Mechanistic insights into translational modulation of selected RNAs by RNA helicase A

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Arnaz K Ranji, M.Sc.

Graduate Program in Molecular Genetics

The Ohio State University

2011

Dissertation Committee

Dr. Kathleen Boris-Lawrie, Advisor

Dr. Stephen A Osmani

Dr. Mamuka Kvaratskhelia

Dr. Harold Fisk
Copyright by
Arnaz K Ranji
2011
Abstract

The 5' untranslated region (UTR) of mRNAs is the entry gate for the translation machinery. Features of the 5' UTR control the efficiency of mRNA translation. Relatively little is known about the specialized proteins that modulate the entry of the translation machinery. Our previous research has identified a redundant structural element in the 5' UTR of many retroviruses and select cellular RNAs that facilitates access of scanning ribosomes. The ribosome loading is facilitated by interaction with RNA helicase A (RHA). This dissertation describes important open questions. Biochemical, genetic and biophysical approaches were employed to define residues that are necessary for selective recognition of target mRNAs and that tether the ATP-dependent helicase for rearrangement of the complex 5' UTR.

The first of the five chapters of this dissertation introduces the present fundamental understanding and key open issues of the process of mRNA translation. Proper control of translation is essential for healthy cell growth and response to infectious agents. The initiation stage of mRNA translation determines the activity of an mRNA as a productive mRNA template or the alternative outcome as a triaged mRNA that is not a productive mRNA template. Chapter 1 describes the discovery and characterization of the retroviral structural element (PCE) and its effector protein RHA. RHA interacts with
PCE at the 5’ UTR and is necessary for the translation of virion proteins of avian, bovine, simian and human retroviruses and the JunD proto-oncogene. RHA also modulates viral RNA replication and gene expression in two other virus families. The final section of Chapter 1 describes the emerging roles of RHA and other RNA helicases in virus replication.

Three experimental studies that define the mechanism of RHA translational regulation of target mRNA are presented in Chapters 2, 3 and 4. Chapter 2 defined the residues of RNA helicase A required for the selective interaction of RNA helicase A with its cognate mRNA. Results of mutagenesis, biochemical and biophysical assays defined three lysine residues (K54,K55 and K236) within the 300 amino acid amino-terminal domain are necessary. Exogenous expression of this domain is sufficient to block translation activity of endogenous RHA and the lysine mutations eliminated the squelching effect on endogenous RHA. In sum, residues within the amino-terminal double-stranded RNA binding domains confer selective interaction of RHA with PCE RNA that tethers the ATP-dependent helicase for rearrangement of the complex 5’ UTR.

Experiments in Chapter 3 determined residues of RHA that are necessary for translation activity of target RNAs. Sucrose density gradient analysis, localization studies, RNA immunoprecipitations, and translation assays in cells identified roles for the amino-terminal RNA binding domains, central ATP-binding pocket and arginine-rich
carboxy-terminal domains of RHA in recognition and translation of PCE-containing mRNAs. The N-terminal residues required for binding of the isolated N-terminal domain to PCE RNA are also important for binding of full-length RHA to PCE RNA. The N-terminal and helicase domains are required for translation initiation that culminates in polysome loading. Carboxy-terminal residues are required for productive completion of the translation process. RHA mutants that are defective in translation initiation become sequestered in stress granules, irrespective of PCE RNA binding ability. RHA is not a core component of stress granules, but accumulates in stress granules during oxidative stress. The arginine-rich carboxy-terminal residues are necessary for oxidative stress-dependent shuttling of RHA to these cytoplasmic triage granules. This indicates that all three of these RHA domains are required for productive translation of RHA target RNAs.

In Chapter 4 I identified the residues of RHA that interact with PCE RNA in cells. The study established a protein footprinting and mass spectrometric protocol to compare residues protected by RHA expressed in *E. coli* or immunoprecipitated from mammalian cells. The results identified that the conformation of the N-terminal domain of RHA does not differ in the context of the isolated recombinant N-terminal domain or mammalian cells. PCE RNA protects two carboxy residues of RHA (K806 and K1048). Mutagenesis and RNA immunoprecipitation assays in transfected cells determined that these residues are not essential for PCE RNA binding. However, mutation of K806 hampered the
recruitment of RHA to stress granules during oxidative stress, which indicates that this residue could be important for interactions with co-factors that are required for stress granule recruitment. The results demonstrate that our proteomic approach using immunoprecipitated protein can help identify novel residues that are important for RHA function.

The final chapter considers the significance of this research and essential future questions. In closing, this dissertation has determined the molecular basis for the activation of translation by RHA involves selective recognition of target mRNAs by the N-terminal domains, and regulation of both the initiation and post-initiation stages of translation. The dual molecular functions of RNA helicase A in the process of translation provides a potent strategy to fine tune expression of retroviruses and JunD. This process is essential for healthy control of cell growth and virus replication.
Dedication

Dedicated to my family, without whom this would not be possible.
Acknowledgements

I would like to thank my advisor Dr. Kathleen Boris-Lawrie for her guidance and help throughout the duration of my PhD. I appreciated all the effort that went into making me the scientist I am today. Even with her busy schedule she has always found time to meet with me and help me through my latest experimental crisis. I am forever grateful that she took a chance on me and allowed me to join her lab and I will miss her company.

I would also like to thank my committee members Dr. Kvaratskhelia, Dr. Osmani and Dr. Fisk. They have been a pleasure to work with and have looked out for my best interests through the years. I would like to separately acknowledge Dr. Kvaratskhelia who spent several hours going over my data and making brilliant suggestions that have resulted in a fruitful collaboration between the Boris-Lawrie and Kvaratskhelia labs and my first data publication. In addition, I would like to thank Dr. Shkiriabai for his help with protein purification and mass spectrometry. I also benefited from interactions with Dr. Karin Musier-Forsyth and her laboratory and would like to sincerely thank them all.

In my own lab, I am indebted to several members who helped me through my time in this lab. Life would have been a lot less interesting without them. In particular I would like to acknowledge Dr. Cheryl Bolinger for her help and companionship as well as her data that I have used in this dissertation. I would also like to thank Dr. Marcela...
Hernandez who has guided me on numerous occasions and been very generous with her precious time. Finally I’d like to thank the members in the Lairmore, Green and Wu labs for being excellent information resources as well as fun lunch buddies. I will miss our interactions when I move on.

Finally I would like to thank all the friends and family members who have played such an important role in getting me through this process. My parents have always supported me in every decision I made and I am thankful that they encouraged me to do what I really wanted to do in life. Together with my grandfather they were responsible for my being able to leave home to study, first for a masters and then for a PhD. I am very lucky to have the support of so many family members who are always encouraging and never doubted my abilities. So to Mumz, Dada, Masi, Nugi, Beba, Granny, Grandpa, Nonu and Katy… a big thank you. Also, a big thanks to my little sister Shernaz who has always enriched my life with her humor and support. Finally a big thanks to my nearest and dearest friends who have been like part of the family… Cookie, Ali, Zoee and Diloo, thanks for being there for me whenever I needed you.
Vita

1998-2001………………………………. BSc, Life sciences and Biochemistry
   St Xavier’s College, Mumbai, India.

2001-2002………………………………..MSc, Medical Genetics
   Glasgow University, Scotland.

2002-2003………………………………..Medical Technical Officer 1
   Duncan Guthrie Institute of Medical Genetics, Glasgow, Scotland.

2004-present……………………………...Graduate Research Associate
   Department of molecular genetics
   The Ohio State University
   Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular Genetics

   RNA biology

   Molecular Virology

   Post-transcriptional gene expression
Table of Contents

Abstract......................................................................................................................................... ii
Dedication ......................................................................................................................................... vi
Vita ................................................................................................................................................... ix
List of Tables .................................................................................................................................... xii
List of Figures .................................................................................................................................. xiii
Chapter 1: RNA helicases: Emerging roles in viral replication, cellular translation and the host innate response ................................................................. 1

I Brief overview of post-transcriptional regulation ............................................................... 1
Eukaryotic translation ................................................................................................................... 1
Regulation of translation ............................................................................................................. 3

Translational regulation during stress .................................................................................... 3
Translation regulation by miRNA and RBPs............................................................................. 5
UTRs of mRNA regulate translation ....................................................................................... 6
PCE regulates the translation of select retroviral and cellular RNAs ...................................................... 7
RNA Helicase A (RHA): a multifunctional cellular helicase ................................................... 8

II RNA helicases: Ubiquitous players in gene expression, innate response and viral replication ................................................................................................................... 12

Modular domain structure of RNA helicases........................................................................... 12
Induction of the antiviral state .................................................................................................... 13
Catalysis of viral gene expression............................................................................................. 15
DDX3 ............................................................................................................................................... 16
RNA helicase A (RHA)/DHX9 ...................................................................................................... 20
DHX30 ......................................................................................................................................... 23
DDX1 ............................................................................................................................................... 24
RH116 ........................................................................................................................................... 26
DDX24 .......................................................................................................................................... 27
P68/DDX5 ..................................................................................................................................... 27
Mov10 .......................................................................................................................................... 28

Virus-encoded Helicases ............................................................................................................. 30
HCV nonstructural protein 3 (NS3) ........................................................................................... 31
Vaccinia virus nucleoside triphosphate phosphohydrolase II (NPH-II) ........................................ 32
Plum pox virus cylindrical inclusion protein (CI) ......................................................................... 33

Perspectives on the future directions ......................................................................................... 34

Chapter 2. The N-terminal double-stranded RNA binding domains of RNA helicase A are necessary for recognition of viral and cellular translation target mRNAs .... 47

Abstract .......................................................................................................................................... 47
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Significant conservation is observed amongst RNA helicases involved in virus biology</td>
<td>45</td>
</tr>
<tr>
<td>1.2. Overview of the role of cellular helicases in replication cycle of indicated Virus</td>
<td>46</td>
</tr>
<tr>
<td>2.1. Apparent equilibrium dissociation constants for binding of recombinant RHA domains to 5’ fluorescein-labelled RNAs</td>
<td>80</td>
</tr>
<tr>
<td>2.2. Sequence of DNA oligonucleotides used in Experimental Procedure</td>
<td>81</td>
</tr>
<tr>
<td>3.1. Select RHA mutant overexpression does not facilitate PCE activity</td>
<td>135</td>
</tr>
<tr>
<td>3.2. Adox pre-treatment affects mNLS sequestration to stress granules upon oxidative stress</td>
<td>136</td>
</tr>
<tr>
<td>3.3. Select RHA mutants fail to complement PCE activity after downregulation of endogenous RHA</td>
<td>137</td>
</tr>
<tr>
<td>3.4. Sequence of DNA oligonucleotides used in indicated Experimental Procedure</td>
<td>138</td>
</tr>
<tr>
<td>4.1. Comparison of the modified lysine residues of the RHA N-terminal recombinant domain and the mammalian purified full-length Fl-RHA</td>
<td>169</td>
</tr>
<tr>
<td>4.2. Sequence of DNA oligonucleotides used in indicated Experimental Procedure</td>
<td>170</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Illustration of translation initiation</td>
<td>37</td>
</tr>
<tr>
<td>1.2. Illustration of translation elongation</td>
<td>38</td>
</tr>
<tr>
<td>1.3. Dynamics of stress granule assembly</td>
<td>39</td>
</tr>
<tr>
<td>1.4. Domain structure of DExH/D helicases involved in viral replication</td>
<td>40</td>
</tr>
<tr>
<td>1.5. RNA helicases of the RIG-I-like receptor (RLR) family play a crucial role in the innate antiviral response</td>
<td>42</td>
</tr>
<tr>
<td>1.6. Cellular helicases involved in HIV-1 replication</td>
<td>44</td>
</tr>
<tr>
<td>2.1. RHA domain structure and isolation of recombinant N-term, C-term and DEIH polypeptides</td>
<td>67</td>
</tr>
<tr>
<td>2.2. Testing incubation time and temperature</td>
<td>68</td>
</tr>
<tr>
<td>2.3. The conserved lysines in dsRBD1 and dsRBD2 are essential for effective binding of N-term with PCE RNA</td>
<td>70</td>
</tr>
<tr>
<td>2.4. N-term exhibits specific interaction with PCE RNA of viral and cellular origin</td>
<td>72</td>
</tr>
<tr>
<td>2.5. RHA C-term and DEIH exhibit weak and undetectable interaction with PCE RNA, respectively</td>
<td>73</td>
</tr>
<tr>
<td>2.6. N-term binding to PCE is recapitulated and quantified by FA measurements</td>
<td>74</td>
</tr>
<tr>
<td>2.7. C-term lacks specificity for PCE RNA</td>
<td>75</td>
</tr>
</tbody>
</table>
2.8. Mutation of conserved lysine residues eliminates affinity of N-term for PCE RNA………………………………………………………………….76

2.9. DEIH domain lacks detectable binding to PCE RNA…………………77

2.10. Mutation of conserved lysine residues eliminates affinity of N-term for PCE RNA………………………………………………………………...78

2.11. N-term interacts with PCE RNA in cells………………………………… 79

3.1. Description of RHA mutants……………………………………………… 115

3.2. RHA loss of function mutants exhibit differences in polysome association………………………………………………………………………..116

3.3. Certain cytoplasmic RHA mutants localize in granules……………….. 115

3.4. RHA mutants localize to cytoplasmic stress granules but not P bodies……120

3.5. RA9 mutation diminishes the recruitment of RHA to stress granules upon oxidative stress………………………………………………………..122

3.6. RHA is not an essential component for stress granule formation…………123

3.7. Effect of Adox pre-treatment on RHA mutants…………………………125

3.8. PCE RNA requires intact N-terminal RHA residues for association in the cell………………………………………………………………………..127

3.9. RHA mutant plasmids express protein resistant to siRNA downregulation..129

3.10. RHA mutation does not affect nuclear distribution…………………..…..130

3.11. RHA overexpression does not alter global cellular translation or viability..131

3.12. RHA RA9 mutant is associated with puromycin sensitive polysomes……..132

3.13. RA9 mutant RHA shows a defect in elongation…………………………133

4.1. RHA domain structure and isolation of recombinant or
    immunoprecipitated RHA.....................................................159

4.2. Identification of surface exposed lysine residues of recombinant
    N-terminal RHA...............................................................160

4.3. Mass spectrometry of biotin modified full length RHA in complex with
    PCE reveals protected residues.............................................162

4.4. Mutation of protected residues does not affect binding of RHA to PCE
    RNA..........................................................165

4.5. K806 mutation diminishes the recruitment of RHA to stress granules
    upon oxidative stress....................................................167

5.1. Overview of effects of mutations of RHA functional domains...........178
Chapter 1: RNA helicases: Emerging roles in viral replication, cellular translation and the host innate response

The introduction to this dissertation has been divided into two sections. The first section provides a general overview of translation and its regulation. Much of this dissertation focuses on the translation modulation of mRNAs containing a structured RNA element (post-transcriptional control element), by RNA helicase A. Therefore, these elements have been described in detail in the first section. The second section is a published review that deals with the role of RNA helicases in virus biology and summarizes the current known roles of eleven RNA helicases in viral replication.

I Brief overview of post-transcriptional regulation

Eukaryotic gene expression is a highly synchronized process that requires precise coordination of transcription and post-transcriptional events (1). The steady-state protein levels in a cell are often not directly correlated to the steady-state mRNA levels, because the lives of mRNAs are more complicated than simply serving as instructions for protein synthesis (2). In fact, mRNAs are regulated at every step in the post-transcriptional process which culminates in the generation of the proteome (3). RNA binding proteins (RBPs) and small non-coding RNAs such as miRNAs are important post-transcriptional
regulators of mRNA expression (4-7). The RBPs can exert either positive or negative influences on the processing of the mRNA, thus fine tuning mRNA expression (8). Multiple RNA binding proteins can interact with a single transcript simultaneously or sequentially to regulate its fate (3). RBPs are involved in the consecutive steps of post-transcriptional regulation of mRNAs which are capping, splicing, polyadenylation, nuclear export, translation and decay (7-9). Because much of this dissertation focuses on translational regulation, a brief overview is provided in the following paragraphs.

**Eukaryotic translation**

Translational control of mRNAs allows for rapid changes in protein concentration and involves 3 main stages: initiation, elongation, and termination (10). Translation initiation is highly regulated and is the rate limiting step in translation. Initiation involves several factors including eIF2, eIF3 and eIF4F (11). In brief, eIF3, eIF1 and eIF1A associate with the smaller 40s ribosomal subunit and are bound by the ternary complex composed of eIF2-GTP-Met-tRNA (Figure 1.1). This complex, referred to as the 43S initiation complex, is then able to associate with capped mRNA that is associated with the eIF4F complex and eIF4B (11) (Figure 1.1). eIF4F consists of the eIF4E cap-binding protein, the eIF4G large scaffold protein and the eIF4A RNA helicase (11;12). Both eIF4A and eIF4B are required to unwind the structure in the 5' UTR to facilitate binding of the 43S complex (11). Highly structured 5' UTRs require assistance from other RNA helicases to unwind the structure and facilitate the ensuing steps of translation. The 43S complex is then able to scan the mRNA downstream to the initiation AUG codon, where the 60S subunit of the ribosome attaches, and poly-peptide synthesis begins (10). eIF4G
is also able to bind to poly A binding protein (PABP) that is bound to the 3’ end of the RNA circularizing the mRNA and thus facilitating reinitiation (10;13).

Eukaryotic translation elongation is catalyzed by three elongation factors: eEF1A, eEF1B and eEF2 (14). eIF1A-GTP is required for binding of the amino-acyl tRNA to the ribosome, and upon recognition of the mRNA codon by the tRNA anti-codon, GTP is hydrolyzed and eEF1A is released. eEF1B is required to catalyze the exchange of GDP bound to eEF1A for GTP. eEF2 catalyzes translocation of the ribosome along the mRNA and allows elongation of the synthesized polypeptide (14). In brief, elongation commences once the the AUG initiation codon with the bound met-tRNA is positioned at the “peptidyl” P site of the 80S ribosome (Figure 1.2). The elongation factor eEF1A (complexed to GTP) then brings the correct amino-acyl-tRNA to the “amino-acyl-tRNA” A site of the ribosome where recognition of the codon by the tRNA anticodon takes place (14) (Figure 1.2). GTP is hydrolyzed upon binding of the amino-acyl tRNA to the A site and eEF1A-GDP leaves the complex. This allows a peptide-bond to form between the two amino acids. The next step of translocation of the ribosome is catalyzed by eEF2, thereby bringing the peptidyl chain bound tRNA into the P site and the deacylated tRNA into the E site, where it will subsequently be ejected after the next translocation (14) (Figure 1.2). The next cycle begins with eEF1A-GTP bringing in a new amino-acyl-tRNA and the peptide is elongated. eEF1B converts GDP-bound eEF1A to GTP-bound eEF1A, thereby allowing for continuation of elongation. Interfering with the activity of any of the elongation factors will result in inhibited or reduced rates of translation elongation. The small molecule cycloheximide is able to inhibit translation elongation by
occupying the E site of the ribosome, thereby altering the binding of the deacylated-tRNA to the E site (15). This does not allow translocation of the ribosome on the mRNA and inhibits further elongation (15).

Termination of translation occurs when the ribosome detects a stop codon, and requires eukaryotic release factors eRF1 and eRF3 (16). eRF1, eRF3, and GTP form a ternary complex that interacts with the ribosome to initiate translation termination. eRF1 recognizes the 3 universally encoded stop codons in mRNA and catalyzes the release of the synthesized peptide chain, whereas, eRF3 promotes the dissociation of eRF1 from the ribosomal complex allowing for the release of the ribosomal complex (16). In the absence of eRF3, slower release of peptide chains is observed (17).

**Regulation of translation**

Translation is regulated at every step by micro RNAs (miRNAs) or RBPs or both (10). Additionally, translation can be differentially regulated during conditions of stress as described below.

