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Article

# Sindbis Virus Usurps the Cellular HuR Protein to Stabilize Its Transcripts and Promote Productive Infections in Mammalian and Mosquito Cells

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## SUMMARY

**How viral transcripts are protected from the cellular RNA decay machinery and the importance of this protection for the virus are largely unknown. We demonstrate that Sindbis virus, a prototypical single-stranded arthropod-borne alphavirus, uses U-rich 3′ UTR sequences in its RNAs to recruit a known regulator of cellular mRNA stability, the HuR protein, during infections of both human and vector mosquito cells. HuR binds viral RNAs with high specificity and affinity. Sindbis virus infection induces the selective movement of HuR out of the mammalian cell nucleus, thereby increasing the available cytoplasmic HuR pool. Finally, knockdown of HuR results in a significant increase in the rate of decay of Sindbis virus RNAs and diminishes viral yields in both human and mosquito cells. These data indicate that Sindbis virus and likely other alphaviruses usurp the HuR protein to avoid the cellular mRNA decay machinery and maintain a highly productive infection.**

## INTRODUCTION

Cellular RNA decay is a robust process by which the cell rapidly removes unwanted or aberrant RNAs from its transcriptome (Garneau et al., 2007). A significant proportion of cellular gene regulation rests at the level of posttranscriptional control via the selective degradation of mRNAs (Cheadle et al., 2005; García-Martínez et al., 2004). Thus, the cellular mRNA decay machinery serves as an effective control mechanism for the quantity and quality of mRNAs in the cytoplasm. The members of genus *Alphavirus* of the family *Togaviridae* are a group of geographically diverse single-stranded positive-sense RNA viruses (Strauss and Strauss, 1994). The genomic and subgenomic RNAs of the alphaviruses closely resemble the cellular mRNAs produced by RNA polymerase II, as they are $^{7me}$GpppG capped at their 5′ end and 3′ polyadenylated. Therefore, these viral mRNAs likely have the capacity to interface with cellular RNA decay factors during infection. The goal of this study was to determine how these viral transcripts escape surveillance by the cellular mRNA decay machinery.

For the majority of cellular mRNAs, the primary and rate-limiting step of degradation is the removal of the 3′ poly(A) tail by one or more cellular deadenylases (Xu et al., 2001; Wilson and Treisman, 1988). Deadenylation of mRNAs results in translational silencing, as well as serving to expose the 3′ end of the transcript to exonucleolytic degradation by the exosome (Schmid and Jensen, 2008) or prime the transcript for decapping and subsequent 5′-3′ exonucleolytic digestion by XRN1 (Franks and Lykke-Andersen, 2008). Therefore, one effective method for viral transcripts to evade the cellular mRNA decay machinery would be to inhibit the deadenylation step. In fact, Sindbis virus (SinV), the model *Alphavirus*, contains multiple elements in its 3′ UTR that we recently demonstrated are capable of independently repressing deadenylation (Garneau et al., 2008). Using both tissue culture-based assays and a cell-free RNA decay system (Sokoloski et al., 2008a, 2008b), we have established that the repeated sequence elements (RSEs) as well as a ~40 base U-rich element in conjunction with the 19 nt 3′-terminal conserved sequence element (URE/CSE) are capable of repressing deadenylation. The ability of the URE/CSE region to repress deadenylation in vitro was associated with the binding of a 38 kDa cellular *trans*-acting factor. Examination of the 3′ UTRs of other viruses within the *Alphavirus* genus reveals that while the overall 3′ UTR sequences may be divergent, the presence of the URE is well conserved in most *Alphavirus* species (Ou et al., 1982; Strauss and Strauss, 1994), with notable exceptions being O'nyong-nyong, Chikungunya, and Ross River viruses. Taken together, these data confirmed our hypothesis that RNA viruses, such as SinV, do in fact interface with the cellular mRNA decay machinery.

In this study, we determined the mechanism by which SinV represses the degradation of its transcripts during infection of human and mosquito cells. The URE/CSE region of multiple alphaviral 3′ UTRs is bound specifically and with high affinity to the cellular HuR protein, a known regulator of cellular mRNA stability (Hinman and Lou, 2008; Abdelmohsen et al., 2009). This interaction occurs during SinV infection in both *Aedes* and human cells. While the mosquito HuR homolog (aeHuR) is largely cytoplasmic, in human cells SinV infection induces a dramatic translocation of the HuR protein from the nucleus to the cytoplasm, where SinV viral RNAs accumulate. Knockdown of HuR protein in either *Aedes* or human cells significantly destabilizes SinV mRNAs and reduces viral yields. Taken together, these studies establish the HuR protein as a cellular factor required for efficient alphavirus infection. Furthermore, these studies suggest that other viruses may also have evolved ways to

interface with the cellular RNA decay machinery to stabilize their transcripts to promote a productive infection.

## RESULTS

### U-Rich Elements in the 3′ UTR of Multiple Alphaviruses Repress Deadenylation and Bind a Similar Set of Cellular Proteins

Given the major roles of RNA decay in gene regulation and disposal of unwanted transcripts, capped and polyadenylated positive-sense RNA viruses have likely developed some method of successfully interfacing with the cellular RNA decay machinery to stabilize their transcripts during the course of an infection. Stability elements of cellular mRNAs are often located within their 3′ UTRs and have been shown to regulate transcript-specific decay (Garneau et al., 2007). We have previously demonstrated that SinV RNAs, like cellular mRNAs, contain stability elements in their 3′ UTRs (Garneau et al., 2008). These stability elements serve to repress deadenylation in tissue culture models of SinV infection and cell-free systems. Interestingly, we determined that a major stability determinant within the SinV 3′ UTR is a ~60 base U-rich region at the 3′ end, termed the URE/CSE. The URE portion of this region previously had no ascribed function despite being conserved, at least in nucleotide bias, among numerous members of the genus (Strauss and Strauss, 1994).

Given the conservation of the URE/CSE among the alphaviruses, we sought to determine if the repression of deadenylation observed with the SinV URE/CSE was indeed a common property of the URE/CSEs found in other members of the genus. We demonstrated previously that a cell-free mRNA decay system that we developed using mosquito cytoplasmic extracts faithfully reproduced SinV URE/CSE-mediated RNA stabilization (Opyrchal et al., 2005; Garneau et al., 2008; Sokoloski et al., 2008a). Therefore, we used this assay to evaluate the ability of the URE-bearing alphaviruses to repress deadenylation. Internally radiolabeled, capped, and polyadenylated RNA substrates that contained either a nonspecific reporter sequence or an insertion of the ~60 base URE/CSE region of SinV, Venezuelan Equine Encephalitis (VEE), Eastern Equine Encephalitis (EEE), Western Equine Encephalitis (WEE), or Semliki Forest Virus (SFV) were incubated in a cell-free mRNA decay assay with mosquito cell cytoplasmic extract. As shown in Figures 1A and 1B, while incubation of the control RNA substrate in the system resulted in the rapid removal of the poly(A) tail and accumulation of a deadenylated (A0) intermediate (more than 50% of the substrate was completely deadenylated within 9 min), the RNAs containing each of the alphavirus URE/CSE regions exhibited a 5-fold or greater stability relative to the deadenylation rate of the control RNA substrate. This strongly suggests that the URE/CSEs of many alphaviruses are indeed bona fide mRNA stability elements and that the ability to repress deadenylation is a conserved property of these viruses.

Using competition analyses in cell-free assays, we have previously shown that the repression of deadenylation associated with the 3′ UTR of SinV is mediated through the interaction of host protein(s) (Garneau et al., 2008). In addition, using UV crosslinking in conjunction with these competition assays, we are able to correlate the binding of a 38 kDa cellular factor to the SinV 3′ UTR with the repression of deadenylation in our cell-free system. As shown in Figures 1C and 1D, the binding site for this 38 kDa factor maps to the URE/CSE region of the SinV 3′ UTR. Interestingly, the URE/CSE region of the other four alphaviruses that repressed deadenylation in Figure 1A all had a pattern of UV crosslinked proteins nearly identical to the SinV URE/CSE, including the 38 kDa band of interest (Figure 1E). While UV crosslinking of the 38 kDa protein was resistant to the addition of 2.6 μg/μl heparin, the faster-migrating 32 kDa protein band seen in Figures 1D and 1E was effectively competed from the RNA by the addition of the polyanionic competitor, suggesting that its interaction could be nonspecific (data not shown).

