hours of infection (Raghow et al., 1973). Much of the cytopathic effect of SinV infection of these cell lines is due to both apoptosis and necrosis (Nava et al., 1998; Jan and Griffin 1999). In contrast, mosquito cells exhibit no apparent cytopathology and usually establish a persistent state of infection (Stollar et al., 1975; Stevens, 1970; Tooker and Kennedy, 1981). The perturbation of host function in mosquito cells is significantly less than that of mammalian systems (Karpf et al., 1997) and viral budding allows the cell membrane to remain intact throughout the infection.

I) The Molecular Biology of the Alphaviruses

As seen in Fig. 3, infection of the host cell with SinV initiates with the interaction of the viral E2 envelope protein with either cellular heparan sulfate moieties or laminin receptors (Wang et al., 1992; Strauss et al., 1994). In both cases interaction with the cell receptor internalizes the virion into an early endosome. As the endosome progresses towards acidification,a reorganization event on the viral envelope leads to protrusion of the viral E1 protein. The Alphavirus genomic RNA strongly resembles a cellular mRNA; both RNAs have a 5' ^{7me}-GpppG cap structure and a 3' poly(A) tail (Strauss and Strauss, 1994). These features enable the viral genome to act as an mRNA upon entry into the host cytoplasm. Unlike cellular mRNAs these features are added by the viral nsp1 (the viral capping enzyme) and nsp4 (the RNA dependent RNA polymerase and poly(A) polymerase) (Ahola et al 1997; Laakkonen et al., 1996; Sawicki et al., 1990; Tomar et al., 2006)

Translation of the genomic RNA results in the formation of a polyprotein, which upon proteolytic processing forms the individual viral nonstructural proteins. Together these proteins comprise the functional replicase machinery. The nonstructural polyprotein exists primarily in two forms, P123+nsp4, and P1234 which results from read-through of an opal stop codon prior to the nsp4 region and is co-translationally processed into the individual nonstructural proteins by both viral and cellular proteases (Strauss and Strauss, 1994). These proteins direct the synthesis of the viral RNAs. The polyprotein P123 in conjunction with nsp4 directs the synthesis of the negative sense RNA (Shirako

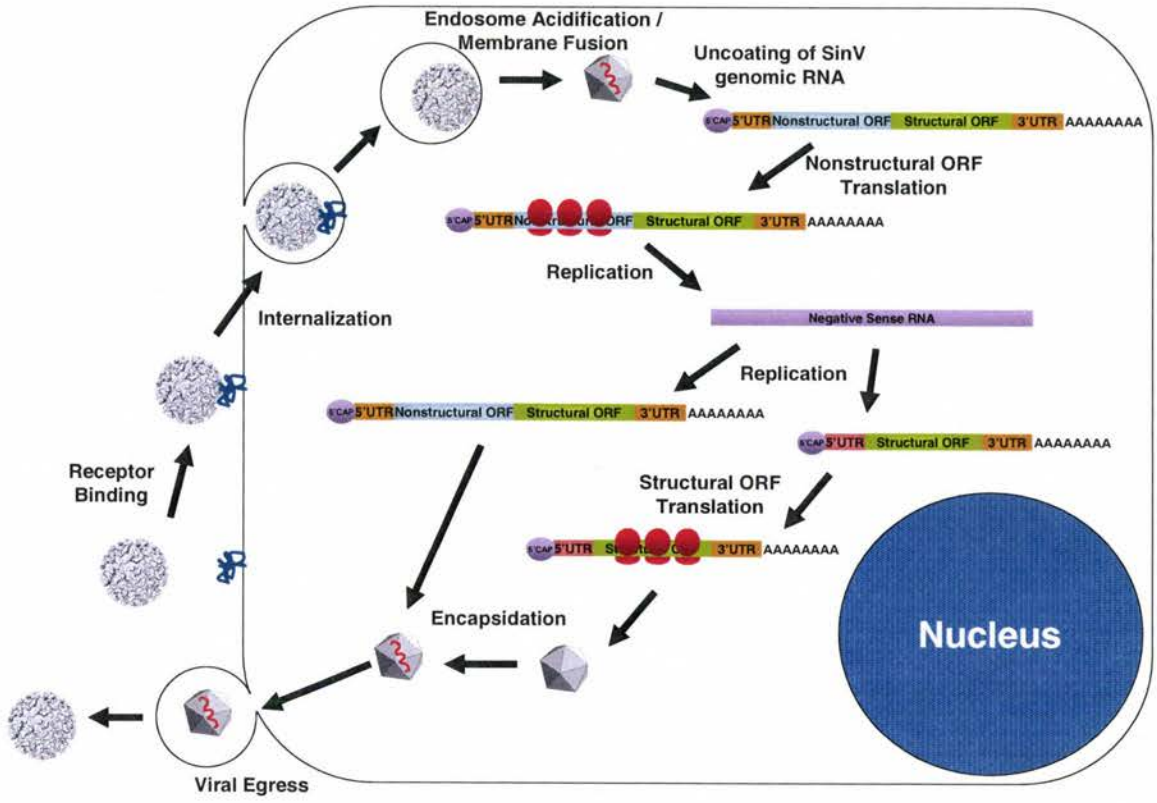


Figure 3- The Life Cycle of Sindbis Virus. The life cycle of SinV initiates upon the interaction of the mature virion with the cell surface receptor. After receptor binding the virion is internalized into the cell via an endosome. Acidification of the endosome results in fusion of the virus envelope with the endosomal envelope and release of the nucleocapsid into the cytoplasm. The viral genome is uncoated and immediately acts as an mRNA to encode the functional replicase. The replicase in turn synthesizes a negative sense copy of the entire viral genome which acts as a template for the synthesis of both subgenomic and genomic viral RNAs. Translation of the subgenomic RNA results in the production of the structural proteins. The capsid protein associates with the viral genome to form the nucleocapsid. The nucleocapsid becomes encircled with membranes derived from the endoplasmic reticulum. The mature virions egress the cell through budding. Images of mature virions were adapted from Zhang et al., 2002b.

et al., 1994, Wang et al., 1994). Cleavage-defective mutants of the P123 protein reveal that cleavage of the P23 junction is necessary to switch from negative sense RNA synthesis to positive sense RNA synthesis (Gorchakov et al., 2008).

SinV nsp1 is membrane bound via palmitoylation of cysteine residues and is posited to anchor the replication complex within membranous invaginations (Laakkonen et al., 1996; Ahola et al., 1999; Salonen et al., 2005). The nsp1 protein is necessary for the initiation of negative strand RNA synthesis (Wang et al., 1991). Examination of the SinV nsp1 protein sequence reveals domains associated with methyltransferase activity (Mi et al., 1989). Furthermore, the nsp1 protein possesses guanylyltransferase activity (Ahola et al., 1997). Alphavirus positive sense RNAs are 5' capped through the concerted actions of the methyltransferase and guanylyltransferase domains of nsp1 (Ahola et al 1997; Laakkonen et al., 1996).

The functions of nsp2 are more diverse than the other nonstructural proteins: the C-terminal portion of the protein has papain-like protease activity, and directs cleavage of the polyprotein in *cis* (Strauss et al., 1992; Hardy and Strauss, 1989). Triphosphatase activity is also a function of the *Alphavirus* nsp2 protein, indicating a role in the capping of the positive sense RNAs (Vasiljeva et al., 2000). Additionally, for Old World alphaviruses, nsp2 expression is associated with host transcriptional shutoff (Gorchakov et al., 2005; Gorchakov et al., 2004; Frolov et al., 1999; Garmashova et al., 2006). Interestingly a significant portion of SinV nsp2 is present in the nucleus; the importance of this localization is currently not understood (Frolov et al., 2009).

The third nonstructural protein, nsp3, is less characterized and its function is currently unknown (Griffin, 2001). The nsp3 protein is phosphorylated and is postulated to localize to the nuclear membrane as exhibited by expression of GFP-nsp3 fusion proteins (Wang et al., 1994). Curiously, nsp3 lacks homology to any characterized protein domains confounding the exploration of its true function.

The final nonstructural protein, nsp4, is included in the initial polyprotein via read through of an opal stop codon (Strauss et al., 1983). SinV nsp4 is the functional RNA polymerase (Sawicki et al., 1990; Barton et al., 1988; Lemm et al., 1998) and possesses poly(A) polymerase-like activites (Tomar et al., 2006). Importantly, replication defective temperature sensitive mutants of the SinV nsp4 protein exist (Barton et al., 1988). In particular, the ts6 mutant SinV has enabled us to selectively inhibit viral RNA synthesis without inadvertently altering the expression of cellular mRNAs (Garneau et al., 2008). This in turn allows us to assess the decay of the viral RNAs in viral infections of cultured cells in several of the experiments presented below.

Following the synthesis of the full length, non-polyadenylated negative sense RNA, the synthesis of the subgenomic mRNA begins. Synthesis of the subgenomic RNA, which encodes the structural proteins, initiates at an internal promoter within the 3' end of the nsp4 ORF, resulting in a subgenomic RNA consisting of the 3' third of the infectious genome (Strauss and Strauss, 1994). Translation of this viral RNA results in the formation of a viral polyprotein that is co-translationally inserted into the lipid bilayer of the endoplasmic reticulum.

Similar to the nonstructural polyprotein, the individual structural proteins are proteolytically processed into their final forms. At the N-terminus of the polyprotein is the capsid protein which remains in the cytoplasmic compartment and coats the viral nucleic acid packaging the viral nucleic acid prior to virus maturation and budding. Additionally, in New World alphaviruses (those found on the American continents), the capsid protein mediates the shutoff of host transcription (Garmashova et al., 2007). Following the capsid protein in the structural polyprotein is the membrane bound type I *trans*-membrane glycoprotein E2. The third protein is the 6k protein which has no ascribed function to date. The final major protein product of the structural polyprotein is the E1 protein. The E1 protein is responsible for cell membrane fusion following endosomal internalization and rearrangement of the viral surface glycoproteins in response to acidification of the late endosome (Wahlberg and Garoff, 1992).

II. Structure of the Alphavirus 3'UTR

An alphavirus 3'UTR consists principally of three distinct elements as shown in Fig. 4 (Ou et al., 1981; Ou et al., 1982). At the extreme 3' terminus, immediately prior to the poly(A) tail, is the 19-nt conserved sequence element (CSE). Adjacent to the CSE is a 40 nucleotide element that is predominantly uridine-rich, termed the U-rich element (URE). The presence of this element is largely conserved, with notable exceptions being o'nyong-nyong (ONNV), chikungunya (CHIKV) and Barmah Forest (BFV) viruses (Ou et al., 1981). The remainder of the 3'UTR consists of a set of repeated sequence elements (RSEs),

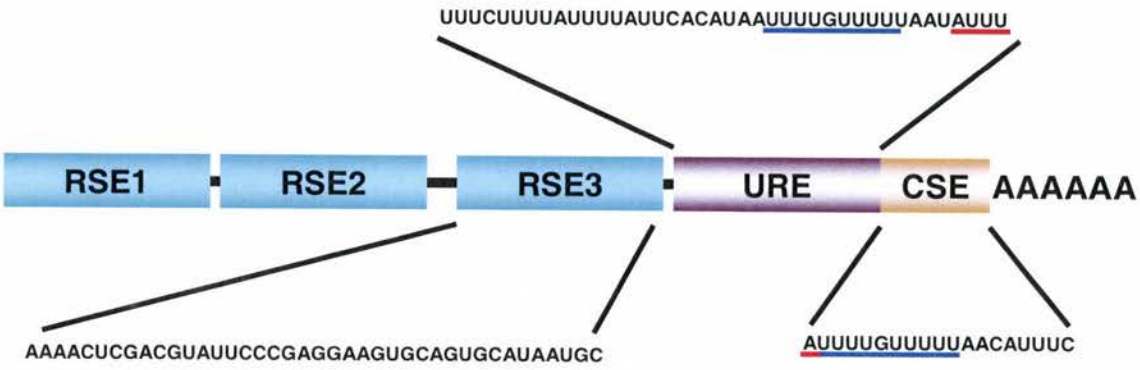


Figure 4- Organization of the Sindbis Virus RNA 3'UTR. The 3'UTR of the SinV RNA consists primarily of three parts. At the 5' end of the 3'UTR is a set of Repeat Sequence Elements (RSEs). The RSEs of the Alphaviruses differ in their sequence and organization amongst the members of the genus (for reference see Fig. 37). Following the RSEs is the approximately 40nt U-rich element (URE) which is present in the majority of the alphaviruses. Finally the 19nt Conserved Sequence Element (CSE) is the 3' terminal part and is present in all members of the genus *Alphavirus*. The sequences for each of the aforementioned elements in Sindbis virus are embedded within the figure. The red underlined sequence denotes the AUUUA pentamer present at the URE and CSE junction. The blue underlined sequence denotes the U_4GU_5 motif.

whose composition and number differ widely amongst the members of the genus (Ou et al., 1982). The RSEs are presumed to have secondary structures and may serve to aid in the recruitment of RNA binding proteins.

Prior to this study only the CSE had a definitively ascribed function (Strauss and Strauss, 1994; Hardy and Rice, 2005; Hardy, 2006). The CSE directs replication of the genomic RNA. Interestingly, deletion of this element results in drastically reduced viral replication but after extensive passaging, rescue mutants with 3' terminal additions reminiscent of the *bona fide* viral 3' end were detected (George and Raju, 2000; Raju et al., 1999).

We hypothesize that similar to cellular mRNAs, viral RNAs may contain elements that influence stability. Examination of the 3'UTR of SinV, and indeed the majority of the Alphaviruses, reveals that the URE resembles cellular AREs.

III. SinV and RNA-Binding Proteins

Interactions between cellular RNA binding proteins and the SinV RNAs have been previously reported. The La protein was found to interact with both negative and positive sense SinV RNAs (Pardigon and Strauss, 1996). In the host cell the La protein aids in the maturation of RNA polymerase III transcripts (Hendrick et al., 1981; Rinke and Steitz 1985; Yoo et al., 1997), tRNA folding and maturation (Copola et al., 2006), histone mRNA stabilization (McClaren et al., 1997) and translational regulation (Crosio et al., 2000). Currently the function of the cellular La proteins interaction with the SinV RNAs is unknown. Additionally hnRNP K associates with SinV components including the nsp2 gene and the

subgenomic RNA. RNAi-mediated reduction of hnRNP K results in decreased viral titer, indicating biological significance (Burnham et al., 2007). Similarly hnRNP A1 binds to the 5'UTR of SinV where it enhances the translation of the viral RNA (Lin et al., 2009). The cellular ZAP protein also impacts the replication and gene expression of SinV RNAs. Over expression of ZAP results in viral inhibition in cell culture models of infection (Bick et al., 2007). The mechanism of action behind ZAP repression of SinV rests at the level of translational arrest and degradation of the target RNA (Bick et al., 2007; Guo et al., 2007). These observations support the notion that SinV RNAs are modulated by the host RNA decay machinery and, similar to cellular mRNAs, stabilize their transcripts via RNA binding proteins.

Rationale

The cellular mRNA decay machinery aggressively monitors and controls the constituency of the host transcriptome. As stated above, the transcripts of many RNA viruses, particularly those of the genus *Alphavirus*, are similar to cellular mRNAs in both function and composition. Despite this similarity, the impact of the cellular mRNA decay machinery on viral biology has not been examined in detail. Since the rate and mechanism of decay for cellular mRNAs is often regulated by the interactions of RNA-binding proteins with elements in the 3'UTRs of cellular mRNAs, we hypothesize that similar events may be responsible for stabilizing SinV RNAs. Evidence in support of this hypothesis is the observation that alphavirus translation is regulated by the ZAP protein (Bick et al., 2007). Nevertheless we propose that the interactions between the alphaviruses and the host decay machinery are not limited to viral restriction, but potentially enhancement as well. Defining the interactions between the cellular mRNA decay machinery and SinV will not only expand our knowledge of viral biology but also may give insight into new therapeutic strategies to treat severe alphavirus infections.

Materials and Methods

Cultivation of Mammalian and Insect Cell Lines

Non-adherent *Aedes albopictus* C6/36 cells were maintained in serum free SF-900II medium (Gibco) in a 6L spinner flask at ~250rpm at 28°C. Cultures were maintained through daily monitoring of cell growth via cell counting using a hemacytometer; cultures were maintained between $8x10^5$ and $1x10^6$ cells per ml prior to harvesting and extract preparation. The doubling time of this particular cell line was approximately 24 hours.

The semi-adherent *Aedes aegypti* Aag2 cell line and derivates of this cell line were cultured in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cell Generation) and 1x Pen/Strep (Hyclone). The cultures were incubated in the absence of supplemental carbon dioxide gas and were held at a temperature of 28°C. Cell growth was monitored microscopically by confluency and the observed doubling time was similar to that for the C6/36 cultures described above.

Human embryonic kidney (293T), Baby Hamster Kidney (BHK-21), HeLa and African Green Monkey kidney (Vero) cells were maintained in MEM/EBSS supplemented with 10% FBS, nonessential amino acids, L-Glutamine and Pen/Strep. Cultures were maintained at 37°C in the presence of 5% CO_2. Cell growth was monitored microscopically by confluency and the doubling times of all of these cells were approximately 24 hours.

The C6/36 cell line adapted to serum free conditions was a gift from Dr. Jon Carlson at Colorado State University. The Aag2 cell line was obtained from Dr. Carol Blair at Colorado State University.

SinV Production and Plaque Titration

Full length infectious SinV transcripts were produced from cDNA clones (pToto1101) of wild type SinV AR339, the temperature sensitive clone ts6 SinV AR339 or the ΔURE mutant ts6 SinV using SP6 polymerase (NEB) as described in Garneau et al. (2008).

Viral titers were determined by plaque assay on confluent Vero cells. Serial dilutions of viral stocks were added to monolayers of Vero cells and after a one hour absorption period the monolayers were overlaid with a 2% carboxy methyl cellulose solution ((w/v) in growth medium). Plaques were allowed to develop for 48 to 72 hours prior to fixation for a minimum of 30 minutes with 7% formaldehyde ((v/v) in phosphate buffered saline (PBS)). After fixation the methyl cellulose / formaldehyde pall was removed. The wells were washed vigorously with cold water and the monolayers were stained with crystal violet staining solution (2% w/v crystal violet / 80% methanol / 18% dH_2O). Cells were briefly destained by rinsing with cold water and allowed to dry by evaporation. Plaques were examined and counted under low power magnification.

Preparation of *Aedes albopictus* (C6/36) and HeLa Cytoplasmic Extracts

HeLa cytoplasmic extracts were prepared as previously described (Ford et al., 1999). For mosquito cell extracts, 3L of C6/36 cells (at a density of $3x10^6$ cells/mL) were harvested via centrifugation for 10 minutes at 300xg in a refrigerated centrifuge. Resulting pellets were resuspended in 50mL of ice cold PBS (Hyclone) and spun again as described above to remove cellular debris.

The packed cell volume (PCV) was noted and 3xPCVs of ice cold Buffer A (10mM HEPES pH 7.9 / 1mM $MgCl_2$ / 10mM KCl) were added to the cell pellet. Following resuspension, the cells were incubated on ice for 10 minutes to allow the cells to swell under osmotic pressure. The swollen cells were then pelleted via centrifugation for 5 minutes at 300xg at 4°C. The supernatant was discarded and 2xPCV Buffer A supplemented with 1mM DTT was added to the swollen cells. The mixture was then transferred to a chilled, appropriately sized, dounce homogenizer (Kontes) and the cells were lysed mechanically via ten strokes using a type B pestle.

The lysed C6/36 cells were transferred to a sterile 50mL conical tube and centrifuged for 10 minutes at 820xg under refrigeration to pellet intact nuclei. The supernatant, consisting of the cytoplasmic milieu, was removed to a fresh tube and supplemented with 0.09 volumes of Buffer B (300mM HEPES pH 7.9 / 30mM $MgCl_2$ / 1.4M KCl). Crude cytoplasmic fractions were then clarified via centrifugation at 100,000xg for 1 hour at 4°C. Cytoplasmic extracts were pooled

and supplemented with 0.25 volumes of 80% glycerol prior to storage at -80°C. Protein concentrations of the C6/36 cytoplasmic extracts utilized in these studies were 6.2-8.5µg/ul.

Generation of Transcription Templates

DNA fragments for the production of RNA substrates used in this study were cloned into the *Eco*RI and *Pst*I sites of pGEM-4 A60. Depending on the size of the construct, or the complexity of the insert, these clones were either derived using a PCR amplification / restriction digestion approach or a direct annealed-oligonucleotide cloning method. Sequences less then 60 nucleotides in length were typically cloned using the annealed-oligonucleotide method. For cloning and PCR, oligonucleotides were purchased from either IDT or Invitrogen.

For the clones generated by conventional PCR the amplified products were digested with the appropriate enzyme and purified by electroelution following electrophoresis on a 1% agarose gel. The resulting purified fragments were cloned into the pGEM-4 A60 transcription vector using T4 DNA Ligase (Fermentas) according the manufacturer's instructions. Positive clones were identified by PCR amplification using SP6 promoter and the HIVE (5'-GAGTGCTCGAGGTAATGCA-3') primers, both of which are independent of the inserted fragment and are present in the transcription vector. Occasionally the PCR primers used to amplify the initial digestion fragment were employed for the screening of colonies. Following verification of positive clones, independent colonies were selected and grown in a large volume of Luria Broth for

subsequent Maxi-prep (Invitrogen) purification. The resulting plasmids were further verified via sequencing prior to being used as transcription substrates.

Cloning of smaller DNA fragments or sequences that do not have available cDNA was performed using an annealed oligonucleotide approach. In this method complementary oligonucleotides were combined and heated to 90°C to dissociate any unintended secondary structure prior to being slowly cooled, allowing for duplexes to form in 1x NEB Buffer #2 (10mM Tris-HCl / 50mM $NaCl_2$ / 10mM $MgCl_2$ / 1mM DTT). After the mixture cooled to room temperature, the dsDNA was phosphorylated at its 5' ends via T4 Polynucleotide Kinase (New England Biolabs) in the presence of 1x T4 DNA Ligase Buffer (50mM Tris-HCl pH 7.5 / 10mM $MgCl_2$ / 1mM ATP / 10mM DTT). The 5' phosphorylated dsDNA fragments were then extracted using PCI (25 volumes equilibrated phenol / 24 volumes chloroform / 1 volume iso-amyl alcohol) and concentrated using ethanol precipitation. The resulting dsDNA was subsequently cloned into the pGEM-4 A60 transcription vector and colonies screened. Positive clones were grown in a large volume of Luria Broth for subsequent Maxi-prep (Invitrogen) purification

Several clones were generated prior to these studies. These transcription templates include the full length SinV RNA 3'UTR as well as the 3xRSE, URE/CSE and URE domains. A detailed description of these clones can be found in Garneau et al., (2008). A list of the oligonucleotides utilized specifically in this study is presented in tabular format in Appendix B.

Transcription templates from the above clones were generated by restriction digestion of the pGEM-4 A60 derived plasmids. For a polyadenylated

RNA substrate the desired clone was digested overnight with *Nsi*I. To generate a template for a non-adenylated RNA substrate the plasmids were digested with *Hin*DIII overnight. After digestion the templates were PCI extracted and ethanol precipitated twice prior to use in an *in vitro* transcription reaction.

In Vitro Transcription Reactions

To obtain internally radiolabeled 5' capped RNA substrates the following protocol was utilized. Reaction mixtures consisted of 1.5µg linear DNA template, 0.5mM CTP, 1mM ATP, 0.05mM GTP, 0.05mM UTP, 0.5mM 7^{me}GpppG cap analog (NEB), 45µCi α^{32}-P UTP (800Ci/mmol, MP Biomedicals), 20U of RNAse Inhibitor (Fermentas) and 10U of SP6 RNA Polymerase (Fermentas) in 1x Transcription Buffer (40mM Tris-HCl pH 7.6 / 10mM NaCl / 6mM $MgCl_2$ / 2mM Spermidine / 10mM DTT). All transcription reactions were assembled at room temperature to prevent precipitation of the DNA template. Transcription reactions were incubated for 1-3 hours at 37°C prior to the addition of dH_2O and an equal volume of PCI. Following PCI extraction the transcribed RNAs were supplemented with ammonium acetate (2M final concentration) and 10µg of yeast-derived tRNA and precipitated in 2.5x volumes of absolute ethanol. As opposed to sodium salts, the use of ammonium acetate reliably precipitates the transcribed RNA while leaving unincorporated nucleotides in the supernatant. The pelleted RNA was then further washed by the addition of 200µl of 80% ethanol and air dried under vacuum in a SpeedVac (Savant). Transcription reactions were resuspended in 10µl of RNA Loading Buffer (7M urea / 20mM

EDTA / 100mM Tris-HCl pH 7.5 / 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol) and heated at 90°C for 30 seconds prior to snap chilling and resolution on a 5% denaturing polyacrylamide gel. Once sufficient resolution of the RNA species was attained, autoradiography was utilized to determine the precise location of the desired RNA species. Slices were removed from the gel and allowed to incubate at room temperature overnight in 400µl of High Salt Column Buffer (HSCB; 400mM NaCl / 25mM Tris-HCl pH 7.6 / 0.1% sodium dodecyl sulfate (SDS)) to passively elute the gel purified RNA. Purified RNA was subsequently PCI extracted and ethanol precipitated. Samples were resuspended in a minimum of 16µl of dH_2O, a single microliter of which was added to 3ml of ScintiSafe Econo scintillation fluid to determine the amount of radiolabeled UTP incorporated into the RNA substrate. The total concentration of the RNA obtained from the transcription was then determined by the specific activity of labeling that was used. Transcriptions routinely were diluted to a stock concentration of 100k Counts Per Minute (CPM) for further use.

For the cold competition analyses mentioned below trace-radiolabeled RNAs were generated using the above protocol with modified reaction conditions. Reaction mixtures consisting of 2µg linear DNA template, 5mM CTP/GTP/UTP/ATP, 0.9µCi α ^{32}P UTP (800Ci/mmol), 20U of RNAse Inhibitor and 10U of SP6 RNA Polymerase were combined in 1x Transcription Buffer. The transcription reactions were incubated at 37°C for 3 hours prior to being processed identically to those described above. Trace labeled RNA competitors were left undiluted until use.

In vitro Deadenylation Assays

200k CPM (~5-40 fmols) of internally radiolabeled RNA substrates were incubated at 28°C in the presence of 6.5µl of 10% polyvinyl alcohol (PVA), 2µl phosphocreatine / ATP (PC/ATP, 250mM / 12.5mM respectively), 2µl poly(A) (500ng/µl; GE Biosciences) and 16µl of C6/36 or HeLa cytoplasmic extract. Five microliter samples were removed at the indicated times and processed using 400µl of HSCB and an equal volume of PCI. Following mixing and centrifugation the aqueous phase was removed to a fresh 1.5mL centrifuge tube containing 2.5x volumes of 100% ethanol and 10µg of yeast-derived tRNA.

Cold competition analyses were performed similar to the standard deadenylation assays with several exceptions. Trace labeled cold competitor RNAs (in molar excess) were added to individual reaction mixtures prior to the addition of RNA substrate and incubation of the reaction. The reaction mixtures were split into two equal parts, with one part being incubated at 28°C for 9 minutes, while the other part was supplemented with 3mM EDTA to inhibit RNA decay for later UV cross-link analysis as described below. The incubated reaction mixtures were processed identically to that described above for the standard deadenylation reaction.

The individual samples were subsequently ethanol precipitated via incubation at -80°C for 10 minutes followed by centrifugation at 16,000xg for 10 minutes. After disposal of the supernatant, nucleic acid pellets were washed by the addition of 200µl of room temperature 80% ethanol and brief centrifugation. Desalted RNA pellets were then dried under vacuum and resuspended in 10µl of

RNA Loading Buffer and heated to 90°C for 30 seconds prior to chilling on ice and electrophoresis.

RNA species were resolved via denaturing electrophoresis on pre-run 6% polyacrylamide gels containing 7M urea. Following sufficient resolution of the RNA substrates, the gels were dried and exposed to a phosphorimager plate (Molecular Dynamics) overnight and analyzed using a Typhoon Trio Variable Mode Imager. Where required, the abundances of the RNA species were determined using the ImageQuantTL (GE Biosciences) software package. Gels shown are representative of a minimum of 3 independent assays involving at least 2 independent extracts.

Ultraviolet Cross-linking and Immunoprecipitation Assays

On ice, 100k CPM (~2.5-20 fmols) of internally labeled RNA substrate were briefly incubated along with 3.25µl 10% PVA, 1µl PC/ATP, 1µl poly(A) (500ng/µl), 1µl 25mM EDTA and 8µl C6/36 or HeLa cytoplasmic extract (~56µg total protein). Samples were transferred to independent wells of a microtiter plate and irradiated with 180mJ of ultraviolet radiation in a UV Stratalinker 2400 (Stratagene). The cross-linked RNA:protein complexes were then transferred to fresh 1.5mL centrifuge tubes and incubated for 15 minutes at 37°C in the presence of 5U RNase One (Promega) and 25µg RNase A (Sigma Aldrich). Equal volumes of 2x SDS Protein Dye (60mM Tris-HCl pH 6.8 / 5% (v/v) glycerol / 2% (w/v) SDS / 100mM DTT, 0.002% (w/v) bromophenol blue) were added to each tube prior to boiling for a total of 5 minutes followed by resolution via SDS-

PAGE. Following sufficient electrophoretic separation the gels were dried on a slab drier and exposed to a phosphorimager screen for analysis.

For immunoprecipitation assays the cross linked, RNase-treated samples were centrifuged at 16,000xg for two minutes to remove insoluble materials. The supernatant was transferred to a fresh 1.5mL tube and incubated in the presence of 300µl of NET-2 buffer (50mM Tris-HCl pH 7.6 / 150mM NaCl / 0.01% (v/v) Nonidet P-40) supplemented with either 4µl of target-specific antibody or control serum. Reaction mixtures were incubated for 1 hour at 4°C on a rotisserie mixer prior to the addition of 25µl formalin-fixed Protein A-positive *Staphylococcus aureus* cells (washed, resuspended as a 50% slurry in NET-2 buffer) (Calbiochem). After 20 minute incubation the samples were briefly centrifuged. The supernatants containing unbound proteins were discarded and the cell pellet was washed vigorously 5 times in 1mL of NET-2 buffer to remove any contaminants. Bound antibody-protein complexes were eluted via the addition of 20µl 2x SDS Protein Loading Dye and boiling for 3 minutes. Radiolabeled proteins were resolved and evaluated as described earlier in this section. Gels shown are representative of a minimum of 3 independent assays involving at least 2 independent extracts.

Electrophoretic Mobility Shift Assays (EMSA)

Two and a half femtomoles of internally radiolabeled, capped, unadenylated RNA substrates were incubated in the presence of 3µl 5x Gel Shift Buffer (70mM HEPES pH 7.9 / 450mM KCl / 10mM $MgCl_2$ / 30% v/v glycerol),

0.15mM spermidine, 10U RNase Inhibitor and up to 10µl of recombinant protein or buffer for a total volume of 15µl. The concentration of the RNA substrates in these assays was constant, while the concentrations of the proteins were increased incrementally. Following incubation at 28°C for five minutes the reactions were transferred to ice following addition of 2.5µg of heparin sulfate. The reactions were allowed to incubate on ice for 5 minutes prior to the addition of 6x EMSA Loading Dye (10mM Tris-HCl pH 7.6 / 60mM EDTA / 0.03% (w/v) bromophenol blue / 0.03% (w/v) xylene cyanol / 60% (v/v) glycerol) to a final concentration of 1x. The samples were loaded onto a native 5% polyacrylamide gel and resolved by electrophoresis for approximately 4 hours at 200V at 4°C. The resulting gels were dried and exposed to a phosphorimager screen overnight prior to examination of the bound and free RNA populations using the ImageQuant software package. Each RNA species was evaluated a minimum of three times, representative gels are shown. The resulting data were used to develop Scatchard plots and the dissociation constants determined from the slopes of the resulting lines.

In Vivo Cross-linking and Detection of Protein-RNA Interactions

Either 293T or Aag2 cells were grown in the presence of complete medium overnight prior to infection with wild-type Sindbis virus at a multiplicity of infection (MOI) of 5. Following sufficient adsorption (1 hour in a minimal volume of medium) the medium was replaced and allowed to incubate at 28°C for 10

hours. The cells were released from the plate via scraping and harvested using centrifugation.

The resulting pellet was washed with 1x PBS twice to remove contaminating medium and serum and resuspended in 10ml of 1x PBS. Formaldehyde was added to a final concentration of 1% from a 37% stock (stabilized with 10% methanol) and the cells were incubated for 10 minutes at room temperature with constant agitation. The formaldehyde cross linking was quenched via the addition of glycine to a final concentration of 0.25M and further incubation for 5 minutes at room temperature.

Cross-linked cells were harvested via centrifugation and washed a minimum of 5 times with 1xPBS prior to resuspension in 1ml RIPA buffer (50mM Tris-HCl pH 7.5 / 150mM NaCl / 1mM EDTA / 1% (v/v) NP-40 / 0.5% (w/v) sodium deoxycholate / 0.05% (w/v) SDS). Since formaldehyde cross-linked cells are resistant to proteases, osmotic lysis and sensitive to heat denaturation, the fixed cells were lysed with three 10-second bursts, each burst followed by an equal rest, from a sonicator (Sonic Dismembrator Model 100, Fisher Scientific) set to 7, with an average output of 12 on ice. Insoluble materials were precipitated from the lysates via centrifugation at 16,000xg at 4°C. The supernatant was transferred to fresh tubes for later use.