**Translational regulation during stress**

Translation can be regulated during stressful conditions in a cell. Stress granules are assembled in cells subject to environmental stress (18). Heat shock, oxidative stress and viral infection, amongst others, all trigger cellular stress leading to translational arrest and rapid polysome disassembly (19;20) (Figure 1.3). Stress granules are translationally inert cytoplasmic centers of molecular triage where mRNAs are stored until their fate is determined (20;21). The mRNAs are then redirected to be translated, degraded or stored
This regulation at the post-transcriptional level allows cells to preferentially translate mRNAs that are required to counter the stress response. The composition of stress granules is dynamic and consists of a large number of proteins and RNAs. Stress induced translational arrest and subsequent storage of mRNAs in cytoplasmic granules allows the cell to mount a survival response to counter the adverse conditions. The presence of free 48S complexes or stalled initiation complexes is essential for stress granule formation. TIA-1 (T-cell internal antigen 1) is an RNA binding protein that promotes the assembly of stress granules by binding to free 48S complexes followed by auto-aggregation of the C-terminal prion like domain of TIA-1. In most cases eIF2α, a key component of translation, is phosphorylated and this inhibits translation initiation by depletion of the eIF2 ternary complex. Furthermore, elongating ribosomes run off translating mRNAs and this results in polysome disassembly and stress granule assembly. Stabilizing elongation complexes using cycloheximide results in inhibition of stress granules whereas promotion of ribosome disassembly using puromycin promotes stress granule formation. This demonstrates that stress granules are in equilibrium with polysomes. In addition, drugs that inhibit translation initiation, by targeting the helicase eIF4A, such as hippocristanol and pateamine A, induce stress granules in the absence of eIF2α phosphorylation. Of the 5 eIF2α kinases known to date, HRI (heme-regulated initiation factor 2α kinase) is responsible for sensing oxidative stress produced by arsenite.
Stress granules are composed of stalled pre-initiation complexes containing mRNA, eIF3, the eIF4F complex, ribosomal subunits and PABP as the core components (21). Many other RNA binding proteins involved in mRNA translation, decay and metabolism also localize to stress granules during the stress response. Some examples of these are TIA, TTP, FXR1, G3BP and staufen (21). While several RNAs are shuttled to stress granules during environmental stress, some RNAs are excluded from stress granules. Certain RNAs like HSP90 and HSP70 are retained on polysomes for preferential translation during stress (23;30). Other RNAs such as β-actin, GAPDH and c-myc are recruited to stress granules (30).

**Translation regulation by miRNA and RBPs**

miRNAs are small non-coding RNAs that can regulate translation by inhibiting protein synthesis, degrading mRNAs, or enhancing mRNA translation (11;31;32). There is evidence from sucrose density gradient analysis that miRNAs inhibit translation at both the initiation and post-initiation stage of cap-dependent translation (11;31). The mechanism of this translational repression is still not completely elucidated; repressed cap recognition or 60S subunit joining, inhibition of elongation and ribosome drop-off have been proposed as possible mechanisms (31). An example of an miRNA enhancing translation of mRNAs is miR10a, which increases translation by interacting with the 5' UTR of the target mRNAs, which include several ribosomal mRNAs (32). RNA-binding proteins play an important role in mediating miRNA-based translational activation or repression. A prominent example is the ARE-binding protein HuR that binds to the 3' UTR of CAT-1 mRNA and derepresses translation of that mRNA by miRNA122 (33).
In addition to the initiation, elongation and release factors, a vast array of RBPs are involved in translational regulation. For example RNA-binding proteins such as DDX6 (34), eIF4E-binding proteins (4E-BPs) (10) and fragile X mental retardation protein (FMRP) (35) repress translation, whereas, proteins such as RNA helicase A (RHA) enhance translation of selected mRNAs (36). While a small percentage of RBPs bind non-specifically to RNA, the majority of RBPs target specific structures and/or sequences in their target mRNA (8;37), and thereby efficiently regulate a selected subset of mRNAs. Such sequences are usually present in the 5' and 3' untranslated regions (UTRs) of the message (8;13;38).

**UTRs of mRNA regulate translation**

The UTRs are required for translation initiation, mRNA stability, and poly A binding (6;13).The length of the 5' UTR, extent of secondary structure, and GC content are all important determinants of its translation rate (39). Structured 5' UTRs may be highly regulated in their translation, as the eIF4F complex might require assistance from additional RNA-binding proteins to unwind the structure. Prominent examples of such regulation by structural elements in the 5' UTR include the internal ribosome entry site (IRES) (40;41), the 5' terminal oligopyrimidine tract (TOP) (13), the iron responsive element (IRE) (38) and the post-transcriptional control element (PCE) (42). The 3' UTR contains AU-rich elements (43) and recognition sites for Puf proteins (in yeast) (8). Interactions between the 5' UTR and 3' UTR RNA binding proteins serve to circularize the mRNA (13). My dissertation research focused on the modulation of translation of PCE containing RNAs by the RNA binding protein RHA.
PCE regulates the translation of select retroviral and cellular RNAs

Initially identified in the 5' UTR of the avian spleen necrosis virus (SNV) (42), PCE is an orientation-dependent, structured RNA element that is required for efficient translation of PCE containing mRNAs. Deletion of the PCE or its presence in the anti-sense orientation results in the loss of protein production from PCE RNAs (42). This RNA element does not affect the amount of steady state RNA, and instead allows the RNA to be efficiently translated on polysomes (42). The PCE has subsequently been identified in a total of 8 human and animal retroviruses including the human immunodeficiency virus-1 (HIV-1) and human T-cell lymphotropic virus 1 (HTLV-1) (36;44-47). It has also been identified in the 5' UTR of a cellular gene; the AP-1 transcription factor junD which is involved in growth control (36). The SNV PCE comprises 165 nucleotides, which have been shown to form a tripartite stem loop structure (A, B and C), determined by chemical modification and enzymatic RNA digestion patterns (48)(Singh, D unpublished data). Stem loops A and C are essential for PCE activity, as deletion of both results in a complete loss of PCE activity, whereas, deletion of either stem loop causes a reduction, but not a complete loss in PCE activity (48). Substitution to disrupt base pairs of both the A or the C loop (termed AC') resulted in a loss of PCE activity, that was restored by compensatory mutations (termed AA’CC’) (48). The loop and bulge regions of the A stem of PCE were also found to be important, as point mutations made in these regions (termed Aall) lowered PCE activity (48). None of these mutations caused a change in steady state accumulation, splicing efficiency or cytoplasmic accumulation of PCE RNA, which is consistent with a function for PCE as
translational regulator (44;48). Experiments to test the level of association of these mutants with polysomes using sucrose density gradient analysis are warranted. A complete deletion of PCE resulted in a dramatic decrease in RNA association with polyribosomes, indicating that PCE is required for ribosome loading onto these RNAs (49). RNA transfection experiments showed that PCE interaction with cellular factors in the nucleus is essential for PCE RNA translation (50). RNA affinity chromatography and mass spectrometry identified RHA as a cellular helicase that interacts with the PCE RNA, but not antisense PCE (36). The following paragraphs describe RHA and its varied functions in cellular and viral gene expression.

**RNA Helicase A (RHA): a multifunctional cellular helicase**

Human RHA (DHX9) was initially isolated from Hela cells and characterized as a DExH helicase family member that translocates on dsRNA in the 3’- 5’ direction (51). It was found to be highly homologous to the *Drosophila* Maleless (MLE) protein that has been implicated in dosage compensation in male development, and bovine NDHII which unwinds RNA in a 3’- 5’ direction (52-54). Further, RHA was shown to unwind dsRNA in an ATPase-dependent manner (55). Initial studies confirmed that RHA is essential for normal gastrulation in mice, and homozygosity for the RHA mutant allele leads to embryonic lethality (56).

RHA is a multidomain protein with a conserved DEIH helicase core containing an ATP-binding site (57). Domain studies show two double-stranded RNA binding domains (dsRBDs) at the N-terminus that are conserved in other double- stranded RNA binding proteins like *E. coli* RNase III, *Drosophila* Staufen and Protein kinase R (PKR) (NCBI
conserved domains database) (58). Co-operation between the two domains for poly (rI.rC) binding was shown which indicates that both dsRBDs are important for RNA binding (57).

The C-terminus of RHA is rich in arginine and glycine residues. This RG-rich domain is conserved in select nucleic acid binding proteins like FMRP, hnRNPA1, and hnRNP U and was found to bind preferentially to single stranded RNAs (57). Deletion of the RG-rich domain of RHA allowed better binding of an N-terminal specific antibody to RHA, which implies a possible interaction with the N- and C-terminal domains of RHA (57). Indeed, an interaction between these two regions of RHA has been detected using recombinant proteins as well as by co-immuoprecipitation from mammalian cells (Jing and Boris-Lawrie unpublished). RHA is mainly nuclear in localization, but immunofluorescence studies showed that it shuttles between the nucleus and the cytoplasm (59). This is due to a bidirectional nuclear transport domain (NTD) in the C-terminal domain (amino acids 1150-1259), that contains both nuclear import and export activity. The nuclear export activity is not leptomycin B sensitive and therefore not CRM1 dependent (60). Deletion of two basic residues of RHA (K1162 and R 1165) results in nuclear exclusion of RHA, which indicates that they are required for import (60). The RG-rich domain of the NTD is a target for the methyl-transferase PRMT1, and radiolabeling studies showed that the RG-rich domain was methylated by PRMT1 in vitro (61). Microinjection experiments showed that methylation of the isolated RG-rich domain was required for nuclear import (61), however, experiments in our lab have shown that mutation of the arginine residues of this domain have no effect on nuclear localization.
(Bolinger, Ranji and Boris-Lawrie unpublished data). The helicase core is described in the second section of this introduction.

RHA was initially shown to play a role in transcriptional regulation. Immunoprecipitation assays showed that RHA interacts with the transcription factor CBP, as well as RNA Polymerase II, thereby serving as a bridging factor between the two, and demonstrating the importance of RHA in CREB-dependent transcriptional induction (62). The N-terminal region of RHA (aa 1-250) was shown to be the CBP interaction domain (62). Mutagenesis studies revealed that the ATP binding or ATP hydrolysis activity of RHA is necessary for transcriptional activation of cAMP responsive genes (62). RHA also interacts with a number of transcription factors: a) RHA bridges breast cancer specific tumor suppressor protein (BRCA1) and Pol II (63); b) a yeast two-hybrid screen uncovered an interaction between RHA and NFκβ p65 which was confirmed by immunoprecipitation assays, and RHA was shown to activate NFκβ mediated gene transcription (64); c) RHA is an important transcription cofactor that enhances the oncogenic activity of EWS-FLI1 (65). In addition, RHA enhances overexpression of MDR1 which causes the multidrug resistance phenotype in drug resistant cancer cells (66), and enhances the expression of the tumor suppressor p16^INK4a (CDKN2A, ARF) (67). In sum, ample evidence has solidified the role of RHA as a transcriptional regulator.

While much was known about the nuclear role of RHA in transcription, the discovery that RHA enhances translation of PCE RNAs uncovered a new role for RHA in the cytoplasm (36). Affinity pulldowns with in-vitro transcribed SNV PCE RNA
followed by mass spectrometry were used to identify the interaction between RHA and PCE RNA. siRNAs against RHA eliminated PCE activity, and overexpression of recombinant RHA rescued PCE activity (36;46). Importantly, RHA was shown to be essential for efficient polysome loading of PCE RNA, although it does not affect its cytoplasmic accumulation (36). The interaction of RHA and PCE is essential for this translation modulation, as mutations of PCE that cause a loss in PCE translation such as AC’ or AC’Aall, were found to loose interaction with RHA as demonstrated by RNA immunoprecipitations (36). Subsequent studies identified that junD, reticuloendotheliosis virus A (Rev-A), human T-cell lymphotropic virus type 1 (HTLV-1) and human immunodeficiency virus 1 (HIV-1) also contain a PCE that is modulated by RHA (36;45;46). The ATPase activity of RHA is required for the translation of these RNAs (46), but, the underlying mechanism of the RHA-PCE translation axis is yet undiscovered. A separate cytoplasmic role for RHA was demonstrated in siRNA loading to the RNA-induced silencing complex (RISC) complex (68). Affinity purification of biotinylated siRNA isolated RISC complex components, and RHA was identified as a RISC binding protein (68). RHA is also able to interact with RISC components Ago2 and Dicer in the absence of siRNA and depletion of RHA reduces RISC activity (68). In sum, RHA has multiple roles in post-transcriptional gene expression in addition to its role in transcription.

Besides a role in cellular gene expression, RHA modulates the cellular interaction with viruses. RHA is one of several RNA helicases that play important roles in the virus
lifecycle. The following section describes in detail the roles of RNA helicases, including RHA, in virus replication.

II RNA helicases: Ubiquitous players in gene expression, innate response and viral replication

RNA helicases are ubiquitous in plants and animals, and all helicases contain the highly conserved DExH/D ATP-binding domain (69-73). RNA helicases play essential roles in a broad array of biological processes. During gene expression, RNA helicases catalyze ribonucleoprotein complex (RNP) rearrangements beginning at gene transcription and continuing during the consecutive steps in post-transcriptional gene expression: pre-mRNA splicing, mRNA export, translation, and turnover(8;74-76). Despite their essential role, the mechanisms used by RNA helicases to mediate RNP rearrangements in gene expression are not fully understood. RNA helicases have been identified in all eukaryotic and prokaryotic cells, but in a minority of virus families (77). Apparently a sufficiently diverse repertoire of cellular RNA helicase proteins is available to support most viral replication strategies. Their role in viral replication is exerted at two focal points: 1) ATPase-dependent catalytic cofactor for viral replication; 2) cellular sensor to trigger, or antagonize, the antiviral state.

Modular domain structure of RNA helicases

Helicases can be classified as RNA or DNA helicases based on the substrate they bind to and remodel. The helicase core is comprised of eight conserved motifs (motifs I, Ia, Ib, II, III, IV, V and VI) (70;73;78;79). Along with DNA helicases, RNA helicases
can be grouped into 5 superfamilies (SF1-SF5) based on conservation of sequence motifs (80). Most RNA helicases fall into SF2 and encode the DEAD (amino acid residues Asp-Glu-Ala-Asp), DExH, DExD, or DEAH motif (81), which are named for the residues of their Walker B motif (motif II) that contains the ATP-binding domain (73;82). SF2 comprises eleven families, five of which are DExH/D helicases (DEAD box, SKI2-like DExH, RIG-I-like DExH, DEAH/RHA and viral DExH) (81). Identified in all eukaryotes examined, the DExH/D box family members are divergent in the amino acid sequences that flank the helicase core. These flanking residues represent interchangeable domains (Figure. 1.4) that reoccur amongst proteins involved in the full scope of gene expression, and determine interaction with protein cofactors or target RNA (58). The RNA helicases may exhibit processive activity that affects long stretches of RNA or rearrangement of short oligonucleotides. They may catalyze RNP remodeling or act as nonenzymatic inducers of the antiviral state, as described below.

**Induction of the antiviral state**

RNA helicases play a prominent role in the cellular response to viral infection. Viral infections are recognized by membrane-bound receptors and cytoplasmic sensors that trigger signaling pathways that operate by cascades of protein interactions. The outcome is the ubiquitinylation, phosphorylation and nuclear translocation of transcription factors that activate expression of interferons, interferon-stimulated genes (ISGs) (83) and inflammatory cytokines (84). One family of cytosolic sensor is the RIG-I-like receptor (RLR) family, which includes retinoic acid-inducible gene 1 (RIG-I/DDX58) and melanoma differentiation-associated gene 5 (MDA-5) (83). Classified as
RIG-I-like DExH family members (DECH variants), these RNA helicases contain amino-terminal caspase activation and recruitment domains (CARD) (Figure. 1.4). Their interaction with viral double-stranded RNA (dsRNA) induces conformational changes that reveal CARD domains for interaction with another CARD-containing adaptor protein, mitochondria antiviral signaling (MAVS; also called interferon-beta promoter stimulator 1, IPS-1) (Figure. 1.5). This leads to activation of the TANK-binding kinase 1 (TBK1)/IκB kinase-ε (IKKe) complex that phosphorylates transcription factors IRF-3 and IRF-7 (85). The phosphorylated IRF-3 and IRF-7 are translocated to the nucleus and activate transcription of antiviral and immunological genes, including IFN-α and IFN-β. Even though RIG-I and MDA-5 encode similar DECH and CARD domains, they recognize different subsets of viral RNA by their divergent structural features or nucleotide composition. RIG-I recognizes free 5’ triphosphate structures and short dsRNAs (<1kb) whereas MDA-5 recognizes longer RNAs (>1 kb in length), such as encephalomyocarditis virus (EMCV) RNA and synthetic poly(I:C) RNA (83;86). RIG-I and MDA-5 signaling converges at the MAVS/IPS-1 adapter protein, which integrates response to a variety of viral infections (83). The complete characterization of the ligands of RIG-I and MDA-5 may identify additional ligands and cofactor interactions involved in IRF signaling. For instance, the activity of RIG-I and MDA-5 can be downregulated by another cytoplasmic DExH helicase, LGP2 (Laboratory of Genetics and Physiology 2). LGP2 shares the domain structure of RIG-I and MDA-5 with the exception of the CARD domains (Figure. 1.4). Preferentially-expressed in mammary tissues and tumors(87), under some circumstances, LGP2 acts to sequester viral double-
stranded RNA from RIG-I and MDA-5 and to attenuate induction of the antiviral state (Figure 1.5) (88). In other cases, LGP2 facilitates the induction of the antiviral state, and residues of the ATPase domain of LGP2 are involved in the viral RNA recognition (86). Results in LGP-2-deficient mice revealed differences in response to two viral infections (89). Compared to control mice, LGP2-deficient mice exhibited reduced sensitivity to lethal vesicular stomatitis virus (VSV) infection, but increased sensitivity to lethal EMCV infection, which are sensed by RIG-I and MDA-5, respectively. A possible explanation is that LGP2 in mice contributes to regulation of RIG-I or MDA-5 activity (90). In sum, recognition of viral RNA by RIG-I and MDA-5 induces the innate immune system, whereas LGP2 interferes with the viral RNA recognition and attenuates induction of the antiviral state, at least in some circumstances. The apparent counterbalance of RIG-1 and MDA-5 by LGP2 sets the precedent that RNA helicases may be adapted to attenuate the antiviral state. The results posit that LGP2 participates in a quality control system to monitor proper induction of the antiviral state.

Catalysis of viral gene expression

Viruses are intracellular parasites that hijack cellular RNA helicases to sponsor their replication (79). Currently, eight cellular RNA helicases have been shown to play a role in viral replication (Figure 1.4). These are DNA or RNA viruses that synthesize their genome within the nucleus of the host cell (91). Additionally, three virus families encode viral RNA helicases: Flaviviridae, Poxviridae, and the plant Potyviridae (79;92;93). The viral RNA helicases share the modular domain structure of cellular helicases, but enjoy
intimate contact with the viral RNP. This review will summarize the properties and activity of the 11 cellular and viral RNA helicases that modulate viral replication.

**Host-encoded RNA helicases**

Biochemical and genetic analysis of viral replication has identified roles for many host-encoded RNA helicases. Specifically, several helicases have been identified by analyzing global changes in host mRNA expression following viral infection (94;95) and small interfering RNA (siRNA) screens to identify genes required for viral replication (96). For example, following HIV-1 infection, changes in gene expression are observed for *dhx9, ddx10, ddx18, ddx21, ddx23, ddx39,* and *ddx52* (94;95). The expression levels of cytosolic sensors *ddx58/RIG-I, mda-5,* and *lgp-2,* are increased by infection with Dengue virus, yellow fever virus, vaccinia virus, Hantaan virus, and Sin nombre virus, consistent with induction of the innate antiviral response (88). The ubiquitous involvement of cellular RNA helicases in virus replication is attributable to their ability to act a scaffold for alternative protein-protein interactions. While some host-encoded RNA helicases, such as RIG-I and MDA-5, induce the cellular antiviral response and limit viral replication, eight host-encoded helicases have been shown to promote virus replication. The following sections describe the structure of these helicases and their role in the virus life cycle.