Taken together, these data strongly suggest that many alphaviruses, despite considerable evolutionary divergence (Strauss and Strauss, 1994; Ou et al., 1982), may have maintained a similar strategy to evade the cellular mRNA decay machinery. Curiously, despite the apparent primary sequence heterogeneity of the *Alphavirus* 3′ UTRs as a whole, the conserved nucleotide bias of the URE (Figure S1A) was sufficient to repress deadenylation and crosslink to similar proteins in each case. The retention of function, despite the fluidity of primary sequence identity, underscores the potential impact of the virus/RNA decay machinery interface on positive-strand RNA virus biology.

### Affinity Purification of SinV URE/CSE-Interacting Factors Identifies the 38 kDa Stability Factor as a HuR Homolog

In order to determine how the 38 kDa cellular factor functions to stabilize alphaviral RNAs, we needed to know its identity. To this end, we used the URE/CSE region of the 3′ UTR that we delineated as being necessary and sufficient for binding of the 38 kDa protein (Figure 1D) in an in vitro affinity purification strategy. Briefly, 5′-biotinylated RNA oligomers consisting of either the 3′-terminal 54 bases of SinV or a nonspecific control sequence were bound to streptavidin-agarose resin. C6/36 *Aedes albopictus* mosquito cell cytoplasmic extract was incubated with the resin, and unbound proteins were removed by rigorous washing. Retained proteins were eluted, resolved using SDS-PAGE, and detected by silver staining. As shown in Figure S2A, several host proteins specifically bound to the SinV USE/CSE RNA oligomer compared to the control. The 38 kDa band was excised and analyzed via tandem mass spectrometry following trypsin digestion.

Given the current lack of a complete *Aedes albopictus* genome, the *Aedes aegypti* genome (Nene et al., 2007) was utilized as a surrogate for the database search of the mass spectrometry data. The analysis revealed with high probability that the 38 kDa factor was a mosquito ELAV superfamily member (AAEL008164) with notable homology to the mammalian HuR protein, a known mRNA stability factor (Hinman and Lou, 2008; Abdelmohsen et al., 2009). Given the high degree of homology between the *Aedes aegypti* hypothetical protein and human HuR (~55% identical according to BLAST analysis) (Figure S3), we have chosen to refer to the *Aedes* protein as aeHuR henceforth.

Since *Drosophila* anti-ELAV monoclonal antibodies failed to recognize *Aedes* ELAV proteins (data not shown), we first needed to develop reliable immunological reagents specific to aeHuR in order to confirm the identity of the 38 kDa factor

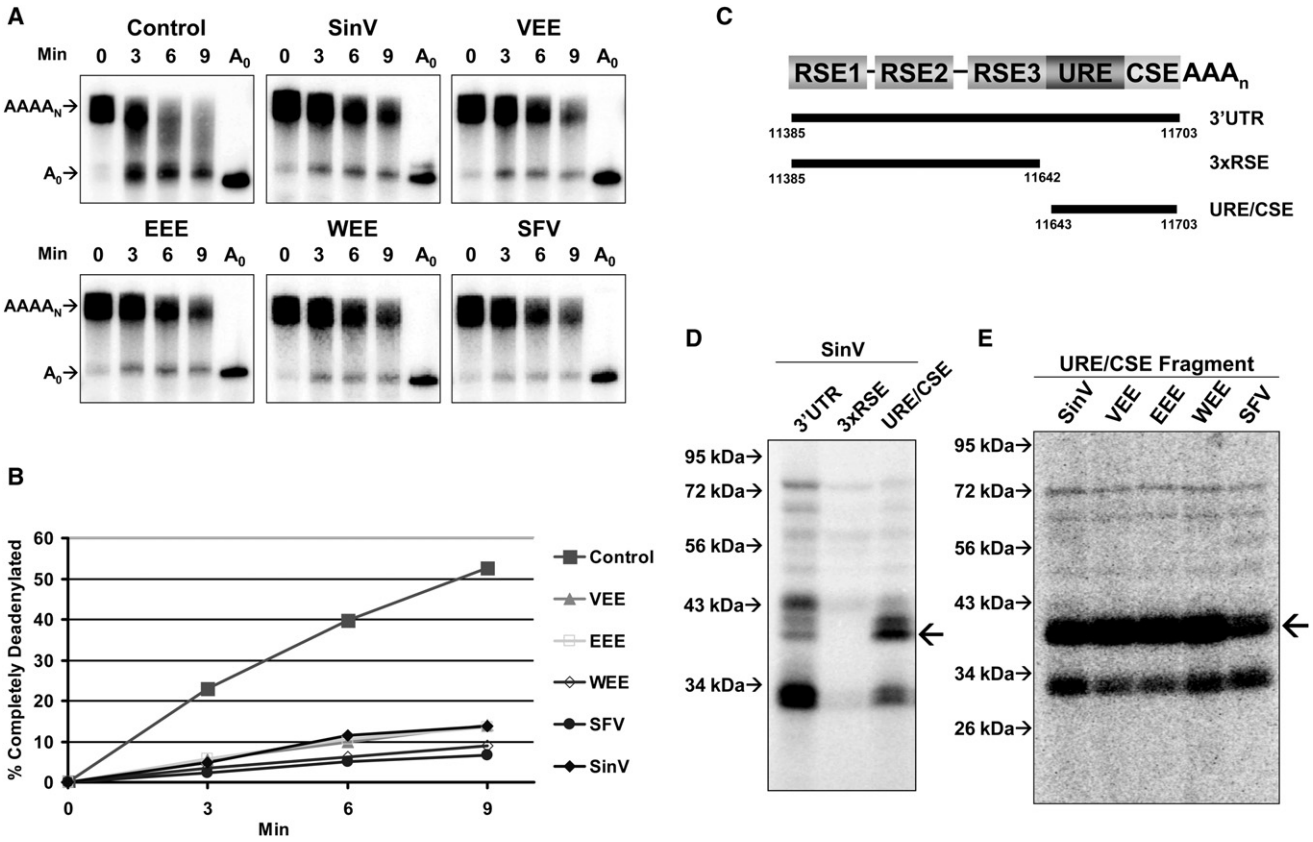



**Figure 1. The Conserved U-Rich URE/CSE Region of the 3′ UTR of Five Alphaviruses Represses RNA Deadenylation/Decay and Interacts with a Common Set of Cellular Proteins**

(A) Capped and polyadenylated reporter RNAs containing either control sequences (control) or the 3′ URE/CSE sequences from Sindbis (SinV), Venezuelan equine encephalitis (VEE), eastern equine encephalitis (EEE), western equine encephalitis (WEE), or Semliki forest virus (SFV) were incubated in a cell-free mRNA deadenylation/decay assay using cytoplasmic extracts from C6/36 cells. Samples were taken at the time points indicated, and RNAs were analyzed on a 5% acrylamide/7 M urea gel. Radioactive RNAs were visualized via phosphorimaging. The arrows on the left indicate RNAs containing a 60 base poly(A) tail ($AAAA_N$) or fully deadenylated products ($A_0$).

(B) Graphical representation of data in (A). Since deadenylation is largely occurring in a processive fashion, the amount of RNA that is completely deadenylated (i.e., migrates with the $A_0$ marker) was compared to the total amount of fully adenylated RNA.

(C) Diagram of the SinV 3′ UTR fragments assayed in (D).

(D) Radioactive RNAs from the entire 3′ UTR of SinV (3′ UTR), the isolated repeated sequence element region (3XRSE), or the terminal 60 nt URE/CSE region (URE/CSE) were incubated with C6/36 cytoplasmic extracts. Samples were irradiated with UV light and treated with RNase, and covalent RNA-protein complexes were separated by 10% SDS-PAGE. Molecular weight markers are indicated on the left, and the arrow highlights the 38 kDa band.