Lysates were pre-cleared to reduce nonspecific interactions by the incubation of 20µl (packed volume) Protein A (or G) Sepharose (GE Healthcare) per 125µl of raw lysate for 1 hour at 4° C on a rotisserie. The Sepharose beads

were removed from solution via centrifugation and the pre-cleared lysate was transferred to a fresh tube and used immediately for immunoprecipitation.

For immunoprecipitation of the candidate cellular factors identified via mass spectrometry (as described below) the anti-aeHuR, anti-HuR (3A2) and control antibodies were pre-bound to Protein A (for anti-aeHuR and control sera) or Protein G (for anti-HuR (3A2) and control sera) Sepharose beads along with 100µg of yeast-derived tRNA for 15 minutes at 4°C. The resin was collected via centrifugation and washed twice with 1ml of RIPA buffer to remove unbound antibodies.

The antibody bound Sepharose beads were added to the pre-cleared lysates described above and allowed to incubate for one hour at 4°C on a rotisserie. The bound cross-linked material was collected from the mixture via centrifugation and washed extensively with RIPA buffer supplemented with 1M urea. The cross-linking of the immunoprecipitated materials was then reversed in a minimal volume of RIPA buffer by heating the samples to 75°C for 45 minutes under gentle agitation. The Sepharose resin was removed from solution via centrifugation and total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions.

Using the purified total RNA from above, cDNA was produced using random hexamers and reverse transcriptase. The resulting cDNA was utilized as a template in conventional PCR amplification of SinV RNA species. Aliquots of each PCR reaction were resolved on a 2% agarose gel and nucleic acid was detected using ethidium bromide.

Purification and Identification of RNA Binding Factors

i) Affinity Purification of RNA Binding Proteins

A total of 15µg of 5' biotinylated RNA (IDT) consisting either of the 3' terminal 50 nucleotides of the Sindbis virus RNA 3'UTR or the nonspecific control substrate utilized in the *in vitro* deadenylation assays described above were incubated for 30 minutes on ice with constant agitation after which 50µl Streptavidin Agarose resin (packed bead volume) (Thermo Scientific) was added to the reaction. Unbound RNAs were washed away with several washes of Buffer D (20mM HEPES pH 7.9 / 100mM KCl / 0.2mM $MgCl_2$ / 1mM DTT / 20% v/v glycerol). 10mg of C6/36 cytoplasmic extract supplemented with 3mM EDTA and 2.5µg heparin sulfate, to retard RNA decay and nonspecific interactions respectively, was added to the above resin to allow formation of the ribonucleoprotein (RNP) complexes under native conditions.

Following removal of the flow-through, unbound proteins were washed away by four washes of 1.5ml Buffer D followed by four similar washes (in volume) with Buffer D supplemented with 250mM NaCl and 0.5% (v/v) NP-40. Affinity purified RNA binding proteins were eluted via the addition of 200µl of HSCB.

The total volume of the eluates were increased to 400µl via the addition of dH_2O prior to the addition of 400µl methanol and 100µl chloroform to precipitate the eluted proteins via centrifugation. Following resuspension of the eluted

proteins in 20µl 2x SDS Protein Loading Dye, the samples were boiled for 10 minutes prior to SDS-PAGE.

After sufficient resolution was obtained the gels were transferred to a Pyrex dish and silver stained via the Shevchenko method (Shevchenko et al., 1996).

ii) Identification of Purified RNA Binding Proteins

Gel slices were removed from the above gels and stored in 1% acetic acid prior to analysis using mass spectrometry. Purified proteins were destained and subjected to trypsin digestion prior to elution from the gel slices and ultimately concentrated prior to mass spectrometry (MS) analysis.

Matrix Assisted Laser Desorption Ionization – Time of Flight (TOF) / TOF (MALDI-TOF/TOF) mass spectrometry was performed by the Proteomics and Metabolomics Facility (PMF) at Colorado State University on the submitted peptide samples.

Briefly, the purified samples were mixed with α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% (v/v) acetonitrile 0.1% (v/v) trifluoroacetic acid) prior to spotting on the MALDI target. Samples were analyzed by an Ultraflex-TOF/TOF mass spectrometer (Bruker Daltonics) in a positive ion reflector mode utilizing a 25kV acceleration voltage. Raw data were processed using the SNAP algorithm from the FlexAnalysis software package version 2.4 (Bruker Daltonics). Monoisotopic peak lists were generated with a signal-to-noise ratio threshold of either 6 or 3, for MS spectra or MS/MS spectra respectively. Additionally, all

MS/MS spectra were smoothed (SavitzkyGolay, width 0.15 m/z, 4 cycles) and base-line subtracted (via the Top Hat algorithm) prior to the generation of peak lists.

Following the removal of contamination peaks, the NCBInr (20070810) database was searched using the Mascot 2.2 search engine. Possible hits were generated using a mosquito taxonomy filter with a mass tolerance of 0.15Da, fragment ion mass tolerance of 0.8Da. Further parameters included a maximum of a single missed trypsin cleavage as well as the presence of oxidation of methionine residues and fixed carbamidomethylation of cysteine residues.

Recombinant Protein Expression

In both of the instances below cDNA was produced from *Aedes aegypti* cells. After DNAse treatment of total RNA extracted from Aag2 cells 0.5µg of total RNA was used in a reverse transcription reaction. RT reactions were performed using ImPromtu RT (Promega) according to the manufacturer's instructions using random hexamers.

i) Aedes aegypti HuR (aeHuR)

The Open Reading Frame (ORF) corresponding to the *Aedes aegypti* HuR gene (AAEL008164-RA) was amplified via PCR from random hexamer-primed cDNA from *Aedes aegypti* total RNA using the primers *ELAVA.aegyptiF* (5'-CATGGATCCATGACCAACAAAGTGCTAGCAGCC-3') and ELAVA.aegyptiR (5'-CATGAATTCTTAATGATCGGCCATTTCGGCG-3'). Amplified products were

subsequently digested with *Bam*HI and *Hin*DIII, gel purified, and ligated into the pGEX-2T-ZQ expression vector (Qian and Wilusz, 1994) to generate the pGEX-aeHUR plasmid. Following the transformation of competent *E. coli* DH5alpha with pGEX-aeHuR positive colonies were detected via a colony screening PCR method. Insertion of the target sequence was further verified via sequencing by the Proteomics and Metabolomics Facility (PMF) at Colorado State University.

Expression of recombinant aeHuR protein was initiated by inoculation of *Escherichia coli* BL21 (DE3) cells transformed with the pGEX-aeHuR plasmid into 100ml of LB broth supplemented with ampicillin and incubation overnight at 30°C. Dense cultures were used to inoculate starter cultures of fresh LB broth at a ratio of 1:10, and further incubated at 30°C until logarithmic growth was attained (typically <1.5 hours). Upon verification of the correct growth phase, as indicated by the optical density (OD) of the culture at 600nm using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech), Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1mM. Induction was allowed to continue for 1.5 hours at 30° C.

Bacterial pellets were harvested by centrifugation at 10,000xg for 10 minutes at 4°C. The resulting pellets were subjected to freezing and resuspended in 5ml of Lysis Buffer (50mM HEPES pH 7.9 / 150mM KCl / 1mM $MgCl_2$ / 1% v/v Triton X-100 / 10% v/v glycerol) supplemented with 1mg/ml lysozyme. Cell pellets were then subjected to four 30-second bursts of sonication with alternating rest periods on ice to homogenize the sample. Insoluble materials were removed via centrifugation at 11,000xg for 20 minutes at 4°C.

Soluble materials contained within the supernatant were then removed to a fresh conical tube containing washed Glutathione S-Transferase-Agarose beads (Sigma) (1ml/L of starting culture) and incubated under constant agitation at 4°C for 1 hour.

Contaminating proteins and nucleic acids were washed from the agarose matrix via the addition of 10ml of Lysis Buffer; this wash was performed a minimum of 5 times to ensure purity of the eluted recombinant aeHuR protein. Elution of the GST-tagged aeHuR consisted of overnight incubation of the bound protein in the presence of thrombin (MP Biomedicals) (10U/ml agarose beads). Following a brief refrigerated centrifugation to remove the agarose matrix the eluted, untagged, aeHuR was collected and dialyzed three times against 500ml of Lysis Buffer. Purified proteins were aliquoted and stored at -80°C.

The purity and concentration of the recombinant aeHuR was determined via SDS-PAGE alongside known amounts of bovine serum albumin. A total of 6mg of aeHuR was supplied to BIOO Scientific for the production of rabbit polyclonal antisera.

ii) Aedes aegypti Squid (aeSquid)

Similar to that described for the expression of aeHuR, the open reading frame corresponding to *A. aegypti* Squid (Squid) protein was amplified using the primers *SquidA.aegyptiF* (5'-CATGGATCCATGGCCGATCAGGATCAAGAGATG-3') and SquidA.aegyptiR (5'-CATCTCGAGTTAGTACGGCGTATGCCTTGG-3') from random hexamer-

primed cDNA. The resulting amplified fragments were gel purified and digested with *Bam*HI and *Eco*RI. Digested PCR products were subsequently ligated into the pTricHis-A vector (Invitrogen). Positive clones were identified by colony screening and confirmed by sequencing. It was determined that splice isoform A of Squid was cloned by this method to form the plasmid pTricHis-aeSquid.

Expression of the aeSquid protein in *E. coli* was similar to that described above for aeHuR protein. Major exceptions included the temperature of incubation, being 37°C rather than 30°C, and the length of the induction period which was extended to 4 hours to allow for adequate expression.

Purification of the aeSquid relied upon a nickel affinity system (Ge Biosciences). Clarified expression lysates were incubated in the presence of washed Ni-NTA resin for 1 hour at 4°C in the presence of 20mM imidazole to decrease nonspecific interactions. Bound samples were then further purified via the use of Lysis Buffer supplemented with 100mM imidazole. Elution of aeSquid involved the incubation of the bound resin in the presence of Lysis Buffer containing 250mM imidazole. Purified aeSquid protein was dialyzed three times against 500ml of Lysis Buffer, aliquoted and stored at -80°C.

As with the expression of aeHuR, the purity and concentration of the aeSquid protein was evaluated by SDS-PAGE. A total of 5mg of aeSquid, of ~80% purity, was supplied to BIOO Scientific for the production of rabbit polyclonal antiserum.

Western Blot Detection of Cellular Proteins

Protein samples suspended in 1x SDS Protein Dye were boiled and resolved via SDS-PAGE. Gels were soaked in PVDF Transfer Buffer (10mM Tris-KOH / 100mM glycine / 10% (v/v) methanol) for 3 minutes prior to being blotted to a PVDF membrane, with a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) run at 18V for a total of 20 minutes. Membranes were trimmed after staining with Ponceau Red to determine the localization of proteins. Membranes were destained with several sterile water rinses. Blocking of the membrane was carried out via the addition of Blotto (2.5% (w/v) dehydrated milk / TBST (100mM Tris-HCl pH 7.5 / 0.9% (w/v) NaCl / 0.1% (v/v) Tween 20) and incubation at room temperature for one hour. Fresh Blotto was added along with an appropriate amount of primary antibody and incubated either for one hour at room temperature or overnight at 4°C. The next morning the primary antibody solution was decanted and the membrane was washed several times with TBST to remove unbound antibodies. A 1:20,000 dilution of an appropriate horse radish peroxidase (HRP) conjugated secondary antibody (diluted in TBST) was added to the membrane and allowed to incubate for one hour at room temperature. Upon the completion of the incubation period the secondary antibody solution was discarded and the membrane was further washed several times with TBST. Chemiluminescent substrate (SuperSignal West Pico Chemiluminescent HRP Kit, Pierce) was added to the washed blots and the reaction was allowed to proceed for 5 minutes prior to being gently blotted dry. Images were captured

using a VersaDoc Imager (Bio-Rad) and evaluated using the QuantityOne software package (Bio-Rad).

Isolation of Nuclear and Cytoplasmic Subcellular Fractions

Either 293T or Aag2 cells were grown to confluence in a 10-cm dish prior to infection with SinV at an MOI of 5, or as otherwise indicated. The medium was removed and replaced with fresh growth medium after a one hour adsorption period. The infection was allowed to proceed for a total of 12 hours prior to the removal the cell culture supernatant and scraping of the cells in 5ml of 1x PBS. The cells were pelleted via centrifugation at 300xg for 10 minutes at 4°C. The resulting cell pellets were resuspended in 1x PBS and washed a second time to remove any contaminating trace medium. The washed pellet was gently resuspended in EBKL buffer (25mM HEPES pH 7.6 / 5mM $MgCl_2$ / 1.5mM KCl / 2mM DTT / 0.1% (v/v) NP-40) and allowed to swell for fifteen minutes on ice. The swollen cells were transferred to an appropriately sized dounce homogenizer and mechanically lysed via 30 swift draws of the pestle. The resulting lysate was centrifuged for 2.5 minutes at 900xg to separate the cytoplasmic supernatant and the nuclear pellet. The cytoplasmic fraction was transferred to a fresh tube and centrifuged at >16,000xg for ten minutes to remove contaminating nuclei. The nuclear pellet was washed twice in EMBK buffer (25mM HEPES pH 7.6 / 5mM $MgCl_2$ / 1.5mM KCl / 75mM NaCl / 175mM sucrose / 2mM DTT) to remove contaminating cytoplasmic materials. After the final wash the purified nuclei were resuspended in 100µl of 0.5% NP-40 (in water). The nuclear fraction was

sonicated briefly to shear the genomic DNA. Both fractions were supplemented with 2x SDS Protein Dye to a final concentration of 1x prior to loading of equal ratios of protein for western blot detection.

Immunofluorescence Assays

Aedes aegypti Aag2 or 293T cells were plated at low confluence on glass coverslips 24 hours prior to treatment. Coverslips were fixed in 4% paraformaldehyde (diluted in 1x PBS) for 15 minutes at room temperature. After removal of the fixing solution the coverslips were permeabilized in methanol for 15 minutes at room temperature and washed by immersion in 70% ethanol for an additional 15 minutes prior to being blotted dry. The fixed and permeabilized cells were then blocked in a solution of 6% bovine serum albumin (BSA, Fraction V) for 1 hour and washed with 1x PBS. Primary antibodies consisting of either the anti-aeHuR polyclonal serum (diluted 1:1,000) or anti-HuR (3A2) (diluted 1:300) were incubated with the coverslips in 0.6% BSA-PBS solution for one hour. The coverslips were washed with PBS and the secondary detection antibody was added at a dilution of 1:10,000 and allowed to incubate for 1 hour. The coverslips were treated with DAPI stain (ProLong Gold Antifade reagent) and affixed with clear acrylic nail polish to glass slides. Imaging was performed using an Olympus IX71microscope using a Retiga 2000R camera (Qimaging) and Slidebook software (Intelligent Imaging Innovations, Inc.) on a Macintosh G5 computer (Apple Computer).

Development of a Selectable Mosquito shRNA Vector

Using PCR the hygromycin phosphotransferase gene of pHyg was amplified using the primers *hphF* (5'-CATACATGTTCATGAAAAAGCCTGAACTCACCGCG-3') and *hphR* (5'-CATCTCGAGCTATTCCTTTGCCCTCGGACGAGTG-3') and digested with *Nco*I and *Xho*I for subsequent ligation into pBiEx-1 (Promega) to form pBiEx-hph. The *Aedes aegypti* U6 promoter described by Konet et al., 2007 was amplified from pAedes1 using the primers *AeU6F* (5'-CATGGGCCCGAATGAATCGCCCATCGAGTTGATACGTC-3') and *AeU6R* (5'-CATGGCGCCAAAAAAAAAAGCTTCAGCTGGGTACCGGATCCATTTCACTACTCTTGCCTCTGCTCTTATATAG-3') and digested with *Apa*I and *Sfo*I. This fragment was then ligated into pBiEx-hph to form pAeSH.

The pAeSH vector was subsequently digested with *BamHI* and *HinDIII* to insert an shRNA (using an annealed oligo method with the oligonucleotides *aeHuR1T* (5'-GATCCCAAAGTGCTAGCAGCCGTATTCAAGAGATACGGCTGCTAGCACTTGTTA-3') and *aeHuR1B* (5'-AGCTTAACAAAGTGCTAGCAGCCGTATCTCTTGAATACGGCTGCTAGCACTTTGG-3')) targeted to the *Aedes aegypti* aeHuR transcript. The resulting anti-aeHuR shRNA vector, pAeSH-aeHuR1, was purified from LB broth culture using an alkaline lysis method (PureLink Hi Pure Plasmid Maxiprep Kit, Invitrogen) and subjected to endotoxin removal with a MiraClean Endotoxin Removal kit (Mirus).

shRNA-Mediated Reduction of Cellular HuR Proteins

In 293T cells the knockdown of HuR was achieved using a commercially available anti-HuR shRNA vector (TRCN0000017277, Sigma Aldrich). Briefly, actively growing 293T cells at a confluency of 60% were transfected with FuGene6 (Roche) according to the manufacturer's instructions. As a control the empty pLKO.1-Puro vector, which lacks a specific shRNA, was transfected in parallel to the anti-HuR vector.

Stable cell lines deficient in aeHuR were developed using a similar transfection based approach. Either pAeSH-ELAV1 or empty pAeSH vector was transfected into wild type Aag2 cells using FuGene6 at a 6:1 ratio of reagent to vector DNA. Forty-eight hours later (roughly 2 divisions) the cells were selected with growth medium supplemented with 300U of hygromycin B. The cultures were maintained for several weeks with occasional medium changes to remove cell debris. Following the formation of independent colonies the cultures were split and transferred to a smaller flask for further cultivation. These cell lines were frozen back to produce low passage number stocks. All experiments involving the Aag2-derived cell lines utilized cells that had been passaged no more than three generations. This was to prevent "curing" of the cell line. The Aag2-derived cell lines, Aag2-EL1 and Aag2-empty, were maintained in selection medium. Prior to experimentation using these cell lines, the medium was changed to no longer include hygromycin B.

Assessment of *In Vivo* Viral RNA Decay Rates

Confluent monolayers of either 293T-derived or Aag2-derived cells were infected with a replication temperature sensitive SinV variant, ts6SinV (Barton et al., 1988) at an MOI of 5 in a six-well plate. SinV absorption was carried out in a minimal volume to enhance viral entry. Following the one hour absorption period the virus containing medium was removed, the cells were washed with PBS and fresh growth medium was added. The infected cells were allowed to incubate under permissive conditions for a period of ten hours. After this period the medium was removed and the cells washed once more with PBS. Pre-warmed (40°C) growth medium was added directly to the cells and they were transferred to non-permissive conditions. Thirty minutes later total RNA was extracted using TRIzol (Invitrogen). This process was repeated every two hours for a total of 10 hours. The harvested total RNA was used to synthesize transcript specific cDNAs to total SinV RNA, genomic SinV RNA and cellular GAPDH mRNA for both the mammalian and the mosquito analyses. Abundances of these transcripts were examined using qRT-PCR using SYBR-Green Supermix (Bio-Rad) and a MyIQ iCycler (Bio-Rad) with a two-step amplification cycle. The subgenomic RNA abundances were calculated by subtracting the levels of genomic RNA from the total viral RNA. Both the genomic and subgenomic RNA levels were plotted with respect to time, and the half lives of the viral RNAs calculated by fitting an exponential regression to the data points.

Quantification of Extracellular SinV Progeny

Confluent monolayers of either 293T-derived or Aag2-derived cells were infected with ts6SinV at an MOI of 5 in a 6-well plate. Following a one hour absorption period (at 28°C) the confluent monolayers were washed twice with 1x PBS and 1ml of fresh growth medium was added. Every three hours a small (200µl) aliquot of supernatant was removed and the monolayers were washed with 1x PBS and the 1ml of fresh medium was replaced. The cells were returned to the incubator and the process was repeated every three hours for a total of 15 hours. The collected supernatants were serially diluted into an appropriate range and 200µl of diluted viral supernatant was added to confluent monolayers of Vero cells in a 12-well plate. The number of plaques observed was used to calculate the titer at each point and growth curves were developed by plotting viral titer with respect to time.

SinV Luciferase Assays

In 12-well plates, confluent monolayers of either 293T or Aag2 cells were infected with a SinV containing a luciferase insert (pToto1101-ts6 Luciferase) or a variant SinV with a deletion of the URE (pToto1101-ts6 Luciferase ΔURE). The virus was allowed to proceed with adsorption for 1 hour in a minimal volume of medium prior to a 1x PBS wash and replacement of the cell culture growth medium. The viral infections proceeded for the indicated times, whereupon the medium was removed, the cells washed with PBS and 200µl of Steady-Glo Luciferase Reagent (Promega) was added. The cell monolayers were disrupted

with a pipette and half of the mixture was transferred to a 500µl thin walled clear PCR tube for analysis on a Turner 20E luminometer. The emitted light was integrated over a period of 15 seconds and multiple readings were taken for each sample.

Total RNA was extracted from the luciferase reaction using TRIzol. The resulting RNA was used to generate transcript specific cDNAs corresponding to the genomic SinV RNA and cellular GAPDH mRNAs after digestion with DNAse I (Fermentas). The abundances of the viral genomic RNAs as determined by qRT-PCR were used to normalize the expression data obtained from above.

Results

During the course of these studies, we reported that in contrast to cellular mRNAs, SinV genomic and subgenomic RNAs degrade in a deadenylation-independent manner during infections of mosquito or mammalian cells (Garneau et al., 2008). Interestingly, deletion of the 3'UTR of the SinV RNA to the minimally required CSE resulted in an activation of deadenylation of viral RNAs during an infection (Garneau et al., 2008). This observation strengthened our hypothesis that viral RNAs contain regulatory elements within their respective 3'UTRs that modulate the interaction of the transcript with the cellular RNA decay machinery.

The SinV RNA 3'UTR Contains Multiple Elements Capable of Repressing Deadenylation *In Vitro*

In order to elucidate the mechanism by which the deadenylation of SinV RNA is repressed in cultured cells, we utilized a cell-free system to reproduce the block of deadenylation. Importantly, both mammalian and mosquito extracts are capable of demonstrating regulated mRNA decay, making them a prime technology for the examination of viral RNA stability (Opyrchal et al., 2005; Ford and Wilusz, 1999).

To reproduce the block to deadenylation observed with the SinV RNA 3'UTR, the entire 3'UTR of the MRE16 strain of Sindbis virus was cloned into the pGEM-4 A60 transcription vector and utilized to produce 5' capped and 3' polyadenylated RNA substrates. As a control, nonspecific sequences derived

solely from the vector were transcribed and used in parallel reactions. RNA substrates were incubated in cytoplasmic extracts derived from *Aedes albopictus* C6/36 cells and the poly(A) tail length of the RNAs was examined via denaturing polyacrylamide electrophoresis in a manner similar to that described previously in (Opyrchal et al., 2005; Opyrchal, 2005). As shown in Figure 5, the 3'UTR of SinV was capable of repressing deadenylation as compared to the non-specific adenylated control substrate in cell-free deadenylation reactions. The rates of deadenylation are often described as either distributive or processive. Distributive deadenylation kinetics are exemplified by the shortening of the poly(A) tail as a population and often in a slow progressive manner. Alternatively, processive deadenylation kinetics consists of the rapid and complete removal of the poly(A) tail from a single substrate. Incubation of the control RNA substrates resulted in the rapid formation of the deadenylated (A0) intermediate, while the SinV RNA 3'UTR exhibited far slower distributive-like deadenylation kinetics. These data indicate that sequences contained in the SinV 3'UTR are acting to repress deadenylation in this cell-free system, reproducing what was seen during the infection of C6/36 *Aedes albopictus* cells (Garneau et al., 2008).

As shown in Fig. 4, the 3'UTR of the SinV RNA contains three distinct elements. Any of these elements individually, or in combination, could potentially direct the repression of deadenylation observed with the SinV RNA 3'UTR. We subdivided the SinV RNA 3'UTR to examine the roles of the individual elements in mediating the repression of deadenylation (Fig. 6A). As shown in Fig. 6B&C,

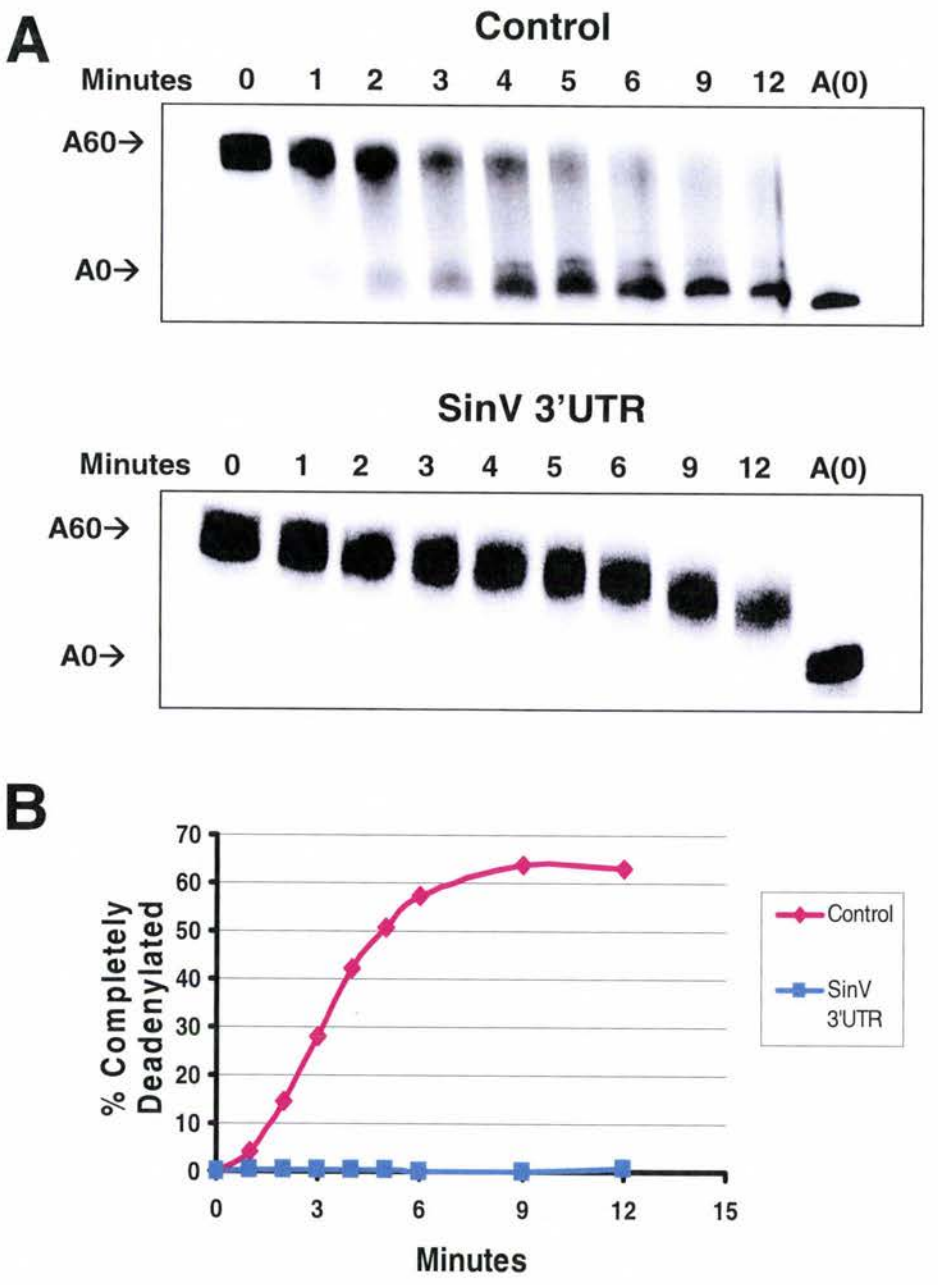


Figure 5- The Repression of Deadenylation by the SinV RNA 3'UTR Observed in Cultured Cells can be Recapitulated in a Cell Free System. (A) Adenylated RNA substrates containing either vector sequences (Control) or the 3'UTR of the SinV RNAs were incubated with *Aedes albopictus* C6/36 cell cytoplasmic extract for the indicated times. Reaction products were examined using 5% denaturing PAGE and visualized by phosphorimaging. The A(0) lane denotes the migration of an unadenylated RNA substrate. Markers on the left denote input RNA substrates that contain a poly(A) tail of 60 residues (A60) or a fully deadenylated RNA (A0). **(B)** A graphical representation of the deadenylation observed in panel A.

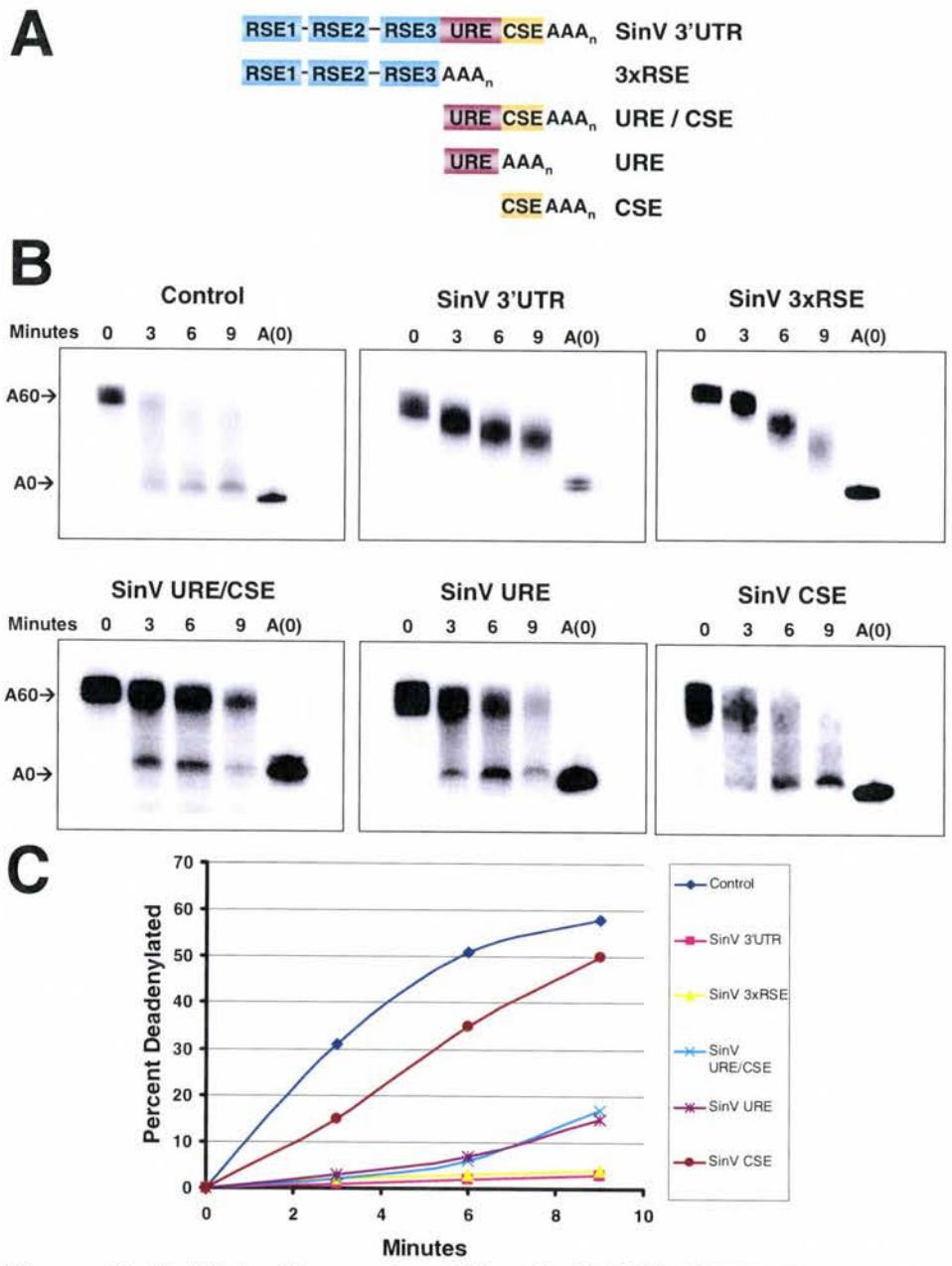


Figure 6- Multiple Elements of the SinV RNA 3'UTR Repress Deadenylation in a Cell Free System. (A) Diagrammatic depiction of the SinV RNA 3'UTR fragments assayed in this study. **(B)** Adenylated RNA substrates containing either vector sequences (Control) or the indicated fragments of the 3'UTR of SinV were incubated in the presence of *Aedes albopictus* C6/36 cell cytoplasmic extract for the indicated times. Reaction products were examined using 5% denaturing PAGE and visualized by phosphorimaging. The A(0) lane denotes the migration of an unadenylated RNA substrate. Markers on the left denote input RNA substrates that contain a poly(A) tail of 60 residues (A60) or a fully deadenylated RNA (A0). **(C)** A graphical representation of the data presented in panel B.

the 3xRSE and the URE/CSE fragments of SinV are both capable of repressing deadenylation.

Further division of the URE/CSE fragment into the URE and CSE reveals that the bulk of the repression of deadenylation observed with the URE/CSE fragment is mediated by the URE rather than the CSE, which exhibits only a minor effect. Interestingly the deadenylation kinetics of RNAs containing the individual components differs substantially. The 3xRSE fragment exhibits distributive-type deadenylation kinetics (Xu et al., 1997; Chen et al., 1995) as demonstrated by the gradual shortening of the poly(A) tail. In comparison to the control (which displays processive-like kinetics), the URE/CSE and URE fragments appear to be more refractory to deadenylation but also exhibit processive-type kinetics, whereby the entire poly(A) tail is removed with no apparent intermediates.