**DDX3**

DDX3 is a 73 kD nucleo-cytoplasmic DEAD helicase that is ubiquitously expressed in a wide range of tissues (97-99). DDX3 has multiple activities in the cell,
which involve ATPase activity and ability to act as a scaffold for alternative protein-protein interactions. The central helicase and DEAD domains are flanked at the N-terminus by a protein-protein interaction domain and a putative nuclear export signal and a glycine-rich C-terminus (Figure. 1.4). DDX3 is highly conserved in metazoans (Table 1). Ded1 is the yeast homolog and is essential for general translation initiation (100). Ded1 was identified in a yeast genetic screen and the ded1-deficiency can be rescued by human DDX3, implicating DDX3 in translation of metazoan RNAs (100;101). Since then, metazoan DDX3 has been observed to modulate gene expression at the transcriptional and post-transcriptional levels, both of which affect the broader processes of cell growth regulation, apoptosis, and the innate response to virus infection (98;102)(103). Members of four virus families use DDX3 to promote their life cycle (Table 2).

**Retrovirus:** Jeang and colleagues identified DDX3 affects HIV-1 posttranscriptional gene expression (99). The exogenous expression of DDX3 increases production of HIV-1 Gag virion structural protein (5 to 12-fold), while suppression of endogenous DDX3 by antisense RNA decreases Gag production (factor of 13) (99). Northern analysis determined that the overexpression of DDX3 did not change the abundance of the unspliced gag transcript or the ratio of unspliced to spliced viral transcripts. The results indicated that DDX3 did not affect HIV-1 transcription or pre-mRNA processing and is likely to enhance Gag production by increasing the Rev-dependent nuclear export of the unspliced gag transcripts. Consistent with this activity,
DDX3 is a binding partner of the CRM1 nuclear export receptor and the HIV-1 viral posttranscriptional trans-activator, Rev (99).

DDX3 expression is enhanced in a HeLa cell line that expresses HIV-1 Tat (99). Two possible explanations are that \textit{ddx3} expression is upregulated by the transcriptional trans-activation activity of Tat, or the post-transcriptional RNA silencing suppressor activity of Tat, which may attenuate miRNA activity against \textit{ddx3} (104;105). Yedavalli et al. explored DDX3 as a target to inhibit HIV-1 replication (106). Two-ring expanded nucleoside analogs were identified that inhibit DDX3 activity, suppress HIV-1 replication, and exhibit insignificant toxicity in cell culture or in mice. These data document that cellular helicases are a feasible target for the development of novel antiretroviral drugs.

**Poxvirus:** DDX3 promotes the innate response to virus infection by its ability to act as a scaffold for protein-protein interactions (107;108)(109;110). DDX3 augments the activity of TBK1/IKKe, which activates IRF-3, IRF-7 and the NF\(\kappa\)B transcription factor to stimulate interferon and interferon response genes that induce the antiviral state (107;108). While the helicase activity of DDX3 is not required, the N-terminal domain of DDX3 is required and associates with IKKe. Notably the same domain is targeted by vaccinia virus K7 protein(107). The crystal structure of K7 in complex with the N-terminal DDX3 peptide reveals that a hydrophobic cleft of K7 residues engage a thumb-like projection of tandem phenylalanine residues (111). Presumably this protein-protein interaction prevents DDX3 from engaging IKKe to promote interferon induction. The
findings suggest that DDX3 is a prime target for viral manipulation of interferon induction (107;108).

**Flavivirus:** Yeast two-hybrid studies initially identified an interaction between DDX3 and the HCV core structural protein (97;101;112). Subsequently RNA interference verified that DDX3 is important for viral RNA replication in HuH-7 liver cells (113). The N-terminal residues of DDX3 are important for interaction with the HCV core and DDX3 interaction is significantly reduced by a single alanine substitution in HCV core (114). However it remains to be determined whether or not the HCV core blocks interaction between DDX3 and IKKε, which would attenuate IRF signalling. Alternatively, HCV core may block interaction between DDX3 and another cellular protein that bolsters replication by another mechanism.

**Hepadnavirus:** Whereas DDX3 enhances replication of HIV-1 and HCV, DDX3 restricts hepatitis B virus (HBV) replication in both hepatoma and non-hepatoma cells. DDX3 binds to HBV polymerase (Pol), which is responsible for reverse transcription of viral RNA to DNA (115) and is incorporated into nucleocapsid with the pre-genome RNA (116). DDX3 ATPase activity, but not helicase activity, is necessary for the inhibitory effect on HBV reverse transcription, which posits that inhibition of viral DNA synthesis occurs at a step following ATP hydrolysis, but prior to RNA unwinding(116). In addition to catalyzing reverse transcription, HBV Pol exhibits an immune modulatory role that is attributed to disrupted IRF signaling (116). Similar to vaccinia virus K7, HBV Pol disrupts the interaction between DDX3 and IKKε. The results suggest that DDX3 is manipulated to evade IFN induction at the expense of viral
reverse transcription. The identification of DDX3 residues essential for the interaction with HBV Pol may provide another selective target for small molecule inhibitors to impair virus replication.

**RNA helicase A (RHA)/DHX9**

Human RHA/DHX9 is an SF2 DEIH helicase that is the homolog of bovine nuclear DNA helicase II and *Drosophila melanogaster* Maleless (117)(54)(53). RHA is the largest and most complex of the helicases involved in viral replication (140 kDa and six domains) (Figure 1.4). RHA executes protein-protein interactions that impact the continuum of gene expression, beginning at transcription and culminating in mRNA translation, and influence cell growth and apoptosis, and the innate response to virus infection(36;71;118-122). RHA contains two double-stranded RNA binding domains (dsRBDs) of conserved α-β-β-β-α topology; the DEIH helicase core; the domain of unknown function (DUF), which is conserved in Ago2; the helicase-associated domain 2 (HA2) of a diversity of RNA helicases; a nuclear transport domain that is recognized by importin-α; and the carboxyl-terminal arginine and glycine-rich residues that are recognized by PRMT1 methylase(61;123). RHA is prominently nuclear(59) and interacts with RNA polymerase II and transcription factors and coactivator that modulate gene transcription(62-64). In the cytoplasm, RHA interacts with structural features of the 5’ untranslated region (UTR) of the junD mRNA to facilitate efficient cap-dependent translation of this protooncogene (36;124;125). Furthermore, the impact of RHA on cellular gene expression may produce cell growth conditions that are advantageous for the viral life cycle. Members of three virus families use RHA to promote their lifecycle.
**Retrovirus:** RHA overexpression has been suggested to increase retrovirus gene transcription, the ratio of spliced to unspliced viral transcripts (126)(60) and nuclear export of unspliced RNA(59)(127). However downregulation experiments with siRNAs and quantitative RNA analysis determined RHA is not required for retrovirus transcription, RNA splicing or nuclear export, but that RHA is necessary for retrovirus translation (42)(36)(46;49). RHA selectively interacts with structural features of the 5’ UTR of many retroviruses and overexpression of RHA increases their translation (42)(36)(46;49). RHA interaction with the 5’ UTR increases polysome association and requires the ATP-binding activity of the DEIH helicase domain (36;46). The molecular basis of RHA translation activity is catalytic rearrangement of the RNP to facilitate ribosome scanning and translation initiation, and possibly protein-protein interaction that secures a circular polysome for efficient translation reinitiation (36). In sum, RHA is necessary for efficient translation of many retroviruses including HIV-1 (Figure. 1.6).

In the case of the simian Mason-Pfizer monkey virus (MPMV), RHA interacts with the 5’ UTR to facilitate translation (44). Additionally, RHA interacts with the 3’ UTR of MPMV in a region necessary for nuclear export of the unspliced viral transcript (127). This region is designated the constitutive transport element (CTE) (59) and facilitates nuclear export by recruiting the TAP nuclear export receptor(128). Conceivably RHA interacts with MPMV RNA in the nucleus and facilitates consecutive remodeling of the viral RNP necessary for nuclear export and subsequent translation in the cytoplasm (129).
Studies on HIV-1 determined that RHA promotes the infectivity of progeny virions (130) (Figure. 1.6). Molecular analysis of the defect determined that RHA in the virus producer cells fosters the reverse transcription activity of the progeny virions (130) (3). Kleiman and colleagues determined the defect is attributable to reduced annealing of tRNA to viral RNA (40% of control) during the virion assembly process (131). Furthermore, RHA is incorporated into the progeny virions, possibly through interaction with the nucleocapsid domain of Gag (130), the HIV-1 RNA, or an adapter protein (46). RHA-deficient virions are less infectious to Hela-based TZM-bl reporter cells (130) and primary T lymphocytes (46). The defect is attributed, in part, to reduced reverse transcription (130) but may also involve replication steps that are downstream of reverse transcription. In sum, RHA appears to be involved in multiple steps in the retroviral life cycle.

**Flavivirus:** Behrens and colleagues identified that RHA binds to the 5’ and 3’ UTRs of bovine viral diarrhea virus RNA and affects viral replication (132). Two other proteins that contain the conserved dsRBD (NF-45 and NF-90/NFAR-1) facilitate viral replication. These findings suggest the dsRBD may be acting as a scaffold to secure the viral RNA in a configuration helpful to replication of the bovine diarrheal virus.

To investigate possible importance of RHA in a human flavivirus, He et al studied HCV replicon cells using RHA downregulation strategies. A lentivector-based inducible RNA interference system administered partial RHA downregulation. Partial RHA downregulation produced a gradual reduction of HCV RNA and protein over 21 days, as
measured by RT-PCR and immunoblot, respectively (133). The results suggest RHA is a necessary host factor for HCV in replicon cells.

**Picornavirus:** Recent study of foot and mouth disease virus (FMDV) determined that RHA downregulation decreases viral replication (134). In infected cells, RHA co-precipitates FMDV replication proteins, 2C and 3A (134). Furthermore, FMDV infection changes the subcellular localization of RHA from prominently nuclear and a uniform distribution, to prominently cytoplasmic and a punctuate distribution. Similar to results in retroviruses and bovine diarrheal virus (36;46;132), RHA interacts with terminal regions of FMDV RNA. Together the results posit the model that FMDV uses RHA to promote viral RNA replication and to facilitate viral protein synthesis. The authors speculate that FMDV uses RHA to connect the 5’ and 3’ UTRs to switch between viral RNA replication to viral protein synthesis, which may occur on a circular polysome (134).

In summary, RHA interacts with the terminal regions of many viral RNAs. Future analysis is warranted to determine the domain(s) of RHA required for interaction with viral RNA and test the possibility that similar residues are required across several virus families. This fundamental investigation will be informative to development of antiviral therapy that selectively discriminates between the cellular and viral RNA targets of RHA.

**DHX30**

To date a single virus, HIV-1, has been shown to interact with DHX30 (isoform 2) (135). DHX30 is a 131 kDa protein with the conserved ATP-dependent helicase domain (DEVH variant) followed by the DUF domain and HA2 domain and one dsRBD
at the amino-terminus (Figure 1.4). The DHX30 domain structure is highly similar to
RHA. Similar to results with RHA, overexpression of DHX30 increases expression of the
HIV-1 provirus (2 to 3-fold) and an HIV-1 LTR luciferase reporter (1.5-4 fold), which is
eliminated by a DEVH mutation (135). In distinction from RHA, DHX30 overexpression
decreased viral RNA packaging into progeny virions (factor of ~10 as measured by RT-
real time PCR on equivalent particle-associated Gag), suggesting proper trafficking of
HIV-1 RNA between the cytoplasm and virions was disrupted (Fig. 1.6). The authors
speculated that DHX30 might cause a modification of the packaging signal by binding to
the 5’ UTR. As expected, the reduction in viral RNA packaging correlated with reduced
infectivity of progeny virions (by a factor of 6). Downregulation of DHX30 did not
produce an effect on HIV-1 gene expression (135), which is likely attributable to
redundant activity by RHA. RHA is abundantly expressed in many types of animal cells
and displays high sequence identity with DHX30 in basic residues that direct RHA
interaction with retrovirus RNA(136). Domain exchange experiments between DHX30
and RHA will be instrumental to understand the specificity and potential functional
redundancy of these RNA helicases.

**DDX1**

Members of three virus families exhibit interaction with DDX1 (Table 2). DDX1
is an 82 kDa ATP-dependent DEAD box family member that contains a SPRY domain
(Figure 1.4). DDX1 exhibits affinity for poly(A) RNA, heterogenous nuclear
ribonucleoprotein K and cleavage stimulatory CSTF-64, which suggests DDX1
contributes to proper 3’ end maturation of mRNAs (137) (138).
**Retrovirus:** In common with DDX3, RHA, and DHX30, DDX1 activity affects HIV-1 replication (Figure. 1.6). Yeast and mammalian two hybrid systems identified DDX1 interacts with HIV-1 Rev (139). DDX1 downregulation reduces the activity of an HIV-1 Rev/RRE-dependent luciferase reporter gene, and RT-PCR determined that nuclear export of unspliced reporter mRNA was impaired. The reduced Rev/RRE activity correlated with disordered Rev subcellular distribution in nucleus and nucleolus (140), consistent with an important role for this host factor in HIV-1 biology. While DDX1 is upregulated in neuroblastoma and retinoblastoma cell lines and tumors (141), expression in astrocytes is low. Astrocytes are nonpermissive for HIV-1 replication and ectopic expression of DDX1 is sufficient to complement the defect in HIV-1 replication (140). The findings that DDX1 facilitates Rev/RRE activity and is a rate-limiting factor for HIV-1 replication in astrocytes indicates that DDX1 is a required host factor for HIV-1 replication (142).

**Polyoma virus:** DDX1 also modulates cell specificity of the human polyoma virus, JC virus (143). DDX1 expression is high in cells susceptible to JC virus infection and low in nonpermissive cells; exogenous DDX1 expression renders nonpermissive cells susceptible to JC virus infection. DDX1 and CSTF64 bind the JC virus transcriptional control region and the outcome is productive expression and accumulation of early and late JC virus transcripts. By contrast, downregulation of DDX1 impairs JC virus gene expression. The results are likely attributable to impaired 3’ end processing that culminates in the decay of viral transcripts.
Coronavirus: Yeast 2-hybrid screens determined DDX1 interacts with coronavirus nonstructural protein 14 (144). Vero cells infected with infectious bronchitis virus exhibited transit of DDX1 from the nucleus to the cytoplasm and colocalization with the coronaviral RNA(144). Mutation of DDX1 DEAD motif to AAAA eliminated the colocalization and the replication of coronavirus, indicating a necessary role for the ATPase-dependent helicase activity of DDX1(144). The results suggest that DDX1 is important for efficient coronavirus replication.

RH116

RH116 has been observed to affect replication of one virus to date, HIV-1 (Table 2). RH116 is a 116 kDa amino acid interferon-induced helicase (DECH variant). Assigned to the RIG-I like DExH box helicase family, RH116 contains two CARD domains (Figure 1.4) and exhibits nuclear accumulation. RH116 is 99.5% identical to MDA-5 and recapitulates its inhibition of cellular proliferation (145). RH116 was identified by a differential display RT-PCR screen for cofactors required for suppression of HIV-1 infection by the synthetic immunomodulator, Murabutide (145). The overexpression of RH116 increases HIV-1 replication in HeLa-CD4 cells coincident with nuclear accumulation of endogenous RH116 (145). A possible explanation for this observation is squelching of a cofactor needed for the antiviral activity of RH116. The remarkable amino acid identity with MDA-5 suggests that RH116 is a cytosolic sensor for HIV-1 under some circumstances. These findings warrant new investigations into the control of RH116 gene expression, the cofactors that control the subcellular localization

**DDX24**

DDX24 is a 96 kDa DEAD box helicase that lacks other conserved motifs (Figure. 1.4) and exhibits amino acid content that is dissimilar to typical DEAD box family members (146). Proteomic analysis identified DDX24 coprecipitates with HIV-1 Gag. This interaction is mediated by the Gag nucleocapsid domain and is disrupted by RNase, indicating that the interaction is RNA-dependent (130). DDX24 also coprecipitates with HIV-1 Rev (91). The downregulation of DDX24 changes the subcellular localization of Rev from nucleolar to nuclear and moderately increases Rev/RRE-dependent nuclear export of unspliced HIV-1 mRNA (2-3 fold) (91). Despite the increase of viral transcript in the cytoplasm, the infectivity of HIV-1 progeny virions was reduced on Hela-based TZM-bl reporter cells. Real time PCR determined reduced abundance of virion-associated RNA, which is sufficient to account for the reduction in titer (91). In sum, DDX24 appears to be important for proper trafficking of the viral RNA for assembly into virions (Table 2, Figure. 1.6).

**P68/DDX5**

P68 is a DEAD box helicase that contains a p68HR domain that is characteristic of p68-like RNA helicases (Figure. 1.4). Named for its molecular weight of 68 kDa, p68 cross reacts with monoclonal antibody to SV40 large-T antigen (pAB204 epitope), which suggests these proteins display a similar epitope (147-149). In common with SV40 large-
T antigen, p68 affects cellular proliferation and tumor development, and mechanistically p68 intersects the processes of transcriptional regulation, RNA splicing, and the processing of pre-ribosomal RNA (150-155). Using results from a yeast two-hybrid screen, Goh et. al. determined that p68 interacts with HCV NS5B RNA-dependent RNA polymerase(156). Expression of NS5B of HCV changes the subcellular localization of endogenous p68 from the nucleus to the cytoplasm (156), similar to the effect of FMDV on RHA (134). Downregulation of p68 reduces the transcription of negative strand HCV RNA. Taken together, p68 is an important host factor for HCV at the level of HCV gene expression (Table 2).

**Mov10**

Moloney leukemia virus 10 homolog (Mov10) contains a putative SF1 helicase (DEAG variant) and lacks other defined domains (Figure. 1.4). Mov10 appears to be heavily post-translationally modified because of substantial difference between the predicted and observed molecular weights (114 kDa and 130 kDa, respectively). Mov10 is required for RNA interference-mediated gene silencing by RISC (157;158); however RNA interference is successfully employed to test the effect of Mov10 on virus replication (below). Mov10 is expressed in a variety of cell types including lymphocytes and embryonic stem cells (159;160). Mov10 in HeLa cells coprecipitates with RNA-induced silencing complex (RISC) components Ago1 and Ago2 and colocalizes with Ago proteins in cytoplasmic processing bodies (157). Virus interaction with Mov10 has the potential to interface with antiviral RNA silencing and influence the viral replication efficiency.
Hepatitis delta virus (HDV, unassigned family): The downregulation of Mov10 or the Mov10 binding partner, Ago2, is sufficient to reduce HDV replication (161). HDV replication requires RNA-directed transcription and host RNA-dependent RNA polymerase catalytic activity. In principle, these processes are similar to RNA-directed RISC loading to miRNA target RNA and RNA-dependent catalytic activity of RISC. We posit a corollary model in which Mov10 facilitates remodeling of the incoming viral RNA to produce a transcription initiation competent RNP.