(E) The URE/CSE fragments of the 3′ UTR from the indicated virus were incubated with C6/36 cytoplasmic extracts and subjected to UV crosslinking analysis, as described for (D). See also Figure S1.

implicated in viral RNA stability. Recombinant aeHuR protein was prepared in *E. coli* and used to generate polyclonal antisera in rabbits. As seen in Figure S2B, these antibodies specifically detected a 38 kDa protein in a western blot of C6/36 mosquito cell cytoplasmic proteins. We next assessed whether this anti-aeHuR sera would recognize and specifically precipitate the 38 kDa protein crosslinked to the SinV URE/CSE that we previously correlated with repression of deadenylation. As seen in Figure 2A, the 38 kDa crosslinked band was specifically immunoprecipitated using aeHuR antisera but not with control preimmune sera. Similar data were obtained for immunoprecipitation of the 38 kDa protein crosslinked to the URE/CSE of the other four alphaviruses analyzed in Figure 1 (Figure S4A).

Next, we wished to examine whether the URE/CSE region of the SinV 3′ UTR was capable of interacting with the mammalian HuR protein. Using UV crosslink/immunoprecipitation assays with HeLa cytoplasmic extract, we confirmed that this was indeed the case. The URE/CSE element of SinV (Figure 2B) as well as the URE/CSE elements of VEE, EEE, WEE, and SFV (Figure S4B) were capable of crosslinking to HuR protein. Nonspecific control RNAs fail to crosslink to HuR in these assays (Garneau et al., 2008 and data not shown). Therefore, the ability of HuR protein to interact with all five alphavirus 3′ UTRs is conserved across both mammalian and vector mosquito species.

Finally, while aeHuR and HuR are clearly capable of interacting with the alphaviral URE/CSE elements in cell-free assays, we sought to extend this observation to tissue culture cells during the course of an infection. Either 293T (human embryonic kidney) or Aag2 (*Aedes aegypti*) cells were infected with wild-type SinV

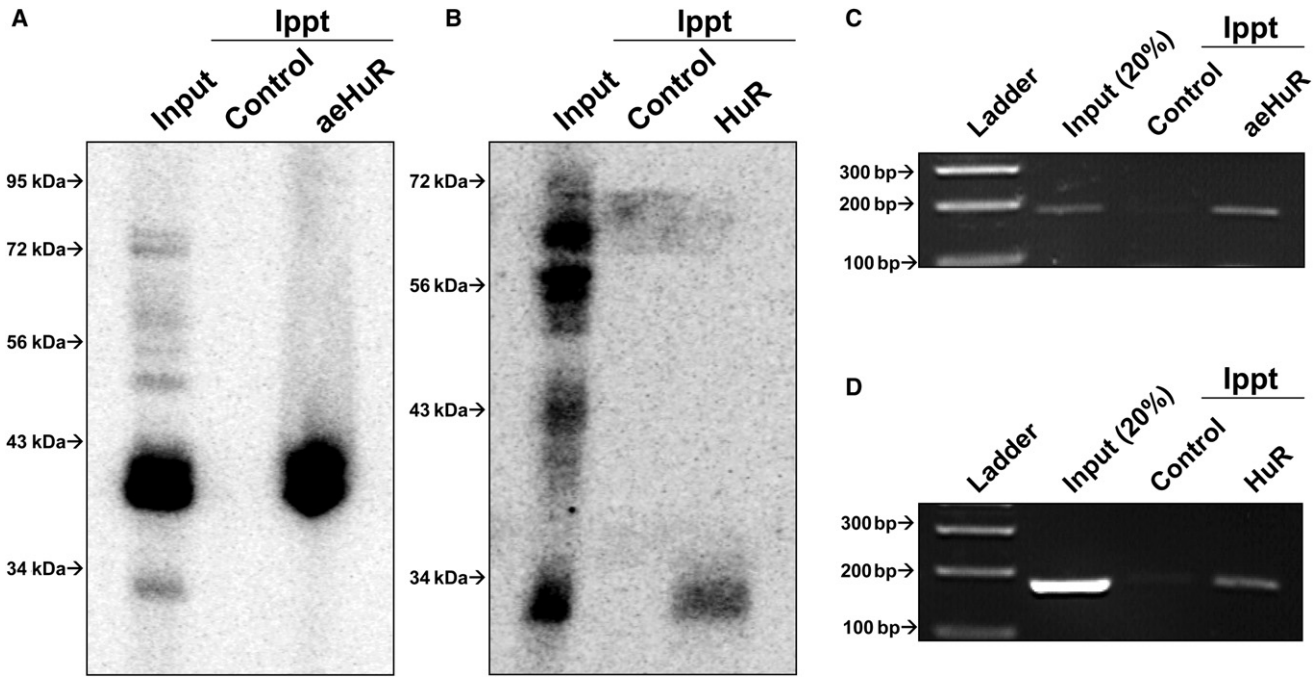



**Figure 2. HuR Protein Interacts with SinV RNAs in Both Cell Extracts and Cultured Cells**

(A) Radiolabeled RNA containing the SinV URE/CSE was UV crosslinked to C6/36 cytoplasmic proteins as described in Figure 1D and either loaded directly onto a 10% SDS-PAGE gel (Input lane) or immunoprecipitated using either a control preimmune antibody (Control lane) or an aeHuR-specific antibody (aeHuR lane) prior to electrophoresis. Radiolabeled proteins were detected by phosphorimaging.

(B) Identical to (A), except that HeLa cytoplasmic extract was used as the starting material and the immunoprecipitation was done with anti-human HuR antibodies.

(C) Aag2 cells were infected with SinV for 12 hr, formaldehyde was added to stabilize protein-RNA complexes, and samples were immunoprecipitated using either a preimmune serum (control lane) or anti-aeHuR antibodies. Crosslinks were reversed and SinV-specific RNAs were detected in the samples using RT-PCR via electrophoresis on a 2% agarose gel containing ethidium bromide.

(D) Identical to (C), except 293T cells were used instead of Aag2 cells and human-specific HuR antisera was used for immunoprecipitation. See also Figure S2.

at an moi of 5. At 12 hr postinfection (hpi), formaldehyde was added to the cells to stabilize protein:RNA complexes. Cell lysates were prepared and immunoprecipitation analyses were performed using anti-aeHuR sera, anti-HuR (3A2) antibodies, or control preimmune sera (to detect nonspecific interactions). Following reversal of the crosslinking, SinV genomic and subgenomic RNAs were detected in immunoprecipitated samples using RT-PCR. The retention of SinV RNA with specific anti-aeHuR and HuR antibodies (Figures 2C and 2D, respectively) but not the control preimmune sera or with antibodies against unrelated proteins (e.g., DCP2, tubulin [data not shown]) clearly indicated that SinV RNA indeed interacts with these ELAV superfamily members during the course of an infection in tissue culture cells.

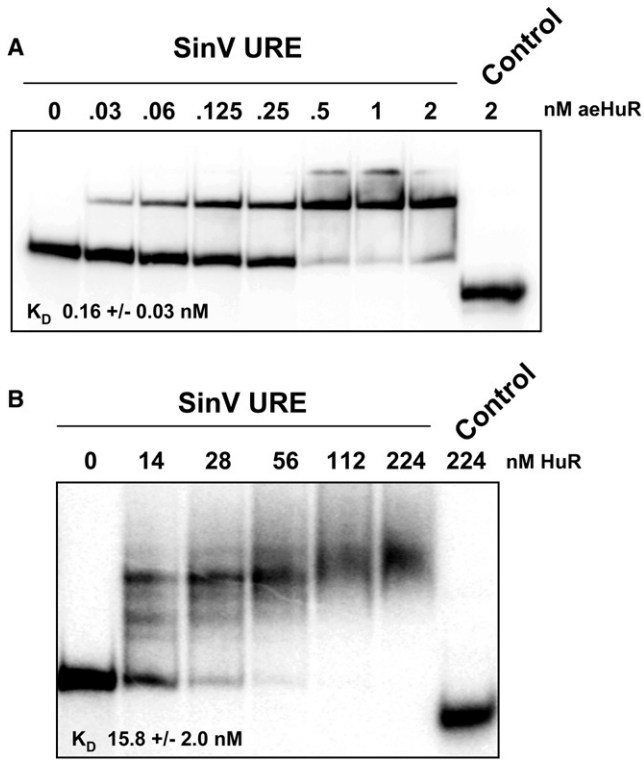
Taken together, these data identify an interaction between the SinV 3′ UTR URE/CSE element and HuR proteins in both cell-free assays and tissue culture models of infection. Furthermore, conservation of the interaction of the URE/CSEs of SinV, VEE, EEE, WEE, and SFV with HuR proteins indicates that alphaviruses have evolved this interaction for an important reason—perhaps to successfully elude the host mRNA decay machinery.