Taken together these data indicate that there are multiple elements present in the 3'UTR of the SinV RNA which act to repress deadenylation *in vitro*. Interestingly, possible combinatorial effects on stabilization may be present as each of the individual component parts represses deadenylation less effectively than the entire viral 3'UTR. It should be noted the subcomponents of the SinV RNA 3'UTR are not acting in an identical fashion to preserve the poly(A) tail. Moreover these findings confirm the reliability of our cell-free RNA decay assay and provide us with a tool to examine mechanistically the stability of the viral 3'UTR.

The 3'UTRs of Clinically Important Alphavirus RNAs Also Repress Deadenylation

While SinV is the model *Alphavirus*, its impact on human health is relatively minor when compared to other members of the genus. For instance, VEEV, WEEV and EEEV viruses are more commonly associated with severe disease in both human and animal populations. Upon infection these viruses may cause encephalitis, occasionally leading to death, and more often permanent neurological sequelae in their at-risk populations. We hypothesized that these clinically important alphaviruses, such as VEEV, EEEV, WEEV and Semliki Forest virus (SFV) may also repress deadenylation both *in vivo* and *in vitro*. Unfortunately, biosafety concerns have limited the availability of these infectious agents for experimentation. Despite this limitation, as previously demonstrated, our *in vitro* assay is capable of reliably recapitulating the repression of deadenylation observed in cell culture models for SinV. Division of the 3'UTR had revealed that multiple elements were present in the 3'UTR of the SinV RNA. A major block in deadenylation was determined to be the 3' 40 nucleotides consisting of the URE immediately adjacent to the CSE as exhibited by the processive deadenylation kinetics observed in a small population *in vitro*. Examination of these sequences in several alphaviruses did not reveal any highly conserved sequence motifs outside a general U-rich bias (Fig 7A). We next sought to determine if the UREs from both Old World and New World alphaviruses repressed deadenylation similarly. We hypothesized that repression of deadenylation would be common amongst the URE-bearing alphaviruses.

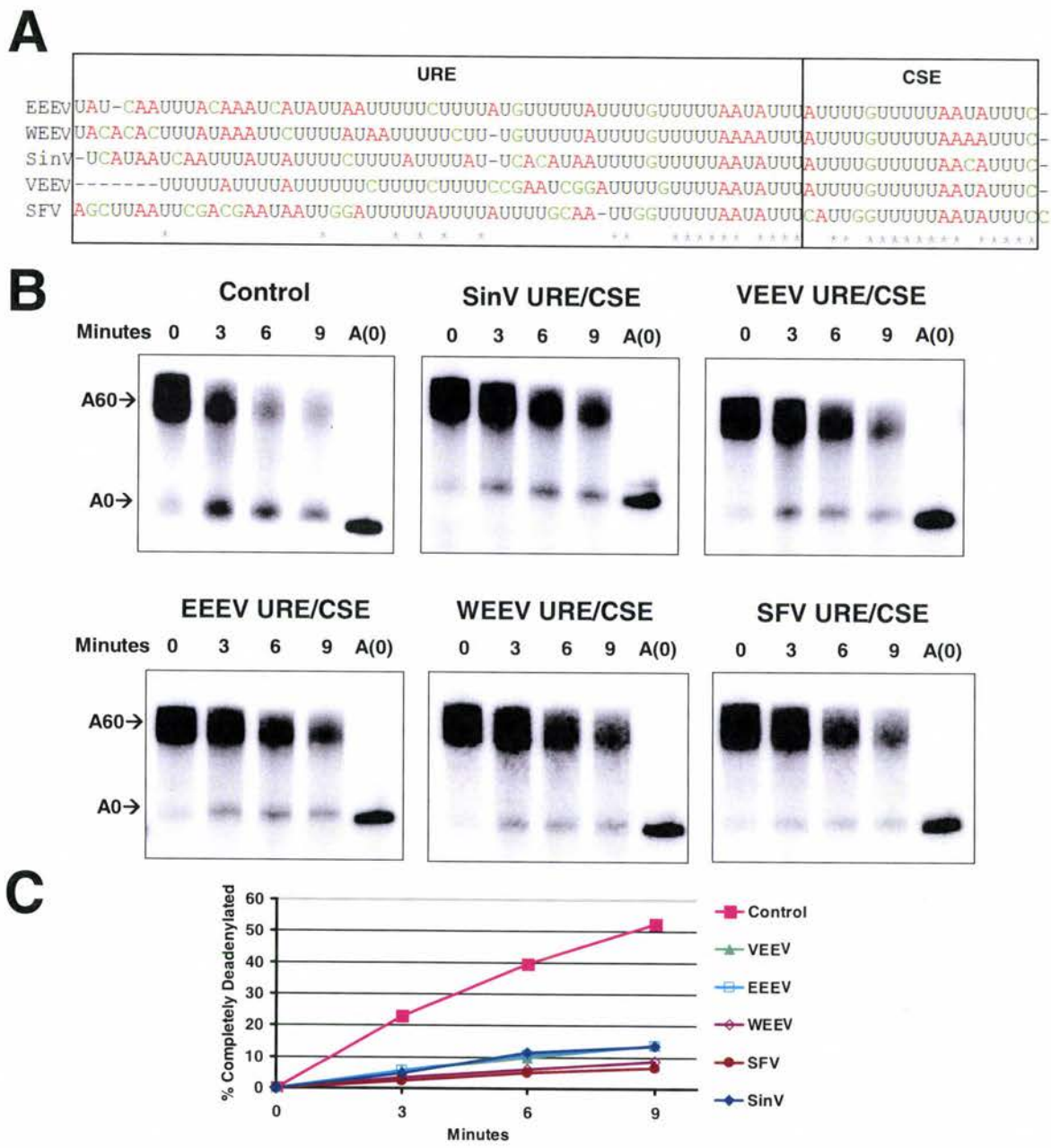


Figure 7- Several Alphavirus URE/CSE Fragments Repress Deadenylation in a Cell Free System. (A) Alignment of the URE/CSE fragments examined in panel B. Asterisks denote conserved residues. Colors are added for nucleotide emphasis. **(B)** Adenylated RNA substrates containing either vector sequences (Control) or the Alphaviral URE/CSE fragments indicated above were incubated in the presence of *Aedes albopictus* C6/36 cell cytoplasmic extract for the indicated times. Reaction products were examined using 5% denaturing PAGE and visualized via phosphorimaging. The A(0) lane denotes the migration of an unadenylated RNA substrate. Markers on the left denote input RNA substrates that contain a poly(A) tail of 60 residues (A60) or a fully deadenylated RNA (A0). **(C)** Graphical representation of the data observed in panel B.

As with the previous assay, 5' capped and 3' polyadenylated RNA substrates consisting of the URE/CSE fragments of VEEV, EEEV, WEEV and SFV RNAs were incubated in our *in vitro Aedes albopictus* mRNA decay system. As exhibited in Fig. 7B, RNA substrates containing the UREs of these other alphaviruses also demonstrated remarkable stability relative to the control substrate. A graphical representation of the rates of deadenylation is shown in Fig. 7C.

These findings support our hypothesis that the URE is an RNA stability element found in the 3'UTRs of many alphaviruses, including those associated with significant clinical illness. Furthermore, it strongly suggests that our examination of the SinV RNA 3'UTR will give insight into the biology of these other alphaviruses by illuminating a conserved function of the *Alphavirus* 3'UTR.

SinV RNA 3'UTR Mediated Repression of Deadenylation is Transcript-Specific and Mediated by a Cellular *Trans*-Acting Factor

The repression of deadenylation observed both in cell culture and *in vitro* could be potentially due to a *cis*-acting feature of the viral 3'UTR or to the binding of cellular factors. *Cis*-elements capable of stabilizing the poly(A) state of a transcript have been previously described, notably the PAN-ENE element (Conrad et al., 2006; Conrad and Steitz, 2005). To determine which of these mechanisms was responsible for the stabilization of the viral 3'UTR, we chose to utilize competition analysis. By varying the RNA substrate and the competitor

concentrations we could examine both the possibilities of a *cis*- and a *trans*-acting feature. First, excess cold competitor RNAs of either the control substrate or the SinV RNA 3'UTR were concomitantly incubated with the unstable control RNA substrate. As depicted in Fig. 8 panels A and B, the addition of neither excess control nor excess SinV RNA 3'UTR competitor altered the rate of deadenylation of the unstable control substrate. These data indicate that the repression of deadenylation associated with the SinV RNA 3'UTR was transcript-specific. As the repression was not extended towards other transcripts during co-incubation, the elements in the 3'UTR of SinV are unlikely to be acting by enforcing general inhibition of the deadenylation machinery.

The converse experiment, whereby the stable polyadenylated SinV RNA 3'UTR was incubated in the presence of excess SinV RNA 3'UTR competitor RNA was more revealing. Addition of nonspecific competitor RNA failed to appreciably change the repression of deadenylation associated with the stable SinV RNA 3'UTR as shown in Fig. 8C. In contrast, addition of excess SinV RNA 3'UTR competitor abrogated the repression of deadenylation *in vitro*. (Fig. 8D).

The activation of deadenylation in the presence of excess SinV RNA 3'UTR competitor strongly suggests that a limiting cellular factor was necessary for stabilization of the poly(A) tail *in vitro*. Interestingly, the decay kinetics of the SinV RNA 3'UTR in the presence of specific competitor RNAs were distributive-like. This may be potentially explained by the presence of multiple elements (the RSEs and URE) that regulate the stability of the SinV RNA 3'UTR. It appears that

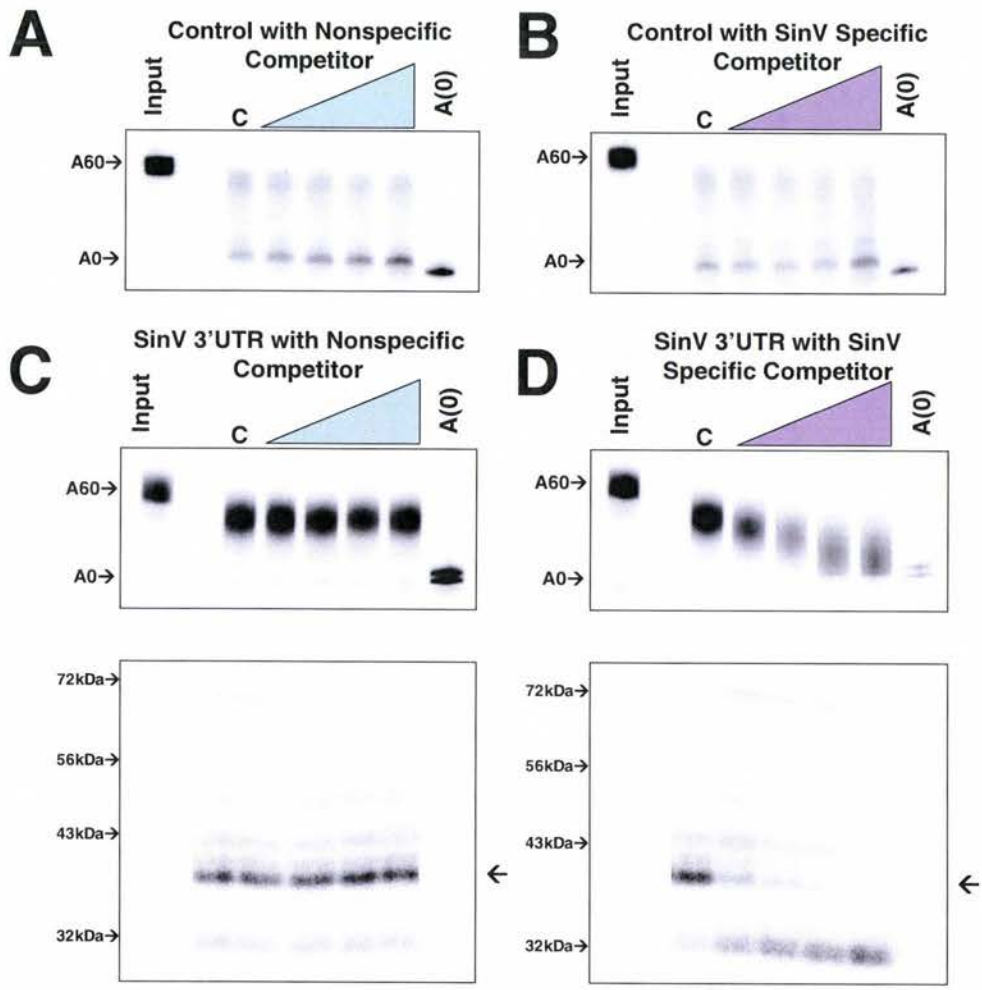


Figure 8- Addition of Excess Competitor RNAs Activates the Deadenylation of the SinV RNA 3'UTR in a Cell Free System. (A) Adenylated Control RNA substrates were incubated with *Aedes albopictus* C6/36 cell cytoplasmic extract for 9 minutes in the presence of increasing nonspecific competitor RNAs. The lane denoted with "C" indicates a reaction incubated with water instead of competitor RNA. Concentrations of the excess competitor RNAs began at 12.5x (molar excess) and increased 2-fold incrementally. RNA species were examined using phosphorimaging following 5% denaturing PAGE. **(B)** Identical to panel A with the exception that the competitor RNA contained the SinV RNA 3'UTR. **(C)** Essentially the same as panel A with the major exception that the RNA substrate contained the 3'UTR of SinV. Below is a paired UV cross-linked examination of the RNA-binding proteins. The reaction mixtures from the above competition analyses were split with one half being UV irradiated to form covalent mRNP complexes. The RNA was degraded by the addition of RNase and the radiolabeled protein species examined using 10% SDS-PAGE. The arrow indicates the 38kD species of interest. **(D)** Identical to panel C with the exception that the competitor RNA contained the SinV RNA 3'UTR. Gels are representative of two independent replicates.

the repression associated with the URE has been relieved, and the decay kinetics are similar to that observed with the RSE domains.

In order to determine whether relief of repression correlated with dissociation of specific RNA-binding proteins, the deadenylation reaction mixtures were split and one half was subjected to UV cross-linking following addition of EDTA to inhibit RNA decay. Several host factors (since the extracts are uninfected) are observed binding the SinV RNA 3'UTR and these factors range widely in molecular weight. In the presence of limiting amounts of RNA substrate the predominant band is at approximately 38kDa. Increasing the amount of RNA substrate used (as observed in the successive UV cross-links; for example Fig. 9) increases the number of bands observed and diminishes the intensity of the 38kDa factor. As exhibited in Figure 8C, the addition of nonspecific competitor does not significantly affect the pattern of RNA binding proteins for either the unstable control substrate or the stable SinV RNA 3'UTR. In contrast, as exhibited in Figure 8D, the addition of increasing amounts of SinV RNA 3'UTR specific competitor results in the removal of a 38kDa cellular factor correlating with the activation of deadenylation. Curiously a second factor of approximately 32kDa in molecular weight is observed to associate with the viral 3'UTR in the absence of the putative 38kDa stability factor.

Thus we conclude that the stability of SinV RNA 3'UTR is at least in part a function of a *trans*-acting mechanism. Furthermore the repression of deadenylation *in vitro* is correlated with the binding of a cellular 38kDa factor. In

the absence of this factor, a binding site is made available for a 32kDa host factor on the SinV RNA 3'UTR.

The 38kDa Factor Binds to the URE/CSE Fragment of the SinV RNA 3'UTR

Following the correlation of binding of a 38kDa cellular factor to the SinV RNA 3'UTR with the repression of deadenylation *in vitro,* we next aimed to refine our knowledge of the SinV:38kDa protein interaction. Utilizing the SinV RNA 3'UTR fragments shown diagrammatically in Fig. 6A in conjunction with UV cross-linking, we were able to delineate the region recognized by the 38kDa host factor.

The binding of the 38kDa cellular factor is associated with the URE/CSE fragment of the SinV RNA 3'UTR. Interestingly both components of this fragment, the URE and CSE, cross-link to the 38kDa factor (Fig. 9). Additionally, the 32kDa factor appears to cross-link to all regions of the viral 3'UTR, suggesting a possible lack of binding specificity for this factor.

As demonstrated earlier the URE/CSE regions of VEEV, EEEV, WEEV and SFV RNAs are also capable of repressing deadenylation *in vitro*. We therefore wished to determine if this repression to deadenylation correlated with the binding of a 38kDa factor. As shown in Figure 10, the URE/CSEs of all 4 clinically significant alphaviruses RNAs also show UV cross-linking patterns nearly identical to that of SinV. To demonstrate that the same 38kDa factor was binding the VEEV and SinV RNAs we used cross-competition analysis (Fig. 11).

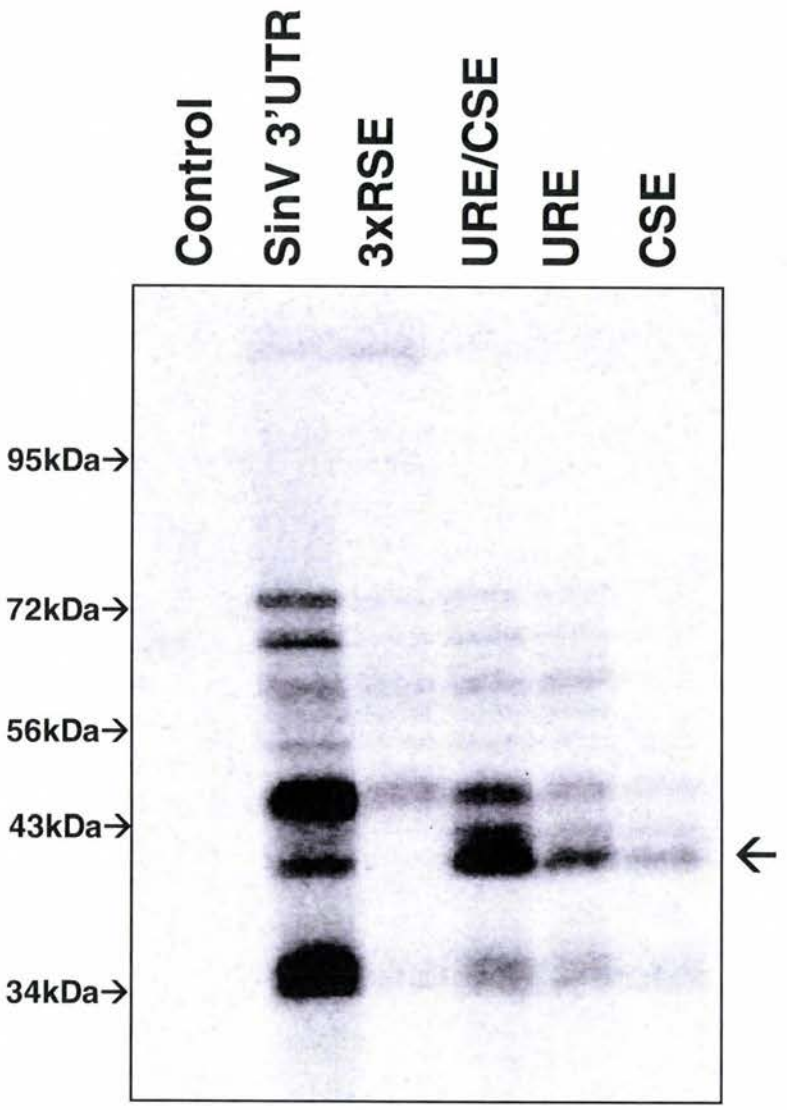


Figure 9- Ultraviolet Cross-Linking of the SinV RNA 3'UTR Elements Reveals the Binding of the 38kD Factor to the URE/CSE. Equimolar amounts of the indicated unadenylated SinV RNA 3'UTR fragment RNA substrates were incubated in the presence of *Aedes albopictus* C6/36 cell cytoplasmic extract prior to being irradiated with UV light to form covalent mRNP complexes. The control lane consisted of the control RNA substrate utilized in Fig. 5. The RNA was degraded by the addition of RNase and the radiolabeled protein species examined using 10% SDS-PAGE. The arrow indicates the 38kD species of interest. Gel shown is representative of three independent UV cross-link reactions.

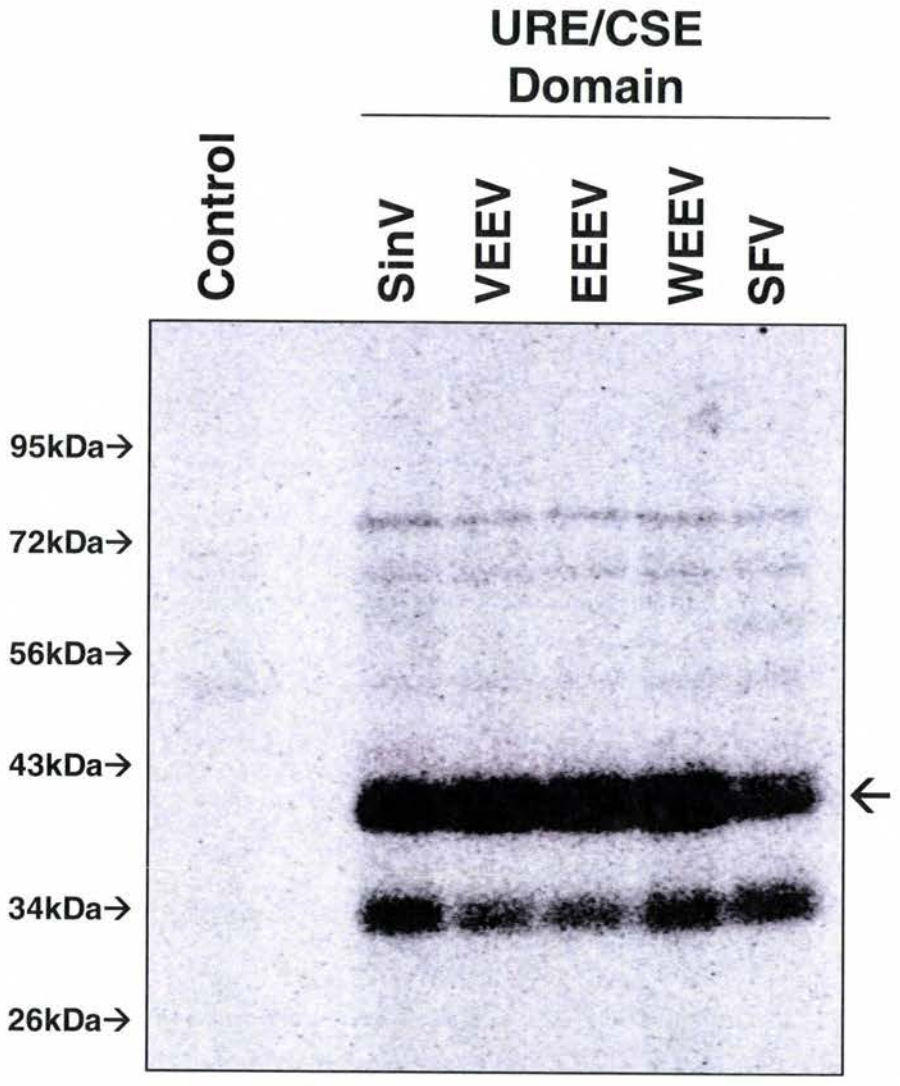


Figure 10- Ultraviolet Cross-linking of the URE/CSE Domains of Several Alphavirus RNAs Reveals Conservation of the Interaction with the 38kD Factor. Equimolar amounts of unadenylated Alphavirus URE/CSE fragments from the Sindbis (SinV), Venezuelan equine encephalitis (VEEV), eastern equine encephalitis (EEEV), western equine encephalitis (WEEV) and Semliki Forest (SFV) viruses RNAs were incubated in the presence of *Aedes albopictus* C6/36 cell cytoplasmic extract prior to being irradiated with UV light to form covalent mRNP complexes. The control lane consisted of the control RNA substrate utilized in Fig. 5. The RNA was degraded by the addition of RNase and the radiolabeled protein species examined using 10% SDS-PAGE. The arrow indicates the 38kD species of interest. Gel shown is representative of three independent replicates.

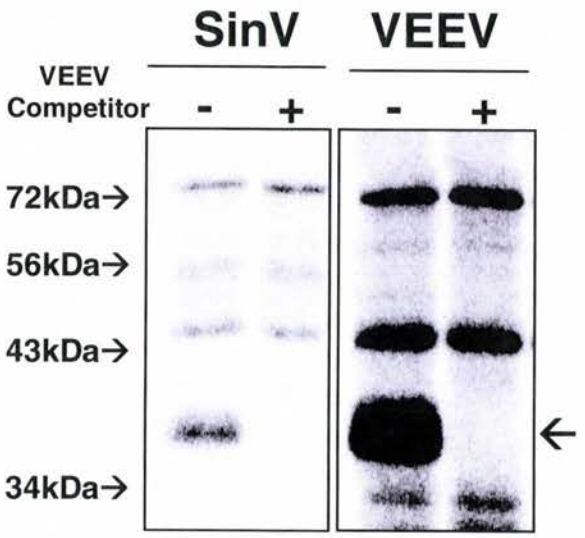


Figure 11- Cross Competition of the 3'UTRs of SinV and VEEV RNAs Reveals Interaction with the Same 38kD Host Factor. The 3'UTRs from Sindbis virus (SinV) and Venezuelan equine encephalitis virus (VEEV) RNAs were incubated with *Aedes albopictus* C6/36 cell cytoplasmic extract in the presence (+) or absence (-) of VEEV 3'UTR competitor RNA prior to being irradiated with UV light to form covalent mRNP complexes. The RNA was degraded by the addition of RNase and the radiolabeled proteins examined using 10% SDS-PAGE. The arrow indicates the 38kD protein of interest. Gel shown is representative of two independent replicates.

UV cross-linking of either the SinV or VEEV viral 3'UTRs in the presence of excess unradiolabeled VEEV RNA 3'UTR confirmed that the 38kDa factor was indeed shared between the two alphavirus RNAs as shown in Fig. 11. These data indicate that the 38kDa putative stability factor interacts within the URE/CSE fragment of the SinV RNA 3'UTR. Further analysis indicates that both the URE and CSE are capable of interacting with the 38kDa factor. This observation is interesting since the URE fragment affords far greater stability to an RNA substrate than the CSE, which exhibits only modest stability as compared to the control RNA substrate. Both regions exhibit a general U-rich nature, suggesting that the 38kDa factor may have a preference for uridine. The conservation of RNA binding patterns for evolutionarily divergent alphaviruses strongly suggests that this interaction is important to Alphavirus biology.

The RSEs of SinV Repress Deadenylation in a Context-Dependent Manner *In Vitro*

As described earlier, the 3xRSE fragment of the SinV RNA 3'UTR was capable of repressing deadenylation *in vitro*. Nevertheless, as shown above in Figs 8 and 9, the binding of the 38kDa putative stability factor correlates with the presence of the URE/CSE region of the viral 3'UTR. Given these observations we conclude that the RSEs are repressing deadenylation *in vitro* through an alternative mechanism from the URE/CSE:38kDa interaction. The 3xRSE fragment of SinV consists of a set of 3 Repeated Sequence Elements that are projected to exhibit significant secondary structure (Ou et al., 1982). To examine

the stability imparted by this region of the viral 3'UTR we chose to further divide the 3xRSE substrate into a single RSE, namely the third RSE.

In our mosquito *in vitro* decay system, a single SinV RSE conferred moderate stability onto an RNA substrate with distributive-type decay kinetics similar to the 3xRSE fragment (Fig. 12A; Garneau et al., 2008). We next sought to determine if the RSEs were capable of blocking deadenylation *in vitro* by a *cis*-acting *or trans*-acting mechanism. To examine this phenomenon we incubated polyadenylated RNA substrates containing the third RSE of the SinV RNA in the presence of recombinant *Aedes aegypti* PARN (obtained from John Anderson, Colorado State University) in lieu of cytoplasmic extract. As shown in Fig. 12B in the absence of other factors the third RSE of SinV was capable of repressing deadenylation in this reconstituted system. These data, along with the observation that competition assays using the RSE fragment failed to activate deadenylation of RNA substrates, suggests that the RSE is repressing deadenylation by a *cis*-acting mechanism.

We next questioned if the RSE exhibited any contextual dependence. If the RSE is shifted towards the 3' end of the RNA substrate, thereby extending the distance between the RSE and the 5' terminus, we observed deadenylation of the previously stable fragment (Fig. 12B). These data suggest that the repression of deadenylation mediated by the RSE in our mosquito *in vitro* decay system may be dependent on the position and or context of the RSE. The possible mechanisms and implications of these observations are discussed later.

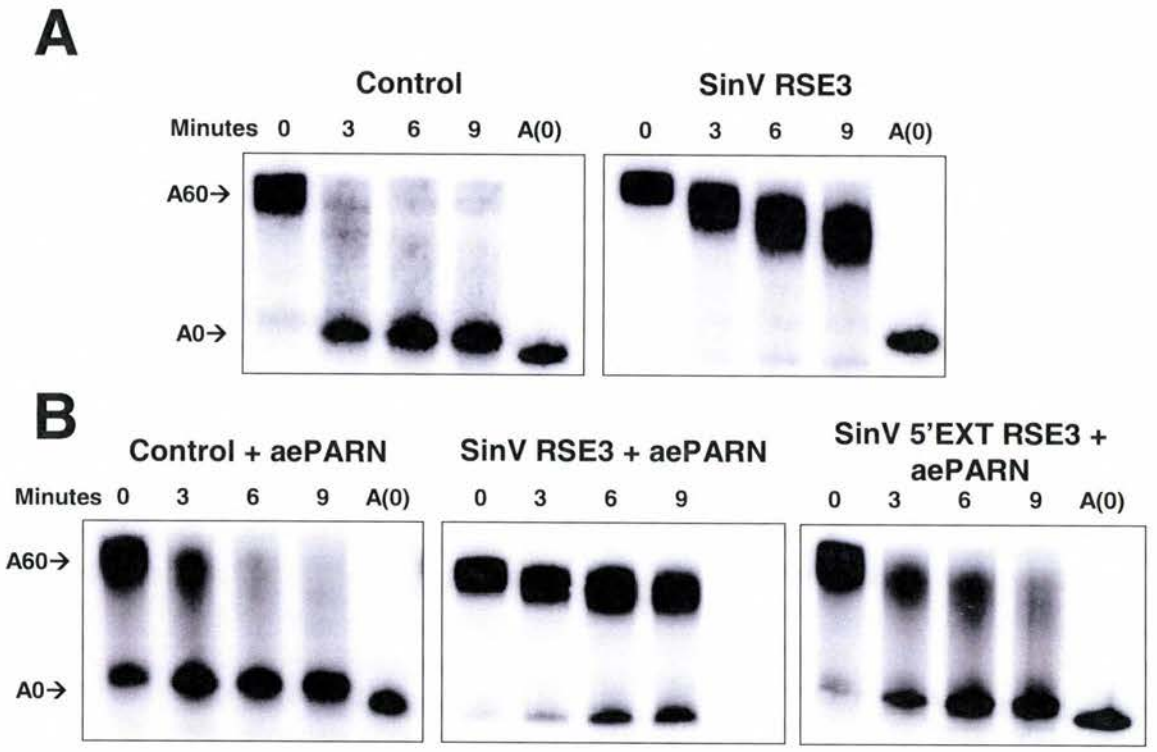


Figure 12- A SinV RSE Represses Deadenylation *in vitro* in a Context Dependent Manner. (A) Adenylated RNA substrates containing either vector sequences (Control) or the indicated fragments of the 3'UTR of the SinV RNA were incubated in the presence of *Aedes albopictus* C6/36 cell cytoplasmic extract for the indicated times. Deadenylation products were examined using 5% denaturing PAGE and visualized by phosphorimaging. **(B)** Control or RNA substrates containing RSE3 of the 3'UTR of the SinV RNA either 15 nucleotides from the 5' terminus or 60 nucleotides from the 5' terminus (SinV 5'EXT RSE3) were incubated in the presence of 4µgs of recombinant aePARN protein. The A0 lane denotes the migration of an unadenylated RNA substrate. Markers on the left denote input RNA substrates that contain a poly(A) tail of 60 residues (A60) or a fully deadenylated RNA (A0). Gels shown are representative of two independent replicates.

These data, taken in conjunction with the above observations of our ability to compete away the repression of deadenylation associated with the entire 3'UTR, leading to distributive-like kinetics, suggest that the primary mediator of stability is the URE, not the RSEs, *in vitro*. It is for this reason that we have chosen to focus our efforts on characterizing the role of the URE in both viral RNA decay and replication.

Affinity Purification of SinV URE/CSE Binding Factors Reveals that the 38kDa Protein is an ELAV Superfamily Member

Delineation of the minimal binding elements of the 38kDa factor allowed for the development of an affinity purification scheme. Purification and mass spectrometric analysis of the specifically bound RNA-binding proteins would identify candidate proteins for further examination. Unfortunately, to date, characterization of the *Aedes albopictus* genome is largely incomplete. To overcome this challenge, the *Aedes aegypti* genome was used as a surrogate for the bioinformatic analysis of candidate protein factors.