Retrovirus: The downregulation of endogenous Mov10 reduces HIV-1 infectivity. However, exogenous expression of Mov10 in primary or transformed cells severely reduces infectivity of progeny virions (158;159;162). These non-reciprocal results suggest an appropriate balance of Mov10 and cofactors is responsible for control of virus replication. Recently published studies determined Mov10 is a restriction factor against several retroviruses: simian influenza virus, murine leukemia virus, feline immunodeficiency virus, equine infectious anemia virus and HIV-1 (158;159;162). Mov10 of human, simian and murine origin exhibit antiretroviral activity, consistent with their significant conservation among various species (158) (Table 1). HIV-1 infection of human cell lines (A3.01, CEM-T4, CEM-SS) reduces endogenous Mov10 (158), which is reminiscent of virus-associated degradation of the APOBEC3G restriction factor by Vif (163). Mov10 interacts with APOBEC3G in primary CD4+ T cells and 293T cells, although Vif does not downregulate Mov10 activity (159).

Similar to RHA and DHX30, Mov10 interacts with the nucleocapsid domain of HIV-1 Gag in an RNA-dependent manner and is packaged in virions (Figure. 1.6).
Restriction occurs at an early, post-entry step in viral replication that occurs before reverse transcription is initiated (Figure 1.6). Results from producer cells indicate that proteolytic processing of nascent virions is reduced (162), which may indicate defective viral core uncoating or translocation to the nucleus. The issue remains uncertain of whether or not the anti-HIV activity requires ATPase–dependent helicase activity of Mov10. Mutation of the DEAG helicase domain eliminated anti-HIV-1 activity in one study, (158) but not in another investigation (159). Both studies used 293T cells to produce virus, but they employed different cells to measure virus titer: osteosarcoma HOS-derived HIV GFP GHOST reporter cells and HeLa, respectively. The use of different target cells may contribute to the conflicting outcomes. The restriction activity of Mov10 in species from mouse to man and against many retroviruses raises the possibility that Mov10 restricts replication of other viral families. Either way, deciphering the fundamental mechanisms of Mov10 activity may provide a target for development of selective anti-viral molecules.

**Virus-encoded Helicases**

While most viruses do not encode their own helicase, 3 virus families encode a viral helicase. The viral helicases display the familiar domain structure of cellular helicases (Figure 1.4). The viral helicases are essential for the replication of these viruses and in some cases are emerging targets for anti-viral therapy (see below). This section focuses on properties of the viral RNA helicases.
HCV nonstructural protein 3 (NS3)

HCV encodes a single open reading frame that is translated to a 3000 amino acid polyprotein and cleaved into 10 mature proteins. NS3 is a 70 kDa DExH box protein (DECH variant) that resembles SF2 family helicases (77;164). NS3 contains five domains: two N-terminal domains that comprise a protease and three distal domains that comprise the helicase (77;165) (Figure. 1.4). Until recently, the NS3 helicase and protease domains were considered functionally independent, however, new data indicate the protease domain exhibits a positive charge that enhances the interaction of the helicase domain with RNA (166;167). NS3 RNA helicase activity is essential for HCV replication and is not replaced by cellular helicases (167;168). NS3 RNA helicase affects two steps in the HCV replication cycle. During RNA-dependent RNA replication, NS3 is required to unwind the double-stranded RNA intermediate, which may enable movement of HCV NS5b polymerase (165;169;170). Second, NS3 assists in virus assembly and is likely attributable to ability to act as a scaffold for interaction with viral or cellular cofactors. Point mutation of the NS3 helicase domain (both subdomains) demonstrated a role for NS3 helicase in infectious virus assembly, independent of a role in HCV RNA replication (171). Homologous NS3 proteins are encoded by two other Flaviviruses, yellow fever virus and dengue virus (81). In sum, NS3 helicase activity is essential for Flavivirus replication and is a potential therapeutic target against the significant human pathogen, HCV.

HCV affects nearly 180 million people worldwide and is responsible for about 50-75% of liver cancer cases (165;172). Current therapy against HCV is ineffective in about
20% and 60% of patients chronically infected with HCV genotypes 2/3 and genotype 1, respectively (173). HCV helicase has been actively pursued as a target to inhibit HCV (174;175). Several studies to identify small molecule, nucleic acid, and antibody inhibitors of HCV NS3 have been described in detail (77;165) and clinical trials have involved combinations to target HCV protease and polymerase (165). The continued mechanistic investigation of NS3 and ready availability of a cache of candidate compounds are essential tools to develop an efficacious treatment for HCV infection.

**Vaccinia virus nucleoside triphosphate phosphohydrolase II (NPH-II)**

Vaccinia virus encodes a 74 kDa helicase that contains a DEVH ATP binding domain and a DUF domain, which is designated nucleoside triphosphate phosphohydrolase II (NPH-II) (Figure. 1.4). NPH-II is an RNA-dependent NTPase that is activated by single-stranded RNA, and can hydrolyze all four ribonucleoside triphosphates (176). Homology is observed between the helicase domain of NPH-II, three yeast PRP splicing proteins (PRP16, PRP2 and PRP22), RHA/DHX9 and the *Drosophila* homolog Maleless (177). NPH-II unwinds RNA substrates or RNA-DNA hybrids in a processive and directional fashion with a small step size of around 6 base pairs (178).

Essential for vaccinia virus replication, NPH-II is incorporated into the vaccinia virion (177). Fathi and Condit uncovered several temperature sensitive mutations of NPH-II that generate non-infectious progeny virions at non-permissive temperature (179;180). Gross and colleagues determined the temperature sensitive mutations produced defects in early transcription of the DNA template (181). DNA-mediated marker rescue of NPH-II temperature sensitive viruses revealed the ATPase and helicase
activity of NPH-II are essential for vaccinia virus replication (182). Mutations that block RNA unwinding without significantly affecting the ATPase activity are lethal, possibly by promoting genomic instability. The authors posited that NPH-II facilitates productive viral transcription by preventing R loop formation behind the elongating RNA polymerase, which otherwise may result in genomic instability (183). Homologous NPHII proteins are encoded by *Poxviridae* members variola virus and fowlpox virus (81). In sum, NPH-II provides essential function(s) in the viral life cycle, which apparently are lacking from cellular RNA helicases or cannot be provided in an accessible configuration. NPH-2 viral RNA helicase activity is a potential therapeutic target to attenuate poxvirus replication and pathogenesis.

**Plum pox virus cylindrical inclusion protein (CI)**

Plum pox virus (PPV) CI is a 70 kDa DExH box RNA helicase (DECH variant) that contains the Poty_potyviridae polyprotein domain (PP) (Figure. 1.4). PPV is a member of the *Potyviridae*, which is the most numerous group of plant viruses. Of unknown function; Poty_PP is ~300 amino acids, rich in basic residues and conserved in polypeptides across *Potyviridae*. CI protein is produced by cleavage of the viral polyprotein and exhibits NTP-dependent helicase activity (184). PPV CI helicase activity was the first reported for an RNA virus (184). CI is a catalyst necessary for viral replication and a scaffold to promote the spread of the virus from cell to cell, and these functions are genetically separable (185-187). DECH helicase and NTPase mutations disrupt viral replication (185). Mutations that impair viral cell-to-cell spread retain helicase and NTPase activity (187) but impair necessary virus-host protein interactions.
Homologs of CI are encoded by potyviruses tobacco-mosaic virus and the lilly-mottle virus (81), which suggests CI activity is conserved among PPV.

**Perspectives on the future directions**

The study of RNA helicases is fueling fundamental understanding of cell biology and the virus-host interface. The versatile roles of RNA helicases in virus biology are attributable to the linkage of the conserved catalytic domain to different substrate recognition domains. This modular configuration is similar in principle to the separable domains of transcription factors: activation domain and DNA binding domain. The functional versatility of RNA helicases encompasses their well-characterized roles as cytosolic receptors to recognize viral infection and trigger activation of the antiviral state, to control of gene expression that results in proper utilization of the viral RNA for translation and assembly into virions, to their manipulation by viral proteins to block induction of the antiviral state.

This fundamental information has generated fresh perspectives to address new open issues of significant potential impact on cell biology, immunology and viral pathogenesis. Future aims include: i) to determine the complete list of ligands that trigger the antiviral state and definition of their biophysical requirements for productive interaction; ii) to define all of the RNA helicases that interface with viral gene products to manipulate the antiviral state and their molecular mechanism; iii) to characterize the features of cellular helicases and viral helicases that determine selective interaction with RNA substrates; iv) to execute effective strategic application of the fundamental knowledge of RNA helicases to identify anti-viral drugs with minimum toxicity to human
and animal patients. In closing, recent achievements to understand the basic science of RNA helicases are being applied to develop selective, efficacious inhibitors of significant human pathogens, HCV and HIV-1. The continued progress to define the molecular mechanisms of RNA helicases at the virus-host interface is rocket-fuel to attain efficacious drugs against human and animal pathogens.
Figure 1.1. Illustration of translation initiation. The initiation factors eIF3, eIF1 and eIF1A bind to a free 40S subunit of the ribosome and then bind the ternary initiation complex comprising eIF2, met-tRNA and GTP. Separately, 5'-capped mRNA is bound by the eIF4F complex comprising eIF4A, eIF4G and eIF4E, through the interaction of eIF4E with the cap. eIF4B also associates with this complex and together with the helicase eIF4A, remodels the structure of the 5' UTR allowing the eIF3-40s-ternary complex to bind. The 40S ribosome, once bound to mRNA is able to scan downstream to the initiation AUG codon that is recognized by the met-tRNA anticodon. The 60S subunit is then able to join the complex and assemble the 80S ribosome that initiates translation. PABP binds the 3' poly-A tail of the mRNA and also the eIF4G subunit bound to the 5' end, resulting in mRNA circularization. This figure is adapted from Pestova et al, 2010, Nat rev mol cell biology.
Figure 1.2. Illustration of translation elongation. Translation elongation begins once the 80S ribosome (shown in purple) positions itself such that the AUG initiation codon with the bound met-tRNA is at the “peptidyl” P site of the ribosome. The elongation factor eEF1A (complexed to GTP) then brings the next amino-acyl-tRNA to the A site of the ribosome, GTP is hydrolyzed and eEF1A-GDP leaves the complex. This allows peptide-bond formation to occur between the two amino acids. The next step of translocation of the ribosome is catalyzed by eEF2, thereby bringing the peptidyl chain bound tRNA into the P site and the deacylated tRNA into the E site where it will subsequently be ejected after the next translocation. A new cycle begins with eEF1A-GTP bringing in the next amino-acyl-tRNA and the peptide is elongated. eEF1B is required to convert GDP-bound eEF1A to GTP-bound eEF1A, thereby allowing for continuation of elongation.

Figure 1.3. Dynamics of stress granule assembly.
In the presence of stress, eIF2α is phosphorylated which leads to depletion of the ternary complex comprising eIF2-GTP-tRNA. The absence of the ternary complex allows Tia-1 to bind to the mRNA. Tia-1 is able to self aggregate and results in the formation of discrete foci known as stress granules.
This figure has been taken from Anderson et al 2002, Journal of Cell Science Reproduced with permission of the Company of Biologists
Figure 1.4. Domain structure of DExH/D helicases involved in viral replication.
Upper rectangle: RNA helicases of the RIG-I-like receptor family are components of the innate antiviral response that attenuates viral replication. Lower rectangle: RNA helicases that promote viral replication. Protein sequences were retrieved from the NCBI conserved domains database and domains determined by annotation and query of the Uniprot database. The predicted molecular weight (kDa) and number of amino acid residues (aa) are indicated. Jagged edges indicate interruption by additional sequence or domains. Domains are: CARD, Caspase-activation and recruitment domain; DExDc, DEAD-like helicase superfamily ATP binding domain; HELICc, Helicase superfamily C-term domain associated with DExH/D box proteins; RIG-I_CRD, Regulatory domain of RIG-I; RS/GYR, Arginine/serine, glycine tyrosine rich domain; dsRBD, Double-stranded RNA binding domain; HA2, Helicase-associated domain of unknown function; DUF, Domain of unknown function; RG-rich, arginine and glycine-rich domain; SPRY, SP1a and RYanodine receptor domain; p68HR, Characteristic of p68-like RNA helicase; Peptidase_S29, serine protease domain with trypsin-like fold; Poty_PP, Observed in polyproteins of the Potyviridae.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecule Mass (kDa)</th>
<th>Domain Structure</th>
<th>Number of Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIG-I</td>
<td>(102 kDa)</td>
<td></td>
<td>925 aa</td>
</tr>
<tr>
<td>MDA5</td>
<td>(117 kDa)</td>
<td></td>
<td>1025 aa</td>
</tr>
<tr>
<td>LGP2</td>
<td>(75 kDa)</td>
<td></td>
<td>679 aa</td>
</tr>
<tr>
<td>DDX3</td>
<td>(73 kDa)</td>
<td></td>
<td>662 aa</td>
</tr>
<tr>
<td>RHA</td>
<td>(140 kDa)</td>
<td></td>
<td>1270 aa</td>
</tr>
<tr>
<td>DHX30</td>
<td>(131 kDa)</td>
<td></td>
<td>1194 aa</td>
</tr>
<tr>
<td>DDX1</td>
<td>(82 kDa)</td>
<td></td>
<td>740 aa</td>
</tr>
<tr>
<td>RH116</td>
<td>(115 kDa)</td>
<td></td>
<td>1025 aa</td>
</tr>
<tr>
<td>DDX24</td>
<td>(96 kDa)</td>
<td></td>
<td>859 aa</td>
</tr>
<tr>
<td>p53</td>
<td>(68 kDa)</td>
<td></td>
<td>614 aa</td>
</tr>
<tr>
<td>Mov10</td>
<td>(114 kDa)</td>
<td></td>
<td>1003 aa</td>
</tr>
<tr>
<td>HCV NS3</td>
<td>(70 kDa)</td>
<td></td>
<td>631 aa</td>
</tr>
<tr>
<td>vacciniaNPHIII</td>
<td>(74 kDa)</td>
<td></td>
<td>675 aa</td>
</tr>
<tr>
<td>PPV CI</td>
<td>(70 kDa)</td>
<td></td>
<td>635 aa</td>
</tr>
</tbody>
</table>

**Figure 1.4.** Domain structure of DExH/D helicases involved in viral replication
RNA helicases of the RIG-I-like receptor (RLR) family play a crucial role in the innate antiviral response. RLR family members, retinoic acid-inducible gene 1 (RIG-I/DDX58) and melanoma differentiation-associated gene 5 (MDA-5) are cytoplasmic sensors that detect viral RNA and trigger induction of the antiviral state. RIG-I and MDA-1 recognize viral RNA as foreign by features including free 5’ triphosphate ends, short double-stranded RNA (dsRNA) and long dsRNA (>1kb), respectively. RLR family member, laboratory of genetics and physiology 2 (LGP2) can antagonize this activity (dashed line) by regulation that is poorly understood. Interaction with their RNA ligands induces conformational changes in RIG-I and MDA-5 that favor interaction with mitochondria antiviral signaling (MAVS), also called interferon-beta promoter stimulator 1 (IPS-1). MAVS/IPS-1 activates the TANK-binding kinase 1 (TBK1)/IκB kinase-ε (IKKε) complex, which phosphorylate IFN-regulatory factor (IRF)-3, IRF-7 and NF-κB (not shown). The IRFs dimerize, translocate to the nucleus and bind to target genes. Transcriptional activation of interferon (IFN) genes leads to production of IFNs (α and β), which are secreted from the cell. IFN binding to the IFN receptor activates the janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. The JAK and tyrosine kinase 2 (TYK2) phosphorylate one another, the receptor and STAT2. STAT2 binds STAT1 and triggers phosphorylation and STAT1/2 dimerization. The STAT heterodimer translocates to the nucleus with IRF-9. They activate transcription of interferon stimulated genes (ISG), which generate the anti-viral state that attenuates viral replication.
Figure 1.5. RNA helicases of the RIG-I-like receptor (RLR) family play a crucial role in the innate antiviral response
Figure 1.6. Cellular helicases involved in HIV-1 replication. DExH/D helicases with a known function in HIV-1 replication are indicated beside the corresponding step in the replication cycle of HIV-1: 1) Receptor-mediated entry; 2) Reverse transcription of viral genomic RNA to DNA and integration into host chromosome to render the provirus; 3) Transcription of HIV-1 provirus and accumulation of viral mRNA; 4) Post-transcription includes RNA processing and export from the nucleus; 5) Translation of viral transcripts on polysomes; 6) Assembly of viral structural and enzymatic proteins and unspliced viral transcript into progeny virions that bud from the plasma membrane.
Table 1.1. Significant conservation is observed amongst RNA helicases involved in virus biology.

| DExD/H family member | Pan troglodytes | Mus musculus | Rattus norvegicus | Canis lupus familiaris | Bos taurus | Gallus gallus | Danio rerio | Drosophila melanogaster | Anopheles gambiae | Caenorhabditis elegans | Plasmodium falciparum | Schizosaccharomyces pombe | Schizosaccharomyces cerevisiae | Kluyveromyces lactis | Eremothecium gossypii | Magnaporthe grisea | Neurospora crassa | Arabidopsis thaliana | Oryza sativa |
|----------------------|----------------|--------------|-------------------|---------------------|-----------|--------------|-------------|---------------------|-----------------|-------------------|-----------------|-------------------|----------------------|-------------------|----------------|----------------|----------------|----------------|----------------|-------------|
| DDX3                 | 99             | 99           | 99                | 99                 | 99        | 94           | -           | 61                  | 51              | 54                | 54              | -                 | -                   | -                   | 55                | -                | -                | 45             |
| RHA/DHX9             | -              | 90           | 90                | 95                 | 95        | -            | -           | -                  | 53              | 53                | 45              | -                 | -                   | -                   | -                 | -                | -                | -              |
| DHX30                | 99             | 98           | 98                | 98                 | 98        | 79           | 53          | -                  | -               | -                 | -                | -                 | -                   | -                   | -                 | -                | -                | -              |
| DDX1                 | 100            | 98           | 98                | 98                 | 97        | 93           | 85          | 61                  | 63              | 50                | 36              | -                 | -                   | -                   | -                 | -                | -                | -              |
| RH116                | -              | 80           | 80                | 84                 | 84        | 63           | 58          | -                  | -               | -                 | -                | -                 | -                   | -                   | -                 | -                | -                | -              |
| DDX24                | 99             | 80           | 82                | 85                 | 84        | 60           | 54          | 41                  | 43              | 39                | 39              | -                 | 40                  | 36                  | 37                | 36               | 36               | 37             |
| P68/DDX5             | -              | 99           | 99                | 100                | 100       | 91           | 86          | 60                  | 62              | 56                | 56              | 58                | 58                  | 59                  | 57                | 60               | 61               | 60             |
| Mov10                | 100            | 91           | 92                | 95                 | 92        | 51           | 47          | -                  | -               | -                 | -                | -                 | -                   | -                   | -                 | -                | -                | -              |

*Protein sequences were retrieved from the NCBI HomoloGene and BLAST databases, date 30 June 2010. The percentage of amino acid identity relative to the human homolog was determined. *Homolog not annotated in database.
<table>
<thead>
<tr>
<th>RNA Helicase</th>
<th>Virus</th>
<th>Role in replication cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DDX3</strong></td>
<td>HIV-1</td>
<td>Binds CRM1 and HIV-1 Rev and increases Gag protein production</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>Interacts with vaccinia virus K7 protein, which blocks transcriptional activation of the interferon and interferon responsive genes</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B virus (HBV)</td>
<td>Restricts viral replication by binding to HBV Polymerase and inhibiting reverse transcription of HBV RNA to DNA</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (HCV)</td>
<td>Important for HCV RNA replication</td>
<td></td>
</tr>
<tr>
<td><strong>RHA</strong></td>
<td>Retroviruses: HIV-1, human T-cell leukemia virus type 1, bovine leukemia virus, spleen necrosis virus, feline leukemia virus, Mason-Pfizer monkey virus (MPMV)</td>
<td>Associates with post-transcriptional control element (PCE) in the 5’ untranslated region and facilitates translation of these RNAs</td>
</tr>
<tr>
<td>MPMV</td>
<td>Facilitates annealing of tRNA to viral RNA during virus assembly</td>
<td></td>
</tr>
<tr>
<td>Bovine viral diarrhea virus</td>
<td>Incorporated into HIV-1 virions and facilitates virion infectivity</td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td>Associates with the constitutive transport element (CTE) in the 3’ UTR that binds the TAP nuclear export receptor; may augment release of CTE-containing RNA from the nuclear pore complex</td>
<td></td>
</tr>
<tr>
<td>Foot and mouth disease virus</td>
<td>Binds to the 5’ and 3’ termini of viral RHA; enhances replication</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Required for HCV replication</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Binds to the 5’ and 3’ termini of viral RHA; enhances replication</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2. Overview of the role of cellular helicases in replication cycle of indicated virus