## AeHuR and HuR Interact with the URE with High Affinity

The next question we wished to address was how effectively viral transcripts interact with the cellular HuR protein. We utilized electrophoretic mobility shift assays (EMSA) to determine the binding affinity of aeHuR and HuR for the URE region of the 3′ UTR of our set of alphaviruses. As shown in Figure 3A, recombinant aeHuR interacted with very high affinity to the URE/CSE of SinV (mean dissociation constant 0.16 nM). Recombinant human HuR binds the URE/CSE of SinV with similarly high affinity (Figure 3B). These high-affinity interactions were also specific, as recombinant aeHuR or human HuR both failed to interact with a nonspecific control RNA. We next assayed RNA substrates containing the URE elements from VEE, EEE, WEE, and SFV by EMSA and obtained dissociation constants for aeHuR binding in a range similar to that obtained for the SinV URE (Figure 3C). Interestingly, the affinity observed for aeHuR and human HuR interactions with the tested alphavirus 3′ UTR elements was comparable to published affinities for HuR with cellular mRNAs (Nabors et al., 2001). Therefore, we conclude that five alphaviruses tested all contain a high-affinity binding site for HuR from various species in their 3′ UTRs.

## Infection with SinV in 293T Cells Results in Striking Relocalization of HuR to the Cytoplasm

While the data presented above suggest that alphavirus transcripts bind HuR with a relative high affinity, the majority of HuR in a mammalian cell resides in the nucleus rather than the

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| Observed Dissociation Constants for aeHuR | |
|---|---|
| Alphavirus | Mean $K_D$ (nM) |
| Sindbis Virus | 0.16 +/- 0.03 |
| Venezuelan Equine Encephalitis Virus | 0.35 +/- 0.15 |
| Eastern Equine Encephalitis Virus | 0.17 +/- 0.11 |
| Western Equine Encephalitis Virus | 0.20 +/- 0.10 |
| Semliki Forest Virus | 1.61 +/- 0.16 |

**Figure 3. Mosquito and Human HuR Proteins Bind to Alphaviral URE-Containing RNAs with High Affinity**

(A) RNA EMSA analysis using the indicated amount of purified recombinant aeHuR protein and radiolabeled RNAs containing the SinV URE element. The control RNA lane represents a nonspecific, vector-derived control transcript.

(B) Same as in (A), except that recombinant human HuR protein was used.

(C) Tabular summary of the results obtained via EMSA analysis using the indicated alphavirus URE element and recombinant mosquito aeHuR protein. See also Figures S3 and S4.

cytoplasm, where it can be accessed by viral RNAs (Kim et al., 2008). The subcellular localization of aeHuR has never been examined. Therefore, HuR could very well be limiting during an alphaviral infection and thus have only a minor role.

In order to begin to address this issue, we first assessed the subcellular localization of aeHuR in mosquito cells by immunofluorescence assays using the antibodies we developed (Figure S2B). As shown in Figure 4A, aeHuR is disseminated throughout the Aag2 cell, with a significant amount present in the cytoplasm. The subcellular distribution of aeHuR in *Aedes albopictus* (C6/36) cells was similar to that observed in Aag2 cells (data not shown). Therefore, we conclude that unlike human cells, a substantial proportion of aeHuR is present in the cytoplasm of mosquito cells and should therefore be readily accessible to alphavirus transcripts during infection.

Given the difference in HuR subcellular localization between human and mosquito cells, we next addressed whether cytoplasmic HuR protein may indeed be a limiting factor during infection. Interestingly, HuR has been shown to relocalize from the nucleus to the cytoplasm in reaction to stimuli that cause a stress response in cells (Kim et al., 2008). Therefore, we tested the hypothesis that SinV infection may cause HuR to relocalize to the cytoplasm. Human embryonic kidney cells (293T) were infected with SinV, and the subcellular localization of HuR was assessed at 6 and 12 hpi. As seen in Figure 4B, while HuR is largely nuclear at the start of the infection, there is a dramatic relocalization to the cytoplasm by 6 hpi. Furthermore, at 12 hpi, the majority of HuR has been shuttled out of the nucleus to the cytoplasm. There was a direct association between the cells that were infected with SinV (as determined by FISH analysis using a probe for the SinV E1 region) and relocalization of HuR to the cytoplasm (Figure 4C). Similar data were obtained at mois of 5, 10, or 20 (data not shown). The relocalization of HuR from the nucleus to the cytoplasm during a SinV infection could also be demonstrated by subcellular fractionation and western blot analysis (Figure 4D). The relocalization of HuR protein to the cytoplasm is a specific phenomenon, as PABPN1 (Figure 4D) as well as the abundant nuclear protein nucleophosmin (data not shown) both remain confined to the nucleus throughout the SinV infection. Finally, aeHuR maintained its relative nuclear/cytoplasmic distribution during SinV infection of mosquito cells (data not shown), suggesting that HuR relocalization is specific to mammalian cells that contain low levels of cytoplasmic HuR prior to infection.

In conclusion, these data demonstrate that both aeHuR and HuR are present within the cytoplasm of infected cells. In mosquito cells this is due to the natural cytoplasmic localization of aeHuR. Within human 293T cells, viral-induced relocalization of HuR serves to increase the available concentration of HuR in the cytoplasm.

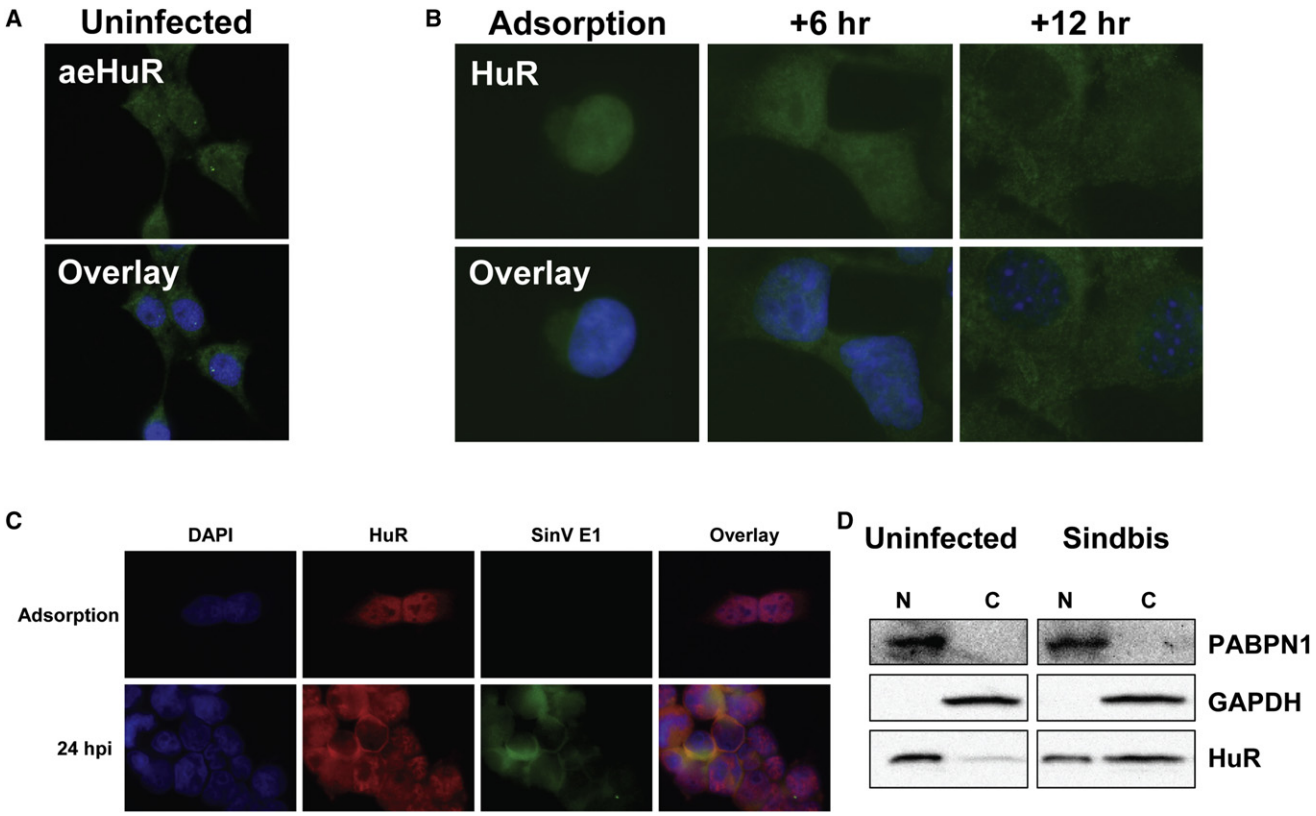


**Figure 4. HuR Relocalizes to the Cytoplasm during SinV Infections of Human Cells**

(A) *Aedes aegypti* Aag2 cells were grown on glass coverslips and stained for anti-aeHuR.