The relative stability of the 38kDa protein – SinV RNA interaction in the presence of varying salt and detergent concentrations was examined using UV cross-linking and the URE/CSE fragment. It was found that the protein-RNA interaction was stable in the presence of at least up to 250mM NaCl and 0.5% NP-40 (data not shown). A set of 5' biotinylated RNA oligomers consisting of the control RNA substrate or the URE/CSE fragment of the SinV RNA (Fig. 13A) was incubated in the presence of C6/36 cytoplasmic extract. After incubation at

A

Control Affinity Purification Oligo-
5'-Biotin-GAAUCGAGCUCGGUACCCGGGGAUCCUCUAGAGUCGACCUGCAG-3'

SinV Affinity Purification Oligo-
5'-Biotin-UAAUCAAUUUAUUAUUUUCUUUUAUUUUAUUCACAUAAUUUUGUUUUUAA-3'

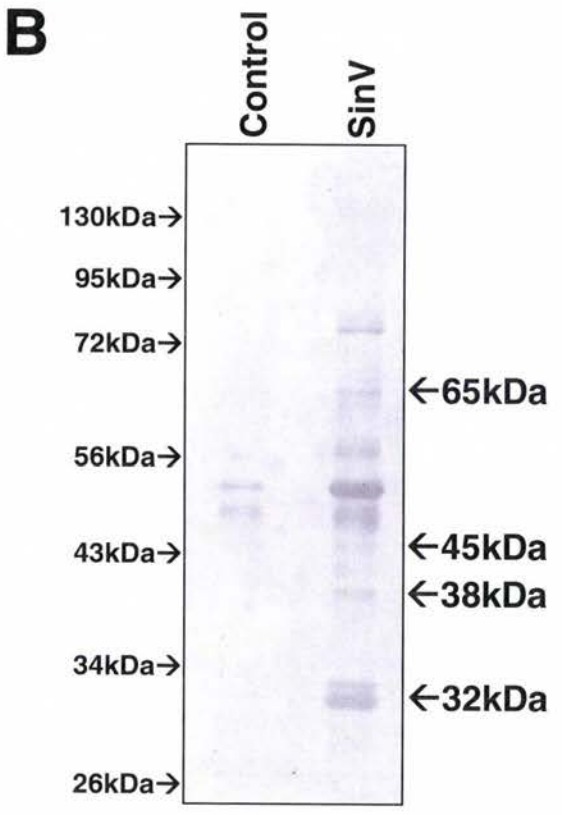


Figure 13- Affinity Purification Using the SinV URE/CSE Fragment Yields Several Cellular Factors. (A) Sequences of the 5' biotinylated oligonucleotides used for the affinity purification displayed in panel B. (B) Equivalent amounts of either Control or SinV affinity purification oligos were incubated in the presence of *Aedes albopictus* C6/36 cell extract prior binding to streptavidin agarose. After rigorous washing the purified RNPs were eluted via the addition of SDS and high salt. The purified proteins were precipitated and resolved using 10% SDS-PAGE. Post-electrophoresis the gel was silver stained. The arrows denote the various protein bands analyzed by mass spectrometry.

4°C to allow formation of RNP complexes, the biotinylated RNAs were bound to streptavidin-agarose beads and washed several times with Buffer D prior to several additional washes with a high stringency buffer (Buffer D supplemented with 250mM NaCl and 0.5% NP-40). The bound proteins were eluted via the addition of HSCB, concentrated and resolved by 10% SDS-PAGE. As exhibited in Fig. 13B, several proteins were indeed selectively bound to the SinV RNA URE/CSE element when compared to the control RNA substrate. The 38kDa protein was selected by its empirical molecular weight as observed by UV cross-linking. The 32kDa protein was chosen using similar criteria. Other factors (at 65kDa and 45kDa) were chosen for analysis due to their appearance in UV cross-linking assays.

Following destaining and in-gel trypsin digestion the candidate proteins were analyzed using a Bruker Daltonics MALDI-TOF/TOF mass spectrometer. The resulting molecular weights and peptide fragments were utilized in a bioinformatic search of the *Aedes aegypti* database.

As shown in Fig. 14A, the results from the mass spectrometry analysis performed on the 38kDa species revealed an exceptional degree of amino acid identity for an ELAV superfamily member with notable homology to HuR, a known mammalian stability factor (Brennan and Steitz, 2001). The statistical analysis for this identification was found to be significant (with a P-value of < 0.05, as indicated by MASCOT analysis), indicating that the purified protein was likely a *bona fide* HuR homolog. Alignment of human and *Aedes aegypti* HuR

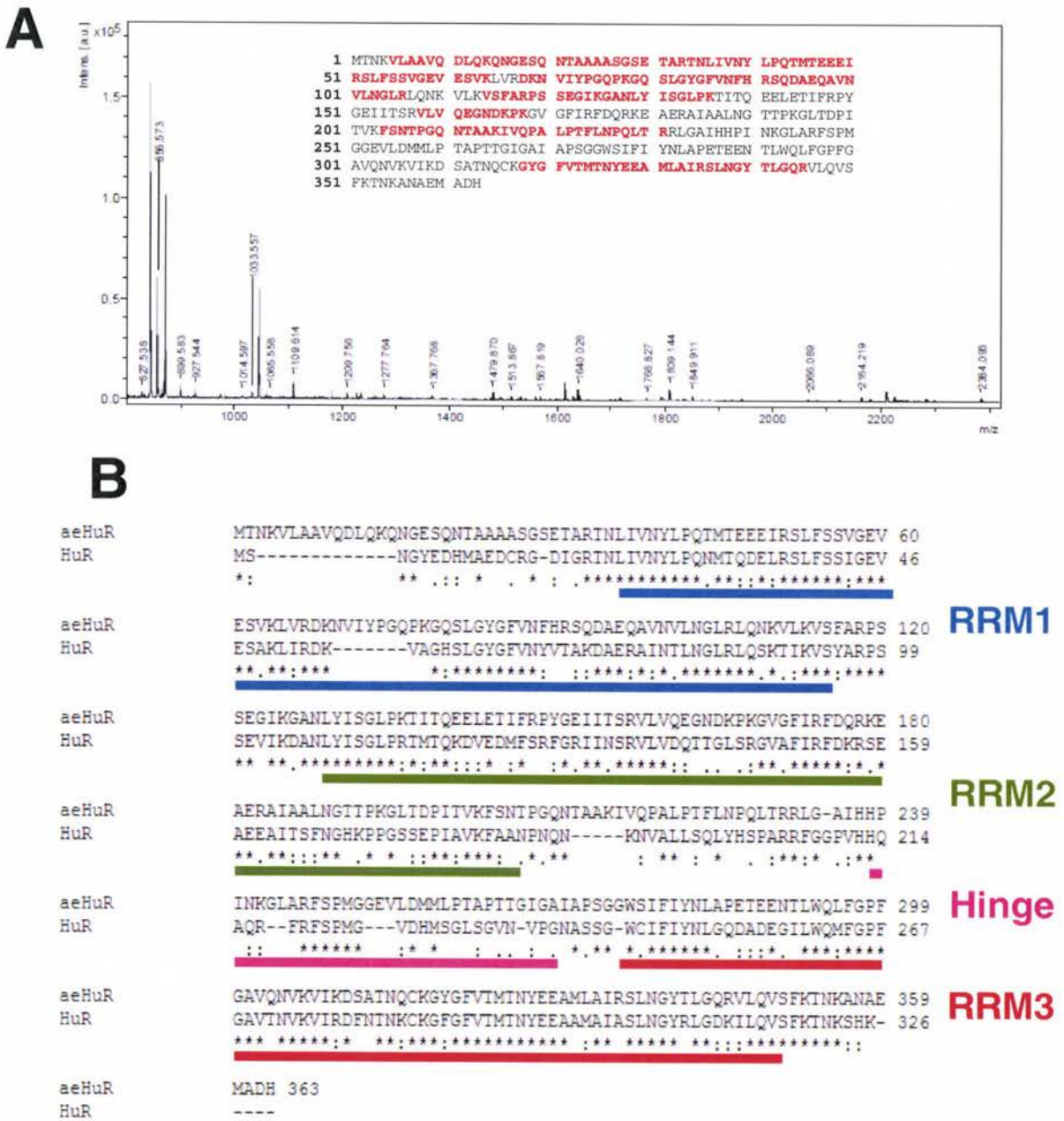
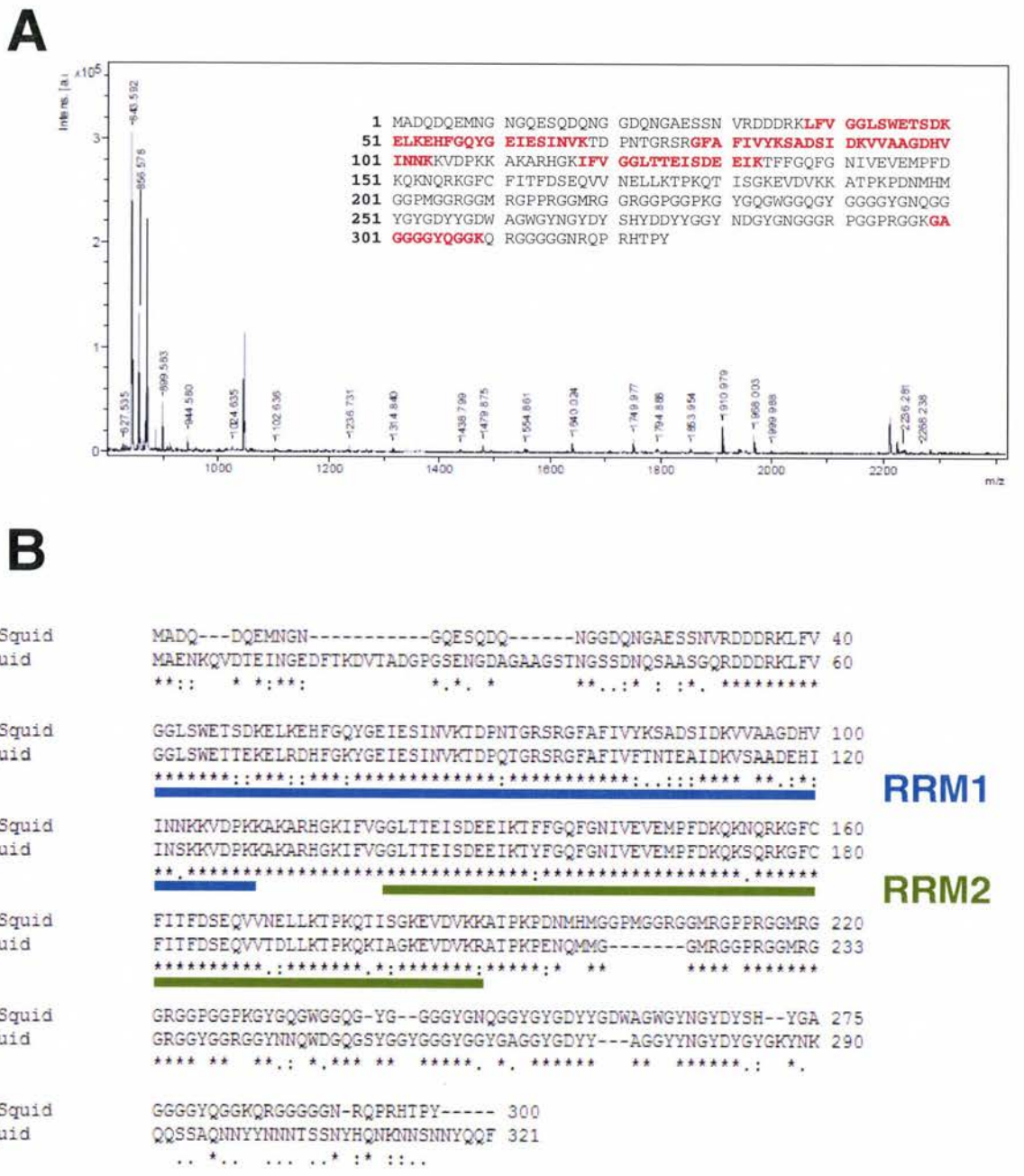


Figure 14- Mass Spectrometric Analysis Reveals Homology of the 38kDa Protein with a Known mRNA Stability Factor. (A) Spectrum obtained from the excised 38kDa band in Fig. 13B. Embedded is a sequence coverage map of the mosquito homology hit identified by Mascot search of the *Aedes aegypti* genome. (B) Alignment of cellular HuR proteins from *Aedes aegypti* (aeHuR) and *Homo sapiens (HuR)*, showing high degree of similarity. The individual RRMs are underlined as well as the Hinge region. The alignment was generated using ClustalW.

proteins revealed a high degree of similarity (Fig. 14B), with 55% identity and 68% similarity. Notably the RRM motifs are highly conserved amongst the two HuR proteins. Interestingly, regions required for the nuclear import of mammalian HuR proteins are not conserved in the *Aedes aegypti* HuR protein (Fig 14B). Given this high degree of homology, we have chosen to refer to the 38kDa protein as aeHuR henceforth.

As demonstrated in Fig. 15A the analysis of the 32kDa candidate band revealed homology to mammalian hnRNP A1/B2 proteins. Once again, the statistical significance of these analyses (P-value of < 0.05) pointed to a high probability that the excised band was indeed the mosquito homolog of this factor. A further bioinformatic search determined that the 32kDa protein also had a significant similarity to *Drosophila* Squid (Fig. 15B), a protein associated with polarized localization of mRNAs and translational modulation in developing *Drosophila melanogaster* embryos.

Two other proteins associated with the viral URE/CSE fragment were identified via mass spectrometry. It should be noted however, that the binding of these factors did not correlate with stability as observed in our competition analyses. The mass spectrometric analysis of the 65kDa band purified with the viral sequence was revealed to be a mosquito homolog of the cytoplasmic Poly(A) Binding Protein (PABPC1). This finding is curious, given that the RNA substrate utilized in the affinity purification above lacked a poly(A) tail. It is possible that the PABPC1 homolog is interacting with either the viral RNA, or is co-purifying via a protein:protein interaction. It should be noted that interactions



B

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aeSquid         MADQ---DQEMNGN----------GQESQDQ------NGGDQNGAESSNVRDDDRKLFV 40
Squid           MAENKQVDIEINGEDFTKDVTADGPGSENGDAGAAGSTNGSSDNQSAASGQRDDDRKLFV 60
                **::   * *:**:          *.*. *       **..:* : :*. *********

aeSquid         GGLSWETSDKELKEHFGQYGEIESINVKTDPNTGRSRGFAFIVYKSADSIDKVVAAGDHV 100
Squid           GGLSWETTEKELRDHFGKYGEIESINVKTDPQTGRSRGFAFIVFTNTEAIDKVSAADEHI 120
                *******::***::***:************:***********:..:::**** **.:*:
                ═══════════════════════════════════════════                    RRM1

aeSquid         INNKKVDPKKAKARHGKIFVGGLTTEISDEEIKTFFGQFGNIVEVEMPFDKQKNQRKGFC 160
Squid           INSKKVDPKKAKARHGKIFVGGLTTEISDEEIKTYFGQFGNIVEVEMPFDKQKSQRKGFC 180
                **.*********************************:******************.******
                ═══════                ═══════════════════════════════          RRM2

aeSquid         FITFDSEQVVNELLKTPKQTISGKEVDVKKATPKPDNMHMGGPMGGRGGMRGPPRGGMRG 220
Squid           FITFDSEQVVTDLLKTPKQKIAGKEVDVKRATPKPENQMMG-------GMRGGPRGGMRG 233
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aeSquid         GRGGPGGPKGYGQGWGGQG-YG--GGGYGNQGGYGYGDYYGDWAGWGYNGYDYSH--YGA 275
Squid           GRGGYGGRGGYNNQWDGQGSYGGYGGGYGGYGAGGYGDYY---AGGYYNGYDYGYGKYNK 290
                **** **  **.: *.*** **  *****. *. ******   **  ******.:  *.

aeSquid         GGGGYQGGKQRGGGGGN-RQPRHTPY----- 300
Squid           QQSSAQNNYYNNNTSSNYHQNKNNSNNYQQF 321
                  .. *..  ... ..* :* ::..
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Figure 15- Mass Spectrometric Analysis Reveals Homology of the 32kDa Factor with the Drosophila Squid Proteins. (A) Spectra obtained from the excised 32kDa band in Fig. 13B. Embedded is a sequence coverage map of the mosquito homology hit identified by Mascot search of the *Aedes aegypti* genome. (B) Alignment of Squid from *Aedes aegypti* (aeSquid) and *Drosophila melanogaster* (Squid), showing similarity. The RRMs are underlined. The alignment was generated using ClustalW.

between PABPC1 and HuR have been previously observed (Nagaoka et al., 2006). Additionally, a fourth factor at approximately 45kDa was identified as the mosquito La homolog; an interaction between La and the RNAs of SinV has been previously established (Pardigon and Strauss, 1996).

Immunoprecipitation Confirms the Identities of the 38kDa and 32kDa Factors

Despite having identifications of the 38kDa putative stability factor and the 32kDa alleged instability factor by mass spectrometry, the proteins observed via UV cross-linking may not be the same. Confirmation of the identities of the factors was approached using immunoprecipitation of proteins cross-linked to the SinV 3'UTR elements. In our experience the cross reactivity of *Drosophila* antibodies to mosquito proteins aeHuR, aeSquid and La is quite poor (data not shown). To overcome this challenge, we therefore needed to develop reliable immunological reagents.

The open reading frames corresponding to aeHuR (AAEL008164-RA) and aeSquid (AAEL005515) were amplified using PCR from cDNA produced from random hexamer-primed *Aedes aegypti* total RNA. The resulting aeHuR and aeSquid fragments were gel purified and cloned into pGEX-2TZQ (Qian and Wilusz, 1994) or pTricHis-A, respectively. These clones were then transformed into *E. coli* BL21 (DE3) and induced to express recombinant aeHuR and aeSquid.

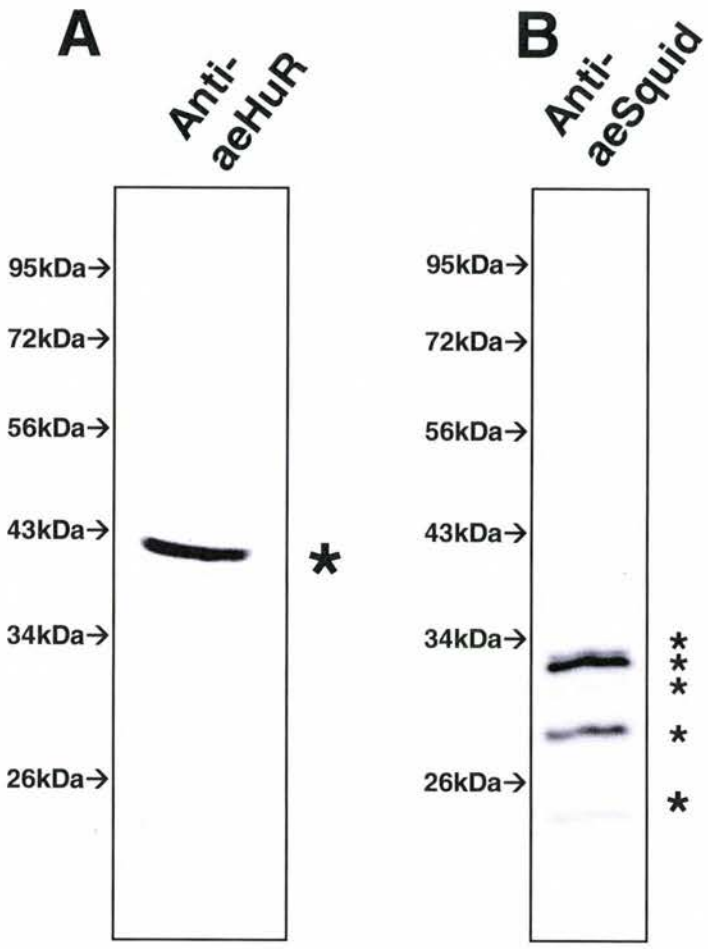

Figure 16- Polyclonal Sera Developed from Recombinant aeHuR and aeSquid Demonstrate High Specificity in Western Blotting. (A) A total of 25ug of *Aedes albopictus* C6/36 cell cytoplasmic extract was resolved by 10% SDS-PAGE prior to western blotting. After transferring the proteins to PVDF membrane the membrane was probed for aeHuR using the anti-aeHuR antibody developed in this study at a dilution of 1:1000. The band corresponding to aeHuR is indicated by an asterisk. (B) Identical to panel A with the exception that anti-aeSquid was employed as the primary antibody. The five putative isoforms (as determined by molecular weight) are indicated by asterisks.

Recombinant proteins were batch purified on glutathione agarose or nickel resin for aeHuR and aeSquid respectively and used to immunize rabbits to generate polyclonal antisera. For the anti-aeHuR serum we generated, a single species of ~38kDa was detected in C6/36 cytoplasmic extract as observed by western blotting (Fig. 16A). As shown in Fig. 16B, the anti-aeSquid antibody produced a set of bands upon western blotting to C6/36 cell lysates, likely corresponding to the multiple isoforms of Squid seen in *Drosophila* (Norvell et al., 1999) or degradation products. Preimmune control sera failed to detect any protein species via western blotting (data not shown).

We used these newly developed polyclonal antibodies to confirm the identities of the 38kDa and 32kDa factors on cross-linked samples. The URE/CSE elements of the five alphaviruses used above (Fig. 7) were incubated in the presence of C6/36 *Aedes albopictus* cytoplasmic extracts and irradiated with UV light to form RNP complexes. Following RNase treatment of the cross-linked RNPs the samples were diluted in NET2 buffer and polyclonal serum specific to either aeHuR or aeSquid was added. The RNP:antibody complexes were then removed from solution via formalin fixed protein A-positive *S. aureus* cells and centrifugation. The purified complexes were vigorously washed and the immunoprecipitated species were resolved in parallel to the input cross-linked materials. As exhibited in Fig. 17, the 38kDa species associated with the repression of deadenylation by the SinV 3'UTR *in vitro* was confirmed to be aeHuR. The aeHuR protein also bound to the URE/CSE regions of VEEV, EEEV, WEEV and SFV RNAs. We next were curious if this interaction was unique to

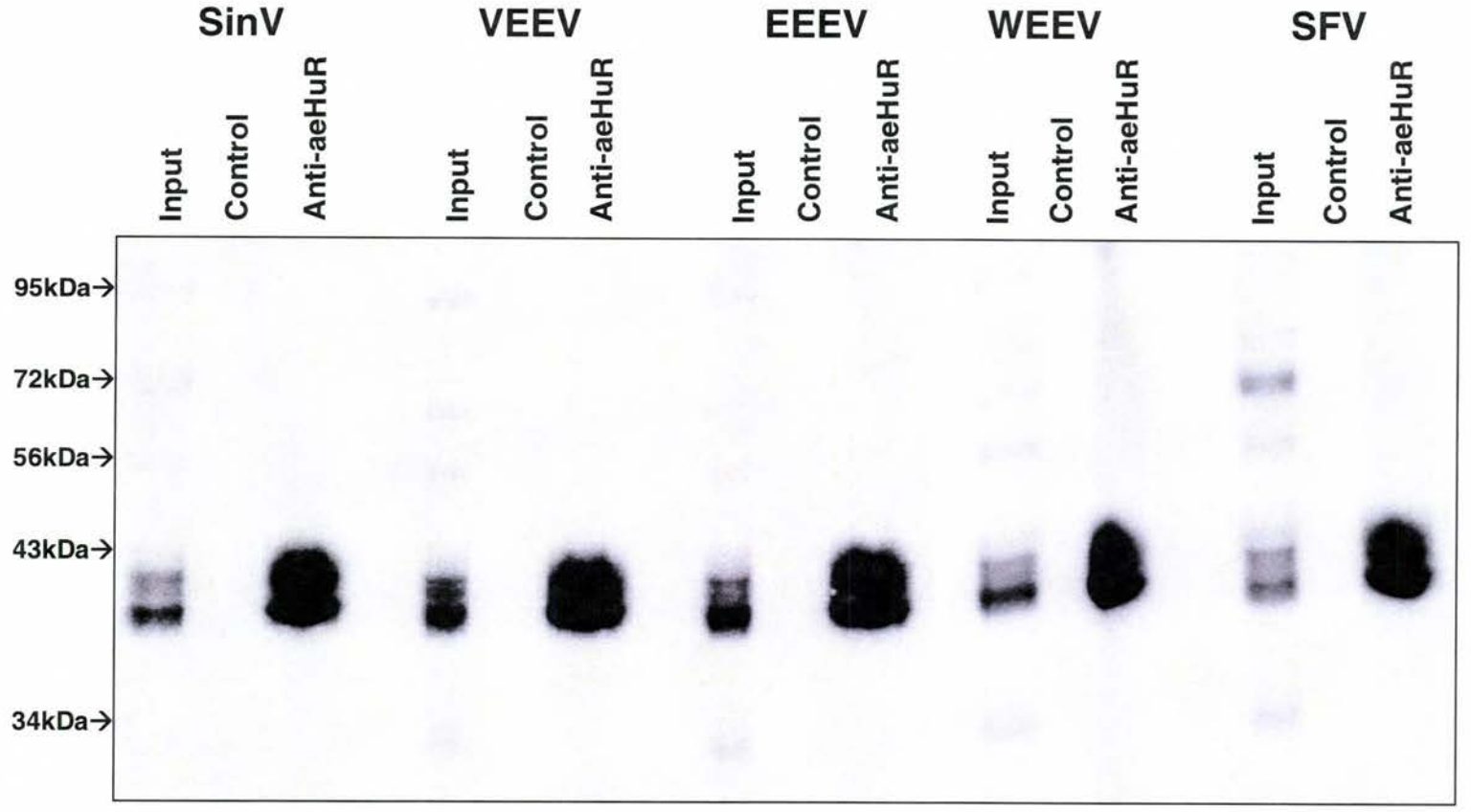

Figure 17- Immunoprecipitation of aeHuR Confirms the Identity of the Cross-Linked 38kD Factor as aeHuR. The URE/CSE fragments of the SinV, VEEV, EEEV, WEEV and SFV RNAs were UV cross-linked to their associated proteins in *Aedes albopictus* C6/36 cytoplasmic extract. After RNase treatment the radiolabeled RNP components were immunoprecipitated using either preimmune (Control) serum or anti-aeHuR serum prior to resolution using 10% SDS-PAGE. Input lanes represent 5% of the UV cross-linked proteins used in the immunoprecipitations. Radiolabeled / immunoprecipitated proteins were detected via phosphorimaging. Gels shown are representative of at least two independent replicates.

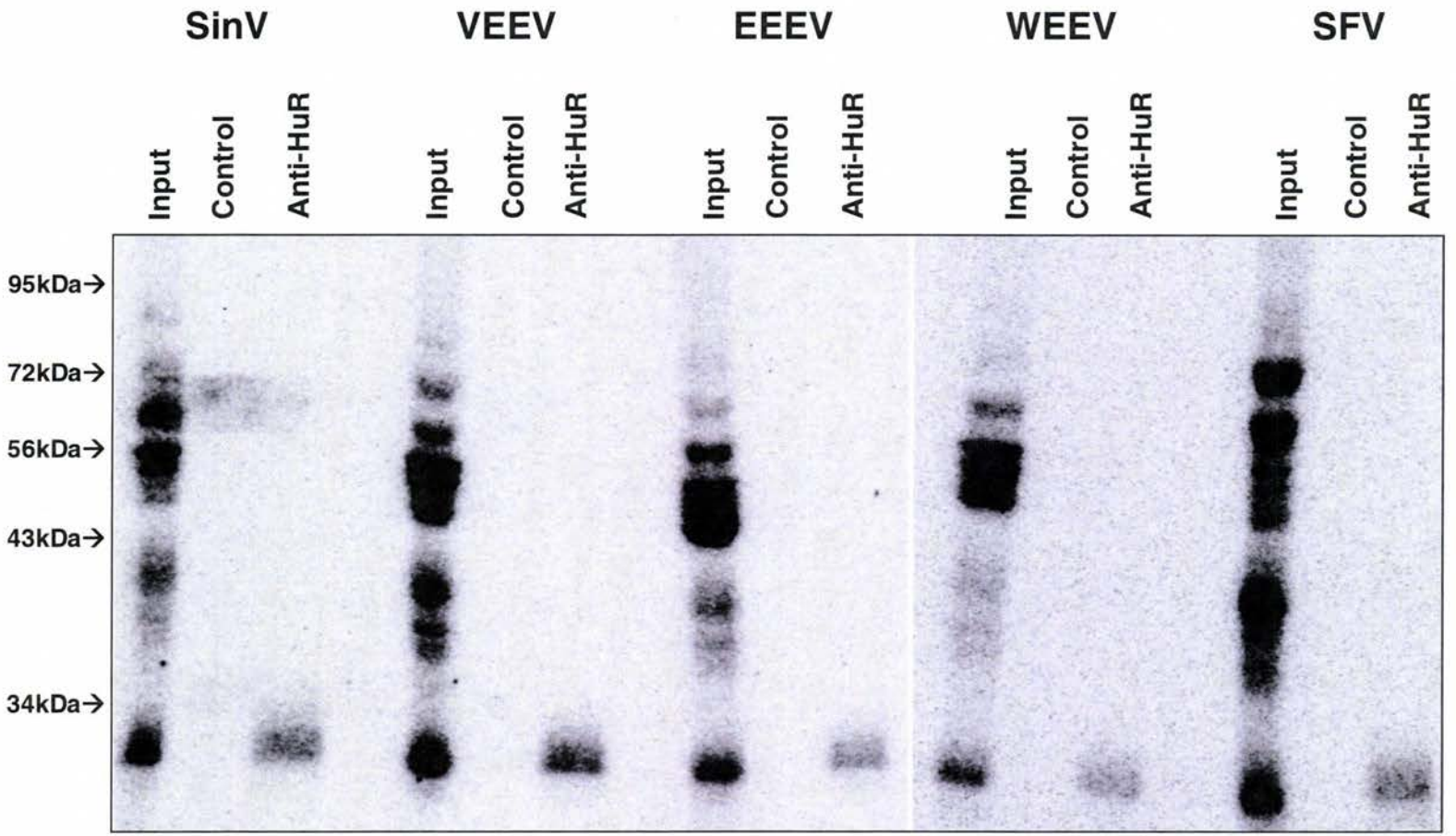

Figure 18- Immunoprecipitation Analysis Confirms the Interaction of Human HuR and the Alphaviral URE/CSE Domain. The URE/CSE fragments of the SinV, VEEV, EEEV, WEEV and SFV RNAs were UV cross-linked to their associated proteins in HeLa cytoplasmic extract. After RNase treatment the radiolabeled RNP components were immunoprecipitated using either normal mouse IgG (Control) serum or anti-HuR (3A2) monoclonal antibody prior to resolution using 10% SDS-PAGE. Input lanes represent 5% of the UV cross-linked proteins used in the immunoprecipitations. Radiolabeled / immunoprecipitated proteins were detected via phosphorimaging. Gels shown are representative of at least two independent replicates.

mosquito systems or could be generalized to mammalian cells. Using the commercially available 3A2 anti-HuR antibody we confirmed the interaction of human HuR and the SinV URE/CSE region in HeLa cell cytoplasmic extracts. Furthermore, interaction between the human HuR protein and the URE/CSE fragments of the VEEV, EEEV, WEEV and SFV RNAs was also observed (Fig. 18). From these data we conclude that the interaction of aeHuR and HuR is conserved amongst the URE-bearing members of the genus *Alphavirus*.

As depicted in Fig. 19, the 32kDa cross-linked protein was identified as the *Aedes* Squid homolog using a similar immunoprecipitation approach with the anti-aeSquid antibody. However we chose to not pursue this protein-RNA interaction any further in this study due to concerns regarding its specificity. As seen in Fig. 20, the interaction of aeSquid with the SinV RNA 3'UTR is likely a nonspecific interaction as the addition of heparin sulfate, a polyanionic competitor, effectively reduces the cross-linking of the 32kDa species in extracts. Note that the interaction with aeHuR is not affected by the addition of heparin sulfate in these assays.

Taken together these data confirm that the 38kDa and 32kDa cellular factors are aeHuR and aeSquid, respectively. Significantly, the binding of HuR with the alphavirus RNA URE/CSE element occurs *in vitro* in mammalian extracts as well.

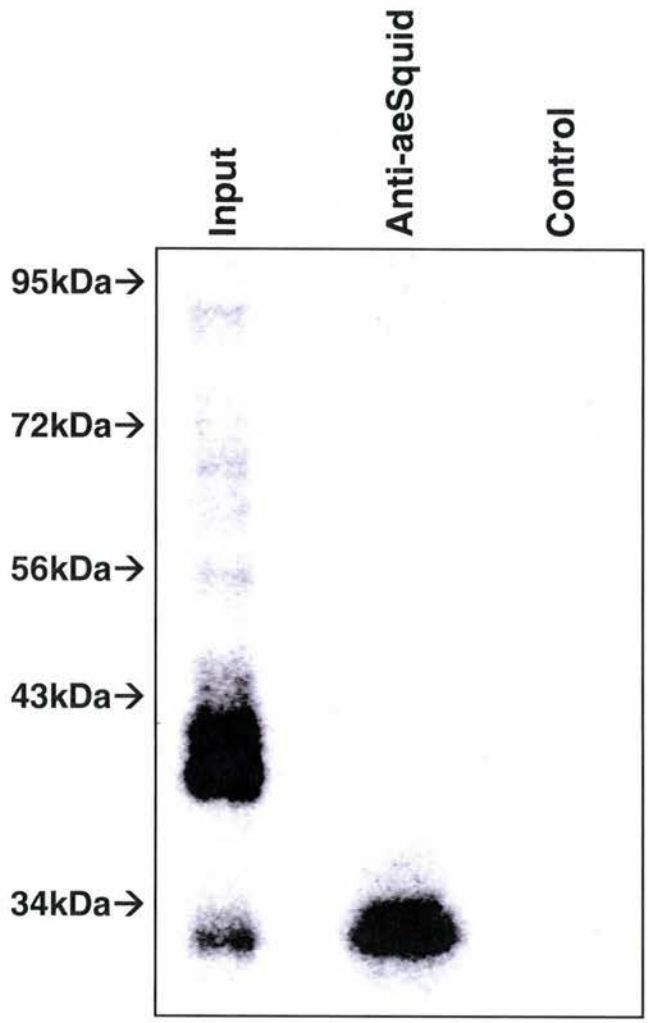

Figure 19- Immunoprecipitation Confirms the Identity of the Cross-Linked 32kD Factor as aeSquid. The URE/CSE domain of the SinV RNA was UV cross-linked to its associated proteins in *Aedes albopictus* C6/36 cytoplasmic extract. After RNase treatment the radiolabeled RNP components were immunoprecipitated using either preimmune (Control) sera or anti-aeSquid sera prior to resolution using 10% SDS-PAGE. The input lane represents 5% of the UV cross-linked proteins used in the immunoprecipitations. Radiolabeled / immunoprecipitated proteins were detected via phosphorimaging. The radiograph shown is representative of two independent replicates.