Continued
Table 1.2. continued

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Virus/Host</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DHX30</strong></td>
<td>HIV-1</td>
<td>Overexpression increases steady state HIV-1 RNA and decreases virion RNA in progeny virions</td>
</tr>
<tr>
<td><strong>DDX1</strong></td>
<td>HIV-1</td>
<td>Interacts with HIV-1 Rev; affects nuclear export of unspliced reporter mRNA</td>
</tr>
<tr>
<td></td>
<td>JC virus</td>
<td>Binds the JC virus transcriptional control region; facilitates productive expression of early and late JCV transcripts</td>
</tr>
<tr>
<td></td>
<td>Infectious bronchitis virus</td>
<td>Interacts with coronavirus non-structural protein 14 and colocalizes with the viral RNA</td>
</tr>
<tr>
<td><strong>RH116</strong></td>
<td>HIV-1</td>
<td>Overexpression increases steady state HIV-1 mRNA</td>
</tr>
<tr>
<td><strong>DDX24</strong></td>
<td>HIV-1</td>
<td>Interacts with HIV-1 Gag; downregulation moderately reduces nuclear export of unspliced HIV-1 mRNA; reduces viral RNA in virions; reduces the RNA infectivity of progeny virions</td>
</tr>
<tr>
<td><strong>p68</strong></td>
<td>HCV</td>
<td>Interacts with HCV NS5B; required for transcription of negative strand viral RNA</td>
</tr>
<tr>
<td><strong>Mov10</strong></td>
<td>Hepatitis delta virus</td>
<td>Required for viral replication</td>
</tr>
<tr>
<td></td>
<td>Retroviruses: HIV-1, simian influenza virus, murine leukemia virus, feline immunodeficiency virus, equine infectious anemia virus</td>
<td>Activity as antiviral restriction factor</td>
</tr>
<tr>
<td></td>
<td>HIV-1</td>
<td>Interacts with HIV-1 Gag, packaged into virions and affects proteolytic processing; overexpression or downregulation reduces infectivity of progeny virions</td>
</tr>
</tbody>
</table>
Chapter 2. The N-terminal double-stranded RNA binding domains of RNA helicase A are necessary for recognition of viral and cellular translation target mRNAs

Abstract
The DExH protein RNA helicase A (RHA) plays numerous roles in cell physiology, and post-transcriptional activation of gene expression is a major role among them. RHA selectively activates translation of complex cellular and retroviral mRNAs. While RHA requires interaction with structural features of the 5' untranslated region (UTR) of these target mRNAs, the molecular basis of activation by RHA of selected cellular and viral mRNAs is poorly understood. RHA contains a conserved ATPase-dependent helicase core flanked by two α-β-β-α double-stranded RNA binding domains (dsRBDs) at the amino-terminus and repeated arginine-glycine residues at the carboxyl terminus. The individual recombinant N-term, central helicase and C-term domains were evaluated for their ability to specifically interact with cognate RNAs by in vitro biochemical measurements and mRNA translation assays in cells. The results demonstrate that N-terminal residues confer selective interaction with retroviral and junD target RNAs. Conserved lysine residues in the distal α-helix of the dsRBDs are necessary to engage structural features of retroviral and junD 5' UTR. Exogenous expression of the N
terminus co-precipitates junD mRNA and inhibits the translation activity of endogenous RHA. The results indicate the molecular basis for the activation of translation by RHA is recognition of target mRNA by the N-terminal domain that tethers the ATP-dependent helicase for rearrangement of the complex 5' UTR.

**Introduction**

RNA helicase A (RHA) is a ubiquitous DEIH superfamily 2 helicase that is necessary for translation of retroviral and selected cellular mRNAs (36;45;46). A unifying feature of RHA-responsive mRNA templates is a structurally complex 5' untranslated region (UTR) that is a platform for alternative RNA-protein interactions that modulate gene expression (188). All retroviruses require a complex 5' UTR for productive viral replication (188). Alternative 5' UTR RNA-RNA and RNA-protein interactions generate alternatively spliced mRNAs that are templates for synthesis of viral proteins, or unspliced genome-length RNA that is dimerized and assembled into virions. The later serves as template for reverse transcription upon infection. RHA is necessary for the translation of the junD proto-oncogene and the retroviruses that infect a variety of metazoan host cells (36;42;44-46). RHA interacts with structural features of the 5' UTR and facilitates polyribosome loading and productive translation of junD and retroviral mRNAs (36;45;46). JunD is an AP1 transcription factor that is necessary for healthy cell growth and whose expression is stringently controlled at transcriptional and posttranscriptional levels (124). The necessary role of RHA in translation of junD and retroviruses posits the hypothesis that conserved features of RHA determine recognition of these target mRNAs.
Site-directed mutagenesis and reporter assays have determined structural features of the complex 5’ UTR that are necessary for RHA interaction and translation activity (36;45;46;48). RHA-dependent translation activity is conferred by orientation-dependent activity of the ~150 nt 5’ terminus, which is designated the post-transcriptional control element (PCE) (13;14). PCE activity is attributable to two functionally redundant stem loop structures (A and C) (42;45). Mutations that disrupt structural features of A and C (mutAC) in spleen necrosis virus (SNV), an avian retrovirus, eliminate translation activity and detectable interaction with epitope-tagged RHA (FL-RHA) in cells, whereas compensatory mutations restore translation activity and interaction with FL-RHA (36;45;46;48). These observations provide a framework to determine the residues of RHA necessary for functionally relevant interaction with PCE.

The architecture of RHA is complex with a conserved ATPase-dependent helicase domain that is flanked by motifs frequently observed in functionally distinct post-transcriptional regulatory proteins (58;71;189). The N-terminal residues contain two repeats of a double-stranded RNA binding domain (dsRBD) that is observed widely among RNA-interactive proteins (5), e.g. the dsRBD in protein kinase R (PKR) and Dicer direct interaction with generic double-stranded viral RNA and small duplex RNAs, respectively (58;190). By comparison, RHA selectively recognizes the complex 5’ UTR of a subset of cap-dependent mRNA templates (188). The possible role for the dsRBDs in the selective recognition of RHA target mRNAs is unknown.

The C terminus of RHA is highly conserved from canine species to Caenorhabditis elegans (NCBI homolog database) and is characterized by repeated
arginine and glycine (RG) residues that are substrates for methylation by PRMT1 (61). A similar RG-rich domain is observed in nucleolin and hnRNPA1 and interacts with protein cofactors that tether target mRNA (191-193). Moreover, the RG-rich domain of fragile X mental retardation protein directly engages target RNA at G quartets (194;195). These findings suggest the possibility that the RG-rich domain of RHA is important for interaction with target mRNAs.

DExH/D box proteins have been identified amongst all prokaryotes and eukaryotes examined and affect all steps in RNA metabolism (70;71). Named for the amino acid residues that are adjacent to the ATP-binding region (74;82), the helicase domain spans 350-400 amino acids and exhibits RNA binding capability (72;73;196). For instance, mutations in the helicase domain of RIG-I disrupt binding of RIG-I to poly (I:C) or in vitro transcribed dsRNA (196). The possibility that the helicase domain of RHA binds target mRNA remains to be determined.

Herein individual recombinant RHA domains were evaluated for binding activity to PCE in comparison with non-functional PCE and generic double-stranded RNAs (dsRNAs). The results of biochemical and biophysical experiments show that the N-terminal domain exhibits higher binding affinity for PCE than for non-functional mutant RNA or control dsRNA. Highly conserved surface exposed lysine residues were required for selective interaction with PCE RNA. By comparison, the isolated DEIH domain lacked detectable binding to PCE RNAs tested in our study, and the C-terminal RG-rich domain bound to negative control dsRNAs with greater affinity than to the PCE RNAs tested here. In cells, the N-terminal domain directs interaction with PCE mRNA and its
exogenous expression blocked the translation activity of endogenous RHA. Our results demonstrate that the N-terminal residues are necessary for recognition of PCE RNA and that this interaction is necessary for translation activation of selected cellular and viral mRNAs.

Materials and methods

Plasmid construction

Plasmids to express recombinant proteins were generated by PCR with the following primer pairs and template DNAs, and gel-eluted PCR product and indicated plasmid were digested with the indicated restriction enzymes. The primer sequences are provided in Table 2. All plasmid constructions were verified by restriction mapping and DNA sequencing. pGex2TN-term: primers KB1109, KB1110 and pcDNAFLRHA (C.G Lee, University of Dentistry and Medicine of New Jersey), EcoRI digestion and ligation to pGEx2T (GE). pGex2TDEIH: primers KB1733, KB 1734 and template pcDNAFLRHA, EcoRI and BamHI digestion and ligation to pGex2T. pT7.7HisC-term: primers KB1661, KB1662 and template pcDNAFLRHA, NdeI and HindIII digestion and ligation to pT7.7 (USB). pFLAG-N-term: primers KB1726, KB1727 and template pcDNAFLRHA, EcoRV and NotI digestion and ligation to pcDNA3.1. PCR-based site-directed mutagenesis (Stratagene) introduced indicated mutations by the indicated primers and template DNA: pGEX2TK54A/55A: KB1674, KB1675 and pGex2TN-term; pGEX2TN-termK236E: KB1440, KB1441 and pGex2TN-term; pGEX2T N-termK54A/55A/236E: KB1440, KB1441 and pGEX2TN-termK54A/55A. To generate pMH110, junD sequences were amplified by PCE with primers KB1377, KB1378 and template genomic
DNA from HEK293 cells; gel-elution, EcoRI digestion and ligation to pCRII-TOPO (Invitrogen).

**In vitro transcription**

T7 RNA polymerase was used to synthesize EMSA RNA probes (Promega). Template DNA was generated by PCR with the following primer/template DNA combinations. SNV PCE: primers KB588, KB 664/pYW100 (42); mutant SNV PCE: KB1352, KB588/pAC’Aal (36); junD PCE: KB 1702, KB 1378/pMH110. Primer sequences can be found in table 2. *In vitro* transcripts were generated in RiboMAX™ Large Scale RNA Production System (Promega) in the presence of $\alpha-^{32}$P UTP/$\alpha-^{32}$P CTP (Perkin Elmer), treated with DNase (Promega), separated on 8% denaturing urea gels and eluted in passive gel elution buffer (Ambion). RNAs were precipitated in 95% ethanol and 0.3 M NaOAc at -80°C for 20 min, collected by centrifugation, resuspended in diethylpyrocarbonate-treated water and monitored by scintillation counting. Details of template and primer DNA sequences are provided in Table 2.

**Expression and purification of recombinant proteins**

Preparations of expression plasmids for RHA domains: N-term, DEIH and C-term, are described in Supplemental Procedures. The proteins were expressed in BL21 codon plus optimized cells (Stratagene). After induction with 1 mM isopropyl-β-D-thiogalactopyranoside (USB) for 2.5 hours at 33 ºC, cells were resuspended in PBS with 10 µl/ml protease inhibitor cocktail (Sigma), 1 µl/ml 1 M dithiothreitol and lysed by Aminco french pressure cell at 5000 units of pressure. Soluble proteins were harvested by centrifugation in Sorvall RC5C (SS-34 rotor) at 12,000 rpm for 20 min at 4 ºC. Wild-type
and mutant N-term domains were purified from the soluble protein lysate on Glutathione Sepharose beads (Pierce). Lysate was exposed to the beads overnight, followed by four washes with 50 mM HEPES, 150 mM NaCl pH 7.0, one wash with 50 mM HEPES, 500 mM NaCl pH 7.0, and one wash with thrombin cleavage buffer (20 mM HEPES pH 8, 0.15 M NaCl and 2.5 M CaCl$_2$). Biotinylated thrombin (10 U) was used to release N-term from the GST tag and was removed from the solution by incubation with streptavidin. For purification of DEIH domain, soluble protein lysate was incubated with 2 ml Glutathione Sepharose (GE-healthcare Bio-sciences AB) for 30 min at room temperature. The beads were then washed with 20 ml buffer containing 50 mM HEPES, pH 7.3, 500 mM NaCl, 5 mM DTT, 1 mM EDTA. The DEIH domain was eluted from the beads using buffer containing 50 mM HEPES pH 7.3, 25 mM reduced glutathione, 500 mM NaCl, 5 mM DTT, 1 mM EDTA. Glutathione from the protein preparation was removed by dialysis of protein fractions in the same buffer without glutathione. C-term domain was purified from the soluble protein lysate on Ni Sepharose (GE-healthcare Bio-sciences AB). After one h incubation of the lysate with 2 ml Ni-beads at room temperature, non-specifically bound proteins were removed by extensive washing of the beads with 20 ml buffer containing 50 mM HEPES pH 7.4, 500 mM NaCl, 7.5 mM CHAPS, 20 mM imidazole and 4mM β-mercaptoethanol. C-term was then eluted in 50 mM HEPES pH7.4, 500 mM NaCl, 7.5 mM CHAPS, 500 mM imidazole, 5 mM EDTA, 4 mM β-mercaptoethanol, and dialyzed against the same buffer without imidazole. Protein preparations were evaluated for purity by PAGE and quantified using BioRad DC protein assay.
Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described (36) with recombinant protein and 100,000 cpm of in vitro transcribed α$^{32}$P-labeled RNA. Control experiments to establish equilibrium conditions varied incubation time and temperature and RNA and protein concentrations. in EMSA buffer (2% glycerol, 0.8 mM EGTA, 0.2 mM EDTA, 2 mM Tris pH 7.6, 14 mM KCl, 0.2 mM Mg(OAc)$_2$). Electrophoresis was performed at 4°C in 5% native Tris borate-EDTA acrylamide gels, which were fixed, dried and exposed overnight in a phosphoimager cassette. EMSAs were repeated with at least 3 independent preparations of protein and RNA. For competition experiments, α$^{32}$P-labeled PCE RNA and recombinant protein were combined prior to addition of unlabeled competitor RNA.

Fluorescence anisotropy (FA) measurements

Synthetic RNA oligonucleotides labelled at the 5' nucleotide with fluorescein were purchased from Dharmacon. To fold RNA, 3 µl of 20 µM RNA was incubated in 5.25 µl DEPC-treated water, 3.75 µl 100 mM HEPES pH 7.5 and 1.5 µl 1 M NaCl at 80°C for 2 min, 60°C for 2 min followed by addition of 1.5 µl 100 mM MgCl$_2$. DEPC-treated water was added to a final volume of 300 µL and the mixture was incubated on ice for 30 min. FA measurements were performed in triplicate in Corning 3676 low volume 384-well black nonbinding surface polystyrene plates with 20 nM RNA and indicated amounts of recombinant protein in EMSA buffer. Reactions were incubated for 30 min at room temperature in the dark to allow samples to reach equilibrium. Samples were excited at 485 nm, and the emission intensities at 530 nm from the parallel and perpendicular planes were measured on a Spectramax M5 plate reader system (Molecular Devices) that
measures significant anisotropy changes above a cut-off of 0.04. The equilibrium dissociation constants \((K_d)\) were obtained by fitting the binding curves to a single binding-site model on KaleidaGraph as described previously (197). Weighted averages and standard deviation were calculated as described (198).

**RNA coprecipitation**

HEK 293 cells were grown in Dulbecco’s Modified Eagle Media supplemented with 10% fetal bovine serum and 1% Antibiotic-antimycotic (Gibco). For transfection, \(1 \times 10^6\) HEK 293 cells in 10-cm dish were cultured overnight and 5 µg of the respective plasmid was transfected in duplicate with FuGene6 (3:1 w/v FuGene6: DNA) following manufacturer protocol (Roche) at 37 °C in 5% CO\(_2\) for 48 h. Cells were harvested in PBS, resuspended in 100 µl of polysome buffer (100 mM KCl, 5 mM MgCl\(_2\), 10 mM HEPES pH 7.0, 0.5% NP40, 1 mM DTT) supplemented with RNAs e out (Invitrogen) and protease inhibitor cocktail of serine-, cysteine-, and aspartic-proteases, aminopeptidase (Sigma). Immunoblotting with rabbit anti-FLAG antiserum (Sigma) verified protein expression. The soluble lysate was incubated with anti-FLAG M2 monoclonal antibody-conjugated agarose beads (Sigma) that were equilibrated in NT2 buffer (50 mM tris-HCl pH7.4, 150 mM NaCl, 1 mM MgCl\(_2\), 0.05% NP40) overnight at 4°C. The mRNP complexes were washed with NT2 buffer. An equivalent aliquot of the input lysates and beads (15µl) were immunoblotted with anti-FLAG antiserum to verify similar immunoprecipitation efficiency. RNA was extracted from the beads with Trizol (Invitrogen) following manufacturer’s protocol. As described (36), reverse transcription
(RT) reactions used random hexamer primer and PCR utilized gene-specific primers (KB1303/KB1304 for junD and KB750/KB752 for gapdh (Table 2).

**RHA translation reporter assay**

As described (45;188), 1 × 10^6 HEK 293 cells in duplicate 10-cm dishes were cultured overnight and transfected with 1 µg pYW100 and the indicated RHA expression plasmid. Cells were harvested in PBS from duplicate wells for protein and RNA analysis. Protein was detected by HIV-1 Gag ELISA (Zeptometrix) and immunoblot with FLAG (Stratagene) and RHA (Vaxron) antiserum. RNA was detected by RT-realtime PCR analysis with gene specific primers (KB1614/KB1615 for gag; KB1252/KB1253 for actin) (46) (Table 2).

**Results**

**Specific interaction of N-term with PCE of viral and cellular origin**

Human RHA is composed of three domains that are observed in functionally distinct post-transcriptional regulatory proteins (Figure 2.1A). The helicase domain is necessary for RHA translation activity on PCE target mRNA (36;45;46). However, the domain(s) necessary for RHA recognition of PCE RNAs is unknown. Therefore, to test whether selective interaction is attributable to the N-terminal, DEIH, or C-terminal domain, epitope-tagged recombinant proteins were expressed in *E. coli* and purified by affinity chromatography (Figure 2.1A, 2.1B). EMSAs were used for initial screening of PCE binding activity and then fluorescence anisotropy was employed for quantitative assessment of RNA affinity. EMSAs were carried out with the following ^32^P-labeled transcripts: 165-nt SNV PCE and 138-nt junD PCE; non-functional 162-nt SNV mutAC;
and negative control dsRNAs tRNA$^{\text{Lys}}$ and 7SL RNA. Equilibrium binding conditions were determined by varying incubation temperature and time. As shown in Figure 2.2A, incubation at 30 min or 60 min produces similar binding patterns indicating equilibrium binding by 30 min (199) and subsequent incubations were performed at 30 min.