(B) Human 293T cells were treated with anti-HuR antibodies and DAPI after the indicated progression of time from the start of infection.

(C) Human 293T cells were treated with DAPI, anti-HuR antibody, and FISH analysis using a SinV E1 region probe at the indicated time postinfection with SinV.

(D) 293T cells that were either uninfected or 24 hr postinfection with SinV were separated into nuclear and cytoplasmic fractions. Fractions were probed for the presence of PABPN1 (nuclear marker), GAPDH (cytoplasmic marker), or HuR by western blotting. See also Figure S3.

## Knockdown of HuR Results in Increased SinV RNA Decay and a Reduction in Viral Titer

Finally, while aeHuR and HuR bind to alphaviral 3′ UTRs with high affinity and the aeHuR-viral RNA interaction correlates with increased viral RNA stability in our cell-free RNA decay assays, it is still crucial to demonstrate whether HuR truly plays a role in viral RNA stability and the efficiency of viral replication in living cells. Therefore, we used a shRNA knockdown approach to assess the effect of a reduction of the cellular levels of aeHuR and HuR on SinV infection.

In three independent experiments, 293T cells were either transfected with HuR-specific shRNA vectors or mock transfected using pLKO-1-puro vector DNA and, 12 hr later, were infected with a variant of SinV that contains a temperature-sensitive mutation in its polymerase (SinV-ts6). At 10 hpi (which was 22 hr posttransfection with the shRNA vectors), cells were switched to the nonpermissive temperature to inhibit viral transcription, and total RNA was recovered at various intervals to assess viral RNA half-lives by quantitative RT-PCR. During the infection, the level of HuR in cells was reduced an average of ~50%–60% compared to mock-treated 293T cells based on quantitative RT-PCR (Figure 5A) or western blot analyses (Figure S5). The relative abundances at each time point for both viral RNA species over the three independent experiments were averaged and used to calculate the mean rate of decay for both the genomic and subgenomic RNA species in control 293T cells and HuR knockdown cell lines. These values were plotted with respect to time, and an exponential regression curve was fitted to the data points. As shown in Figure 5B, the mean half-life of both the genomic and subgenomic RNAs was significantly decreased in the HuR-depleted 293T cells, indicating an increased rate of viral RNA decay. A comparable increase in the rate of viral RNA decay was observed in a pool of stably transfected Aag2 mosquito cells that were knocked down for aeHuR (Figures 5C and 5D). Similar data were obtained when samples were analyzed using an RNase protection assay (data not shown).

To assay viral replication in a HuR-deficient environment, 293T cells were treated with anti-HuR shRNA vectors or mock treated as described above. Twenty-two hours after transfection, cultures were infected with SinV-ts6 at an moi of 5, and aliquots were removed over the next 15 hr for determination of virus yield by plaque titration. As shown in the one-step growth curve in Figure 6A, the growth kinetics of SinV were significantly delayed in HuR-deficient 293T cells, and a >10-fold repression in viral growth was observed. A statistically significant 5-fold

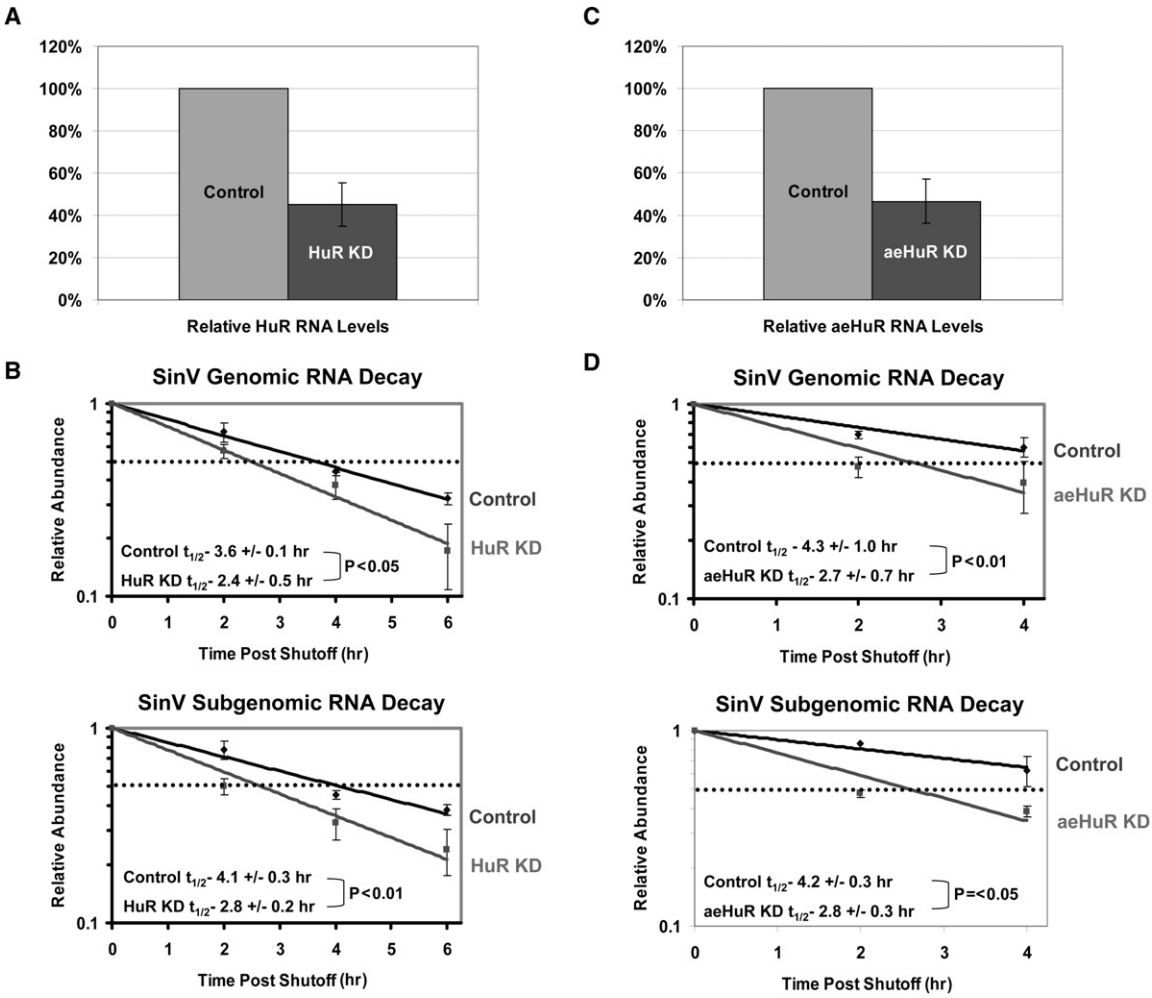


**Figure 5. Knockdown of HuR or aeHuR Protein Destabilizes SinV RNAs**

(A) Quantification of HuR knockdown efficiency in 293T cells using quantitative RT-PCR.

(B) RNA half-life analysis of SinV genomic RNA (top) or subgenomic RNA (bottom). Cells were infected with SinV-ts6 virus for 10 hr and shifted to 40°C to block viral transcription. Samples were taken at the times indicated and analyzed for genomic and subgenomic RNA levels by quantitative RT-PCR.

(C) Quantification of aeHuR knockdown efficiency in Aag2 cells by quantitative RT-PCR.

(D) Same as (C), except Aag2 cells were used. Half-lives represent the data obtained from three independent experiments. The error bars represent standard deviations of the means. See also Figure S5.

reduction in the growth kinetics of SinV was also observed in a stable pool of Aag2 cells that were knocked down for aeHuR (Figure 6D). Note that these HuR and aeHuR knockdown cells were viable and showed no apparent growth defects that could overtly account in an indirect way for any of the observations made in this study.