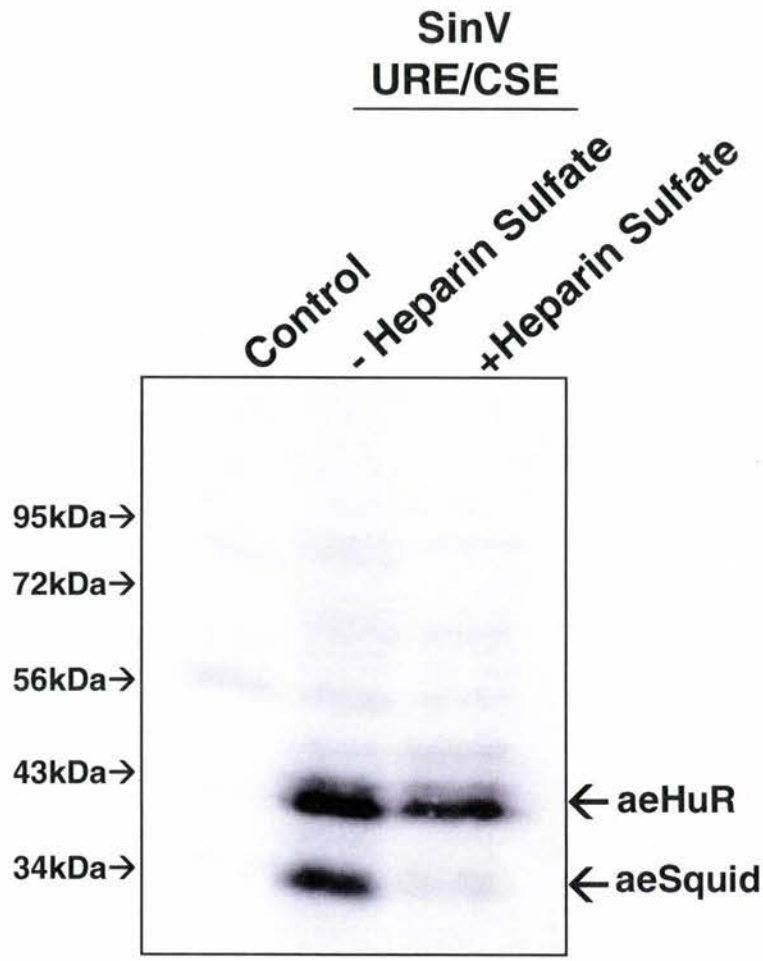

Figure 20- The Polyanionic Competitor Heparin Sulfate Successfully Competes Binding of the 32kD Factor. Either the Control RNA substrate (Control) or the URE/CSE fragment of the SinV RNA was subjected to UV cross-linking in *Aedes albopictus* C6/36 cell cytoplasmic extract in absence or presence of heparin sulfate. After RNase digestion the radiolabeled RNP components were resolved using 10% SDS PAGE. Molecular weights are indicated to the left and relevant proteins are indicated to the right. The gel shown is representative of two independent UV cross-linking analyses.

EMSA Analysis Reveals a High Affinity Interaction Between the HuR Proteins and the Alphavirus RNA Stability Elements

Following confirmation of the identities of our candidate factors by immunoprecipitation, we next sought to examine the affinity of the HuR protein for its viral RNA binding site. Using purified recombinant aeHuR protein (Fig. 21), we assayed the interaction between aeHuR and the SinV RNA 3'UTR elements in an EMSA. As exhibited in Fig. 22A the entire SinV RNA 3'UTR bound with high affinity to aeHuR with a dissociation constant (K_D) of 0.07 (+/- 0.02) nM. Curiously, the presence of two shifted species suggests that multiple aeHuR proteins are associated with the SinV RNA 3'UTR either through RNA:protein or protein:protein interactions. The 3xRSE fragment of SinV failed to appreciably interact with the recombinant aeHuR in our assays (Fig. 22B), confirming our observations that the 3xRSE does not interact with aeHuR via UV cross-linking (Fig. 9). Interestingly, as shown in Fig. 22C, the URE/CSE region was also bound by aeHuR with high affinity (K_D of 0.16 (+/- 0.03) nM) displaying two shifted species similar to those observed with the full length SinV RNA 3'UTR. Indeed the isolated URE of the SinV RNA was capable of being bound by aeHuR with high affinity (Fig. 22D) with an exhibited K_D of 0.17 (+/- 0.18) nM. Finally, as demonstrated in Fig. 22E, the CSE, a common feature of all alphavirus RNAs, was capable of binding aeHuR with a lesser affinity, as indicated by a K_D of 4.7 (+/- 1.0) nM. These interactions were found to be specific, as aeHuR failed to appreciably bind to a control RNA substrate. Examination of the UREs of the other alphaviruses used in this study revealed high affinity interactions between

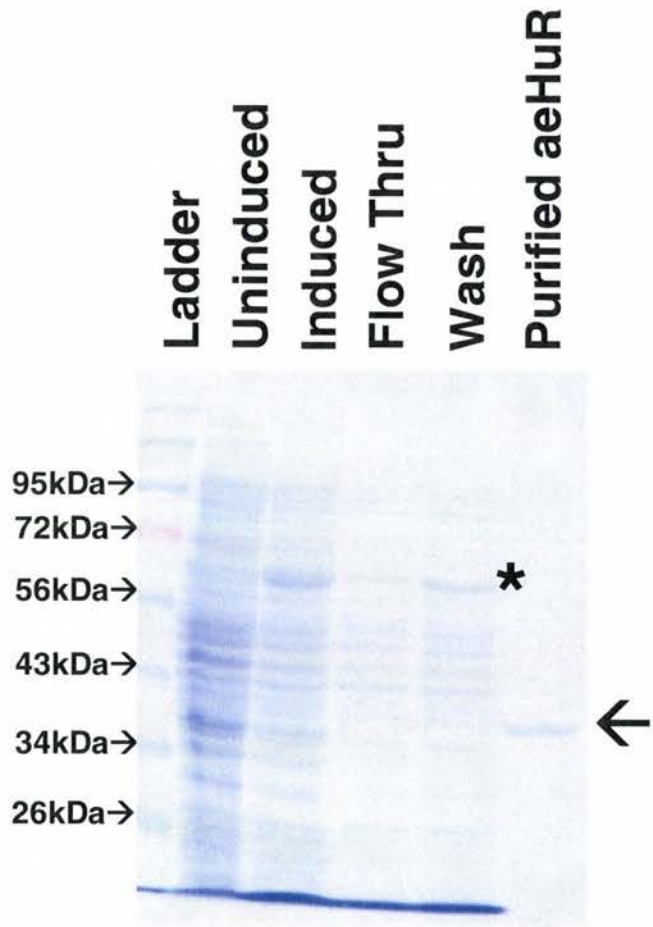

Figure 21- Purification of Recombinant aeHuR. Recombinant aeHuR was expressed in *E. coli* BL21(DE3) cells and purified over glutathione agarose. Recombinant aeHuR was released from the resin via thrombin cleavage, which removed the 26kD GST tag. Samples were resolved by 10% SDS-PAGE and stained with Coomassie blue. The asterisk denotes the uncleaved form of GST-aeHuR while the arrow indicates the final purified aeHuR protein.

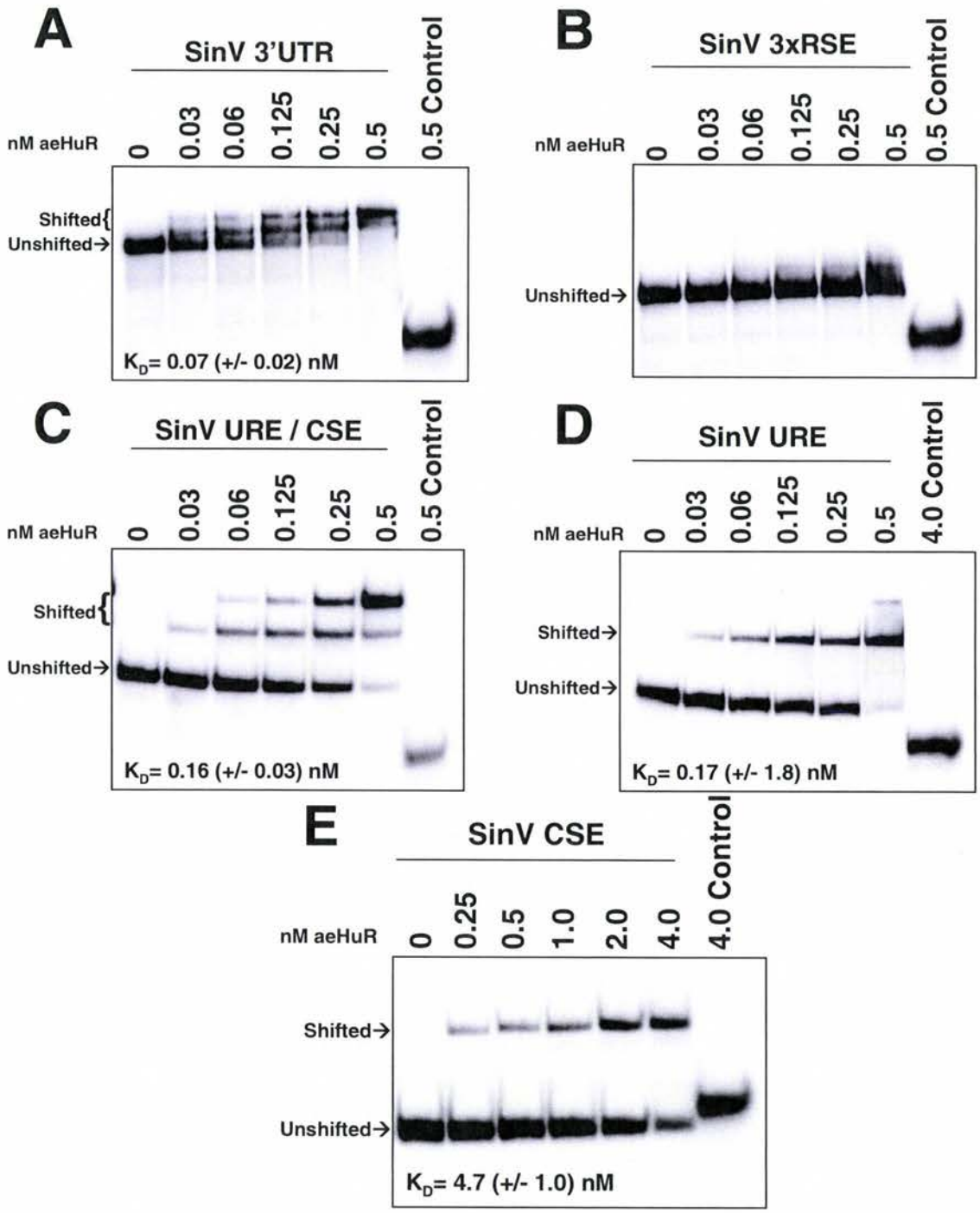

Figure 22- EMSA Analysis Reveals High Affinity Interactions Between aeHuR and the U-Rich Domains of the SinV RNA 3'UTR. Equal molar amounts of unadenylated internally radiolabeled RNA substrates consisting of the various SinV RNA 3'UTR domains were incubated with the concentrations of recombinant aeHuR indicated above each lane. Complexes were resolved on a 5% native polyacrylmide gel and visualized via phosphorimaging. Shifted and Unshifted species are noted to the left of each panel. Control RNA substrates consisted of vector-derived sequences. Dissociation constants are embedded on each gel.

aeHuR and the URE/CSEs of all URE-bearing Alphaviruses including VEEV (K_D=0.35 (+/- 0.15) nM, EEEV (K_D=0.17 (+/- 0.11) nM, WEEV (K_D=0.20 (+/- 0.10) nM and SFV (K_D=1.61 (+/- 0.16) nM (Fig. 23).

EMSA analysis of the SinV URE with recombinant human HuR also revealed a high affinity interaction (Fig 24). Interestingly the observed dissociation constant (K_D=4.4 (+/-1.4) nM) indicates that the affinity of this interaction is comparable to if not greater than the affinity for normal cellular targets (Nabors et al., 2001). Taken together, these data indicate that Alphaviruses have evolved a high affinity interaction with cellular HuR proteins. Additionally these data confirm our earlier observations that this interaction is due to the URE/CSE regions of the SinV RNA 3'UTR and not the 3xRSE region of the RNA.

The observation that two shifted species are observed when examining the full length SinV RNA 3'UTR or the URE/CSE domain indicates that multiple HuR proteins are associated with the viral RNAs. Given that HuR is known to oligomerize on bound mRNAs (Kasashima et al., 2002) we sought to examine if this phenomenon was producing the multiple shifted species observed with the SinV URE/CSE domain. Division of the URE/CSE domain into the URE and CSE revealed that two independent HuR interaction sites were present, indicating that the HuR proteins are likely binding at two distinct sites rather than oligomerizing. Further evidence in support of this assertion is the lack of companion shifted species, making the presence of oligomer HuR unlikely.

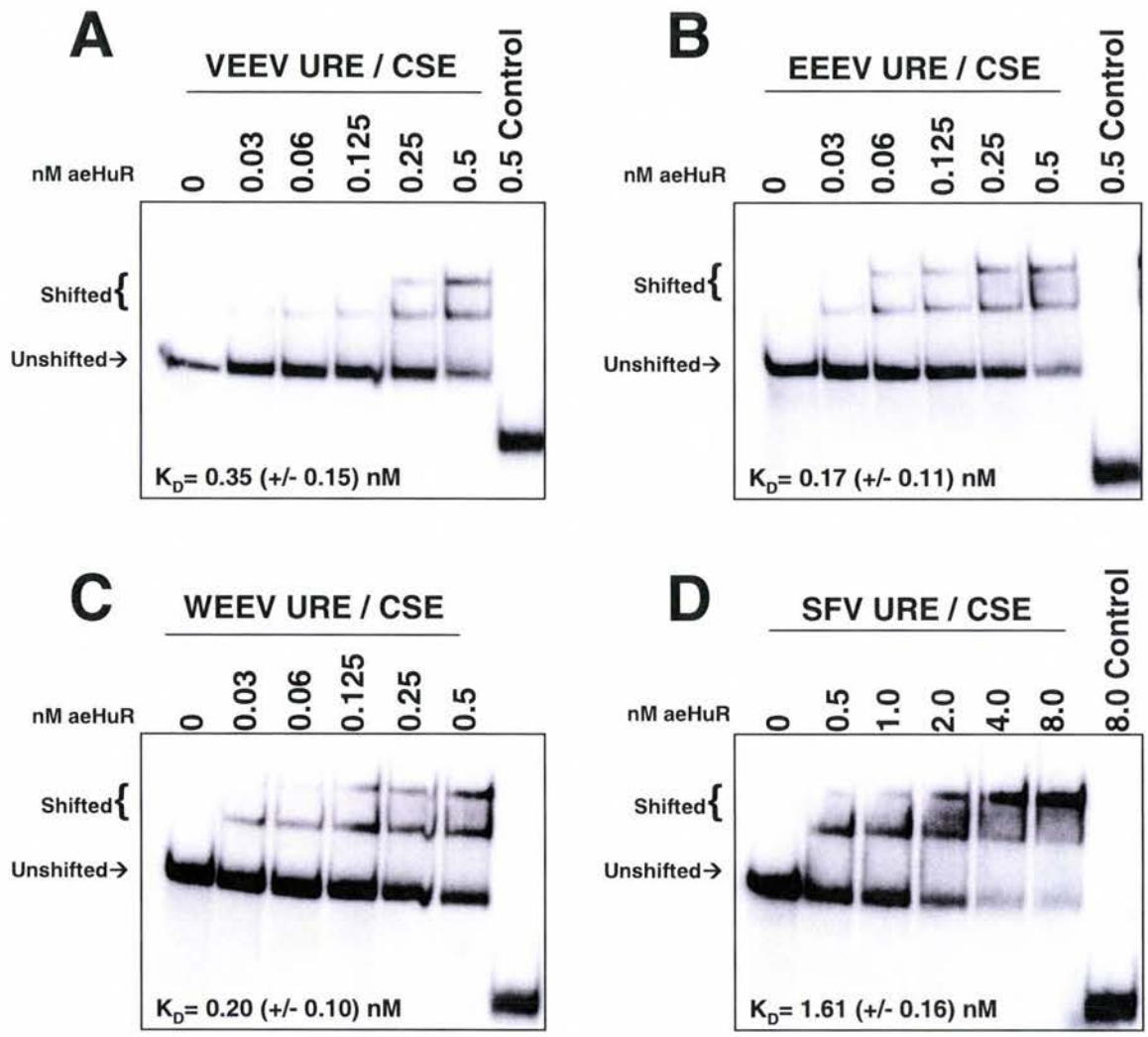

Figure 23- EMSA Analysis Reveals High Affinity Interactions Between aeHuR and the Alphavirus RNA URE/CSE Stability Element. Equimolar amounts of unadenylated internally radiolabeled RNA substrates consisting of the URE/CSE fragments of VEEV, EEEV, WEEV and SFV were incubated with the amounts of recombinant aeHuR indicated above each lane. Complexes were resolved on a 5% native polyacrylmide gel and visualized via phosphorimaging.Shifted and Unshifted species are noted to the left of each panel. Control RNA substrates consisted of vector sequences. Dissociation constants are embedded on each gel. Images shown are representative of at least three indepdendent replicates.

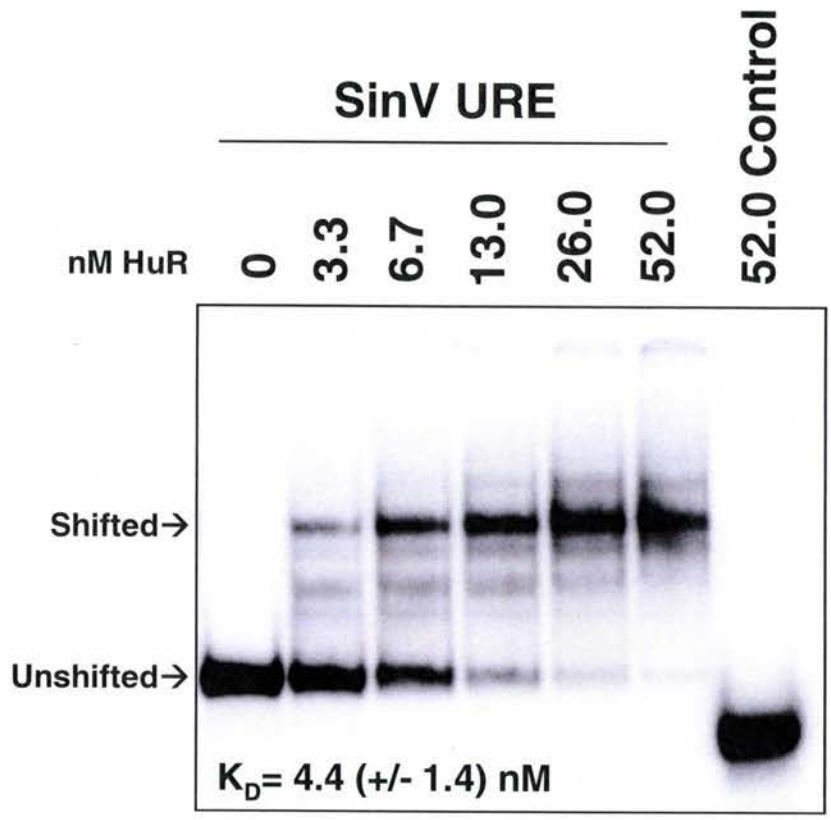

Figure 24- EMSA Analysis Reveals High Affinity Interactions Between Human HuR and the SinV URE. The URE from the SinV RNA was incubated in the presence of human HuR at the concentrations indicated above each lane prior to native PAGE. After sufficient resolution was obtained the gel was dried and examined using a phosphorimager. The Shifted and Unshifted species are indicated to the left of the panel. Control RNA substrates consisted of vector sequences. The calculated dissociation constant is embedded on the gel and represents data from three independent replicates.

aeHuR and HuR Interact with SinV RNAs During Infections of Cultured Cells

The discovery of the URE/CSE:aeHuR/HuR interaction using cell extracts strongly suggests but does not directly demonstrate that the protein-RNA interaction occurs during a viral infection. In order to proceed with the investigation of the biological roles of these cellular factors we first needed to corroborate these interactions in living cells. Authentication of this interaction can be achieved using a reversible cross-linking approach in conjunction with viral infection in cultured cells.

Either Aag2 or 293T cells were infected with wild type SinV at an MOI of 5. Infected cells were collected and washed in PBS prior to formaldehyde cross-linking. Formaldehyde treatment effectively halts the rearrangement of mRNP complexes and also stabilizes these interactions for later rigorous purification. Fixed mRNPs were then bound to anti-aeHuR, anti-HuR or control sera and purified from the lysate. The cross-links were reversed via heating and the released RNA was purified using organic phase separation. The purified RNA was then utilized in an RT-PCR reaction to detect the presence of viral nucleic acids. As exhibited in Fig. 25, the positive sense SinV RNAs were detected in the immunoprecipitations of aeHuR and HuR but not the control sera. It should be noted that the primers used in this experiment detect both the SinV genomic and subgenomic RNAs. The amplification of SinV nucleic acid following formaldehyde cross-linking and stringent immunoprecipitation of aeHuR and HuR further confirms the interaction of the cellular HuR proteins and viral RNAs in live cells.

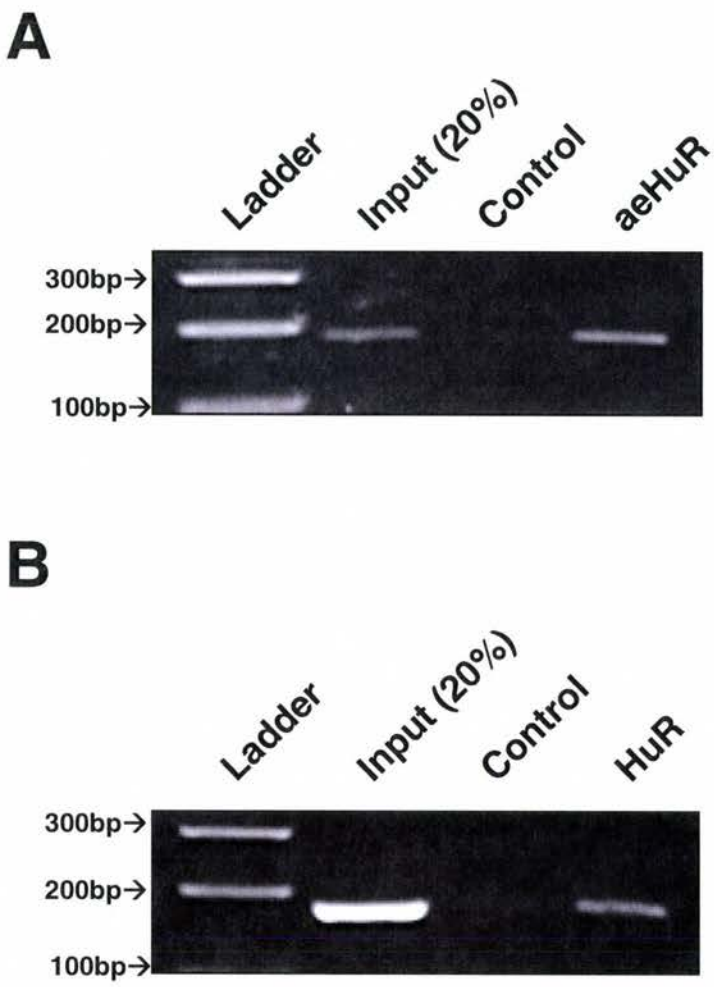

Figure 25- Reversible Cross-Linking Indicates an Interaction Between the Cellular HuR Proteins and SinV RNAs in Cultured Cells. (A) *Aedes aegypti* (Aag2) cells were infected with SinV at an MOI of 5 prior to cross-linking with 1% formaldehyde. Following disruption of the cell membrane, cross-linked RNP complexes were immunoprecipitated with either preimmune (Control) or anti-aeHuR sera. The cross-linking of the precipitated RNPs was reversed via heating and the viral RNAs detected using RT-PCR. The amplified products were resolved on a 2% agarose gel and visualized with Ethidium Bromide. (B) 293T cells were infected with SinV at an MOI of 5 and processed identically to panel A with slight modifications. Normal mouse IgG (Control) or anti-HuR (3A2) monoclonal antibodies were utilized in the immunoprecipitation. Gels are representative of a minimum of two independent replicates.

Additionally these data provide further evidence for the conservation of this interaction between both mammalian hosts and vector mosquitoes.

SinV Infection Perturbs HuR, but not aeHuR, Localization in Cultured Cells

Mammalian HuR is largely nuclear in localization (Fan and Steitz, 1998a), at least in unstimulated cells. Since the localization of aeHuR has never been examined, it was plausible that the subcellular localization of aeHuR may be different from its mammalian counterparts. Examination of the *Aedes aegypti* aeHuR Nuclear Localization Sequence (NLS), as defined by comparison to those of mammalian HuR (Fan and Steitz 1998), reveals that several of the key amino acids within the motif are not conserved (as shown in Fig. 14B). This suggests that mosquito aeHuR and mammalian HuR may have different patterns of localization. Using our polyclonal anti-aeHuR serum the localization of aeHuR in Aag2 cells was examined. As shown in Fig. 26, the localization of aeHuR in Aag2 mosquito cell lines was diffuse with approximately equal accumulation in the nucleus and cytoplasm.

In contrast to aeHuR, mammalian HuR is predominantly nuclear in localization prior to SinV infection (Fig. 27). Following selective lysis of the plasma membrane of infected 293T cells the cytoplasmic and nuclear fractions were separated and blotted to detect the localization of HuR. Fraction integrity was monitored by blotting for PABPN1 (a nuclear marker) and GAPDH (a cytoplasmic marker). Infection with SinV induced relocalization of HuR from the

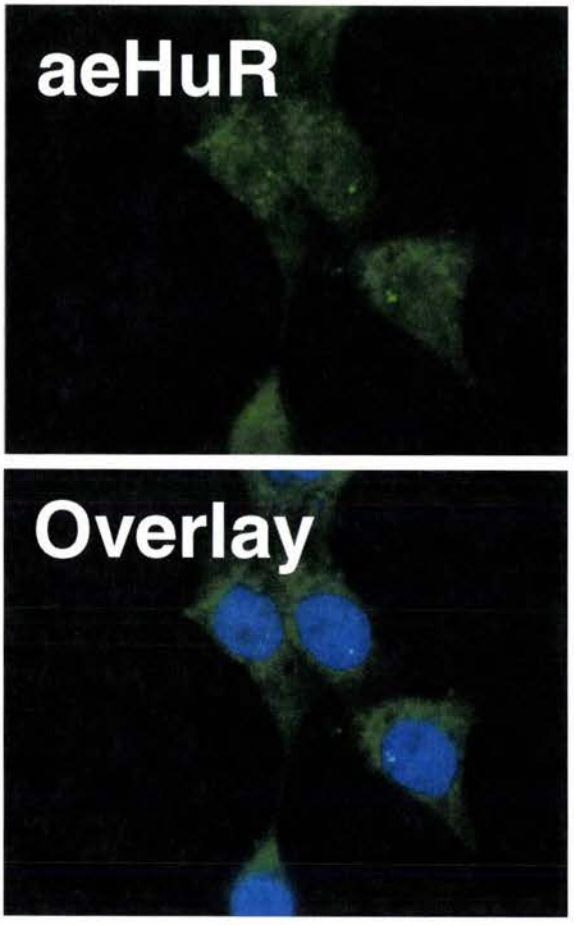

Figure 26- aeHuR Protein Exhibits Diffuse Localization in *Aedes aegypti* Aag2 cells. Aag2 cells were grown on glass coverslips prior to fixation and permeabilization. Cellular aeHuR protein was detected using the anti-aeHuR polyclonal antibody developed in this study. Nuclei were stained using DAPI. Background levels of fluorescence as determined by preimmune serum were subtracted from the above images. (Images courtesy of Dr. A. M. Dickson).

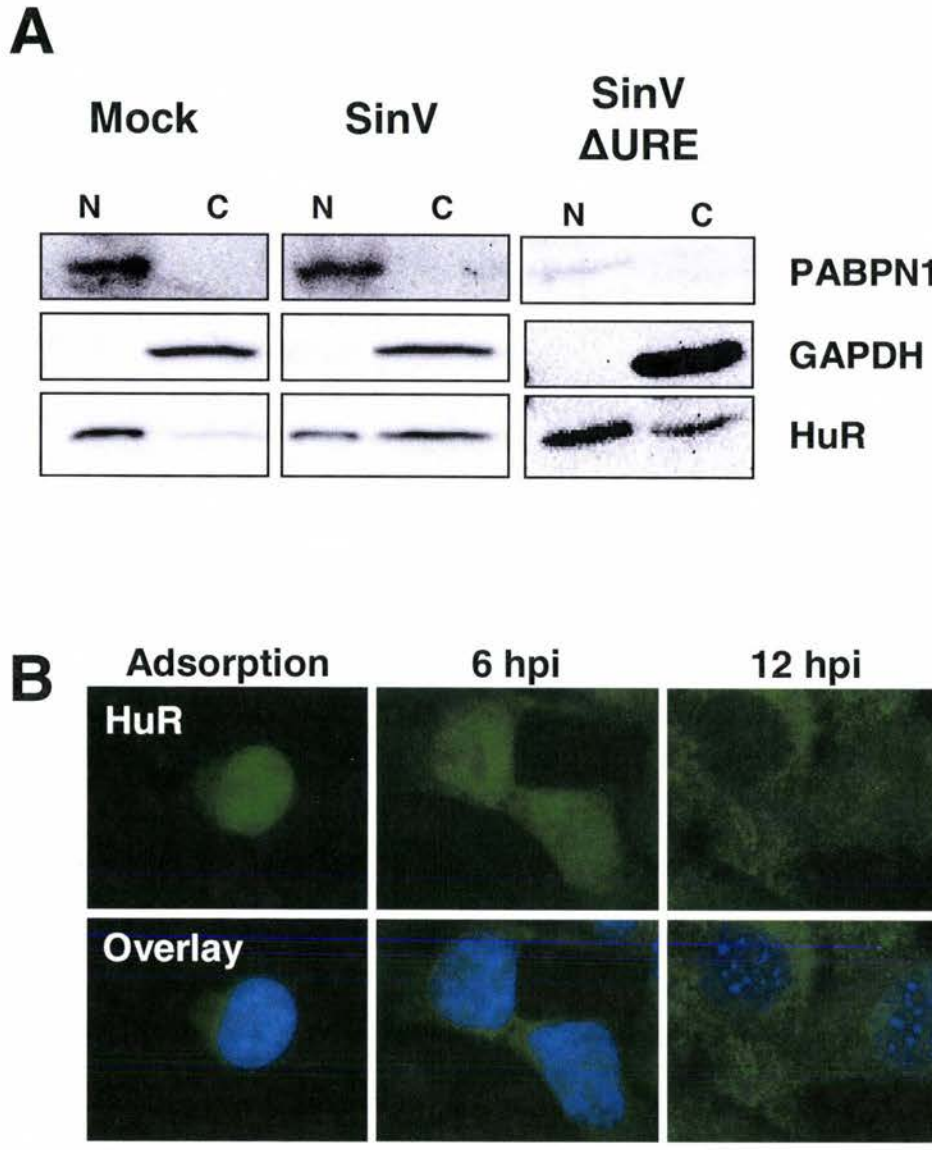

Figure 27- SinV Infection Results in the Relocalization of Mammalian HuR from the Nucleus to the Cytoplasm. (A) 293T cells were infected with SinV at an MOI of 5. After overnight incubation the cells were collected and separated into cytoplasmic and nuclear fractions. Equivalent amounts of the two fractions were loaded and resolved on a 10% SDS-PAGE gel. Following transfer to PVDF membrane the samples were probed for PABPN1 (K-18) as a nuclear marker and GAPDH as a cytoplasmic marker in addition to HuR (3A2). (B) 293T cells were grown on glass coverslips prior to fixation and permeabilization. Following blocking the cells were probed for the cellular HuR protein using the anti-HuR (3A2) monoclonal antibody. Nuclei were stained using DAPI. Background levels of fluorescence as determined by normal IgG were subtracted from the above images.Images courtesy of Dr. A. M. Dickson.

nucleus to the cytoplasm after 24 hours of incubation (Fig. 27A). The relocalization of HuR from the nucleus to the cytoplasm in infected cells was also observed by immunofluorescence as shown in Fig. 27B.