RNA EMSA detected interaction of N-term with $^{32}$P-labeled junD PCE and SNV PCE, as indicated by the shift in probe mobility and diminished unbound probe (Figure 2.3A and 2.3B). By contrast, N-term did not interact significantly with 76-nt $^{32}$P-labeled tRNA$^{\text{Lys}}$ (Figure 2.3C). The specificity of the N-term-PCE interaction was evaluated by competition with unlabeled SNV PCE and junD PCE. As shown in Figure 2.4, robust interaction of N-term and $^{32}$P-labeled SNV PCE (compare lanes 1 and 2 or 9 and 10) was eliminated by addition of SNV PCE RNA in 10-, 50- and 100-fold excess (lanes 3-5, and 11-13). Moreover, unlabeled junD PCE also competed for N-term interaction with $^{32}$P-labeled SNV PCE (lanes 6-8), indicating N-term has a similar binding affinity for SNV PCE and junD PCE. By contrast, N-term interaction with $^{32}$P-labeled SNV PCE was not competed by tRNA$^{\text{Lys}}$ in 10-, 50- or 100-fold excess (lanes 14-16). The results indicate that N-term exhibits specific interaction with viral and cellular PCEs.

Similar experiments assessed DEIH and C-terminal domains for RNA binding activity. The DEIH domain lacked detectable binding to $^{32}$P-labeled junD PCE or SNV PCE in our assay (Figure 2.5A, 2.5B). C-term exhibited weak interaction with $^{32}$P-labeled junD PCE or SNV PCE that required high protein concentration (10 to 20 µM) (Figure 2.5C, S2D). Moreover non-functional mutant PCE exhibited slightly higher affinity for
C-term (Figure 2.5E). These results indicate that isolated DEIH and C-term RNA binding activity is not selective for PCE RNA.

FA assays using synthetic 5’ fluorescein-tagged SNV PCE RNAs were carried out to verify the RNA binding trends and measure the RNA affinity. Because of the length constraints of chemical RNA synthesis (<100 nt), shorter RNAs were used for these studies. The 96-and 98-nt functionally redundant stem loop structures, PCE$^{AC}$ and PCE$^{AB}$, which are necessary for RHA translation activity (48) and for precipitation of epitope-tagged RHA in cells (36) were chosen for this work. Control dsRNAs were again used to compare RNA binding. The mutAC structural mutant of PCE$^{AC}$, which lacks translation activity and does not coprecipitate RHA in cells, was used to measure the expected reduction in binding affinity. The minihelix$^{Lys}$ 35-nt RNA derived from the acceptor-TΨC stem of human tRNA$^{Lys,3}$ (200) and the human 7SL 27-nt hairpin RNA provided negative control RNAs that lack PCE activity. PAGE confirmed the integrity of the synthetic RNAs (data not shown) and the FA of each RNA (20 nM) was measured with titration of each recombinant domain (25 to 3000 nM). Consistent with the EMSA, N-term bound PCE$^{AB}$ and PCE$^{AC}$ with comparable affinity (Figure. 2.6A, 2.6B, Table 1). In contrast, N-term exhibited lower affinity for mutAC by a factor of 3, indicating the loss of functional activity by mutAC is attributable to weakened, but not eliminated binding ability for N-term. Moreover, N-term and negative control dsRNAs minihelix$^{Lys}$ RNA and mini7SL RNA exhibited anisotropy changes < 0.04, which is below the threshold value for statistically significant interaction. (Figure 2.6C, 2.6D, 2.6E, Table 1). By contrast, C-term exhibited binding to all RNAs examined (Figure 2.7). As expected
from the EMSA, C-term exhibited weak affinity for PCE\textsuperscript{AC} and PCE\textsuperscript{AB} (Table 1). However, slightly increased affinity was observed for mutAC. Moreover, C-term exhibited 4-5-fold higher affinity for minihelix\textsuperscript{Lys} and 7SL negative control dsRNA (Table 1). The results indicate that C-term exhibits greater affinity for control dsRNA than for PCE RNA. These results argue against C-term conferring selective recognition of PCE RNA. Similar analysis of DEIH determined less than detectable affinity for PCE\textsuperscript{AB}, PCE\textsuperscript{AC}, and mutAC by this assay (Figure 2.8, Table 1). In sum, the results of EMSA and FA measurements indicate that N-term, but not DEIH or C-term, is essential for selective recognition of PCE RNA.

**N-term is necessary for RNA binding**

All dsRBDs exhibit the α-β-β-β-α topology and conserved lysine residues (58). The crystal structure of the African clawed frog Xlrbpa dsRBD, a homolog of human PKR, in complex with a 10-nt double-stranded RNA, revealed these basic residues are surface-exposed (201). In RHA, these residues correspond to Lys-54 and Lys-55 in dsRBD1 and to Lys-236 in dsRBD2. To assess their potential roles in PCE interaction, the following mutant proteins were prepared: i) the triple mutant containing K54A, K55A and K236E substitutions (referred hereafter as K54A/55A/236E N-term); ii) the double mutant containing K54A and K55A (designated as K54A/55A N-term); and iii) the single K236E mutant (termed as K236E N-term). The RNA binding affinity of these proteins was assessed by the EMSA and FA measurement.

EMSA results determined K54A/55A/236E N-term produced a severe reduction in interaction with junD PCE or SNV PCE compared to N-term (compare Figure 2.3D,
2.3E and 2.3A, 2.3B). By contrast, K54/55A N-term and K236E N-term exhibited relatively minor reduction in interaction with SNV PCE (compare Figure 2.3F, 2.3G and 2.3B). Similar trends were observed in the FA assays. K54A/55A/236E N-term severely reduced affinity for PCE\textsuperscript{AB} or PCE\textsuperscript{AC} (Figure 2.9A and 2.9B, Table 1), while K54A/55A N-term or K236E N-term exhibited weak interaction (Figure 2.9C, 2.9D, 2.9E, 2.9F, Table 1). The FA measurements (Table 1) determined that K54A/55A in dsRBD1 or K236E in dsRBD2 produced a minor (factor of <2) reduction in binding affinity to PCE\textsuperscript{AB} RNA or PCE\textsuperscript{AC} RNA relative to N-term. However, K54A/55A/236E N-term eliminated detectable interaction with PCE\textsuperscript{AB} or PCE\textsuperscript{AC}. In sum, the results indicate that conserved lysine residues of both dsRBD1 and dsRBD2 contribute to PCE RNA interaction. Mutation of the conserved lysine residues in both dsRBD1 and dsRBD2 are necessary to eliminate interaction with PCE RNA.

**N-term is sufficient for interaction with PCE RNA in cells**

RNA coprecipitation was used to determine whether or not PCE RNA binding activity of N-term is recapitulated in cells. HEK293 cells were transfected with plasmids that express FLAG-tagged N-term or K54A/55A/236E N-term in four independent experiments. Western blot verified equivalent expression of FLAG-tagged N-term and K54A/55A/236E N-term and also that the proteins were immunoprecipitated (Figure 2.10A and 2.10B). Total RNA and RNA isolated from IPs with FLAG antibody were harvested and evaluated by RT-PCR with primers specific for junD and gapdh (Figure 2.10C). As expected, total RNA preparations displayed junD and gapdh transcripts (Figure 2.10C lanes 1,3,5). N-term IP RNA exhibited junD but lacked detectable gapdh
transcript (lane 4), as observed previously for FL-RHA (36). K54A/55A/236E N-term IP RNA exhibited severe reduction in junD (lane 6). The negative control FLAG immunoprecipitate lacked detectable junD RNA (lane 2). No amplification products were detected in reactions that lacked reverse transcriptase (RT-) (lanes 7-12). The results demonstrate that the N-term is sufficient for interaction with PCE RNA in cells and that the interaction is severely reduced by the lysine mutations.

**N-term dominantly interferes with translation of PCE reporter RNA**

Because N-term binds to PCE RNA in 293 cells (Figure 2.10), we hypothesized that it could inhibit translation of PCE target RNA by endogenous RHA. To test this, HEK 293 cells were transfected with PCE reporter plasmid and increasing amounts of plasmids that express FLAG-tagged N-term or K54A/55A/236E N-term. Western blot with FLAG antiserum verified comparable expressions of N-term and K54A/55A/236E N-term; control anti-tubulin anti-serum verified equivalent protein loading (Figure 2.11A, 2.11B). To determine PCE activity HIV-1 Gag protein expression was measured by ELISA and gag RNA levels were determined by quantitative RT-PCR (45). The results of three independent transfection experiments indicated that PCE activity was decreased in response to N-term (p <0.03) (Figure 2.11A). By contrast, PCE activity is not affected by K54A/55A/236E N-term (Figure 2.11B). In sum, the RNA immunoprecipitation and translation assay results demonstrate that PCE binding activity of N-term interferes with translation activity of endogenous RHA, presumably by blocking productive interaction with endogenous RHA.
Discussion

We have demonstrated that the N-term domain of RHA contributes to selective recognition of viral and cellular PCE RNA. Exogenously expressed N-term interacts with PCE target RNA and requires the conserved lysine residues within RHA dsRBD1 and dsRBD2. We observed that PCE interaction with exogenously expressed N-term blocked RHA translation activity. Conversely, mutations of its conserved lysine residues eliminated interaction with PCE RNA in cells and did not block translation of PCE RNA. Our results indicate that the N-term basic residues of RHA are necessary to engage the RNA structure and deliver the ATP-dependent helicase activity that is necessary for translation of PCE mRNA (46). This N-term interaction is selective for functional PCE because the binding activity of the nonfunctional PCE (mutAC) is reduced by a factor of 2-3, and binding to negative control dsRNA is not detectable.

The conserved lysine residues are positioned in the second conserved α-helix of the dsRBD α-β-β-β-α topology. The crystal structure of the Xlrβpa dsRBD-RNA complex determined the second highly conserved α-helix positions the lysine residues for interaction with the RNA backbone (201). In RHA dsRBD1, PKR and TAR RNA binding protein (TRBP), the amino acid conservation of this α-helix is 65%, whereas conservation of the β-sheets is <20%. We postulate that the divergent residues of the β-sheets provide conformational or electrostatic interactions that are unique among the dsRBDs and facilitate their affinity for a particular category of target RNA. The position of the β-sheets on top of the α-helices directs solvent-exposed side chains away from the
RNA. These residues are strong candidates for interaction with important protein cofactors.

Our results document that, despite the wide conservation in RNA binding proteins, the dsRBD are necessary for interaction with functionally distinct populations of substrate RNAs. Our results are similar in principle to the selective interaction of the budding yeast Rnt1p dsRBD with an AGNN tetraloop that was directed by the first α-helix of the dsRBD (202;203). *Drosophila* Staufen dsRBDIII contacts the stem loop of dsRNA at the first α-helix (204). Thus, the conserved α-β-β-β-α topology is essential for contacting the target mRNA but is malleable to accommodate variable residues that generate the sequence or structure-specific recognition.

In this study, we observed that mutations of the conserved lysine residues introduced separately in dsRBD1 or dsRBD2 reduced, but did not eliminate affinity for PCE RNA. By contrast, mutation of either dsRBD was sufficient to eliminate RHA translation activity on retrovirus PCE RNA (data not shown). Possible explanations are as follows. 1) PCE RNA binding of the lysine residues of both dsRBD1 and dsRBD2 is necessary to reshape the conformation of the β-sheets to facilitate interaction with protein co-factor(s). This scenario is similar in principle to augmentation of eIF4A activity by interaction with eIF4B during cap-dependent translation initiation (205). 2) Alternatively, an initial interaction with protein cofactor(s) is necessary to rearrange the lysine residues for productive interaction with PCE RNA. Notably, the results of this and previous studies (36;46) determined direct binding of RHA to PCE. However, the possibility
remains that cofactor interaction refines intramolecular RNA structure to enable productive ribosome scanning of PCE mRNA.

We have shown that the dsRBDs play a predominant role in the selectivity of the RHA-PCE interaction. Our EMSA and FA measurements failed to detect RNA binding activity for the DEIH domain to PCE RNA, and revealed a low, nonspecific RNA binding affinity for C-term. Additionally, co-incubation with C-term did not enhance or inhibit the binding of N-term to the PCE RNA (data not shown). An important future direction is to assess the possible role of these domains in the context of the full length RHA. In the cellular environment, similar or increased RNA affinity may be conferred by domain interactions that produce conformational changes or cofactor interaction. In the case of heterogeneous nuclear ribonucleoprotein A1, the C-terminal RGG-rich domain interacts with itself and other hnRNP proteins to influence activity (192). Likewise the RGG-rich domain of nucleolin is required for interaction with ribosomal protein L3 (191;193). Similar to RHA, PKR possesses two amino-terminal dsRBD, and they interact with the distal kinase domain (206). For PKR, the engagement of generic dsRNA by dsRBD reveals the kinase domain in an active conformation (206). By analogy, our results indicate selective engagement of PCE RNA is necessary for RHA translation activity. The possibility remains that the amino-terminal dsRBD(s) interact with distal RHA domains; future experiments to test this model are warranted. The results will contribute to understanding the essential role of RHA in normal cells and the apparent contribution of RHA dysfunction to neoplastic growth (207-209). The outcomes will fuel
fundamental understanding of RNA helicases across cell biology and at the virus-host interface.
Figure 2.1. RHA domain structure and isolation of recombinant N-term, C-term and DEIH polypeptides. (A) Depiction of the 1269 amino acid RHA and amino acid coordinates of recombinant domains evaluated in this study. (B) SDS-PAGE analysis of recombinant protein preparations visualized by coomassie staining.
Figure 2.2. Testing incubation time and temperature. Representative results of two electrophoretic mobility shift assays demonstrate that (A) there is no observable difference in binding of N-term to $^{32}$P-SNV PCE at 30 minutes (lanes 1-7) versus 60 minutes (lanes 8-14) indicating that the sample has come close to reaching equilibrium (B) there is not a marked difference in binding of N-term to $^{32}$P-SNV PCE on ice (lanes 1-7) versus room temp (lanes 8-14).
Figure 2.2. Testing incubation time and temperature.
Figure 2.3. The conserved lysines in dsRBD1 and dsRBD2 are essential for effective binding of N-term with PCE RNA. Electrophoretic mobility shift assays (EMSAs) of indicated recombinant protein and $^{32}\text{P}$-labeled RNA. (A) Wild type N-term and $^{32}\text{P}$-junD PCE. (B) Wild type N-term and $^{32}\text{P}$-SNV PCE. (C) Wild type N-term and $^{32}\text{P}$- tRNA$\text{Lys}$. (D) K54A/55A/236E N-term and $^{32}\text{P}$- junD PCE (E) K54A/55A/236E N-term and $^{32}\text{P}$-SNV PCE. F) K54A/55A N-term and $^{32}\text{P}$-SNV PCE. (G) K236E N-term and $^{32}\text{P}$-SNV PCE.
Figure 2.3. The conserved lysines in dsRBD1 and dsRBD2 are essential for effective binding of N-term with PCE RNA
Figure 2.4. N-term exhibits specific interaction with PCE RNA of viral and cellular origin. Electrophoretic mobility shift assay of N-term and $^{32}$P-labeled SNV PCE RNA (0.02 μM) upon addition of unlabeled SNV PCE (lanes 3-5 and 11-13) or junD PCE (lanes 6-8), or tRNA$^{Lys}$ (lanes 14-16). The concentrations of competitor RNA were 0.2; 1; 2 μM, respectively. The protein concentration was 1 μM and shifts all $^{32}$P-SNV PCE RNA.
Figure 2.5. RHA C-term and DEIH exhibit weak and undetectable interaction with PCE RNA, respectively. Representative results of 3 or more electrophoretic mobility shift assays with indicated recombinant protein and 32P-labeled RNA. (A) DEIH and 32P-junD PCE. (B) DEIH and 32P-SNV PCE (C) C-term and 32P-junD PCE (D) C-term and 32P-SNV PCE (E) C-term and 32P-mutant SNV PCE.
Figure 2.6. N-term binding to PCE is recapitulated and quantified by FA measurements. Representative FA assays to measure binding affinity of N-term to indicated RNA. (A) PCE\textsubscript{AB}. (B) PCE\textsubscript{AC}. (C) mutAC (D) minihelix\textsuperscript{Lys}. (E) 7SL RNA. Anisotropy changes greater than 0.04 are statistically significant.
Figure 2.7. C-term lacks specificity for PCE RNA. Representative FA assays to measure binding affinity of C-term to indicated RNA. (A) PCE$^{\text{AB}}$. (B) PCE$^{\text{AC}}$. (C) mutAC (D) minihelix$^{\text{Lys}}$. (E) 7SL RNA.
Figure 2.8. Mutation of conserved lysine residues eliminates affinity of N-term for PCE RNA. Representative FA assays to measure binding affinity of indicated N-term mutant protein and RNA. (A) K54A/55A/236E N-term and PCE\textsuperscript{AB}. (B) K54A/55A/236E N-term and PCE\textsuperscript{AC}. (C) K54A/55A N-term and PCE\textsuperscript{AB}. (D) K54A/55A N-term and PCE\textsuperscript{AC}. (E) K236E N-term and PCE\textsuperscript{AB}. (F) K236E N-term and PCE\textsuperscript{AC}.
Figure 2.9. DEIH domain lacks detectable binding to PCE RNA. Representative FA assays with DEIH and indicated RNA. (A) PCE^{AB}. (B) PCE^{AC}. (C) mutAC.
Figure 2.10. Mutation of conserved lysine residues eliminates affinity of N-term for PCE RNA. Representative immunoprecipitation (IP) assay to compare PCE RNA coprecipitation with N-term proteins. Empty FLAG plasmid, N-term (FLAG tagged), or K54A/55A/236E N-term (FLAG-tagged) were expressed in transfected HEK 293 cells and FL-tagged proteins were subject to IP with FLAG antibody (mouse) conjugated to agarose beads. Immunoblot of equivalent aliquots of input lysate or immunoprecipitate on agarose beads determined the IP efficiency was similar for each N-term protein. RNA was harvested from the remaining beads and subjected to RT-PCR with junD or gapdh primers. (A) Western blot with Flag-specific antiserum (rabbit) detected equivalent expression of N-term and K54A/55A/236E N-term. The 9 amino acid FLAG peptide was not resolved. (B) Western blot with Flag-specific antiserum (rabbit) detected IP was similar for N-term and K54A/55A/236E N-term. (C) PCR amplification products from indicated RNA preparation incubated with or without reverse transcriptase (RT+ or -). Representative samples of total RNA and RNA isolated from IP samples were evaluated with junD- or GAPDH-specific primers. The junD amplification product is 232 bp and the lower band is primer dimer.
Figure 2.10. Mutation of conserved lysine residues eliminates affinity of N-term for PCE RNA.
Figure 2.11. Exogenously expressed N-term dominantly inhibits translation of PCE reporter RNA. HEK293 cells were transfected with pYW100 SNV PCE reporter plasmid and increasing amounts of indicated N-term expression plasmid. Total cell protein was subjected to Gag ELISA and Western blot with FLAG and Tubulin antiserum. Total cellular RNA was subjected to RT-real-time PCR and control reactions lacked RT. PCE reporter activity was determined in three independent assays summarized graphically as Gag protein relative to gag RNA standardized to actin. Error bars indicate standard deviation. Asterisks indicate statistically significant difference (p= < 0.03). (A) PCE activity is inhibited by FL-N-term (B) PCE activity is not changed by FL-K54A/55A/236E N-term.
Table 2.1. Apparent equilibrium dissociation constants for binding of recombinant RHA domains to 5' fluorescein-labelled RNAs

FA measurements were carried out as described in Experimental Procedures. Values represent the average of three or more independent experiments and standard deviations are indicated.