Taken together, these data demonstrate that both aeHuR and HuR are important cellular factors for efficient alphavirus infection in tissue culture cells. Even a modest ~50%–60% reduction in aeHuR or HuR abundance resulted in a significant destabilization of SinV RNAs. In order to verify this using a complementary set of experiments, the URE region of the 3′ UTR of SinV that binds to the HuR protein (Figure 3) was deleted. As seen in Figures 6B and 6E, this ΔURE SinV-ts6 variant showed a significant repression in viral growth compared to SinV-ts6 containing a wild-type 3′ UTR in either 293T or mosquito Aag2 cells, similar to the repression observed in HuR knockdown cells in Figures 6A and 6D. Furthermore, the ΔURE SinV-ts6 variant virus did not demonstrate any additional growth defects in 293T or Aag2 cells that were knocked down for HuR (Figures 6C and 6F). Taken together, these results confirm that aeHuR and HuR are indeed viral RNA stability factors that act through a specific binding site in the viral 3′ UTR and help determine the outcome of an infection. Additionally, these data elucidate a previously unappreciated facet of *Alphavirus* biology that potentially could be exploited for the development of effective antiviral strategies.

## DISCUSSION

The cellular HuR protein has been identified as an important stability factor for >50 cellular mRNAs (Wilusz and Wilusz, 2007). In response to cellular proliferation or stimulation by

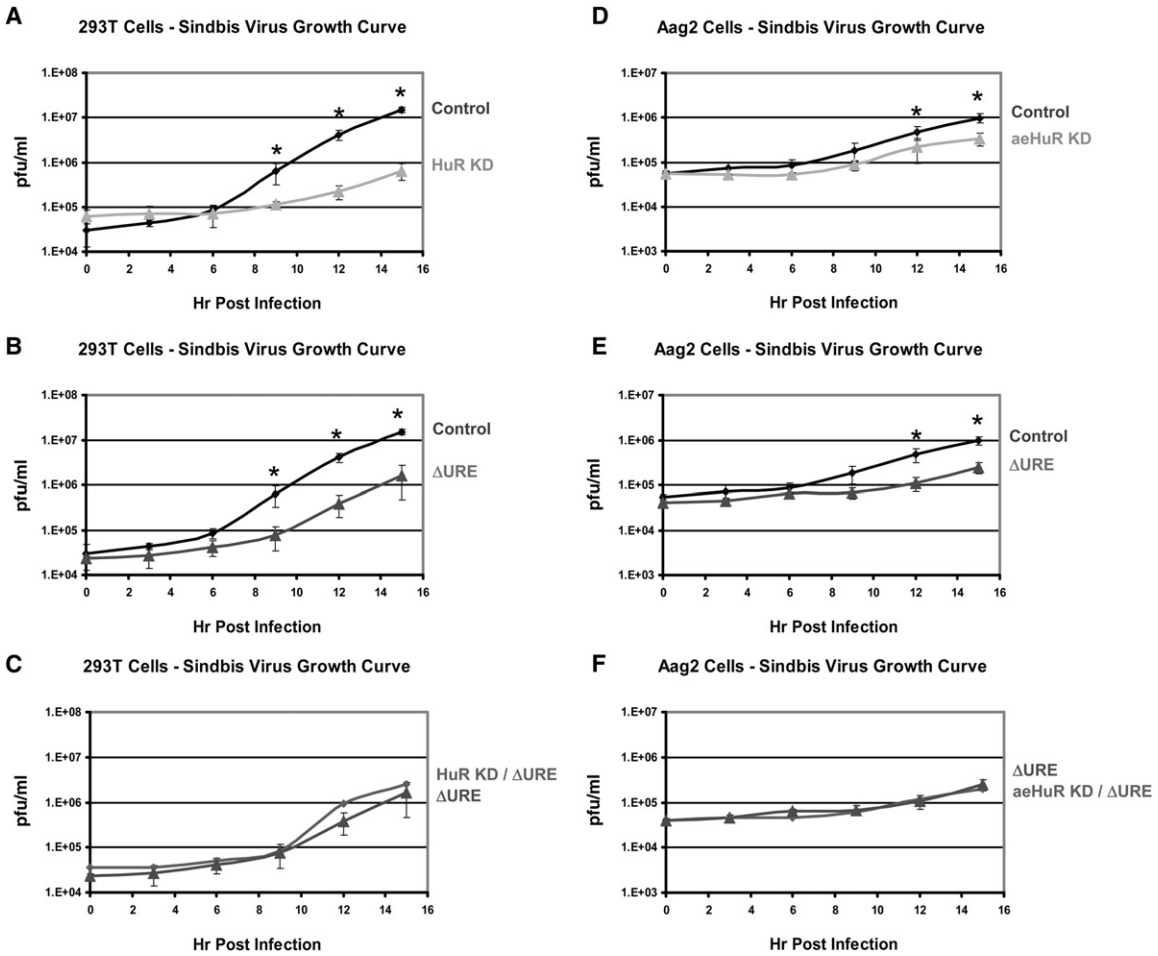


**Figure 6. Knockdown of HuR or Deletion of Its Binding Site in SinV RNAs Substantially Reduces the Yield of SinV Progeny Virions in 293T or Aag2 Cells**

(A) Mock-transfected wild-type 293T cells (control) or 293T cells that were knocked down for HuR protein using shRNAs were infected with SinV-ts6. Extracellular virus was isolated at the times indicated, and viral titers were obtained by plaque formation on Vero cells.

(B) 293T cells were infected with SinV-ts6 or a ΔURE SinV-ts6 variant that lacks the high-affinity HuR-binding site. Extracellular virus was isolated at the times indicated, and viral titers were obtained by plaque formation on Vero cells.

(C) Same as (A), except the infections were done using a ΔURE SinV-ts6 variant that lacks the high-affinity HuR-binding site.

(D–F) Same as (A), (B), and (C), respectively, except that the experiments were done in Aag2 cells. All panels are graphs of the mean values obtained in three independent replicates. The error bars represent standard deviations of the means. The asterisks indicate significant differences as determined by t test (p value $\leq$ 0.05). See also Figure S5.

a variety of factors (stress, immune modulation, etc.), HuR protein will often relocalize from the nucleus to the cytoplasm and play a vital role in regulating the stability and translation of select populations of mRNAs (Zhang et al., 2009; Antic et al., 1999; Abdelmohsen et al., 2008; Fan and Steitz, 1998). This study demonstrates a function for the cellular HuR protein in supporting a SinV infection (and likely other alphavirus infections) in both mammal and vector host cells.

Our working model for how HuR promotes SinV infections is shown in Figure 7. HuR interacts with high affinity to a U-rich region near the 3′ end of SinV mRNAs and stabilizes the transcripts during infection. When SinV infects mammalian cells, HuR is largely nuclear. However, by 6 hpi, when levels of viral RNA synthesis are high, the protein has been induced by the virus to relocate to cytoplasm, where it is readily available for binding to SinV transcripts. Knockdown of HuR protein results in reduced stability of SinV mRNAs and a significant reduction in the yields of progeny virions. Thus, these studies suggest that HuR is an important host factor that is usurped by viruses to protect their transcripts from the major pathways of the cellular RNA decay machinery. Furthermore, these studies clearly document the importance of the interface between viral mRNAs and the cellular RNA decay machinery.