We next sought to examine if the high affinity HuR binding site, the URE, was perhaps responsible for the retention of HuR in the cytoplasm. This possibly could explain the relocalization event as the SinV genomic and subgenomic RNAs are both very abundant during viral infection. To explore this possibility we utilized a SinV lacking the URE in its 3'UTR, namely ΔURE SinV. While this virus may still bind HuR given the presence of the CSE, the interaction between the viral RNA and the cellular HuR proteins should be reduced greater than 25 fold as determined by gel shift analysis for the host HuR proteins. As observed in Fig. 27A, infection with the ΔURE SinV mutant still resulted in the relocalization of the cellular HuR protein to the cytoplasm.

Taken together these data indicate that the cellular HuR proteins are available to the SinV RNAs during infection of both mosquito and mammalian cells. The diffuse nature of aeHuR suggests that the availability of aeHuR is consistent throughout the life cycle of SinV. In contrast, the movement of HuR from the nucleus to the cytoplasm in SinV infection of mammalian cells implies greater opportunity for the SinV RNA to interact with the cellular HuR protein at later stages of viral infection.

Development of a Selectable Mosquito shRNA Vector and Selection of aeHuR Deficient Aag2 Cell Lines

To examine the effect of aeHuR on SinV RNA decay and viral replication we first needed to develop a reliable technology to allow us to knockdown aeHuR expression in mosquito cells. Given the poor transfection efficiencies of mosquito cell lines, we elected to develop a selectable mosquito-specific shRNA vector named pAeSH.

Using PCR, the hygromycin phosphotransferase (hph) gene was amplified from pHyg (Gritz and Davies, 1983) and inserted into the *Nco*I and *Xho*I sites of the multiple cloning site of the pBiEx-1 (Promega) insect expression vector to generate pBiEx-hph. In order to drive shRNA expression, the *Aedes aegypti* U6 promoter fused to a multiple cloning site was amplified from pAedes.1 (Konet et al., 2007) and was cloned into the *Sfo*I and *Apa*I sites of pBiEx-hph to generate pAeSH. As exhibited in Fig. 28, pAeSH contains a multiple cloning site allowing for the insertion of shRNAs targeting any desired mRNA.

To reduce the intracellular concentrations of aeHuR, an shRNA specific to aeHuR was cloned into the *Bam*HI and *Hin*DIII sites of pAeSH. This plasmid, pAeSH-aeHuR1, was then transfected into Aag2 cells using FuGene 6 according to the manufacturer's instructions. Stable cell colonies were pooled and selected over a two week period via the addition of 300U/ml of hygromycin B to the growth medium. As shown in Fig. 29, this pooled stable cell line expressed approximately 40% of the aeHuR observed in the empty pAeSH-transfected control cell line as detected by qRT-PCR.

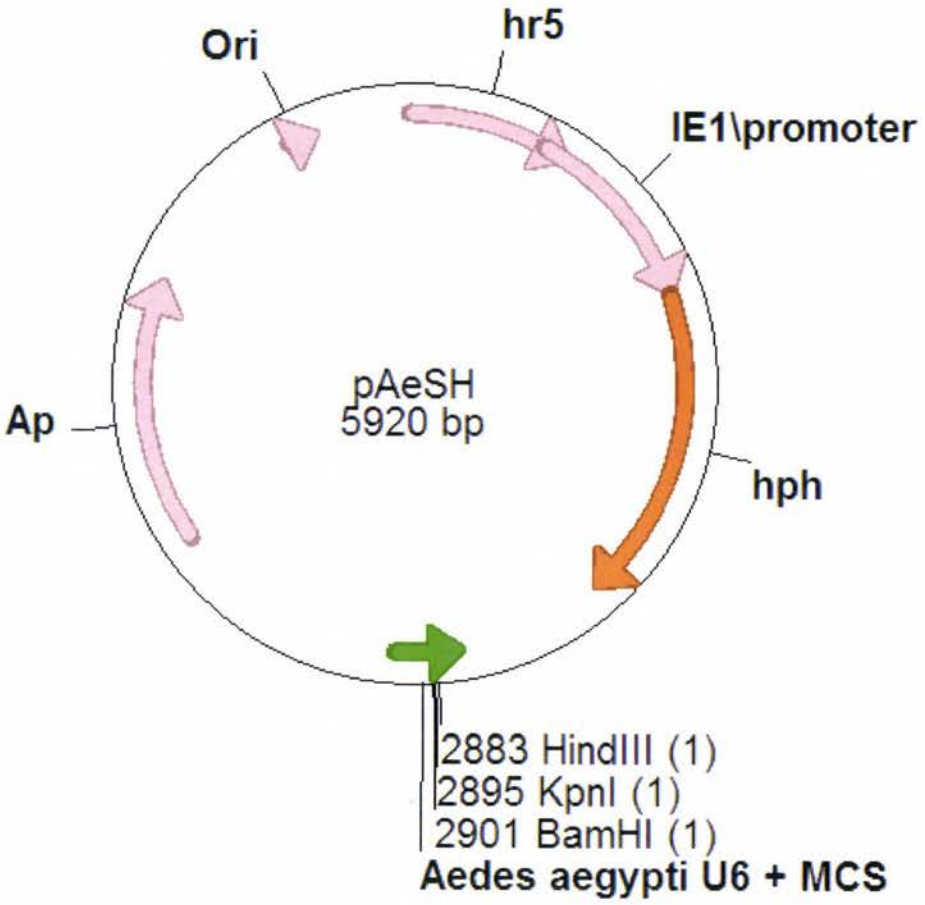

Figure 28- Plasmid Map of the pAeSH Selectable Mosquito shRNA Vector. Plasmid map of the pAeSH shRNA vector. Notable features include: **(AP)** Ampicillin resistance marker, **(Ori)** pUC origin of replication, **(hr5)** Baculoviral transcriptional enhancer, **(IE1\promoter)** Baculoviral Immediate Early promoter, **(hph)** Hygromycin Phosphotransferase gene, **(*Aedes aegypti* U6 + MCS)** *Aedes aegypti* U6[1] promoter driving expression of a downstream Multiple Cloning Site. Unique restriction enzyme cleavage sites for the cloning of shRNAs are indicated.

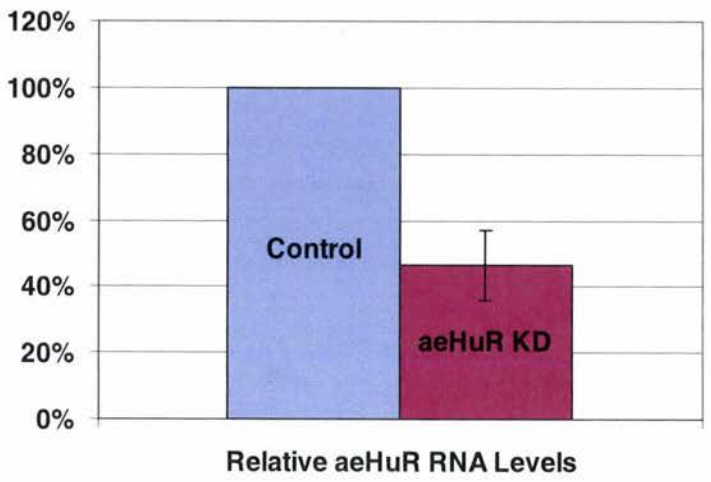

Figure 29- Transfection of an Anti-aeHuR shRNA Encoding pAeSH Results in Decreased aeHuR mRNA Levels. Total RNA from Aag2-derived stable cell lines with either an anti-aeHuR shRNA encoding plasmid or an identical vector lacking the specific shRNA (Control) were analyzed by qRT-PCR for the detection of aeHuR transcript levels. Percent abundance of aeHuR mRNA relative to that detected in the pAeSh control cell line for three independent replicates is reported.

shRNA Mediated Knockdown of the Cellular HuR Proteins Increases the Rate of SinV RNA Decay in Cultured Cells

While the interaction between the cellular HuR proteins and the SinV RNAs has been established from the evidence above, the biological significance of these interactions is still unknown. In order to examine the significance of this interaction we utilized RNAi to selectively reduce the intracellular levels of both aeHuR and HuR. From this approach we were able to examine the impact of the cellular HuR proteins on viral RNA stability.

Members of the ELAV superfamily are required for cellular growth (Ghosh et al., 2009). Indeed the cellular HuR proteins are responsible for stabilizing numerous cellular mRNAs and regulating the cell cycle (Blaxall et al., 2002; Brennan and Steitz, 2001; Wang et al., 2000; Wilusz and Wilusz, 2007; Levy et al., 1998; Linker et al., 2005; Fan and Steitz, 1998b; Atasoy et al., 2003; Peng et al., 1998). The necessity of these proteins to direct normal cellular function may have impacted the level of knockdown we could successfully attain for the cellular HuR proteins while still maintaining cell viability and minimizing indirect effects. Cells transfected with the anti-HuR shRNA vector exhibited slower growth than those treated with control vectors (data not shown). Therefore the shRNA-mediated reduction of the cellular HuR proteins must be performed within a short timeframe such that the cellular processes, as measured by cell division, were not significantly negatively impacted.

As shown in Figure 30A, transfection of 293T cells with anti-HuR shRNA-containing vector reduced the levels of HuR approximately 55% at 24 hours post

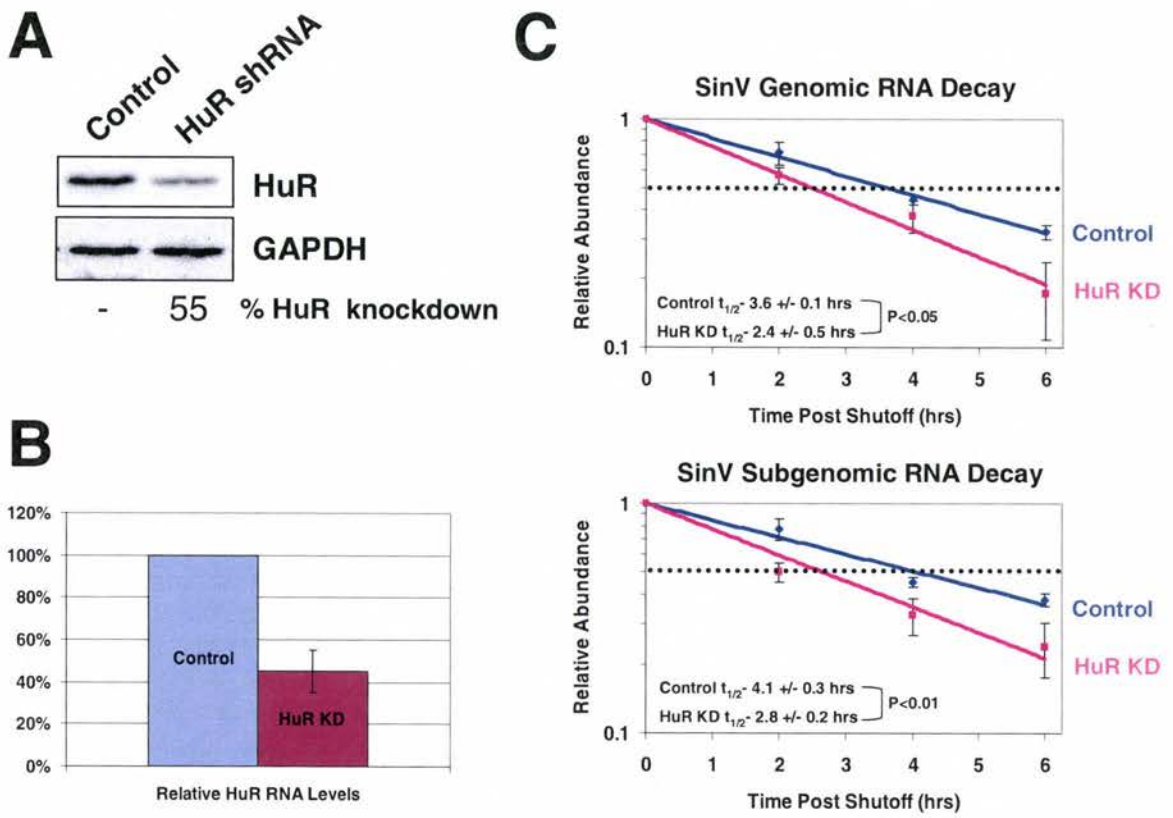

Figure 30- RNAi Knockdown of HuR Results in Increased Viral RNA Decay. (A) 293T cells were transfected with either an anti-HuR shRNA encoding plasmid or an identical vector lacking the specific shRNA (Control) with FuGene 6 according to the manufacturer's instructions. Western blot indicating the relative expression of HuR with shRNA treatment to control treatment at the start of viral infection as determined by densitometry. (B) Twelve hours after transfection the cells were infected with SinV at an MOI of 5 and incubated for 10 hours prior to raising the temperature of incubation from 28°C to 40°C to inhibit viral transcription. Total RNA was extracted and examined using qRT-PCR to measure the HuR or viral RNAs relative to cellular GAPDH mRNA. The graph represents HuR transcript levels at time point 0 of the half life analysis. (C) Graphical analysis of the abundances of the genomic and subgenomic viral RNAs with respect to time post temperature shift. The mean of three independent replicates is shown, with error bars representing the standard deviation. Mean half lives are indicated alongside standard deviations and P-values as determined by a two-tailed Student's t-test.

transfection as compared to cells transfected with an identical vector lacking a specific shRNA as shown by western blotting. Similar reductions in HuR transcript levels were detected via qRT-PCR, Fig. 30B. To measure the rate of viral RNA decay the anti-HuR shRNA transfected cells were infected with a temperature-sensitive mutant of SinV. Infection was allowed to progress and total RNA was extracted at the indicated time points and used to generate cDNA. The resulting cDNA was used in qRT-PCR reactions to determine the abundances of the viral transcripts.

The abundances of the SinV genomic and subgenomic RNAs were averaged over three independent replicates and plotted with respect to time. As exhibited in Fig. 30C, the mean half lives of the genomic and subgenomic SinV RNAs in control cells were found to be 3.6 (+/- 0.1) hr and 4.1 (+/- 0.3) hr respectively. RNAi mediated reduction of the HuR levels to approximately 55% of control resulted in a statistically significant reduction in the stability of the SinV transcripts to 2.4 (+/- 0.5) hr for the genomic and 2.8 (+/- 0.2) hr for the subgenomic RNA.

Infection of the Aag2 derived cell lines with the temperature-sensitive SinV enabled us to examine the rates of decay for the SinV transcripts in the presence of reduced levels of aeHuR. In the hygromycin B-selected stable anti-aeHuR shRNA transfected Aag2 cells the level of aeHuR mRNA was reduced ~60% as indicated by qRT-PCR (Fig. 29). As exhibited in Fig. 31, the half lives of the SinV genomic and subgenomic RNAs in control cells were 4.3 (+/- 1.0) hr and 4.2 (+/- 0.3) hr respectively. Similar to the observations reported for the mammalian cell

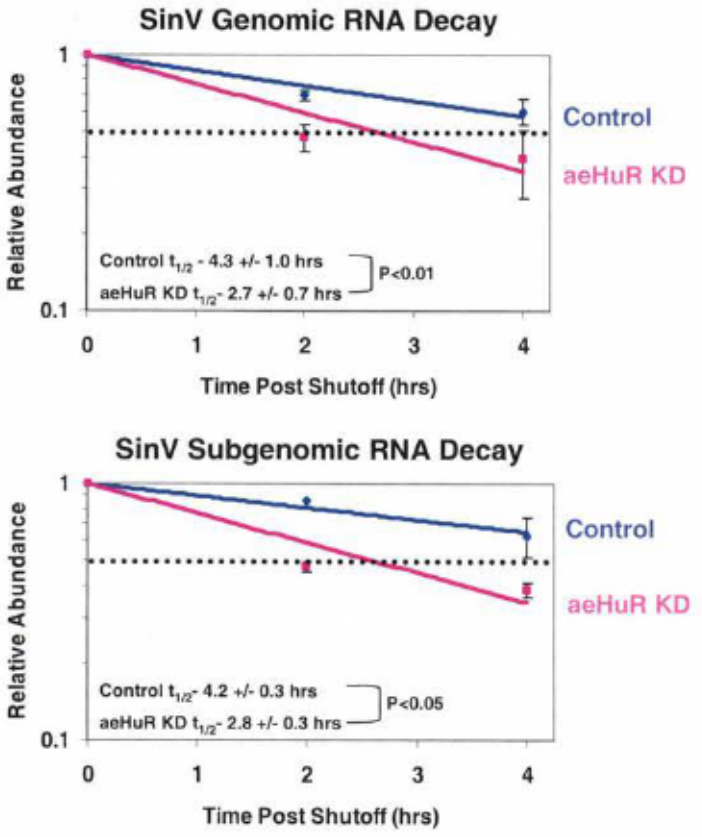

Figure 31- RNAi Knockdown of aeHuR Results in Increased Viral RNA Decay. Aag2-derived stable cell lines transfected with either an anti-aeHuR shRNA encoding plasmid or an identical vector lacking the specific shRNA (Control) were infected with SinV at an MOI of 5 and incubated for 10 hours prior to raising the temperature of incubation from 28°C to 40°C to inhibit viral transcription. At the timepoints indicated total RNA was extracted and examined using qRT-PCR to measure the viral RNA species relative to cellular GAPDH mRNA. The graphs represent analysis of the abundances of the genomic and subgenomic viral RNAs with respect to time. The mean of three independent replicates is shown, with error bars representing the standard deviation. Mean half lives are indicated alongside standard deviations and P-values as determined by a two-tailed Student's t-test.

line above, shRNA-mediated reduction of aeHuR resulted in a statistically significant increase in the rate of decay. The observed half lives of the genomic and subgenomic viral RNAs in the anti-aeHuR shRNA transformed cell line were 2.7 (+/- 0.7) hr and 2.8 (+/- 0.3) hr, respectively. These data demonstrate that even a modest reduction of the cellular HuR levels, typically a ~2-fold reduction, resulted in a significant decrease in the stability of the viral transcripts. Interestingly, this interaction appears to be biologically significant in cultured cells from both the human host and the mosquito vector as these modest reductions in HuR protein abundance yielded statistically significant increases in the rates of viral RNA decay.

shRNA Mediated Knockdown of Expression of the Cellular HuR Proteins or Ablation of the URE Decreases the Production of Viral Progeny in both Mammalian and Mosquito Cultured Cells

The increase in the rate of viral RNA decay in the HuR-deficient cell lines demonstrates a biological function of the HuR protein:SinV RNA interaction. Despite the confirmation of interaction and description of function, it is still unclear if the HuR protein:SinV RNA interaction plays a major role in viral replication. To ascertain if viral yield was adversely affected by the reduction of HuR, we next examined the levels of viral progeny in both the aforementioned mammalian and mosquito systems.

Using the same shRNA-vector transfected 293T cells, and Aag2-derived stable cell lines, we assayed viral production in the presence of diminished

cellular HuR proteins. Rather than infecting the transfected 293T cells with SinV after a twelve hour period the cells were cultured for an additional twenty-two hours prior to infection with SinV. This extended incubation period was intentional, with the goal of attaining levels of cellular HuR protein consistent with those observed during the half-life assays. After the removal of unbound SinV via washing with PBS, samples of cell culture supernatants were removed at regular intervals, the infected cells were gently washed and the medium replaced. The supernatant samples were assayed by plaque formation in Vero cells and the number of plaques were noted and used to calculate the concentration of infectious virus.

As depicted in Fig. 32A, the levels of viral progeny produced in HuR-deficient 293T cells were approximately 10-fold less than the empty shRNA vector transfected control. Similarly a 5-fold effect was noted in the Aag2 aeHuR shRNA transfected cell line (Fig. 32B). Statistical analyses of three independent growth curves demonstrated a statistically significant reduction (with a minimum P-value of <0.05) of viral progeny in both cell lines. Taken together these data strongly imply that the cellular HuR proteins are vital factors for viral infection. Furthermore, these data suggest that the HuR protein may be more important to viral infection in mosquito cells than mammalian cells as observed by the relative effects on viral titer.

The observation that HuR reduction results in diminished viral progeny gives biological importance to the SinV RNA:HuR protein interaction. To further examine the significance of this interaction, we sought to delete the URE of SinV

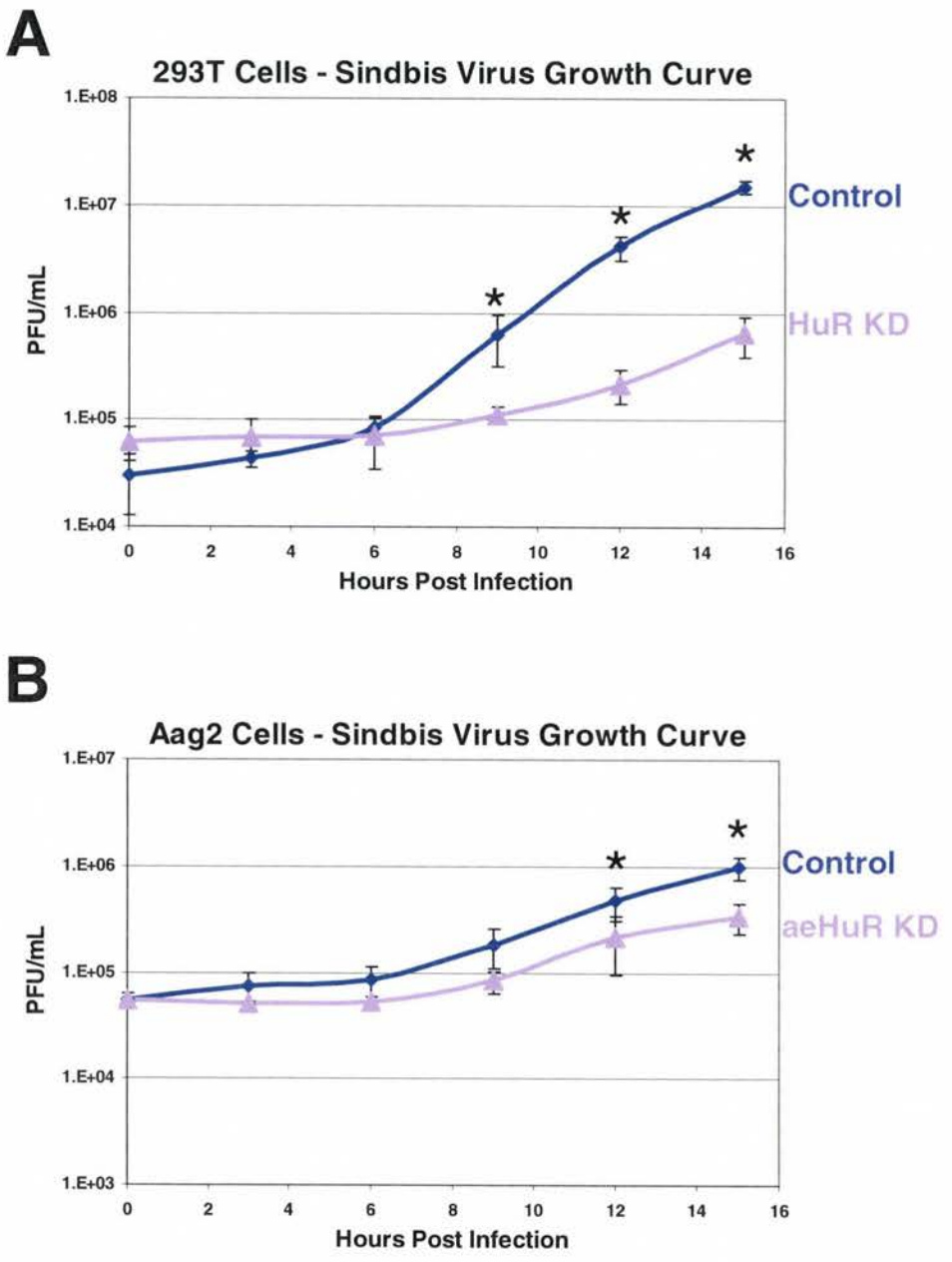

Figure 32- shRNA Reduction of HuR and aeHuR Results in Decreased SinV Progeny. (A) One-step growth curves of viral titer with respect to time in 293T cells transfected with either an anti-HuR shRNA (HuR KD) or a vector lacking an shRNA sequence (Control). **(B)** One-step growth curves of viral titer with respect to time in Aag2-derived cells stably transfected with either an anti-aeHuR vector (aeHuR KD) or a vector lacking a specific shRNA (Control). Data shown are the means of three independent replicates, P-values of equal to or less than 0.05 are indicated with an asterisk.

to effectively reduce the level of interaction between the viral RNAs and the cellular HuR proteins. While entire removal of the HuR binding sites is impossible given the necessity of the CSE, removal of the URE alone will presumably reduce the HuR interaction ~25-fold, as exhibited by the difference in dissociation constants for the URE/CSE and CSE (Fig. 22). Furthermore, during the course of these studies we have reported that the CSE alone was not sufficient for the repression of deadenylation in both mammalian and mosquito cell culture models (Garneau et al., 2008). As shown in Fig. 33, the infections of both control 293T and control Aag2 cell lines with the ΔURE SinV resulted in decreased viral progeny, 10-fold and 5-fold, respectively. Once again, analyses of three independent replicates supported the significance of these observations. Furthermore, when we infected the anti-HuR shRNA and anti-aeHuR shRNA transfected cells described above with either the SinV with a wild type 3'UTR or the ΔURE SinV similar viral titers were observed, (Fig. 34).

Taken together these results indicate that the viral RNA:HuR interaction increases the production of progeny virus during infection of cultured cells. In the presence of reduced HuR protein levels, as compared to control treatments, either the replication or translation or both of the SinV RNAs is diminished, resulting in decreased viral growth. Excitingly, the ΔURE SinV gave very similar results, indicating that the effects of HuR knockdown were indeed significant and that the effects of HuR are predominantly mediated via its association with the URE.

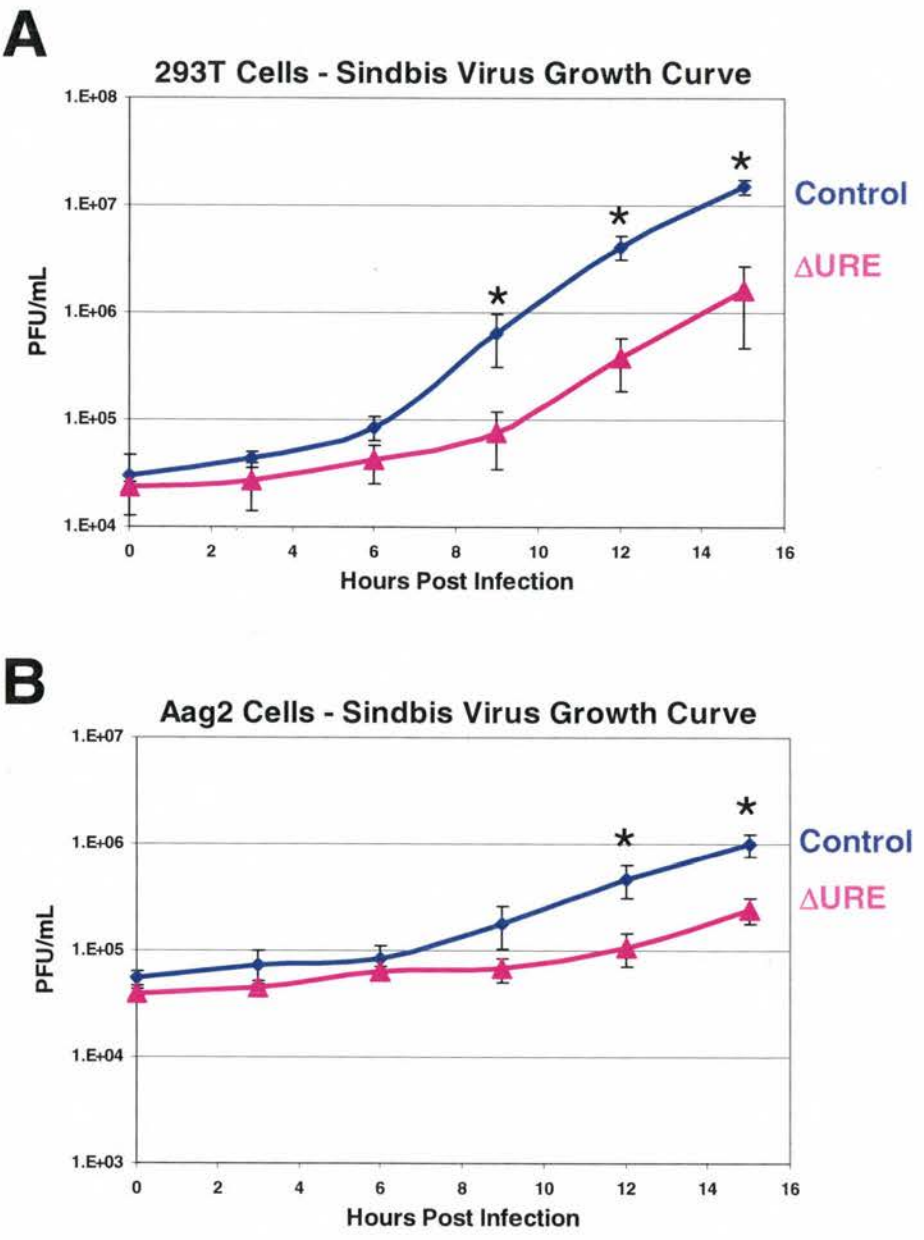

Figure 33- Deletion of the URE from the SinV RNA 3'UTR Results in Decreased SinV Progeny. (A) One-step growth curves of viral titer with respect to time in 293T cells infected with either SinV with a wild type 3'UTR (Control) or a SinV with a 3'UTR lacking the URE (ΔURE). **(B)** One-step growth curves of viral titer with respect to time in Aag2 cells infected with either SinV with a wild type 3'UTR (Control) or a SinV with a 3'UTR lacking the URE (ΔURE). Data shown are the means of three independent replicates, P-values of equal to or less than 0.05 are indicated with an asterisk.

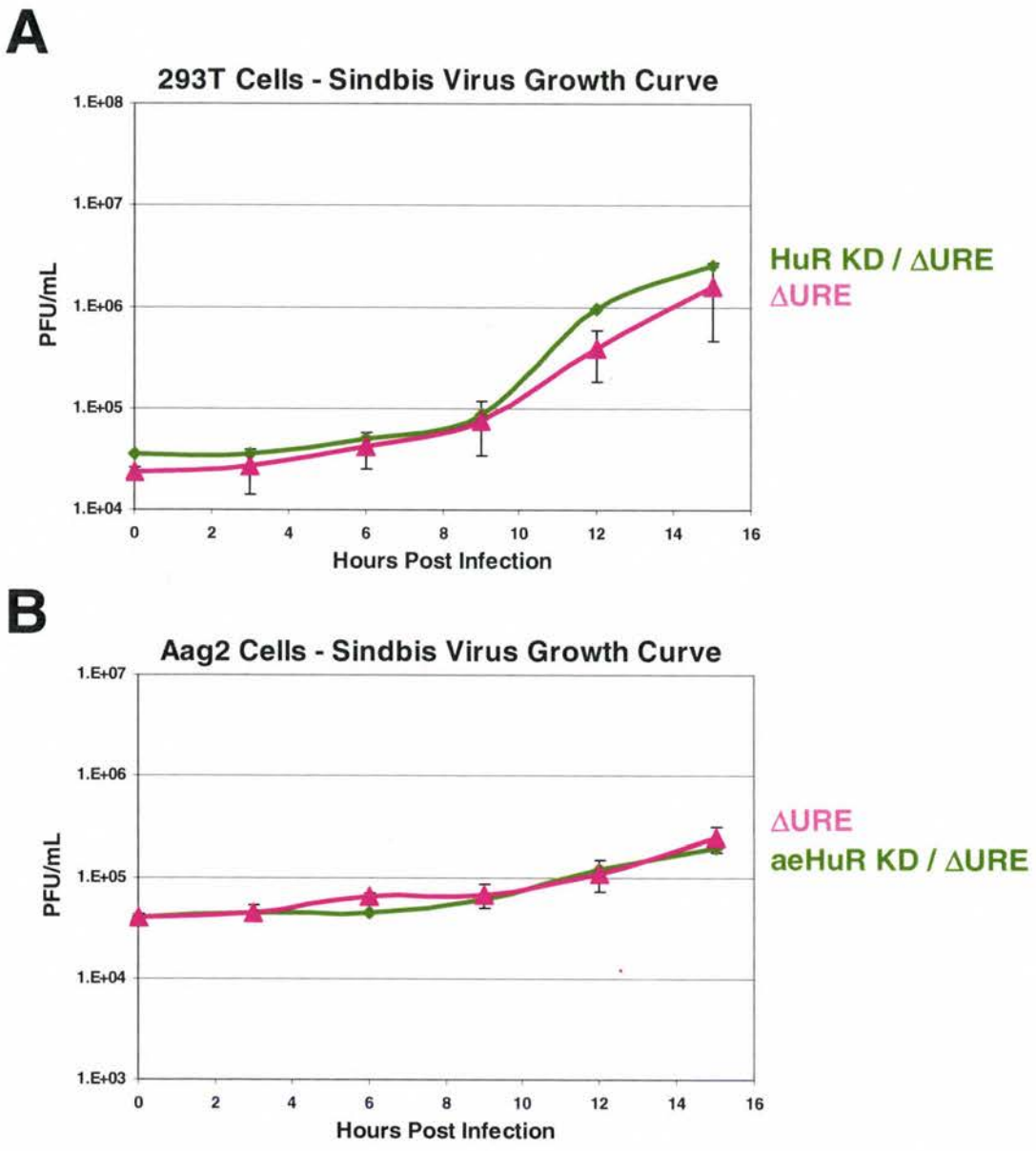

Figure 34- Infection of HuR Deficient Cells with the ΔURE SinV Results in Similar Titers Compared to Wild Type SinV. (A) One-step growth curves of viral titer with respect to time in 293T cells either transfected with an anti-HuR shRNA vector (HuR KD / ΔURE) or a vector lacking an shRNA sequence (ΔURE) were infected with SinV with a 3'UTR lacking the URE. **(B)** One-step growth curves of viral titer with respect to time in Aag2-derived cells transformed with an anti-aeHuR shRNA (aeHuR KD / ΔURE) or a vector lacking an shRNA sequence (ΔURE) were infected with SinV with a 3'UTR lacking the URE. Data shown for the ΔURE SinV growth curves are the means of three independent replicates with the error bars being the standard deviation. Data for the HuR / aeHUR knockdowns infected with the ΔURE SinV are representative.