<table>
<thead>
<tr>
<th>RNA</th>
<th>N-term</th>
<th>C-term</th>
<th>DEIH</th>
<th>K54A/55A/236E N-term</th>
<th>K54A/55A N-term</th>
<th>K236E N-term</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCE&lt;sub&gt;AB&lt;/sub&gt;</td>
<td>284 ± 33</td>
<td>1170 ± 92</td>
<td>&lt;MD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>414 ± 47</td>
<td>437 ± 53</td>
<td></td>
</tr>
<tr>
<td>PCE&lt;sub&gt;AC&lt;/sub&gt;</td>
<td>348 ± 50</td>
<td>1590 ± 170</td>
<td>&lt;MD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>515 ± 85</td>
<td>721 ± 120</td>
<td></td>
</tr>
<tr>
<td>mutAC</td>
<td>829 ± 160</td>
<td>993 ± 190</td>
<td>&lt;MD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>minihelix&lt;sub&gt;Lys&lt;/sub&gt;</td>
<td>&lt;MD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>222 ± 37</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>7SL</td>
<td>&lt;MD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>305 ± 48</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>b</sup> <MD, less than minimum detectable.

<sup>c</sup> ND, not determined.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB588</td>
<td>GTACTACGGATTCAGTCCGG</td>
</tr>
<tr>
<td>KB 664</td>
<td>AAATCTAGACGATAGAATTCTAATACGACTCACTATAGGGGTGCCGTCCTACACATTTGTTT</td>
</tr>
<tr>
<td>KB 1352</td>
<td>AGACGATAGAATTCTAATACGACTCACTATAGGGGTGCG</td>
</tr>
<tr>
<td>KB 1702</td>
<td>AAGGTAATACGACTCAGTATAGGGAGGAGCCGGCCGCCG</td>
</tr>
<tr>
<td>KB 1109</td>
<td>TTTTCGCAATTCCCATGCGTGATTTAAAAATTTTCTG</td>
</tr>
<tr>
<td>KB 1110</td>
<td>AAAAAAGGAATTCCGAGGCAAAATCTCAAAGATTAGC</td>
</tr>
<tr>
<td>KB 1674</td>
<td>GGGAATTTCCACCAATTCAGCAGATGCACAAGAC</td>
</tr>
<tr>
<td>KB 1675</td>
<td>GCTTTGTGCTCTGCTGTCAATTGGTGAATTTCCC</td>
</tr>
<tr>
<td>KB 1440</td>
<td>GGATCAAATAAGGAATTGCGACACAGTCC</td>
</tr>
<tr>
<td>KB 1441</td>
<td>GGACTGTCGCTGCGAAATCTCTATGATCC</td>
</tr>
<tr>
<td>KB 1733</td>
<td>AAGGATCCGGGAGCTACTGAGATGTGGGC</td>
</tr>
<tr>
<td>KB 1734</td>
<td>AAGAATTCTCAGTCAGCCGACGCCTCGCCTTTCCC</td>
</tr>
<tr>
<td>KB 1661</td>
<td>CTTAGGATCTCAGTTTGGAATTCCTATTTATAGCGG</td>
</tr>
<tr>
<td>KB 1662</td>
<td>CTCTCCTCT</td>
</tr>
<tr>
<td>KB 1663</td>
<td>CGTATCCAGCCATATGCATCATCATCATCATCATCATCAT</td>
</tr>
<tr>
<td>KB 1377</td>
<td>CCGAGGCTATAAGAGGCGC</td>
</tr>
<tr>
<td>KB 1378</td>
<td>CCTCCCGCTCCCCGCGCG</td>
</tr>
<tr>
<td>KB 1303</td>
<td>GTCGCCCAATCGACATGGACAGC</td>
</tr>
<tr>
<td>KB 1304</td>
<td>GCCGCCTGTGTGAGGCTGAGG</td>
</tr>
<tr>
<td>KB 750</td>
<td>CTTTGGATCGTGAGGACTC</td>
</tr>
<tr>
<td>KB 752</td>
<td>GTTGCTGTAGCCAAATTCGTTG</td>
</tr>
<tr>
<td>KB 1252</td>
<td>TCACCCACACTGTCGCCCATCTACGA</td>
</tr>
<tr>
<td>KB 1253</td>
<td>CAGCGGAACCAGCTCATTGCAATGG</td>
</tr>
<tr>
<td>KB 1614</td>
<td>GTAAGAAAAAGGCACAGCAAGCAG</td>
</tr>
<tr>
<td>KB 1615</td>
<td>CATTGCCCTTGAGGTTCTG</td>
</tr>
</tbody>
</table>

**Table 2.2. Sequence of DNA oligonucleotides used in experimental procedures**
Chapter 3. Distinct residues of RNA helicase A modulate initiation and post-initiation stages of translation of target RNAs

Abstract

RNA helicase A is required for translation of select retroviral and oncogene RNAs. Features of the N-terminal domain of RHA are required for specific recognition and translation of RHA target RNAs. Herein, we investigate residues of RHA that are necessary for translation of RHA target RNAs. Sucrose density gradient analysis, functional assays, sub-cellular localization studies and RNA co-immunoprecipitation studies were used to understand features of the mechanism of the PCE-RHA translational axis. RHA utilizes its N- and C-terminal RNA binding domains as well as ATPase activity to specifically bind to PCE-containing mRNA and facilitates polysome loading. Ribosomal profile analysis indicated that RHA is required for two stages in translation of mRNAs. N-terminal and catalytic amino acids affect a pre-initiation stage of translation and mutation of these amino acids results in sequestration of RHA in stress granules. C-terminal residues affect a post-initiation stage of translation. C-terminal residues are also required for recruitment of RHA to stress granules under conditions of oxidative stress. This study identified RHA functional domains necessary for selected translation of PCE RNAs.
Introduction

The complex untranslated regions of oncogene and retroviral mRNAs are highly structured and require helicase activity to facilitate their remodeling to initiate translation. Mammalian eIF4A is a non-specific helicase that couples RNA unwinding with ribosome movement (210;211). eIF4A acts in concert with eIF4B and eIF4G to unwind RNA secondary structure distal to the 5' 7-methyl guanosine cap structure and is necessary for activity of the 43S translation initiation complex (212;213). Despite the activity of eIF4A, RNA secondary structures of $\Delta G < -60$ kcal/mol impede scanning ribosomes and reduce protein production (214;215). In this case, eIF4A may require supplementation by another helicase to facilitate translation. The DEIH box protein RNA helicase A was identified to bind to oncogene and retroviral mRNA by proteomic screening and is necessary for translation of these RNAs (36). Two \(\alpha\beta\beta\alpha\) domains at the N-terminus of RHA are necessary for specific interaction of RHA with these RNAs (136). We postulate that RHA is a candidate supplement to eIF4A activity.

RNA helicase A (RHA, also known as DHX9) is a multifunctional nucleocytoplasmic shuttle protein (216-218). Dysregulation of RHA has been linked to several cancers (209;219;220), and affects retrovirus gene expression and reverse transcription (221-226). Consistent with this disease association, RHA is necessary for translation of select cellular growth control genes including junD, and avian, simian and human species retroviruses including human T cell lymphotropic virus type 1 (HTLV-1) and HIV-1 (46;223;227). The RHA RNA response element is a 5' terminal triple stem-loop structure termed the post-transcriptional control element (PCE). RHA interacts with the complex
structure of PCE in both the nucleus and cytoplasm, and this RNA-protein interaction comprises a positive RNA switch necessary for efficient translation (228).

Mutation of conserved lysine residues in the N-terminal domain of RHA (K54, K55 and K236) destroys binding of the isolated N-terminal domain to RNA both \textit{in vitro} and \textit{ex vivo} (136). Further, mutation of these residues eliminates the squelching effect of increasing amounts of N-terminal RHA on endogenous RHA activity (136). The RG (arginine and glycine) rich domain at the C-terminus of RHA is conserved amongst certain other RNA binding proteins such as fragile X mental retardation protein (FMRP) and hnRNPA1. The arginine residues in this domain are substrates for methylation by PRMT1, which affects the cellular localization of RHA (Bedford, smith 2004). The N-terminal and RG rich domains flank the central DEIH helicase core that is conserved amongst several other RNA helicases like DDX3, DDX1 and p68 (226). The ATP binding residue in this helicase core (K417) is required for translation of HIV RNA that contains a PCE in the 5' UTR (46;62).

Analysis of the avian spleen necrosis virus (SNV) PCE identified nuclear education as a pre-requisite for translational enhancement (229); whether or not this is due to nuclear interaction with RHA remains an open issue. RHA depletion is associated with reduced polysome association of PCE-containing RNA independent of a change in cytoplasmic accumulation or RNA abundance (223;230). A working model is that RHA is recruited to PCE RNA and arranges RNA-protein interactions to efficiently engage the protein synthesis machinery. We expect that tight regulation of the translational activity of RHA in the cytoplasm is crucial for appropriate expression of PCE-containing viral
and cellular genes, which in turn control appropriate cell and virus growth. The RHA-PCE interaction appears to be mechanistically distinct from DEVH protein DHX29, which associates with 40S ribosomes and is expected to remodel 43S ribosomal complexes to facilitate entry of mRNA with complex secondary structure into the mRNA-binding cleft (231). Furthermore, DHX29 is predominately localized with 40S ribosomes in sucrose density gradients and not with polysomes (232). RHA is predominately polysome associated and its downregulation results in decrease in polysome loading of RHA target RNA (233). This difference suggests that DHX29 acts early in initiation, while RHA is required for pre- and post-initiation events.

Translational control of mRNAs involves 3 main stages: initiation, elongation, and termination (10). Translation initiation is highly regulated and is the rate limiting step in translation. Initiation involves several factors including eIF1, eIF1A, eIF2, eIF3 and the eIF4F complex comprising eIF4G, eIF4A and eIF4E which are required for UTR remodeling and assembly of 80S ribosomes at the AUG start codon of translation (11). Translation elongation is catalyzed by 3 elongation factors: eEF1A, eEF1B and eEF2, that are required for ribosome transit on the mRNA (234). eIF1A-GTP is required for binding of the amino-acyl tRNA to the ribosome, and upon recognition of the mRNA codon by the tRNA anti-codon, GTP is hydrolyzed and eEF1A is released. eEF1B is required to catalyze the exchange of GDP bound to eEF1A for GTP. eEF2 catalyzes translocation of the ribosome along the mRNA and allows elongation of the synthesized polypeptide (14). Termination of translation occurs when the ribosome detects a stop codon, and requires eukaryotic release factors eRF1 and eRF3 (16). eRF1, eRF3, and
GTP form a ternary complex that interacts with the ribosome to initiate translation termination.

Drugs that inhibit translation initiation by targeting the helicase eIF4A such as hippuristanol and pateamine A induce stress granule formation (26-28). Stalled initiation complexes or the presence of free 48S complexes gives rise to stress granules (23). We hypothesize that inhibiting the initiation activity of RHA would lead to stress granule formation in a similar manner. Stress granules are assembled in cells subject to environmental stresses such as heat shock, oxidative stress and viral infection, and lead to translational arrest and rapid polysome disassembly (18;19;21). They are translationally inert cytoplasmic centers of molecular triage where mRNAs are stored until their fate is determined (20;21;21). The composition of stress granules is dynamic and consists of a large number of RNA-binding proteins and RNAs (18;21;235). Stabilizing elongation complexes using cycloheximide results in inhibition of stress granules, whereas, promotion of ribosome disassembly using puromycin promotes stress granule formation (20;24).

The objective of this study was to determine the residues of RHA that are necessary for translation of RHA target RNAs. The results indicate that RHA requires its N-terminal RNA binding residues and the ATP-binding residue to efficiently associate with polysomes, and therefore for translation initiation of target RNAs. The C-terminal residues are required for a post-initiation event in translation, and are also therefore, required for translation of target RNAs. We propose that RHA utilizes its N- and C-
terminal RNA binding domains and ATPase activity to specifically interact with and remodel PCE-containing mRNAs and facilitate their translation.

**Materials and methods**

**Plasmid construction**

All primer sequences are described in Table 4. RHA siRNA-resistant Fl-RHA (WT) was generated by two rounds of site-direct mutagenesis (SDM) on pcDNA3.1-Fl-RHA (C.G Lee, University of Dentistry and Medicine of New Jersey) using primers KB1218 with KB1219 and KB1216 with KB1217 to create silent mutations. To create ∆dsRBDI&II, SDM was performed on WT using primers KB1088 and KB1089 to delete both dsRBDs. ∆dsRBDI was created with primers KB1676 and KB1677 on WT. ∆RG was created with primers KB1658 and KB1659 on WT to delete amino acids 1168-1269. K236E was created with primers KB1440 and KB1441. K54A/K55A was created with primers KB1676 and KB1677. K417R was created with primers KB1073 and KB1074. K417A was created with primers KB1678 and KB1679. mNLS was created with primers KB1077 and KB1078 to delete amino acids K1162 and R 1165. Five rounds of SDM were performed to create RA9 using primers KB1393 with KB1394, KB1395 with KB1396, KB1418 with KB1413, KB1414 with KB1415, and KB1416 with KB1417. All mutations were verified by DNA sequencing (The Ohio State University Plant Microbe Genomics sequencing facility, Columbus, OH). HTLV-1 proviral clone ACHneo was previously described (236).
**Transfection and cell culture**

Cos7 cells were grown in Dulbecco’s Modified Eagle Media (Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic (Gibco) prior to transfection. For PCE functional assays, Cos7 cells were transfected with siRNAs and subsequently with Fl-RHA mutants and HTLV-1 proviral plasmid. For siRNA transfection, cells were seeded in 10cm dishes at $1 \times 10^6$ cells/dish. After ~24 h, cells were incubated with a mixture of Oligofectamine (Invitrogen, to a final concentration of 85.7 nM) and 50 µl of 20 µM siRNAs (Dharmacon) targeted to two regions of RHA or scrambled sequences in OptiMEM media (Gibco) without serum. FBS was added 4 h post-treatment and cells were incubated at 37°C in 5% CO$_2$. After 24 h, the siRNA-treated cells were split into 6 well plates at $2 \times 10^5$ cells/well and incubated at 37°C in 5% CO$_2$ for 24 h. The siRNA treatment was repeated to sustain downregulation of endogenous RHA, using 5 µl of scrambled siRNAs or the RHA target siRNAs (2.5 µl of each) per well. After addition of serum (10% final volume), duplicate wells of cells were transfected with 1.5 µg Flag-tagged RHA plasmids plus 0.5 µg HTLV-1 ACH$^{\text{neo}}$ molecular clone plasmid using FuGENE 6 at a ratio of 3:1 FuGENE 6:DNA (w/v) following manufacturer’s protocol (Roche) and incubated at 37°C in 5% CO$_2$ for 48 h. Upon harvesting, media was combined from duplicate wells and used for p19 Gag enzyme-linked immunosorbent assay (ELISA) (Zeptometrix). Adherent cells were rinsed with PBS and duplicate wells combined then separated into two eppendorf tubes for subsequent RNA and protein isolation as described below.
For immunofluorescence assays, Cos7 cells were seeded on glass coverslips in 6 well plates at 1 × 10^5 cells/well. After 24 h, cells were transfected with 1 µg Fl-RHA plasmid (WT or mutant) using FuGENE 6 as described above. Assays for stress granule or P body colocalization required co-transfection of 500 ng GFP-G3BP1, 500 ng TIA-YFP or 500 ng Dcp1a-RFP (GFP-G3BP1, YFP-TIA-1 and RFP-Dcp1a obtained from N. Kedersha). To perform MTS assay, Cos7 cells were plated in triplicate in 6 well plates at a density of 2 × 10^5 cells/well. After 24 h, cells were transfected with 1.5 µg of indicated Fl-RHA plasmid using FuGene 6 as above, then incubated at 37°C in 5% CO_2 for 24 h. Cells were then split into 3 wells/sample of a 96 well plate and 3 replicate plates at a density of 10,000 cells/well in a volume of 200 µl complete DMEM to be used for readings on that day and readings on day 2 and day 3. Before each time point reading, 40 µl of Cell titer 96 AQueous non-radioactive cell proliferation reagent (Promega) was added to each well, incubated for 3 h at 37°C in 5% CO_2, then 490 nM OD measurement taken. 

**Ribosomal profile analysis**

Cultures of HEK 293 cells were grown in Dulbecco’s Modified Eagle Media (Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic (Gibco) prior to transfection. Cells were seeded at 5 × 10^6 cells/dish in 2 150 mm dishes. After 24 h, cells were transfected with 15 µg of Fl-RHA plasmid (WT or mutant) using FuGENE 6 as above or 10 µg of Fl-RHA plasmid (WT or mutant) by CaPO_4 method. CaPO_4 transfection was carried out as follows: for each 150 mm plate of cells, 10 µg plasmid was diluted in 750ul water plus 250ul 1M CaCl_2, vortexed briefly, followed by addition of 1 ml 2X HEPES buffered saline then incubation at room temperature. After 20 min, preparations were
added to cells and incubated at 37°C in 5% CO₂ for 5 h. Cells were washed twice with PBS then 20 ml of DMEM with 10% FBS (Gibco) and 1% antibiotic (Gibco) was added. Cells were incubated at 37°C in 5% CO₂ for 48 h. Prior to harvesting, cycloheximide was added to the cell media and incubated for 20 min at 37°C in 5% CO₂ at a final concentration of 0.1µg/mL. For samples treated with puromycin, puromycin (Sigma) was added to the cell media at a final concentration of 400 µM and incubated for 40 min prior to addition of cycloheximide for a total incubation time of 1 hr. Cells were then rinsed and collected in PBS, pelleted by centrifugation at 1540 rpm (Forma Scientific centrifuge model 5682) for 4 min, and lysed in 450 µl gradient buffer (10 mM HEPES, 10 mM NaCl, 3 mM CaCl₂, 7 mM MgCl₂) plus 50 µl 5% NP40 solution, 1 µl 1M DTT, and 80 U RNaseOUT (Invitrogen) as previously described (237). For samples treated with RNase A, 0.5 µg/mL RNase A was added to both the PBS used to pellet cells and also to the cell lysate. All lysates were separated on a 15-47.5% sucrose gradient by centrifugation at 36,000 rpm (Beckman L-80 ultracentrifuge, SW41 rotor) for 2.25 h at 4°C. Gradients were fractionated and A₃₅₄ absorbance detected on an ISCO Foxy Jr. system. Proteins were isolated from the fractions by TCA precipitation followed by 3 acetone washes and dried in a speed vacuum. Pellets were resuspended in 35 µl water and 35 µl 2× SDS loading dye, boiled for 5 min and subjected to SDS-PAGE and western blotting as described below.

For testing the elongation defect the sucrose density gradient analysis was performed as described with certain modifications. Cells were seeded at 3 × 10⁶ cells/dish in 2 150mm dishes. After 24 h, cells were transfected with 15 µg of Fl-RHA plasmid (mNLS RHA or
RA9/mNLS) with FuGENE 6 (4:1 w/v FuGENE 6: DNA) following manufacturer protocol (Roche) at 37 °C in 5% CO₂ for 48hrs. Prior to harvest cycloheximide was added to the cell media at a final concentration of 0.1 µg/mL for CHX+ samples, but not for CHX- samples and incubated for 20 min at 37°C in 5% CO₂. Immunoblotting was performed with antibodies to rabbit FLAG (Sigma) or mouse monoclonal α- tubulin (Santa Cruz).

**Protein analysis**

Cells collected in PBS were pelleted by centrifugation at 1100 g for 3 min, resuspended in 1% NP40 or RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA), iced for 10 min, then centrifuged at 13,400 g for 10 min at 4°C to collect proteins. For western blot analysis, proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (BioRad). Immunoblotting was performed with rabbit polyclonal anti-RHA (Vaxron), mouse monoclonal anti-β-actin (AbCam), mouse anti-Tubulin (Santa Cruz), mouse anti-Flag (Stratagene) or rabbit anti-FLAG (Sigma) antibody. HRP-linked secondary antibodies were from Amersham and visualization was performed with ECL+ Western blotting detection reagent (Amersham) and imaged on a Fuji LAS4000.