It is interesting to note that unlike mammalian HuR, a substantial amount of aeHuR protein is cytoplasmic in both Aag2 *Aedes aegypti* and C6/36 *Aedes albopictus* cells. This may reflect an innate difference in the way HuR functions in insects versus vertebrates in terms of finding its RNA substrates and helping to define RNA regulons (Keene, 2007). Thus, it will be interesting to characterize the roles and regulation of aeHuR in the mosquito

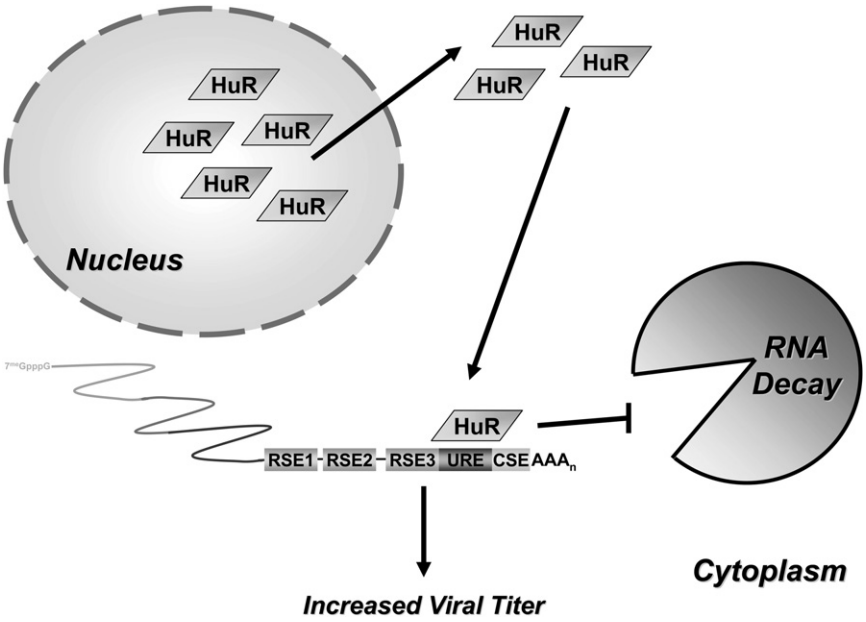


**Figure 7. A Model for the Role of HuR in Viral Gene Expression during Alphavirus Infection**

system, as further comparative analyses may give significant insight into its mechanisms of action. Curiously, SinV infection does not alter the subcellular localization of aeHuR in mosquito cell lines as it does in mammalian cells (data not shown). This may be important for preventing the dramatic changes in cellular gene expression influenced by relocalization of HuR (Zhang et al., 2009), thus reducing cytopathology in the mosquito, allowing survival of the insect to ultimately serve as an effective vector.

The underlying mechanism for the relocalization of HuR during SinV infection is also unclear. We are currently pursuing two main hypotheses to gain insight into this area. First, a variety of cellular stresses such as heat shock (Gallouzi et al., 2000) and oxidative stress (Mazroui et al., 2008) cause HuR to rapidly shift from the nucleus to the cytoplasm. Perhaps the general stress induced by SinV infection is triggering signaling mechanisms along the same lines as these environmental stresses (McInerney et al., 2005). Alternatively, SinV could be specifically targeting HuR or its transport mechanisms to actively induce HuR protein relocalization. Interestingly, a significant fraction of SinV and SFV nsP2 protein is found in the nucleus of infected cells (Atasheva et al., 2007; Garmashova et al., 2006; Frolov et al., 2009) as well as bound to the ribosome (Ranki et al., 1979) and is associated with significant cytotoxicity. Given the nuclear localization of HuR as well as its role in regulating translation (Kawai et al., 2006), it is tempting to speculate that a viral factor such as the nsP2 protein may be specifically targeting HuR and promoting its relocalization.

The observation that four other alphaviruses (VEE, EEE, WEE, and SFV) in addition to SinV interact with HuR suggests an evolutionary conservation of function that further supports the significance of HuR protein-RNA interactions to a productive alphavirus infection. HuR protein-RNA interactions have also been documented for several other viruses. HuR protein binds to the untranslated regions of hepatitis C virus (HCV) (Spångberg et al., 2000; Harris et al., 2006) and has been recently shown to activate translation (Rivas-Aravena et al., 2009). Interestingly, one study has shown that siRNA knockdown of HuR expression in cells decreased RNA and protein expression from HCV viral replicons (Korf et al., 2005). These observations suggest that HuR could perhaps stabilize the nonpolyadenylated transcripts of HCV and other *Flaviviruses* in a manner at least in part related to what we observed in this study with the alphaviruses. The potential role of HuR protein in infections with retroviruses or DNA viruses appears to be more complex than for the cytoplasmic RNA viruses. In human immunodeficiency virus (HIV) infections, the HuR protein has been shown to interact with the viral reverse transcriptase (Lemay et al., 2008) and to have a negative impact on HIV internal ribosome entry site (IRES)-mediated translation (Rivas-Aravena et al., 2009). Herpesvirus saimiri virus small HSUR RNAs can specifically interact with the HuR protein (Cook et al., 2004), although the functional impact of this interaction is not clear. Finally, HuR protein is upregulated in neoplastic cells that contain human papilloma virus (HPV sequences) (Cho et al., 2006; Sokolowski et al., 1999), and HuR protein has been associated with the posttranscriptional regulation of late HPV gene expression through interactions with the 3′ UTR of late HPV transcripts (Koffa et al., 2000). Determining the mechanistic role of HuR protein in these viral infections may not only give important insights into viral-host interactions but could also help further characterize the underlying mechanisms of HuR function in host cells.

## EXPERIMENTAL PROCEDURES

### Cell Lines, Virus Propagation, and Plaque Titration

BHK-21, Vero, and 293T cell lines were cultured in HyQ MEM/EBSS media with 10% fetal bovine serum (FBS). *Aedes aegypti* Aag2 cells were maintained in Schneider's *Drosophila* medium supplemented with 10% FBS. HeLa S3 spinner cells were maintained in JMEM with 10% horse serum. C6/36 *Aedes albopictus* suspension cells were cultured in SF-900II (GIBCO) serum-free media.

Full-length SinV genomic RNAs were produced by in vitro transcription of either wild-type SinV AR339 or the temperature-sensitive SinV-ts6 AR339 clone (Barton et al., 1988; Bick et al., 2003), as previously described (Garneau et al., 2008). The ΔURE SinV variant, which contained a 30 base deletion of the URE, was constructed using the primers 5′-ATTTTGTTTTTAACATTTCA$_{(37)}$GG GAATTC and 5′-TTATGCAGACGCTGCGTGGCATTATGC to jump from the CSE to the region upstream of the URE in the pToto1101/SinV-ts6 AR339 vector using PCR (Garneau et al., 2008). Viral titers were determined by plaque titration on Vero cells.

### Construction of a Mosquito-Specific shRNA Vector

The hygromycin phosphotransferase (hph) gene was isolated from pHyg (Gritz and Davies, 1983) via PCR using the primers 5′-CATACATGTTCATGA

AAAAGCCTGAACTCACCGCG and 5′-CATCTCGAGCTATTCCTTTGCCCTC GGACGAGTG. PCR products were cut with PciI and XhoI and inserted into pBiEx-1 (Promega) to create pBiEx-hph. An *Aedes aegypti* U6 promoter-driven shRNA expression cassette was generated via PCR from pAedes1 (Konet et al., 2007) using the primers 5′-CATGGGCCCGAATGAATCGCCCAT CGAGTTGATACGTC and 5′-CATGGCGCCAAAAAAAAAAGCTTCAGCTGGG TACCGGATCCATTTCACTACTCTTGCCTCTGCTCTTATATAG. The PCR product was cut with ApaI and SfoI and ligated into pBiEx-hph to create a selectable mosquito shRNA vector, pAeSH, that allows for the insertion of any shRNA into the multiple cloning site present downstream of the U6 promoter. Targeted shRNAs to *Aedes aegypti* aeHuR were designed, and the following oligos were inserted into the BamHI and HindIII sites of pAeSH: 5′-GATCCC AAAGTGCTAGCAGCCGTATTCAAGAGATACGGCTGCTAGCACTTGTTA and 5′ AGCTTAACAAAGTGCTAGCAGCCGTATCTCTTGAATACGGCTGCTAGCA CTTTGG, to create pAeSH-aeHuR1.

### Preparation of RNA Substrates

Oligos containing sequences derived from the 3′ UTRs of VEE, EEE, WEE, and SFV (Figure S1B) were inserted into the EcoRI and PstI sites of pGEM4-A60 (Garneau et al., 2008). Transcription templates were generated via digestion with HindIII or NsiI for the nonadenylated and adenylated species, respectively. Internally radiolabeled, capped RNA substrates were transcribed by SP6 polymerase and purified as described (Wilusz and Shenk, 1988).