The URE of the SinV RNA 3'UTR Enhances Translation of the SinV Genomic RNA

In addition to the known roles of HuR protein in modulating mRNA stability, the cellular HuR proteins enhance the translation of bound mRNAs (Mazan-Mamczarz et al., 2003). We therefore sought to determine if a SinV with the HuR binding site, the URE, deleted from the 3'UTR of the genomic RNA exhibited decreased translation in cultured cells when compared to a SinV with a wild type genomic RNA. Confluent monolayers of 293T and Aag2 cells were infected with SinV encoding Luciferase inserted into the nsp3 gene (Bick et al., 2003) with either an intact 3'UTR or 3'UTR lacking the URE.

As indicated in Fig. 35A, the deletion of the URE drastically reduced the level of luciferase activity per genomic RNA at early times during infection when compared to a SinV with a wild type 3'UTR in 293T cells. Similarly the expression of luciferase from a SinV lacking the URE was decreased in Aag2 cells, as shown in Fig. 35B. Interestingly, in contrast to that observed with 293T cells, the expression levels of luciferase from the wild type SinV initially decrease at 2 hours after adsorption in Aag2 cells. Currently the cause(s) of this effect are unknown and the levels of luciferase activity in the earliest time point may be aberrant. Taken together, these data indicate that the URE of the SinV RNA 3'UTR enhances the translation of the viral RNA during an infection.

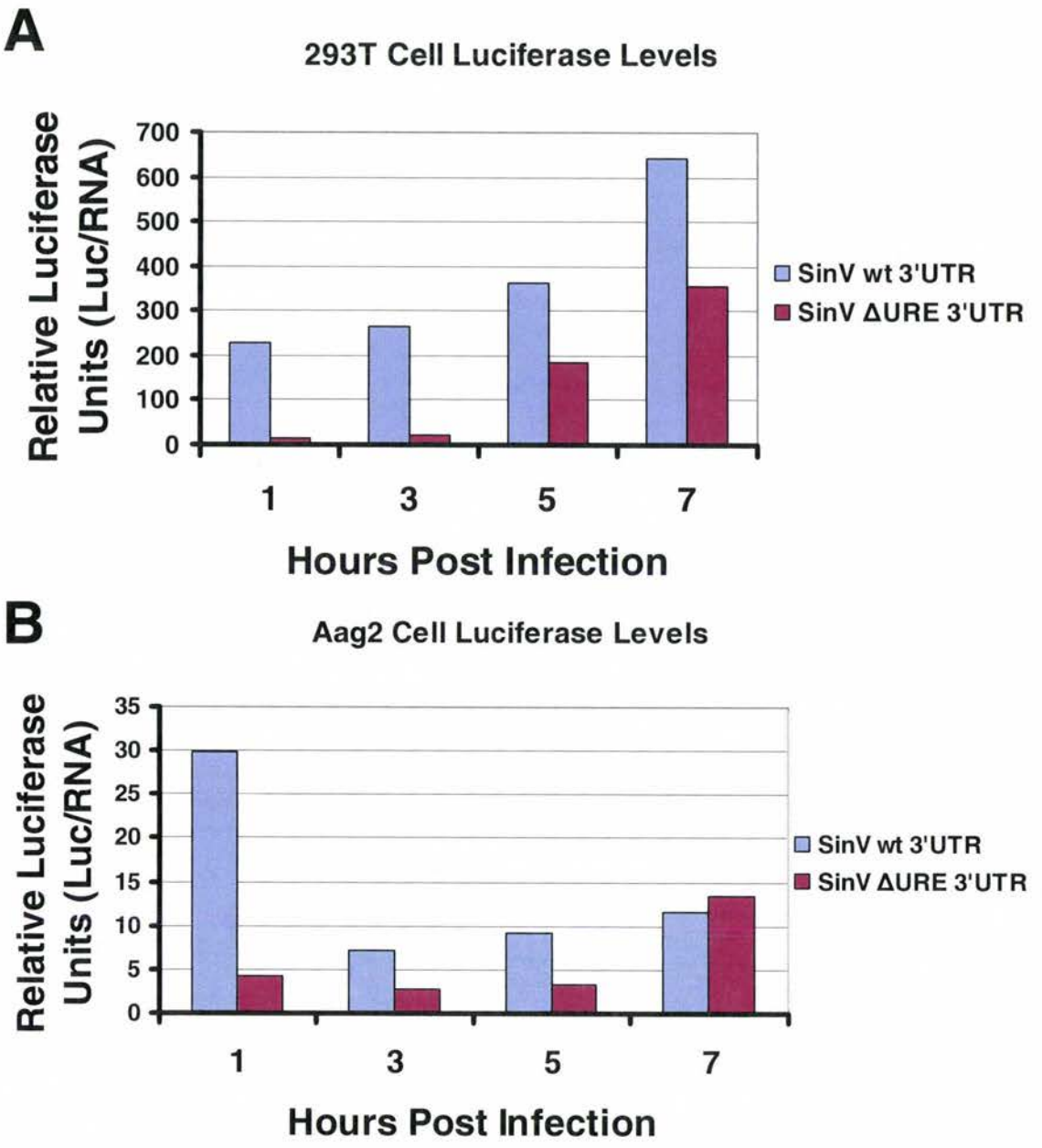

Figure 35- Deletion of the URE from the SinV RNA 3'UTR Results in Diminished Genomic Translation at Early Times in Infection. (A) 293T cells were grown in a 12-well dish and infected with either a SinV with a wild type 3'UTR or a SinV with a 3'UTR lacking the URE. Luciferase levels were measured using a luminometer and normalized to the abundance of the genomic viral RNA. **(B)** Identical to that described in panel A with the exception that Aag2 cells were utilized instead. Graphs are representative of two independent experiments.

Discussion

SinV clearly interacts with the cellular mRNA decay machinery in tissue culture systems. As reported in Garneau et al. 2008, the degradation of SinV RNAs was by a deadenylation-independent pathway. Furthermore, truncation of the SinV RNA 3'UTR to the minimally required CSE resulted in the activation of deadenylation in tissue culture models. The primary purpose of this dissertation project was to examine the mechanism behind the observed repression of deadenylation. To this end, we utilized a cell free RNA decay system pioneered in our lab. We were able to recapitulate the repression of deadenylation associated with the SinV RNA 3'UTR using a cell-free system.

Examination of the repression of deadenylation in our cell free extract system revealed the interaction of the mammalian and mosquito HuR proteins with the viral RNAs. This interaction correlated with the repression of deadenylation *in vitro* and was determined to be biologically significant during infection of cell models. Our current model for the stabilization of the SinV transcripts (Fig. 36) involves the binding of HuR to the 3'UTR of the SinV RNA where it may serve several functions- 1) HuR stabilizes the SinV RNAs, perhaps by interacting with the cellular decay machinery, 2) interaction with HuR enhances viral titer.

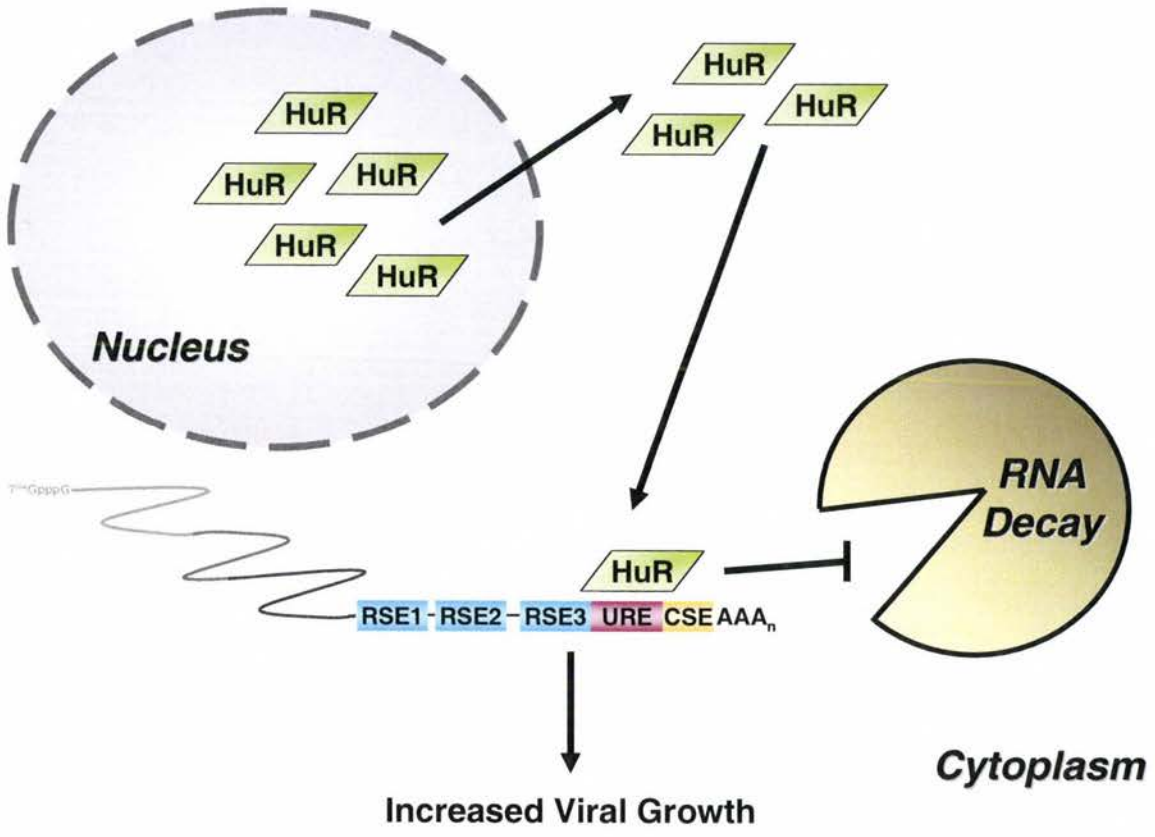

Figure 36- Proposed Model of the Functions of the HuR:SinV RNA Interaction

The SinV RNA 3'UTR Represses Deadenylation *In Vitro*

During the course of this study we reported an interaction between the host mRNA decay machinery and SinV RNAs in cell culture models (Garneau et al., 2008). In contrast to what was observed for most cellular mRNAs, the degradation of the SinV transcripts was deadenylation-independent. Since many cellular mRNAs contain regulatory elements in their 3'UTRs, we chose to examine the role of the viral 3'UTR with respect to viral RNA decay. Truncation of the 3'UTR of the SinV RNA to the minimal elements required for replication activated deadenylation of the viral RNAs in cell culture models of viral infection. In order to determine the mechanistic basis for this stability we employed a cell-free cytoplasmic decay system pioneered in our lab (Ford et al, 1999). Assessment of the 3'UTR of the SinV RNA in our cell-free RNA decay system revealed that the SinV RNA 3'UTR was capable of repressing deadenylation (Fig.5). The recapitulation of the block to deadenylation observed in cultured cells in this cell-free system enabled us to elucidate the mechanisms responsible for this repression.

I. Repression of Deadenylation is Due to Multiple Elements *In Vitro*

As described in the introduction, the Alphavirus RNA 3'UTR consists of three components – RSE, URE and CSE. At the 5' portion of the 3'UTR is a set of RSEs, which prior to this study have had no ascribed function. In most alphavirus RNAs a U-rich (URE) region is observed with notable exceptions of ONNV, CHIKV and BFV. The URE is conserved in nucleotide bias as depicted in

Fig. 7A. The final element, which is also the most conserved, is the 19-nt CSE which functions in replication. Division and examination of the individual domains of the SinV RNA 3'UTR revealed that multiple elements in the 3'UTR are capable of repressing deadenylation. Interestingly the 3xRSE and URE domains were capable of repressing deadenylation independently of each other (Fig. 6). Further examination of these two elements revealed them to be acting by different mechanisms to repress deadenylation.

II. The SinV RNA RSEs Act in Cis to Repress Deadenylation

The 3xRSE domain of SinV, which consists of three individual RSEs, repressed deadenylation and promoted distributive-like decay kinetics. RSEs are commonly found in the 3'UTRs of the Alphavirus RNAs, but they differ in nucleotide sequence and organization. For instance the RSEs of the SinV RNA are divergent in nucleotide sequence from those found in VEEV RNAs (Fig. 37). Furthermore the organization of the RSEs widely differs amongst the members of the genus. As an example, while the RSEs of the SinV and WEEV RNAs are similar in sequence, they differ in number with SinV having 3 while WEEV has a pair of RSEs. The SinV RNA RSE3 alone is capable of repressing deadenylation in our cell-free RNA decay system. Examination of the stability of RSE3-containing RNA substrates in a recombinant system using aePARN revealed that the RSE3 domain was acting in *cis* to repress deadenylation (Fig. 12). Interestingly the repression of deadenylation associated with the RSE3 is

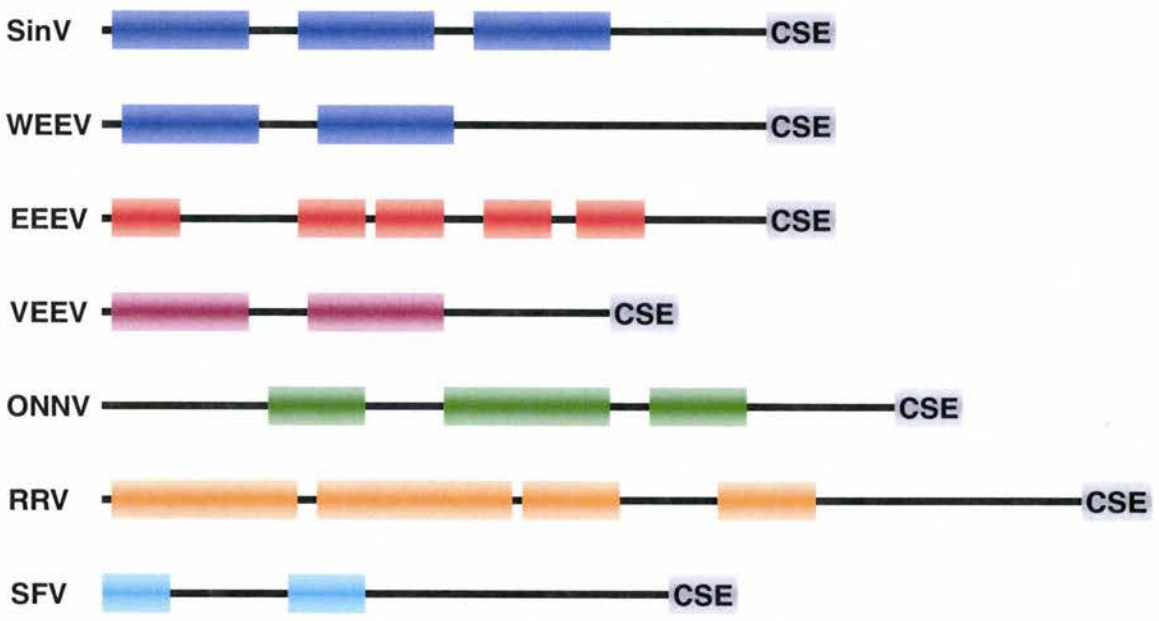

Figure 37- Diagram of RSE Organization and Conservation. Graphical representation of the 3'UTR structure of Sindbis (SinV), western equine encephalitis (WEEV), eastern equine encephalitis (EEEV), Venezuelan equine encephalitis (VEEV), o'nyong-nyong (ONNV), Ross River (RRV) and Semliki Forest (SFV) viruses. Individual types of RSEs are given their own unique colors. Adapted from Strauss and Strauss (1994).

dependent on context, as relocating the RSE3 domain further from the 5' end of the substrate results in the loss of repression in our recombinant assay (Fig. 12B).

The ability of a viral RNA to direct its own decay in *cis* would greatly benefit the virus by regulating the cellular mRNA decay machinery without the necessity of extraneous factors. Indeed, similar elements that repress deadenylation have been previously described in viral RNAs. Notably the PAN-ENE element found in KSHV mRNA represses deadenylation in a *cis*-acting manner (Conrad and Steitz, 2005; Conrad et al., 2006). The current model for the repression of deadenylation via the PAN-ENE element involves the folding back of the poly(A) tail to interact with the PAN-ENE stability element, preventing the host deadenylases from productively interacting with the poly(A) substrate. The *cis*-acting mechanism behind the stability observed with RSE3 is unclear. In our deadenylation assays reconstituted with purified recombinant aePARN, the SinV RSE3- containing RNA substrate is degraded with processive-like deadenylation kinetics. This observation is in contrast to the distributive kinetics of the SinV RNA 3'UTR observed in cell-free cytoplasmic extract systems, indicating that while a minority of the substrate is deadenylated, the vast majority of the population is refractory to deadenylation (Fig. 12B, middle panel). This switch in deadenylation kinetics leading to the formation of fully deadenylated intermediates implies that the RSE3 domain is preventing recognition of the RNA as a substrate for PARN, rather than repressing enzymatic activity. The current mechanism responsible for the RSE making the RNA 'invisible' to the PARN

enzyme is currently unknown. Possible mechanisms include preventing PARN from recognizing either the 5' cap or the poly(A) tail as both are required for PARN function (Martinez et al., 2001; Martinez et al., 2000; Nilsson et al., 2007; Wu et al., 2009; Gao et al., 2000; Dehlin et al., 2000). It is possible that the RSEs form structures that are acting to repress deadenylation. Perhaps as a result of these structures, PARN recognition of the 5' cap or the poly(A) tail is inhibited. Interestingly, repositioning of the RSE3 correlates with a loss of stability and an activation of deadenylation. The reason(s) behind this observation is currently unknown. Repositioning of the SinV RSE3 may cause the secondary structure to erroneously form, resulting in the loss of stability. The RSEs of SinV may be preventing substrate recognition by obscuring a "toe-hold" required for activity. Alternatively, recognition of the 5' cap may be impaired when the RSE3 is close to the 5' terminus. When the RSE3 domain is relocated farther away from the 5' end, the cap structure may become more accessible to the deadenylase, leading to instability. It is possible that the repression associated with the RSE3 domain is an artifact of our *in vitro* assay. In its natural context the RSEs of the SinV RNA are at a considerable distance from the 5' cap structure. However the viral RNAs may adopt a conformation whereupon the cap structure may indeed be spatially close to the 3'UTR. Closer examination of the RSE3 with respect to structure and context could possibly illuminate the mechanism of action for the RSE3 domain.

III. The Alphavirus URE Blocks Deadenylation In Vitro

The second region of the SinV RNA 3'UTR that modulated deadenylation was the URE. As stated earlier, the URE is conserved amongst most members of the genus. Alignment of the UREs of SinV, VEEV, EEEV, WEEV and SFV RNAs reveals little conservation outside of a "UUUUGUUUUU" motif located near the 3' terminus of the URE (Fig. 7A). This motif is highly similar to the reported HuR consensus binding sites (Nabors et al., 2001; Meisner et al., 2004). Examination of the URE/CSE fragments of the aforementioned Alphavirus RNAs revealed that the repression of deadenylation was a common feature of all the tested UREs (Figs. 6 & 7). Taken together these data led us to conclude that the URE is a conserved RNA stability element. Examination of the URE/CSE sequence reveals a single canonical AUUUA pentamer – however it is unlikely to function as a stability determinant as the AUUUA pentamer by itself does not appear to impart function (Lagnado et al., 1994). Furthermore, despite being in the URE, the AUUUA pentamer is not located specifically within a U-rich context, indicating it is not a *bona fide* class I or class II ARE. Ignoring the AUUUA pentamers that lay outside the U-rich tract of the URE, the URE region could be typified as a class III ARE given its U-richness. As discussed in the introduction, AREs modulate the stability of cellular mRNAs via RNA-binding proteins. This classification coupled with the above conservation of function confirms our hypothesis that viral RNAs have evolved to contain mRNA stability elements similar to those observed in cellular mRNAs and further implies that the URE is likely stabilized in a *trans*-acting manner.

IV. The SinV RNA 3'UTR is Stabilized via a Trans-Acting Mechanism

We next sought to determine if the SinV RNA 3'UTR was stabilizing RNAs via a *trans*-acting manner. To this end we evaluated the ability of the SinV RNA 3'UTR to repress deadenylation in the presence of cold competitor RNAs (Fig. 8). The addition of excess SinV-specific competitor activated the deadenylation of an adenylated SinV RNA 3'UTR RNA substrate. This observation led us to conclude that the SinV RNA 3'UTR was acting via a *trans*-acting mechanism to repress deadenylation in our cell free system. Interestingly, the activation of deadenylation via the addition of competitor RNAs resulted in distributive-like deadenylation kinetics. This is perhaps due to the independent function of the RSEs to slow deadenylation, rather than the block associated with the URE. Further examination of these competition reactions using UV cross-linking revealed a correlation between the binding of a 38kDa factor and the repression of deadenylation.

V. UV Cross-Linking Identifies the Binding Site of the 38kDa Factor as the URE and CSE Domains of the SinV RNA 3'UTR

Using UV cross-linking the 38kDa host factor was observed binding to the URE and CSE domains of the SinV RNA 3'UTR (Fig. 9). Inspection of the CSE and URE primary sequences revealed that both the URE and CSE possess the aforementioned "UUUUGUUUUU" (U_4GU_5) motif (Fig. 7A). Interestingly in our deadenylation assays, the CSE exhibited only a minor delay of deadenylation when compared to control substrates. Therefore binding of HuR, or the inclusion

of a single copy of the conserved U_4GU_5 motif does not appear to be sufficient to repress deadenylation on its own. The repression of deadenylation associated with the binding of the 38kDa factor and / or the U_4GU_5 motif may be dependent on other factors capable of interacting with the URE domain but not the CSE. Another possible explanation for these observations is that the associations of the 38kDa factor and these binding domains may be different in their affinity. These possibilities will be discussed in more depth later.

UV cross-linking of the other URE/CSE domains of VEEV, EEEV, WEEV and SFV RNAs revealed a similar pattern of protein binding when compared to the SinV RNA (Fig. 10). Furthermore, cross-competition of the SinV and VEEV 3'UTRs reveals that the similar 38kDa factors are indeed identical (Fig. 11). Taken together these data further strengthen our conclusion that the URE is in fact a conserved viral RNA stability determinant.

VI. The URE is Not Universally Conserved Amongst the Alphavirus Genus

The observation that the SinV RNA 3'UTR is repressing deadenylation via a *trans*-acting factor in our cell free RNA decay assay indicates that the RSE domains are playing a minor role in the repression of deadenylation *in vitro*. Furthermore the ability of the URE domains of several Alphavirus RNAs to repress deadenylation and bind to similar proteins, including a 38kDa factor that correlates with stability, strongly asserts that this function is evolutionarily conserved. However, as stated earlier, several Alphavirus RNAs do not contain the URE, and hence only a single U_4GU_5 motif found in the CSE. Since the CSE

of the SinV RNA did not significantly contribute to repression of deadenylation *in vitro*, we hypothesize that other domains may be present that are serving to direct mRNA stability in these viruses. Similarly it was seen that the CSE alone was not capable of repressing deadenylation in cell culture systems (Garneau et al. 2008).

Examination of the 3'UTRs of ONNV, CHIKV and BFV RNAs demonstrates the absence of the URE domain. Through phylogenetic analysis these viruses are believed to have split from the URE-bearing Alphaviruses several thousand years ago (Powers et al., 2001). Perhaps they have evolved a different element as a result of divergent evolution- in lieu of the URE is a tract that is more G/C rich than the other members of the genus as shown in Fig. 38. Analysis of these sequences reveals a potential "GGGACGUAGG" motif that is conserved amongst the non-URE Alphavirus RNAs. In addition, several other residues are conserved outside of this motif, indicating that the elements present (if any) may be complex. The possibility that this region is acting as a stability determinant has not been directly assessed to date. Perhaps this domain is interacting with other protein factors leading to stability, or other factors that attract the HuR to the RNA in the absence of a U-rich motif. Alternatively it is possible that in these viruses, the RSEs (which differ from the other URE-containing Alphavirus RNAs) play a larger role in determining stability.

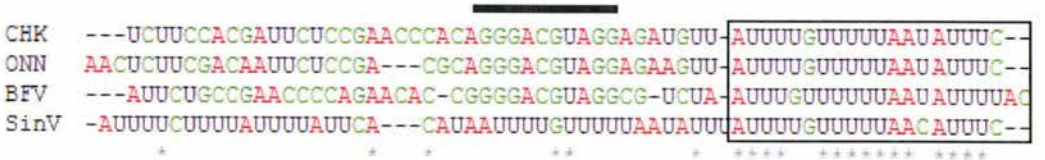

Figure 38- Sequence Alignment of the Alphavirus RNA 3' Terminal Regions with Non-URE Alphaviruses. Alignment of the 3' terminal 60 nucleotides of chikungunya (CHIKV), o'nyong nyong (ONNV), Barmah Forest (BFV) and Sindbis (SinV) virus RNAs. The CSE is boxed and the potential GGGACGUGG motif is indicated with a line.

VII. HuR Binding to Viral RNAs May be a Widespread Phenomenon

The HCV RNA interacts with the cellular HuR protein (Spangberg et al., 2000) and includes a similar motif to those found in the SinV RNA URE/CSE domains. In the 3'UTR of the HCV RNA an U-rich tract exhibits similarity to the consensus cellular HuR binding site (Lopez de Silanes, et al., 2004) and has been shown to mediate binding of the viral RNA to HuR (Spangberg et al., 2000). Reduction of HuR protein levels using RNAi decreases viral titer via an unknown mechanism (Korf et al., 2005). Perhaps the cellular HuR protein is stabilizing the viral RNAs comparable to that described here for the SinV RNAs.

Given the differences of HCV and the URE-bearing Alphaviruses it is apparent that viral HuR binding sites may be more ubiquitous than originally appreciated. It is possible that viral RNAs, like cellular mRNAs, have evolved to contain mRNA stability elements that respond to cellular regulation. As described in the introduction, HuR stabilizes many cellular mRNAs that are directly involved in the host antiviral response. The inclusion of cellular-like viral RNA stability elements could potentially serve as a means by which viruses could regulate the functions of their mRNAs in response to changes in host RNA stabilization. Further identification of HuR-interacting viruses and the characterization of the virus:HuR interactions function will no doubt be an area of future research.

The Cellular HuR Protein Interacts with the SinV RNA URE with High Affinity

As described above, the URE of the SinV RNA binds to the 38kDa factor in mosquito cells correlating with stability. Using this knowledge we affinity purified the RNA-binding proteins that interacted with the URE/CSE domain. Mass spectrometric analysis revealed that the 38kDa protein was likely a mosquito homolog of HuR, a protein with a known role in the stabilization of cellular AREs (Fig. 14). Using recombinant aeHuR and polyclonal antiserum developed to aeHuR we examined the interaction of aeHuR with the SinV RNA 3'UTR.

Using an UV cross-linking / immunoprecipitation approach we were able to confirm the interaction of aeHuR and the human HuR protein with the SinV RNA 3'UTR. We were able to corroborate these observations in cell culture models of infection, strengthening the evidence for a *bona fide* interaction between the HuR proteins and viral nucleic acids. Immunoprecipitation analysis of the URE/CSE domains of the aforementioned Alphaviruses confirms that the 38kDa protein observed binding to all of the sequences is indeed the cellular HuR protein (Figs. 17 & 18). Furthermore these data strengthen our assertion that the URE is an mRNA stability element that, similar to cellular AREs, acts through a cellular mRNA stability factor – HuR.

I. The URE and CSE Interact with the Cellular HuR Proteins with Different Affinities – Implications for RNA Stability

Following confirmation of the identity of the 38kDa factor as aeHuR we aimed to characterize its interaction with the SinV RNA 3'UTR. Using EMSA analysis, we evaluated the interaction of aeHuR with the 3xRSE, URE/CSE, URE and CSE domains. Determination of the dissociation constants of the URE and CSE HuR interactions confirmed our observations that the URE and CSE domains were responsible for HuR binding to the SinV RNAs. High affinity interactions were noted for all of the examined URE-containing Alphavirus RNAs (Figs 22 – 24). Interestingly the dissociation constants for the URE and CSE were different, with the dissociation constant of the CSE being greater than 25-fold that of the URE. These data indicate that the CSE domain on its own is a minor contributor to HuR binding when compared to the URE domain. This potentially explains the difference in deadenylation stability noted between the URE and CSE elements- perhaps the affinity of interaction between the HuR proteins and the CSE is insufficient to completely repress deadenylation. It should also be noted that the inclusion of the CSE domain with the URE domain does not significantly enhance the affinity of HuR beyond what is already observed for the URE domain alone (Fig. 22). Taken together these data further support our conclusions – SinV, and likely all of the URE-bearing Alphavirus RNAs, have evolved an mRNA stability element that interacts with a known mRNA stability factor HuR via a high affinity interaction.

II. HuR Binds to the SinV RNAs with Greater Affinity than Reported Cellular Targets

Comparison of the dissociation constants between the HuR proteins and the SinV RNA 3'UTR and those reported for cellular mRNAs reveals disparity. The dissociation constants are equal to or less than those reported for cellular mRNAs (sometimes as much as 100-fold) (Nabors et al., 2001; Meisner et al., 2004). While these dissociation constants are obviously from independent recombinant protein samples, the range of the relative affinities reported for these interactions are comparable. Perhaps the high affinity observed with the viral RNA is due to the strong similarity of the sequences present in the URE/CSE domain of the SinV RNA with sequences defined by SELEX of the HuR protein (Meisner et al., 2004). Either way these data suggest that during an infection the SinV RNAs may potentially be better targets for HuR binding than cellular mRNAs. This may be due not only to the affinity of the HuR:viral RNA interaction but also the sheer number of viral transcripts present relative to individual cellular mRNAs. The implications of this phenomenon, if true, are wide ranging. HuR is involved in the stabilization of many mRNAs in mammals, most notably those encoding cytokines and other immune mediators (TNFα, IL-3, IL-6, IL-8 and GM-CSF amongst others) (Atasoy et al., 2003; Ming et al., 2001; Winzen et al., 1999; Fan and Steitz, 1998b; Fan and Steitz, 1998a). One could hypothesize that SinV infection was preventing the interaction of HuR with these "normal" targets thereby preventing their stabilization. This in turn could downregulate the host response to viral infection at the level of post-

transcriptional control, enhancing viral infection. Testing this hypothesis would be relatively straightforward – one could examine the decay rates of known HuR-interacting transcripts during an infection. An increase in decay over mock infected cells would indicate potential virus induced cellular mRNA instability. Over expression of HuR or tethering of the HuR protein to a destabilized cellular mRNA would potentially confirm this hypothesis. If the previously destabilized transcripts regained their "native" stability the idea of viral theft of HuR function would be strongly supported.

The Localization of the Mammalian HuR Protein is Altered by SinV Infection

As described in the introduction, the subcellular localization of HuR significantly impacts its function. HuR is predominantly nuclear in localization in unstimulated cells (Ma et al., 1996; Fan and Steitz, 1998a). Infection with SinV induces relocalization of HuR from the nucleus to the cytoplasm in mammalian cells (Fig. 27). Arguably this could serve to promote viral infection by enhancing the likelihood of HuR:SinV RNA interaction by increasing the cytoplasmic concentration of HuR during viral infection.