### Viral RNA Decay Analysis

293T cells were either transfected with a Mission shRNA vector (Sigma Aldrich) specific to HuR (TRCN0000017277) or mock transfected (lacking specific shRNA but still containing transfection reagent) using FuGENE 6. At 12 hr after transfection, cells were infected with SinV-ts6 at an moi of 5. Following a 30 min adsorption period, fresh media were added, and the cells were placed at 28°C for 10 hr. Prewarmed media were then added, and the cells were transferred to 40°C to shut off viral transcription. At the times indicated after shutoff, total RNA was harvested using TRIzol and quantified by quantitative RT-PCR (Garneau et al., 2008) using the primers listed in Figure S1B. Note that since the analysis was done at 10 hpi, shRNA knockdown of HuR was allowed to proceed for 22 hr after transfection.

Determination of SinV RNA decay in Aag2 cells was performed as described above using stable aeHuR-knockdown cell lines selected following transfection of the pAeSH-aeHuR1 or empty pAeSH vector using FuGENE 6. Stable cell pools were selected using 300 U of hygromycin B.

From the half-life regression curves for each independent in vivo RNA decay experiment, the average genomic and subgenomic half-lives observed in the 293T and HuR-deficient cell lines were determined. These average half-lives were subjected to statistical analysis via a two-tailed Student's t test. Abundance of aeHuR and HuR mRNAs was examined using quantitative RT-PCR and the primers listed in Figure S1B. The relative abundances between control transfected cells and shRNA treated cells were compared at time point zero.

### Cell-free RNA Deadenylation Assays

Cytoplasmic extracts were derived from C6/36 cells and HeLa S3 cells as previously described (Sokoloski et al., 2008a, 2008b). 100,000 CPM (4–50 fmoles) of internally radiolabeled, polyadenylated RNA was incubated in the presence of mosquito cytoplasmic extract as previously described (Opyrchal et al., 2005), aliquots were removed at the desired time points, and recovered RNAs were analyzed on 5% acrylamide gels containing 7 M urea. RNAs were analyzed by phosphorimaging.

### Ultraviolet Crosslinking and Immunoprecipitation Assays

100,000 CPM (4–50 fmoles) of RNA substrate was incubated in cell-free RNA decay assays for 1 min using either HeLa or C6/36 extracts in the presence of 0.5 mM EDTA to inhibit RNA decay (Sokoloski et al., 2008b). Following crosslinking of RNA-protein complexes by 254 nm UV light, the mixture was treated with RNase A and RNaseONE, and proteins crosslinked to short radiolabeled RNA oligomers were resolved using 10% SDS-PAGE and visualized by phosphorimaging.

For RNA crosslinking/immunoprecipitation assays, samples were incubated, irradiated, and treated with RNase as described above. Samples were diluted with NET2 buffer (40 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.05% [v/v] NP-40) and clarified by centrifugation. Samples received either control sera or target-specific antibody and were incubated for 1 hr at 4°C. Protein G Sepharose or Pansorbin was then added, antibody-antigen complexes were washed ~5 times with NET2 buffer, and immunoprecipitated proteins were analyzed by 10% SDS-PAGE.

### Cell-Based Coimmunoprecipitation Assays

293T or Aag2 cells were infected with wild-type SinV at an moi of 5. Cells were released using trypsin (293T) or mechanical scraping (Aag2), washed with PBS, resuspended in a 1% formaldehyde solution in PBS, and incubated for 10 min at room temperature. The reaction was quenched using 0.25 M glycine. Cell pellets were washed with PBS and resuspended in RIPA buffer (50 mM Tris-HCl [pH 7.5], 1% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 0.05% [w/v] SDS, 1 mM EDTA, and 150 mM NaCl). Cells were disrupted by sonication on ice, and insoluble materials were removed via centrifugation. Aliquots received anti-HuR, anti-aeHuR, or control (preimmune) antibodies and were incubated at 4°C for 1 hr. Antibody-bound complexes were recovered using protein G or protein A Sepharose after 6X washes with RIPA buffer containing 1 M urea. Formaldehyde crosslinks were reversed by heating at 70°C for 45 min. Isolated RNAs were analyzed by RT-PCR to assess total SinV RNA.

### Affinity Purification and Mass Spectrometry

5′-biotinylated RNA oligos consisting of either the SinV URE/CSE fragment (5′-Bio-UAAUCAUUUAUUAUUUUCUUUUAUUUUAUUCACAUAAUUUUGUU UUUAA) or a nonspecific sequence (5′-Bio-GAAUCGAGCUCGGUACCCGG GGAUCCUCUAGAGUCGACCUGCAG) were incubated with 2.5 mg cytoplasmic extract. Protein-RNA complexes were bound to streptavidin resin, pelleted by centrifugation, and washed five times with 20 mM HEPES (pH 7.9), 225 mM KCl, 2.5 mM $MgCl_2$, 0.1% (v/v) NP-40, and 1 mM DTT. Proteins were eluted by boiling in HSCB buffer, concentrated using methanol, and resolved via 10% SDS-PAGE. Gels were stained and excised bands were analyzed by MALDI-TOF/TOF.

### Expression of Recombinant Proteins

The *Aedes aegypti* open reading frame corresponding to AaeL_AAEL008164 was PCR amplified using the primers 5′-CATGGATCCATGACCAACAAAGT GCTAGCAGCC and 5′-CATGAATTCTTAATGATCGGCCATTTCGGCG. The gel-purified fragment was cut with BamHI and EcoRI and inserted into pGEX-2TZQ (Qian and Wilusz, 1994) to make pGEX-aeHuR. Recombinant protein was prepared from BL21 DE3 *E. coli*, and the GST tag was removed by thrombin cleavage. Recombinant human HuR was a gift from N. Curthoys (Colorado State University).

### Cell Fractionation and Western Blotting

Separation of cells into nuclear and cytoplasmic fractions was performed as described in Hel et al. (1998). Following separation from nuclei, the cytoplasmic fraction was centrifuged at 16,000 × *g* for 10 min at 4°C prior to analysis by western blotting. Antibodies to PABPN1 (K-18) and HuR (3A2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); GAPDH antibodies were obtained from Millipore (Billerica, MA). A polyclonal aeHuR antisera was raised by Bioo Scientific (Austin, TX) in rabbits using the above recombinant aeHuR. Serum specificity was determined via western blot analysis of C6/36 cytoplasmic extract separated on 10% SDS-PAGE.

### Immunofluorescence and FISH Analysis

Aag2 and 293T cells were grown on glass coverslips, fixed in 4% paraformaldehyde, permeabilized in methanol, and rehydrated in 70% ethanol. Coverslips were blocked in 6% bovine serum albumin fraction V (BSA) in PBS for at least 1 hr and washed in PBS. Primary antibody (diluted in 0.6% [w/v] BSA in PBS) was added for 1 hr and washed in PBS, and secondary antibody (diluted as above) was applied for 1 hr. After washing, the coverslips were mounted using ProLong Gold antifade reagent with DAPI. Antibodies used were HuR (3A2) (Santa Cruz Biotechnology), Nucleophosmin NA24 (Thermo Fisher Scientific, Waltham, MA), anti-aeHuR polyclonal serum (this study), Cy2 donkey anti-mouse Ig (Jackson ImmunoResearch; West Grove, PA), Cy5 goat anti-rabbit Ig (GE Healthcare; Piscataway, NJ), and Texas red anti-mouse Ig (Santa Cruz Biotechnology).

For FISH analyses, cells were grown, fixed, permeabilized, and rehydrated as above. Coverslips were washed in PBS followed by a treatment of 40% formamide/2X SSC. The oligonucleotide (5′ Alexa 488-labeled 5′-TGACATTTC AAGGAGCCGCAGCATTT) was diluted in 40% formamide/2X SSC/0.2% (w/v) BSA, added to the coverslip for 2 hr, and washed in 1X SSC.

### RNA EMSAs

Internally radiolabeled unadenylated alphavirus URE/CSE fragments (~1.25 fmoles) were incubated in the presence of recombinant aeHuR or HuR at the indicated concentrations in gel shift buffer (15 mM HEPES [pH 7.9], 100 mM KCl, 2.25 mM $MgCl_2$, 5% [v/v] glycerol). The complexes were allowed to form for 5 min at room temperature prior to the addition of 2.6 μg/μl heparin sulfate. The addition of heparin prior to the incubation with recombinant protein gave similar results. Following a 5 min incubation on ice, protein-RNA complexes were resolved on a 5% native acrylamide gel and analyzed by phosphorimaging. Values obtained for bound versus free RNA were plotted and dissociation constants were calculated from the slope of the linear regression line fitted to the data.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.chom.2010.07.003.

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