I. Possible Mechanisms Inducing HuR Relocalization

Currently the mechanism(s) behind this change in localization is unknown. Possible mechanisms include- i) The recognition of SinV infection as a cell stress, ii) The recognition of dsRNA created as a consequence of viral replication

induces relocalization, iii) Viral proteins may induce relocalization, iv) Binding of HuR to the viral 3'UTR could result in cytoplasmic retention of HuR via RNA:protein interactions, and v) Generalized perturbation of nuclear export / import pathways. The evidence in support (and in contradiction) to each of these models is given below in addition to possible experimental ways of testing these hypotheses.

i. SinV Infection as a Generalized Cell Stress

As indicated above, many cell stresses modulate the localization of HuR. Infection with SinV is undoubtedly a stressful event for the cell. SinV replication and protein synthesis lead to host cell shutoff, which may serve to relocalize HuR via a mechanism similar to that observed with other cell stresses. Therefore it is plausible that HuR is being relocalized by the host response to SinV infection via one (or more) of the cell signaling pathways. As noted in the introduction, these pathways alter the localization of HuR by post-translational modification. Examination of the post-translational modifications of HuR during SinV infection may give insight into this potential mechanism. Inhibition or RNAi-mediated knockdown of the implicated kinases / methylases would then allow one to examine directly the role of each with respect to the relocalization of HuR in response to SinV infection.

ii. Cellular Recognition and Response to Viral dsRNA

A second possible mechanism of relocalization could involve the recognition of viral RNA. Several RNA helicase sensors, such as RIG-I and MDA5, recognize viral RNAs and activate the interferon response (Yoneyama et al., 2005; Kato et al., 2006). It is possible that one (or more) of these helicase sensors is inducing relocalization of HuR as a result of viral RNA recognition. Furthermore, the host cell aggressively monitors the transcriptome for dsRNA. The formation of dsRNA during replication activates the dsRNA-sensing protein kinase (PKR) resulting in phosphorylation of eIF2α and inhibition of translation (Sanz et al., 2009; Ventoso et al., 2006). PKR additionally enhances IFN expression, potentially at the level of post-transcriptional control (Schulz et al., 2010). It is possible that the activation of PKR induces relocalization of HuR. Indeed PKR is known to post-translationally modify HuR by enhancing the cleavage of HuR by caspases 7 and 3, activating a pro-apoptotic pathway (Mazroui et al., 2008, von Roretz et al., 2010). Determination of the role(s) of the viral RNA in the relocalization of HuR should first focus on the formation of dsRNA during replication. Exclusion of dsRNA as a trigger for HuR relocalization is easier to directly assess since it can be directly inhibited using the ts6SinV. If the ts6SinV (which is replication defective at non-permissive temperatures) is used to infect cells under non-permissive conditions, one could examine the localization of HuR in response to a replication-defective viral infection. If HuR failed to relocalize from the nucleus to the cytoplasm, then the formation of dsRNA could be concluded as a significant triggering event. If relocalization was

observed then the activation of PKR could be viewed as a relatively minor contributor. If the presence of the viral RNA alone was enough to induce relocalization then the stimulus may either be the viral RNA itself, or a component of the nonstructural polyprotein. Examination of the roles of the RNA helicase sensors RIG-I and MDA5 would involve RNAi and the examination of HuR localization during viral infection.

iii. HuR Relocalization May be Due to Viral Proteins

A third possible mechanism is that SinV is encoding effectors that induce relocalization of HuR as a result of their expression. This could be due to either direct HuR:viral protein interactions or to modulation of HuR:cellular protein interactions. Of the four SinV nonstructural proteins, nsp2 and nsp3 have the highest likelihood due to their nuclear interactions. In the Old World Alphaviruses nsp2 is the host transcription shutoff factor (Garmashova et al., 2006). Furthermore nsp2 enters the nucleus and associates with nucleoli during infection (Atasheva et al., 2007; Frolov et al., 2009). The precise mechanism of transcriptional arrest is unknown. It is plausible that a secondary activity, or a consequence, of nsp2 expression is perturbation of HuR localization. Nonfunctional mutants of nsp2 have been identified and characterized (Frolov et al., 2009). The function of the SinV nsp3 protein is far more enigmatic. Mutational analysis has revealed domains required for viral synthesis (Park and Griffin, 2009; Wang et al., 1994; Gorchakov et al., 2008; Li et al., 1990). Exogenous expression of nsp3-GFP proteins reveals an association with the nuclear

envelope early in infection (Liang and Li, 2005). Furthermore, nsp3 has been observed associating with the nuclear pore complex (Gorchakov et al., 2008). The functional relevance of this observation has yet to be characterized. One could hypothesize that nsp3 is altering the import or export of HuR during viral infection. These proteins could be acting either by themselves, or through adaptor proteins, such as pp32 and APRIL, to modulate the localization of HuR. Testing of these hypotheses could be accomplished using ectopic expression of the nsp2 and nsp3 proteins. Furthermore, mutants of both of these proteins could be used to examine these hypotheses during viral infection.

In the New World Alphaviruses, the capsid protein possesses many of the activities associated with the Old World Alphavirus nsp2 protein. Interestingly, the VEEV capsid protein interacts with the nuclear pore complex. This interaction is believed to impede nuclear import / export in infected mammalian cells (Atasheva et al., 2008; Atasheva et al., 2010). Perhaps the Old World and New World Alphaviruses have evolved independent mechanisms to modulate the localization of RNA binding proteins. As with the above SinV proteins, ectopic expression of the VEEV capsid protein could test this hypothesis.

iv. Sequestration of HuR via Interaction with the Viral RNAs

Another possible mechanism of relocalization could involve the sequestration of the cellular HuR proteins via an interaction with the high affinity binding sites of the SinV RNAs. This mechanism is the least likely as HuR relocalization is still observed with SinV with the URE deleted from the 3'UTR

(Fig. 27A). While this is not a complete ablation of the HuR binding sites, the remaining HuR binding site in the CSE exhibits ~25-fold weaker binding *in vitro*. A significant reduction of HuR relocalization is not observed upon infection of mammalian cells with the ΔURE SinV as compared to a SinV with a wild-type 3'UTR (data not shown). Further examination of this mechanism could be accomplished in two ways. First, complete removal of the SinV RNA 3'UTR, including the CSE, could be attained using *in vitro* transcription and electroporation. Second, the HuR binding sites could be incorporated into a reporter construct. The localization of HuR could be examined following introduction of these transcripts.

v. HuR Relocalization as a Result of Defective Nuclear Import / Export

Finally relocalization of HuR may be due to a general perturbation of the nuclear import / export pathways. Examination of other nuclear factors including PABPN1 and Nucleophosmin revealed that relocalization of HuR is specific (data not shown). Interestingly CUGBP1 relocalizes from the nucleus to the cytoplasm in response to viral infection (data not shown). Therefore the pathways responsible for moving HuR in and out of the nucleus are likely intact, making this possibility unlikely.

It is possible that multiple mechanisms are inducing the relocalization of HuR as a consequence of viral infection. As indicated above, and in the introduction, SinV infection results in a high degree of host perturbation. Given the presence of multiple potential stimuli that could induce relocalization of HuR

there is a high probability of there being multiple events that lead to HuR perturbation. Determination of the primary driving force behind relocalization is likely to be difficult given the concurrent nature of many viral processes. To efficiently examine the effect of the above stimuli each potential HuR-altering event should be assayed as independently as possible. Currently it is unknown if HuR relocalization is a common consequence to viral infection or a SinV-specific phenomenon. Other cellular proteins relocalize as a result of viral infection. For instance the PTB protein is relocated in Vero cells during Dengue virus infection and in Cytomegalovirus infection (Agis-Juarez et al., 2009; Jiang et al., 2009; Anwar et al., 2009; Gaddy et al., 2010). Understanding the motivation behind HuR relocalization may give us insight as to how to affect viral stability via HuR modulation.

Development and Application of a Selectable Mosquito shRNA Technology

In order to directly assess the role(s) that aeHuR plays during an infection we first needed to develop a system to target the aeHuR mRNA using RNAi. RNAi systems consisting of shRNA vectors have been widely applied to reduce target protein levels in mammalian cells. The absence of similar vectors for exclusive use in mosquito systems represents a disadvantage to the field of arbovirology. To this end we set out to develop a multifunctional tool to generate shRNAs in mosquito cells.

I. The Hygromycin Phosphotransferase (hph) Cassette

A common feature of many mammalian shRNA vectors is the inclusion of a selection cassette encoded alongside a polymerase III based promoter. We could not utilize a mammalian shRNA vector since the promoters driving expression of the selection cassettes would not be optimal in mosquito cells. To this end we chose to utilize the Baculovirus immediate early (IE-1) promoter to drive expression of the hygromycin phosphotransferase (*hph*) gene from pHyg. Hygromycin selection has been used extensively throughout the field and has previously been successful in the development of insect cell lines (Monroe et al., 1992). Driving expression of *hph* with the IE-1 promoter ensures high levels of expression. Furthermore the IE-1 promoter is functionally active in a wide array of insect cell lines (Pullen and Friesen, 1995). Importantly the constitutive expression of *hph* ensures that rigorous selection via Hygromycin B will yield resistant cell clones.

II. The Aedes aegypti U6[1] Promoter Drives shRNA Expression

A second common feature of existing shRNA systems is the presence of a polymerase III-driven promoter typically in the form of a U6 promoter. Once again we chose to maintain the species specificity of the U6 promoter to avoid any possible deleterious effects on shRNA expression. The U6 promoters of *Aedes aegypti* were previously described (Konet et al., 2007). In our system we have utilized the *Aedes aegypti* U6[1] promoter to drive expression of sequences cloned into a downstream multiple cloning site. This particular multiple cloning site was

designed to be identical to that observed in the commercially available pSilencer 4.1-CMV vector (Ambion) allowing the use of Ambion's siRNA designer to select siRNAs. Both of these elements are combined to form the highly versatile pAeSH mosquito shRNA vector.

III. Applications of the pAeSH Vector

While the overall design and composition of the pAeSH mosquito shRNA vector is not novel, it is to our knowledge the first vector of its kind designed specifically with mosquitoes in mind. An obvious detractor to this technology is still the limitations on delivery into mosquito cells. Inclusion of the selection cassette on the same vector as the shRNA cassette has increased the efficiency of the development of cell lines. As exhibited by this dissertation project, this vector may be applied to develop hygromycin resistant cell populations that also express target specific shRNAs. Indeed it is precisely this application that enabled us to select the aeHuR deficient Aag2 cells utilized in this project.

We relied on this technology rather than the use of viral transducing vectors. Many of the described viral transducing systems rely upon the use of an Alphavirus with a second subgenomic promoter. While these systems have shown to be effective (as reviewed in Foy and Olson, 2008) we chose not to employ this technology for two reasons. First, we wished to decrease the HuR protein levels prior to infection so that we could examine the effects of HuR on *de novo* viral infection. Second we didn't want to confound the interpretation of our results by using a virus that may alter the natural progression of viral infection.

We avoided densonucleosis virus-mediated transducing systems for a similar reason.

IV. Future Development of the pAeSH System

Further advancement of this technology could entail altering the vector to be conducive to other delivery strategies. Pseudotyped lentivirus vectors have exhibited infectivity in insect cell lines (Matsubara et al., 1996). Altering the composition of the pAeSH vector could enable the use of a lentivirus-mediated approach to enhance delivery. This could potentially solve the delivery issues observed with mosquito cell cultures and enhance the utility of the pAeSH vector system.

The Cellular HuR Proteins Modulate Viral RNA Decay and Enhance Viral Infection

The reduction of the amount of both mosquito and mammalian HuR using RNAi resulted in the activation of viral RNA decay as exhibited by a decrease of the half lives observed for the SinV genomic and subgenomic RNAs (Figs. 30 & 31). Additionally, in cells with reduced HuR protein levels, decreases in viral progeny were observed. Furthermore deletion of the high affinity HuR binding site, the URE, resulted in a similar decrease of viral titer. Taken together these observations give biological function to the HuR:SinV RNA interactions characterized above.

I. RNAi of the Mosquito and Mammalian HuR Proteins Activates Viral RNA Decay

The activation of viral RNA decay in the presence of decreased cellular HuR protein levels implies that HuR is acting as a viral RNA stability factor (Fig. 30 & 31). As discussed above the SinV RNA 3'UTR, by way of the URE, contains a class III ARE-like sequence. Given the evidence presented above we conclude that this element is acting as a viral RNA stability determinant. Moreover this element is acting to stabilize the viral RNAs in tissue culture cells via the cellular HuR protein. This interaction represents a potential target for the development of antiviral therapeutics.

II. RNAi of the Mosquito and Mammalian HuR Proteins Decreases Viral Titer

An additional consequence of RNAi-mediated reduction of HuR protein levels is decreased viral titer in tissue culture systems (Fig. 32). The basis behind this observation is unclear. It is possible that the activation of viral RNA decay is directly resulting in the decreased viral titers. Due to the normalization process involved with the qRT-PCR analysis of viral RNAs we cannot distinguish, directly, the precise effect of RNA decay on viral titer. Our analysis of the abundances of SinV transcripts relies upon normalization to the cellular GAPDH mRNA. While examination of the GAPDH levels within our assays reveals that GAPDH abundance is largely unaffected during the course of the experiment, it also indicates that there may be differences in GAPDH mRNA abundance between control and HuR-knockdown cell lines. Therefore with the present data we cannot

conclude that the levels of viral RNA are directly affecting viral titer. Alternatively the cellular HuR proteins may be playing an unforeseen role in replication, for instance dictating the switch between translation and replication. Regardless, the reduction of viral titer by approximately 10-fold in mammalian tissue culture systems emphasizes the promise of HuR as an antiviral target. Reductions of these magnitudes are positively correlated with viral clearance of Hepatitis C infections treated with ribavirin (Reichard et al., 1998). Therefore it may be possible to alleviate or prevent severe Alphavirus disease, such as encephalitis, using an HuR-targeting approach.

III. The Cellular HuR Protein Represents a Promising Anti-Viral Target

Modulation of the interaction between the viral RNA and HuR could be accomplished in a multitude of ways. The binding of HuR to the viral RNA could be interrupted by small molecule inhibitors. Compounds with this type of function have been characterized, yet their potency as an antiviral has yet to be evaluated (Chae et al., 2009). A prospective challenge to this approach is that the binding of HuR to cellular targets may also be altered, leading to aberrant cellular function. A potential strategy to overcome this problem is to target the interaction at the level of the viral binding site. Aiming to interfere with the interaction of HuR and the viral RNA by pre-binding (and hence blocking HuR) or disrupting the binding site would potentially alleviate off target effects. This could in effect be mediated either by a protein (such as an RRM deficient HuR protein as described by Fan et al., 1998b) or by a small RNA that is complementary to the virus-

specific HuR binding site. A second potential strategy to prevent viral RNA stabilization by HuR is to preclude HuR from relocalizing from the nucleus to the cytoplasm. As stated earlier the localization of the Hu-family proteins greatly influences their function, thusly this strategy may also yield undesirable consequences.

HuR has also been shown to interact with the HCV RNA. While the direct function of this interaction is currently unknown it may involve post-transcriptional regulation of the HCV genome. Either way HuR appears to be important during HCV infection as RNAi of HuR results in substantial reduction of HCV infection (Korf et al., 2005). This implies that HuR may not be only a target for Alphavirus and HCV intervention, but perhaps a potent pan-viral therapeutic target.

The SinV URE Enhances Translation of the Viral Genomic RNAs

Deletion of the high affinity HuR binding site, the URE, resulted in decreased translation of the SinV genomic RNA. As indicated in Fig. 35, luciferase activity per RNA molecule was significantly reduced at times early in infection. In the cell, HuR-binding increases the association of the bound transcript to polysomes, resulting in enhancement of translation (Gantt et al., 2006; Nguyen et al., 2009; Perlewitz et al., 2010; Kawai et al., 2006; Lu et al., 2009). These data indicate that the HuR:viral RNA interaction may also be enhancing translation. Enhancing the translation of the viral genomes would greatly benefit the replication of the virus. Enhanced translation would lead to increased numbers of replication complexes, and as a result more viral

replication. The implications of this include possible roles in transmission and in pathogenicity as increased viral titer is associated with both. Taken together these data indicate that the URE-containing Alphaviruses, as noted with SinV, may benefit from the binding of HuR at multiple facets of post-transcriptional control: RNA stabilization and translational enhancement.

Possible Mechanisms of HuR Enhancement of SinV Translation

On cellular mRNAs the association of HuR is correlated with the enhancement of translation and is presumed to act through stabilization of the poly(A) tail-cap complex interaction. In the current model this interaction in turn aids in the shuttling of ribosomes leading to increased rates of initiation. Cellular PABP is implicated as a component of this complex. Human HuR is known to interact with PAIP-2, a known interacting partner of PABP. Interestingly, mosquito PABP was found to be associated with unadenylated SinV RNA 3'UTR. Whether this is a consequence of a HuR:PABP interaction or a PABP:SinV RNA 3'UTR interaction is unclear. Either way the possible binding of HuR and PABP on viral RNAs indicates that maybe a translational effect is a component of the HuR:SinV RNA 3'UTR interaction.

The observation that the effect is greater earlier during SinV infection perhaps indicates a temporal effect on the importance of the URE during infection. As indicated by Fig. 35 the difference between the translational efficiencies of the ΔURE SinV and the wild type SinV decreases with time. There are several explanations for this effect- 1) The URE (and perhaps HuR) is more

important at early times during infection where the viral RNAs are few, 2) SinV infection is altering the cytoplasmic abundance of HuR, abrogating the effect of the URE deletion and 3) Replication is changing the functions of the genomic RNAs leading to decreased luciferase production.

i. Differential Replication May Lead to Increased Luciferase from Wild Type SinV Early During Infection

The possibility that SinV has evolved the high affinity HuR-binding URE to jumpstart viral infection is interesting. In our assays, using the MOI of 5, nearly all (as much as 99.8%) of the cells are infected with a single virus. Perhaps SinV has evolved to utilize the URE to enhance the translation of the transcript reducing the amount of time required to assemble functional replicase complexes. Infecting the host cell with multiple copies of the virus would likely increase the amount of replication complexes formed in the cell. Perhaps this would overcome the stall observed with the ΔURE SinV. The effects of MOI on the translational efficiency of the ΔURE SinV have not been examined.

ii. HuR Relocalization as a Result of Viral Infection Diminishes the Role of the URE in Directing Early Translation

We have previously shown that SinV induces the relocalization of HuR from the nucleus to the cytoplasm as a result of infection. It is possible that this relocalization event is influencing the translation of SinV. Perhaps the increased abundance of HuR in the cytoplasm is mitigating the effect of the URE deletion

via the interaction of HuR and the lower affinity CSE-binding site. Since the CSE cannot be deleted without significantly inhibiting the replication of SinV examination of this would require manipulation of the HuR molecule itself. Indeed residues capable of sequestering HuR to the nucleus have been identified (as outlined above), and expression of one of these HuR molecules in lieu of the wild type HuR could be used to examine this potential model of HuR translational regulation. Obviously this approach would take considerable development, as the wild type HuR would have to be eliminated from the proteome. Alternatively the URE (and CSE) could be cloned into a reporter construct and examined for their effects on translation independent of viral infection.

iii. Replication of SinV Genomic RNAs Leads to Diminished Translation Allowing the ΔURE SinV to "Catch Up"

Another possible explanation for the apparent lag in translation of ΔURE SinV genomic RNAs is the assembly of replicase complexes. Translation of the SinV genomic RNA leads to formation of the nonstructural polyprotein which, after proteolytic processing, yields the components of the viral replicase complex. It is possible that assembly of the replicase complex leads to a change in the function of the genomic RNA from mRNA to template. In the wild type SinV the increased translation may be leading to a more rapid switch of the genomic RNA from mRNA to template leading to decreased luciferase per RNA. This may in effect allow the ΔURE SinV genomic RNAs to "catch up" to the wild type translational efficiencies. To examine this possibility one could use a replication

deficient SinV, such as ts6SinV – where under nonpermissive conditions the expression of luciferase would be entirely independent of viral replication allowing the "catch up" hypothesis to be examined. Examination of luciferase expression levels in the absence of replication would eliminate any possible mRNA / template effects.

II. Luciferase Levels Decrease Very Early During Infection of Aag2 Cells

It should also be noted that in mosquito cells a reduction of luciferase activity at 2 hours post infection is observed. The cause of this effect is currently unclear. This phenomenon may be due to several causes: 1) degradation of luciferase may be higher in mosquito cells than mammalian cell lines, inhibiting accumulation, and 2) SinV RNAs may not be as robustly translated in mosquito cells as compared to mammalian cells due to the persistent nature of the infection. Regardless of the drop in luciferase expression at 2 hpi the luciferase levels of the ΔURE SinV remain well below the wild type 3'UTR SinV in the remaining time points.

III. Future Directions

These data further highlight the importance of the URE in viral infection. Similar to the knockdown of HuR using shRNAs the replication of the ΔURE SinV mutants was significantly reduced. Whether the reduction of viral progeny is a result of RNA instability or translational impairment is yet to be characterized.

This represents a logical next step, as understanding the full role of the URE will fundamentally enhance our comprehension of Alphavirus biology.

Conclusion

In conclusion the SinV RNA 3'UTR directs the stability of the SinV RNAs *in vitro* and in tissue culture cells. The SinV RNA 3'UTR was found to block deadenylation, the primary mechanism of cellular mRNA degradation. Repression of deadenylation was found to be due to two independent elements. Both the RSE and URE domains were found to be capable of repressing deadenylation independently of each other *in vitro*. The RSE was determined to be acting in a *cis*-manner, while the URE was found to be acting via a *trans*-acting mechanism. Moreover the URE was a major stability element when compared to the stability observed with the entire SinV RNA 3'UTR.

Examination of the URE revealed a high affinity binding site for the cellular HuR protein. This interaction is likely due to the strong resemblance of the URE to class III AREs, known modulators of cellular mRNA stability. The abundance of the HuR protein was found to directly impact viral RNA stability and viral titer. Deletion of the predominant HuR binding site, the URE, also resulted in decreased viral titer. Furthermore deletion of the URE decreased translation. Taken together these findings indicate that our hypothesis is correct. SinV has evolved a stability element that is highly similar, in both composition and function, to cellular mRNA stability elements.

These studies are the first to functionally characterize an mRNA stability element within the 3'UTR of an Alphavirus in both mammals and mosquito cell lines. Furthermore, understanding the function of the URE, and to a lesser extent

the RSE, has increased the breadth of our comprehension of Alphavirus biology. The implications of these findings are broad – It is highly likely that all viruses must at some level interact with the cellular mRNA decay machinery. Moreover, to preserve, and perhaps enhance, the functions of their RNAs, most RNA viruses likely have evolved regulatory features like that described above for SinV. These elements, in conjunction with their associated effectors, are prime targets for therapeutic intervention. Understanding the role(s) of and mechanisms behind these elements will no doubt be an important area of future research.

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APPENDIX A

Protein Alignment of the *Aedes aegypti* Hu Proteins to *Drosophila melanogaster* ELAV. Protein sequence alignment of the NCBI Protein REFSEQs AAF45517 (meELAV), XP_001658986.1 (aeHuR), XP_001659335.1, XP_001657938.1 and XP_001660922.1. An asterisk denotes conservation while a (**:**) and a (**.**) denote moderate and weak similarity respectively. Alignment generated using ClustalW.

Sequence Cloned	Nucleotide Sequence	Primer Name	Plasmid Name
VEE URE/CSE	5'-AATTCTTTTTATTTTATTTTTTCTTTTCTTTTCCGAATCGGATTTTGTTTTAATATTTCCTGCA-3' 5'-GGAAATATTAAAACAAAATCCGATTCGGAAAAGAAAAGAAAAAATAAAATAAAAAG-3'	VEE3'60 top VEE3'60 bot	VEE 3'60
EEE URE/CSE	5'-AATTCTATCAATTTACAAATCATATTAATTTTTCTTTTATGTTTTTATTTTGTTTTTAATATTTCCTGCA-3' 5'-GGAAATATTAAAAACAAAATAAAAACATAAAAGAAAAATTAATATGATTTTGTAAATTGATAG-3'	EEE3'60 top EEE3'60 bot	EEE 3'60
WEE URE/CSE	5'-AATTCTACACACTTTATAAATTCTTTTATAATTTTTCTTTGTTTTTATTTTGTTTTTAAAATTTCCTGCA-3' 5'-GGAAATTTTAAAAACAAAATAAAAACAAAAGAAAAATTATAAAAGAATTTATAAAGTGTGTAG-3'	WEE3'60 top WEE3'60 bot	WEE 3'60
SFV URE/CSE	5'-AATTCAGCTTAATTCGACGAATAATTGGATTTTTATTTTATTTTGCAATTGGTTTTTAATATTTCCCTGCA-3' 5'-GGGAAATATTAAAAACCAATTGCAAAATAAAATAAAAATCCATTATTCGTCGAATTAAGCTG-3'	SFV3'60 top SFV3'60 bot	SFV 3'60
VEE URE	5'-AATTCTTTTTATTTTATTTTTTCTTTTCTTTTCCGAATC-3' 5'-CCGATTCGGAAAAGAAAAGAAAAAATAAAATAAAAAG-3'	VURR top VURR bot	VURE
EEE URE	5'-AATTCTATCAATTTACAAAATCATATTAATTTTTCTTTTATGTTTTTCTGCA-3' 5'-GAAAAACATAAAAGAAAAATTAATATGATTTTGTAAATTGATAG-3'	EEEURR top EEEURR bot	EURE
WEE URE	5'-ATTCTACACACTTTATAAATTCTTTTATAATTTTTCTTTTGTTTTTCTGCA-3' 5'-GAAAAACAAAAGAAAAATTATAAAAGAATTTATAAAGTGTGTAG-3'	WEEURR top WEEURR bot	WURE
SFV URE	5'-AATTCAGCTTATTCGACGAATAATTGGATTTTTATTTTATTTTGCACTGCA-3' 5'-GTGCAAAATAAAATAAAAATCCAATTATTCGTCGAATTAAGCTG-3'	SFVURR top SFVURR bot	SFURE
SinV RSE3 5'EXT	5'-CAAAACGCAATGTATTCTGAGGAAGCGTGGTGCATAATGCA-3' 5'-AGCTTGCATTATGCACCACGCTTCCTCAGAAATACATTGCGTTTTGCATG-3'	RSE35xT RSE35xB	RSE3 5'EXT

qRT-PCR Detection Oligos		
SinV Total RNA	Forward-	5'-ACCAAAAACGCAGGAGAAGA-3'
	Reverse-	5'-CCTTTCACGTGCAGAGGTTT-3'
SinV Genomic RNA	Forward-	5'-CATCGGTGAGAGACCACCTT-3'
	Reverse-	5'-AACCACGCCTTTGTTTCATC-3'
Human GAPDH	Forward-	5'-AAGGTGAAGGTCGGAGTCAA-3'
	Reverse-	5'-AATGAAGGGGTCATTGATGG-3'
Human HuR	Forward-	5'-AGCAGCATTGGTGAAGTTGA-3'
	Reverse-	5'-GCGGTCACGTAGTTCACAAA-3'
Mosquito aeHuR	Forward-	5'-AAGATTGTGCAGCCTGCTTT-3'
	Reverse-	5'-CGTATCCCTTGCACTGGTTT-3'
Mosquito GAPDH	Forward-	5'-AAGCTGCCAGAGAATGTCGT-3'
	Reverse-	5'-CTTTTCCTCTGCGACCTCAC-3'

APPENDIX C

Abbreviation	
293T	T-antigen transformed human embryonic kindey cell line
Aag2	*Aedes aegypti* cell line
AGO	Argonaute protein
AMPK	AMP-activated kinase
APRIL	Acidic protein rich in leucine
ARE	AU rich element
ATP	Adenosine triphosphate
AUF1	AU rich element binding protein 1
BFV	Barmah Forest virus
BHK-21	Baby hamster kidney cell line
Brd2	Bromodomain containing 2
BSA	Bovine serum albumin
C	Celsius
C6/36	*Aedes albopictus* cell line
CAF1	CCR4-associated factor 1
CARM1	Coactivator associated arginine methylase 1
CCR4	Carbon catabolite repressor 4
CCR4-NOT	Carbon catabolite repressor 4 – negative on TATA complex
cDNA	Complementary DNA
Cdk1	Cyclin dependent kinase 1

C/EBP	CCAAT/ enhancer binding protein
CHIKV	Chikungunya virus
Chk2	Cell cycle checkpoint kinase 2
c-fos	FBJ osteosarcoma oncogene
c-myc	Myelocytomatosis oncogene
COX-2	Cyclooxygenase 2
CPM	Counts per minute
CSE	Conserved sequence element
CTP	Cytidine triphosphate
CUGBP1	CUG binding protein 1
Da	Daltons
DCP	Decapping protein
DCPS	Decapping protein scavenger
Dhh1p	DEAD-box helicase
DNA	Deoxyribonucleic acid
Dom34p	Endoribonuclease, probable pelota transcription factor
dsRNA	Double stranded ribonucleic acid
DTT	Dithiothreitol
E1	Envelope protein1
E2	Envelope protein 2
Edc	Enhancer of decapping
EDTA	Ethylenediaminetetraacetic acid
EEEV	Eastern equine encephalitis virus

eIF4E	Eukaryotic initiation factor 4E
EJC	Exon junction complex
ELAV	Embryonic lethal abnormal vision
EMSA	Electrophoretic mobility shift assay
eRF	Eukaryotic release factor
ErEN	Erythroid-enriched endonuclease
FBS	Fetal bovine serum
Fig	Figure
FHVB2	Flock House virus B2 protein
g	Gravity (on Earth)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCSH	Glutamylcysteine synthetase heavy subunit
GCNF	Nuclear receptor group A subfamily 6
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte macrophage colony stimulating factor
GRE	GU rich element
GST	Glutathione S transferase
GTP	Guanidine triphosphate
GW182	Trinucleotide repeat containing 6A protein
Hbs1p	Hsp70 subfamily B repressor 1
Hedls	Human enhancer of decapping (large subunit)
His3p	Histidine sensitivity
HIVE	HIV primer E

HCV	Hepatitis C Virus
HeLa	Human cervical carcinoma cell line
hnRNP	Heterogenous ribonucleoprotein particle
HNS	HuR nucleocytoplasmic shuttling sequence
hph	Hygromycin phosphotransferase
hpi	Hours post infection
hr	Hours
HSCB	High salt column buffer
HRP	Horseradish peroxidase
Hsp	Heat shock protein
Hu	Human antigen
IFN	Interferon
IL	Interleukin
IRES	Internal ribosome entry site
K_D	Dissociation constant
kDa	Kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
KSRP	KH-containing splicing regulatory factor
L	Liter
La	SS-B autoantigen
LB	Luria broth
Lsm1-7	Like SM protein complex
MALDI-TOF/TOF	Matrix assisted laser desorption ionization time of flight time of flight spectrometry

MAPK	Mitogen-activated protein kinase
mCRD	Major coding region determinant of instability
MDA5	Melanoma differentiation associated gene 5
MK2	MAPK activated kinase 2
mL	Milliliter
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mRNP	Messenger ribonucleoprotein
MS	Masspectrometry
NCBI	National center for biotechnology information
NEB	New England biolabs
NF	Nuclear factor
NGD	No-go decay
nm	Nanometer
NMD	Nonsense-mediated decay
NOT	Negative on TATA
NSD	Non-stop decay
nsP	Nonstructural protein
OD	Optical density
ONNV	O'nyong-nyong virus
ORF	Open reading frame
PABP	Poly(A) binding protein
PAGE	Polyacrylamide gel electrophoresis

PAN	Poly(A) binding protein-dependent poly(A) nuclease
PAN RNA	Poly(A) nuclear RNA
PAN-ENE	PAN enhancer of nuclear retention element
PARN	Poly(A)-specific ribonuclease
Pat1p	Topoisomerase I associated protein
PAZ	PIWI Argonaute and Zwille domain
P-Bodies	Processing bodies
PBS	Phosphate buffered saline
PC	Phosphocreatine
PCBP	Poly(C) binding protein
PCI	Phenol chloroform iso-amyl alcohol
PCR	Polymerase chain reaction
PCV	Packed Cell Volume
PIWI	P-element induced wimpy testes
PKC	Protein kinase C
PMR1	Polysomal ribonuclease 1
PNK	Polynucleotide kinase
PP2A	Protein phosphatase 2A
PPE	Pre-mRNA processing enhancer
PRE	Pyrimidine-rich element
PTB	Polypyrimidine tract binding protein
PTC	Premature termination codon
PVA	Polyvinyl alcohol

PVDF	Polyvinylidine fluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RBP	Ribonucleic acid binding protein
RISC	RNA induced silencing complex
RIG-I	Retinoic acid inducible gene 1
RIPA	Radioimmunoprecpitiation assay
RNAi	RNA interference
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RRM	RNA recognition motif
RSE	Repeat sequence element
RSV RSE	Rous sarcoma virus ribonucleic acid stability element
RT	Reverse transcriptase
SARS	Severe acute repiratory syndrome
SDS	Sodium dodecyl sulfate
SET	SET nuclear oncogene
SFV	Semliki Forest virus
SG	Stress granule
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIRT1	Silent mating type information regulon 1
SinV	Sindbis virus

SKI	Superkiller
SLBP	Stem loop binding protein
SMAUG	Sterile alpha motif domain containing protein
SMG	Small male genitalia
SMG-SURF	SMG1-Upf1-eRF1-eRF3 Complex
SOX	Shutoff and exonuclease
SP6	Bacteriophage DNA dependent RNA polymerase
spp	Species
ssRNA	Single stranded ribonucleic acid
Stm1p	Stimulator of decapping
T4	Bacteriophage T4
TIA-1	T cell intracellular antigen 1
TIAR	TIA-1 related protein
TNF	Tumor necrosis factor
tRNA	Transfer ribonucleic acid
TTP	Tristetraproline
U	Units
UA	Uridine adenosine dinucleotides
µCi	Microcurie
µg	Microgram
UNR	Upstream of N Ras
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

Upf	Upstream frame shift protein
URE	Uridine rich element
UTP	Uridine triphosphate
UTR	Untranslated region
UV	Ultraviolet
UU	Uridine uridine dinucleotides
µg	Microgram
µl	Microliter
v	Volume
VEEV	Venezuelan equine encephalitis virus
VPg	Virus 5' protein covalently linked
WEEV	Western equine encephalitis virus
w/v	Weight per volume
XRN1	5'→3' exoribonuclease 1
ZAP	Zinc-finger antiviral protein