**RNA isolation and quantification**

Cells collected in PBS were pelleted by centrifugation at 1100 g for 3 min then resuspended in Trizol reagent (Invitrogen) following manufacturer’s protocol. Total cellular RNA was subjected to DNase treatment (Promega), extraction with acid phenol
(Ambion) followed by precipitation with ethanol and resuspension in diethyl procarbonate-treated water. Reverse transcription was performed on 200 µg total RNA using Omniscript reverse transcriptase (Qiagen) and random hexamer primer for 1 h at 37°C. Ten percent of the cDNA preparation was used for real-time PCR with primers complementary to HTLV-1 gag or β-actin and Lightcycler 480 SYBR green master mix (Roche) in a Lightcycler480 (Roche). Standard curves to determine mRNA copy numbers were prepared with control reactions using HTLV-1 ACH\textsuperscript{neo} molecular clone plasmid or β-actin plasmid in the range of $10^3$–$10^7$.

**Immunofluorescence and microscopy**

For adox treatment a stock solution of 500 µM adenosine periodate (Sigma) in DMEM was made and added dropwise onto cells to a final concentration of 50 µM 24 hours prior to harvesting. For arsenite treatment 0.1 M Sodium arsenite (Ricca chemical company) was added dropwise to cells to a final conc of 1 mM 30 min prior to harvesting the cells. Where indicated, a final concentration of 2 µM hippuristanol (kind gift from Dr. Jerry Pelletier) was added to the cells for 30 minutes prior to fixing. Where indicated, cells were treated with 50 ng/ml cyclohexamide (Sigma) and incubated at 37°C in 5% CO$_2$ for 1 h prior to fixation. Twenty-four to forty-eight hours post transfection, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X100, then blocked in 10% normal goat serum (Vector Labs) for 45 min at room temperature. Cells were washed 3 × 5min in PBS then incubated in primary antibody solution (1:200 mouse Flag antisera from Stratagene, 1:200 rabbit FLAG antisera from Sigma, 1:100 mouse anti-PABP antibody from Abcam) prepared in normal antibody dilution buffer (Scytek) for 2
RNA coprecipitation

HEK 293 cells were grown in Dulbecco’s Modified Eagle Media supplemented with 10% FBS and 1% antibiotic (Gibco). For transfection, 1 × 10^6 HEK 293 cells in 10 cm dishes were cultured overnight and 5 µg of the respective plasmid was transfected in duplicate with FuGENE 6 (4:1 w/v FuGENE 6: DNA) following manufacturer protocol (Roche) at 37 °C in 5% CO_2 for 48 h. Cells were harvested in PBS, resuspended in 100 µl of polysome lysis buffer (100 mM KCl, 5 mM MgCl_2, 10 mM HEPES (pH 7.0), 0.5% NP40, 1 mM DTT) supplemented with RNAase out (Invitrogen) and protease inhibitors (Sigma). The soluble lysate was incubated with anti-FLAG M2 monoclonal antibody-conjugated agarose beads (Sigma) equilibrated in NT2 buffer (50 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM MgCl_2, 0.05% NP40) overnight at 4°C. The mRNP complexes were washed with NT2 buffer and RNA was extracted with Trizol reagent (Invitrogen) following manufacturer’s protocol. As described (Hartman 2006) reverse transcription
(RT) reactions used random hexamer and PCR utilized junD gene-specific primers KB1303 and KB1304.

**Statistical analysis**

For PCE assays mean fold changes were log-transformed and analyzed by a one-sample t-test to test whether the log-transformed values were different from the baseline controls as indicated in Table 1 and Table 2. Holm’s step down procedure was used to calculate p-values. For confocal experiments one-sided t-tests were performed to assess statistically significant differences. Dunnet’s method was used and p <0.01 was considered statistically different from the control group.

**Results**

We have previously shown that RHA selectively recognizes a complex 5’ UTR structure entitled the post-transcriptional control element (PCE) resulting in robust protein production (238;239). RHA has the following domain structure: two N-terminal αβββα domains that are conserved within multiple key proteins including PKR, Dicer, and ADAR; a central Walker helicase core, and a multi-functional C-terminal RG-rich domain (Figure 3.1) (240). Here, we sought to identify the residues of RHA that are necessary for the translation of PCE RNAs. Flag-tagged RHA expression plasmids that contain deletion and substitution mutations were generated to evaluate the role of conserved RHA domains in facilitating translation of highly structured PCE RNA (Figure 3.1). Mutations were based on previous studies that characterized important roles for these residues in RNA binding, polyribosome association, ATP binding, nuclear localization and PRMT-1 interaction (46;61;62;123;136). Mutants were analyzed by
sucrose density gradients, functional assays, confocal microscopy to determine sub-cellular localization and RNA co-immunoprecipitation assays.

**Conserved N-terminal amino acids are required for RHA polysome association and PCE translation**

Prior studies have indicated that RHA is polysome associated and facilitates polysome association of PCE RNAs (36). Furthermore, features of the two N-terminal conserved domains are necessary for selective recognition of PCE RNA. Mutation of conserved lysine residues in these domains destroys binding of the isolated N-terminal domain to PCE RNA (136). Based on these observations, we hypothesized that disruption of the N-terminal domains of RHA would reduce polysome association of full length RHA and would therefore interfere with PCE translation.

We also tested if these RHA mutants were still able to translate PCE RNA. We have previously shown that the 5' RU5 region of HTLV-1 gag RNA contains an RHA-responsive PCE and that RHA is necessary for translation of HTLV-1 Gag virion protein (223). Herein we used HTLV-1 Gag protein production as a biological reporter for PCE activity.

Two days post-transfection with HTLV-1 provirus, the following cellular components were collected: i) cell supernatant to measure HTLV-1 Gag production by enzyme-linked immunosorbant assay (ELISA); ii) cell lysate for analysis of Fl-RHA and endogenous RHA protein production by immunoblotting; and iii) total RNA to quantify steady-state levels of gag expression. HTLV-1 gag translation efficiency (i.e PCE activity) is represented by the ratio of HTLV-1 Gag protein:gag RNA.
Ribosome profile analysis determined that deletion of one (\(\Delta\text{dsRBDI}\)) or both N-terminal dsRBDS (\(\Delta\text{dsRBDI}\&\text{II}\)) decreased the abundance of RHA in heavy fractions, indicative of reduced polysome association (Figure 3.2A, compare sedimentation pattern of endogenous RHA to mutants). It is possible that the observed loss of polysome association could be a consequence of altered protein conformation caused by the extensive domain deletion. However the substitution mutants K54A/K55A, K236E and K54A/55A/236E also showed reduced polysome association compared to endogenous RHA, demonstrating that these conserved amino acids are critical to polysome association of RHA (Figure 3.2A and 3.2B). Possible explanations are: i) disruption of either dsRBD eliminates association of full length RHA with PCE RNA; or ii) RHA:PCE interaction may occur in a conformation or complex that does not favor efficient polysome association. The observed reduction in polyribosome association indicates that these mutants are blocking a pre-initiation stage of translation. Our results indicate that N-terminal amino acids are essential for efficient polysome loading of RHA.

Conserved N-terminal amino acids are required for translation of PCE-containing RNA. Similar to previous results, overexpression of Fl-RHA increased HTLV-1 Gag protein production compared to cells transfected with an empty vector control plasmid (Table 1) (223). Overexpression of \(\Delta\text{dsRBDI}\) (Table 1) or RHA \(\Delta\text{dsRBDI}\&\text{II}\) (data not shown) did not enhance HTLV-1 Gag protein production compared to empty vector control, indicating that the N-terminal 250 amino acids are necessary for PCE activity. The substitution mutants K54A/K55A and K236E also exhibited loss of PCE activity.
demonstrating that these conserved amino acids are critical to PCE translation by RHA (Table 1).

RHA catalytic domain is necessary for polysome association and PCE translation

A conserved central lysine residue within the RHA helicase core, K417, is critical for RHA to both bind and hydrolyze ATP (241). Previous data has shown that the ATPase-dependent helicase function of RHA is required for translation of HIV-1 mRNA that contains a PCE (46). Ribosome profile analysis determined that the K417R mutation decreased the presence of RHA in heavy fractions, indicative of reduced polysome association (Figure 3.2B, compare with sedimentation pattern of endogenous RHA in Figures 3.2A and 3.2C). This demonstrates that the ATPase activity of RHA is required for polysome loading and mutation of this residue is blocking the pre-initiation step of translation. Overexpression assays demonstrated the importance of the intact ATP-binding K417 residue in PCE translation. In contrast to Fl-RHA (p < 0.001), overexpression of K417R or K417A did not provide statistically significant PCE activity (both mutants were not different from the empty vector control, p = 0.585 and 0.433, respectively) (Table 1). The data indicates that this residue is critical for efficient initiation and therefore PCE translation.

RHA C-terminal residues affect a post-initiation step in PCE translation

To evaluate the necessity of the RHA C-terminal RG-rich domain for PCE activity, the RG-rich domain was deleted after the nuclear localization signal (amino acids 1168-1269, referred to as ∆RG), or 9 arginine residues in the context “RG” were substituted with alanine (referred to as RA9). Ribosomal profile analysis indicated that
both ΔRG and RA9 mutant proteins were present in polysome fractions, similar to endogenous RHA (Figure 3.2C). This indicates that mutations of the C-terminal domain of RHA do not affect the initiation step of translation. However, overexpression of ΔRG not only abolished PCE activity, but, it reduced HTLV-1 gag translation to a level significantly lower than the vector only control (Table 1, p = 0.01). The ΔRG mutation is therefore classified as transdominant. The RA9 mutant yielded a similar trend, although we cannot classify it as a transdominant because it is statistically in the same range as the vector only control (Table 1, p = 0.132). The data indicate that the C-terminal residues of RHA are required for a post-initiation event in PCE translation.

**Select RHA mutants are sequestered in cytoplasmic granules**

Defective initiation complexes result in the formation of cytoplasmic stress granules (23). Since select mutations of RHA are defective in polysome loading, and therefore initiation, we used immunofluorescence to observe the cellular localization of our RHA mutants. Although RHA shuttles to the cytoplasm it is mainly nuclear, which makes it difficult to visualize by confocal microscopy (59). In order to overcome the limitation of visualizing cytoplasmic RHA, we mutated the nuclear localization signal. K1162 and R1165 deletion results in prominent cytoplasmic RHA expression (Figure 3.3A). Ribosomal profile analysis indicated that mNLS is present in heavy polysome fractions, indicating that the mutation does not disrupt the cytoplasmic activity of RHA in translation (Figure 3.3B). Overexpression of cytoplasmic RHA significantly increased HTLV-1 gag translation in comparison to empty vector control (p = 0.006) (Table 1). Because mNLS activity was twice that of Fl-RHA, mNLS was classified as a gain-of-
function mutant. Therefore disruption of nuclear localization does not hamper PCE translation and instead increases PCE activity, possibly due to an excess of RHA available for translation in the cytoplasm.

We then made combination mutants to observe the effects of the mutants on cytoplasmic RHA localization. We initially tested the localization of K236E/mNLS via immunofluorescence. Addition of the K236E mutation to mNLS altered the cytoplasmic distribution of the protein from a diffuse distribution into large cytoplasmic granules, as shown by immunofluorescence staining (Figure 3.3A, compare mNLS to K236E/mNLS). Sucrose gradient analysis of K236E/mNLS determined that a large proportion of the protein was present in the 60S/80S fraction, as compared to mNLS, which is mainly polysome associated (Figure 3.3B). Overexpression assays indicated that K236E/mNLS does not provide statistically significant PCE activity (Table 1, p = 0.130 compared to vector only control) and demonstrate the importance of the K236 residue of RHA for PCE translation. Other selected combination mutants were then tested for cytoplasmic localization. Consistent with K236E/mNLS, K54A/55A/236E/mNLS was sequestered in large cytoplasmic granules (Figure 3.3C). Mutation of K54, K55 and K236 in combination was shown to completely eliminate binding of the N-term domain of RHA to PCE RNA (136). Similar results were obtained with the K417R/mNLS ATPase combination mutant (Figure 3.3C). We also tested a C-terminal combination mutant, RA9/mNLS and found that it was primarily diffused through the cytoplasm with relatively few cells showing the granular phenotype (Figure 3.3C). It is noteworthy that the mNLS mutation itself resulted in occasional sequestration of RHA in cytoplasmic
granules. In order to quantify the percentage of cells displaying a granular phenotype for each mutant, cells were transfected with the combination mutants and visually quantified to determine the ratio of granular versus non-granular cells. Each transfection and quantification was repeated 3 times and results were averaged. P-values were calculated and show that the K236E/mNLS, K54A/55A/236E/mNLS and K417R/mNLS mutants result in a primarily granular phenotype that is statistically different from the mNLS and RA9/mNLS mutants, which show a primarily non-granular phenotype (Figure 3.3D). This phenomenon could be repeated when 1/5th the amount of DNA was transfected into cells, showing that it is not an overexpression phenotype (data not shown). We also saw the same phenotype for each mutant in HEK 293 cells (data not shown) demonstrating that the effect is not cell type dependant. We tested for a change in endogenous RHA localization upon RHA mutant transfection and found no change (data not shown). This result is in agreement with the polysome data that demonstrates that RHA with mutations in the N-terminal or helicase domains is unable to efficiently initiate translation, whereas RHA with C-term mutations is polysomes-associated.

**RHA mutants are sequestered in stress granules independent of eIF2α phosphorylation**

In order to investigate the composition of the cytoplasmic granules, we initially tested co-localization of the representative granular mutant K236E/mNLS with various granule markers by immunofluorescent staining. Stress granules are translationally inert cytoplasmic centers of molecular triage where mRNAs and certain RNA binding proteins are stored until their fate is determined (20;21). The RNA-binding protein TIA-1 is a
widely used stress granule marker (19) Strong co-localization was observed for K236E/mNLS with endogenous TIA-1 in Cos7 cells (Figure 3.4A) (242). Similar co-localization results were observed for K236E/mNLS with a YFP-tagged version of the stress granule protein TIA-1 (Figure 3.4B). Treatment with cycloheximide to disrupt stress granule formation redistributed K236E/mNLS and TIA-1 to a diffuse cytoplasmic pattern (242). Results with K417R/mNLS also demonstrate colocalization of the protein with YFP-tagged TIA-1 and the redistribution to a diffuse cytoplasmic pattern upon cycloheximide treatment. We confirmed the colocalization of all granular mutants with additional stress granule markers such as endogenous PABP (Figure 3.3C) as well as GFP-tagged G3BP-1(Figure 3.4C) demonstrating that all the RHA mutants that display a granular appearance are segregated in stress granules.

It has been reported that another class of cytoplasmic granules, called processing bodies (P bodies), are sites for storage of miRNA-silenced RNA, RNA decay, and more recently, HIV-1 particle assembly (243-245). Co-localization was not observed for any of the granular RHA cytoplasmic mutants with an RFP-tagged version of Dcp1a (Figure 3.4D), which is a resident P body protein involved in mRNA decay (246). These results show that the granular RHA mutants are sequestered in stress granules and not P bodies.

Stress granule formation can occur in an eIF2α-dependent or independent method (19;28). In order to test if the RHA mutants induced stress granule formation in an eIF2α-dependent method, lysates of HEK 293 transfected cells were run on an SDS acrylamide gel and probed using antisera against phospho-eIF2α and eIF2α. Lysate of cells treated with arsenite, a known inducer of eIF2α phosphorylation, was used as a positive control.
Results show that the RHA cytoplasmic mutants do not induce phosphorylation of eIF2α, indicating an eIF2α-independent method of stress granule formation (Figure 3.4E). Inhibition of translation initiation by the small molecule hippuristanol triggers the formation of eIF2 α-independent stress granules by inhibiting eIF4A RNA binding, and thereby translation initiation (28). Since RHA mutants that induce stress granule formation are unable to initiate efficiently, we tested the effect of hippuristanol on the non-granular RHA mutants to determine if they would segregate to stress granules similar to the initiation defective granular mutants. Results show that inhibition of translation initiation upon treatment of cells with hippuristanol causes mNLS and RA9/mNLS to be segregated to stress granules (Figure 3.3C lower two panels). Therefore it appears that overexpression of RHA mutants that are defective in translation initiation could induce stress granule formation.

**RG mutation diminishes the recruitment of RHA to stress granules upon oxidative stress**

Due to the observation that specific RHA mutants co-localize with stress granules, it is possible that RHA is recruited to granules under conditions of cellular stress. Stresses such as oxidative stress or heat shock trigger a sudden translational arrest leading to rapid polysome disassembly and the formation of stress granules (19-21). Arsenite is a well known inducer of oxidative stress leading to stress granule formation and we tested its effect on RHA localization. Results show that mNLS staining was redistributed to a granular pattern (85 % of cells) upon arsenite treatment resembling the K54A/55A/236E/mNLS, K236E/mNLS and K417R/mNLS mutants (Figure 3.5 upper
The granules showed strong colocalization with PABP, used here as a stress granule marker. We noticed some granular cells displayed FLAG staining that appeared intermediate between granular and non-granular; possibly a transition state between the two. Surprisingly the RA9/mNLS mutant showed a far less granular appearance (46% of cells) or segregation into stress granules upon arsenite treatment (Figure 3.5). PABP staining was still granular demonstrating that the RA9/mNLS mutant does not affect PABP localization upon arsenite treatment. Quantification of the number of granular and non-granular cells for each mutant shows that RA9/mNLS has statistically fewer (p value 0.0058) granular cells than mNLS. An example of a non-granular RA9/mNLS cell is shown in Figure 3.5B (lower panel). This result demonstrates the partial requirement of RG-rich domain for localization of RHA to stress granules under conditions of oxidative stress. However, the RGG residues do not appear to be important for eIF2α-independent stress granule formation as demonstrated by the hippuristanol treatment.

**RHA is not an essential component for stress granule formation**

Certain RNA binding proteins are essential for stress granule formation and the downregulation of these proteins results in a diminished response to stressful stimuli (25;247;248). Detection of select RHA mutants in cytoplasmic stress granules led us to test the possibility that RHA was an essential component for stress granule formation. Endogenous RHA was depleted with siRNA (Figure 3.6A) followed by arsenite treatment to induce cellular stress. Immunofluorescence analysis of TIA-YFP (Figure 3.6B) or endogenous PABP (Figure 3.6C), used here as stress granule markers, indicated no difference in the apparent size or number of stress granules in the control (SCi) compared
to RHA (RHAi) siRNA treatments. Based on this observation, we conclude that RHA is not an essential component for stress granule formation.

**Methylation of the RG residues is important for RHA localization in stress granules during oxidative stress**

The RG-rich domain of RHA is known to be methylated and mutation of the RGG motifs to AGG should abrogate the methylation (61;123). There is evidence that inhibition of methylation of the RG residues in other RNA binding proteins results in a change in the localization of the protein upon arsenite treatment (249). Therefore, we hypothesized that the RA9 mutation prevents efficient methylation of RHA RG residues and thereby reduces RHA stress granule recruitment upon arsenite treatment. To test this hypothesis, we used adox, a well characterized general methylation inhibitor, to prevent methylation of RHA. Adox treatment has previously been shown not to represent a significant cellular stress (249). We made several unsuccessful attempts using mass spectrometry and methyl-specific antibodies to determine the methylation state of the RA9/mNLS mutant. Untransfected (Figure 3.7A) or mNLS (data not shown) transfected cells were initially treated with adox in the absence of arsenite to test the effect of adox on cells, using PABP as a marker. Results show that PABP exhibits diffuse non-granular staining, indicating the absence of stress granules. To test if adox affects RHA recruitment to stress granules mNLS or RA9/mNLS transfected cells were treated with 50 µM adox 24 hours post-transfection or left untreated. All cells were then treated with arsenite 48 hours post-transfection, before being fixed and stained for FLAG and PABP. Results for mNLS show a significant increase in the number of non-granular cells with
adox treatment compared to the no adox control (p-values < 0.01) (Figures 3.8A and 3.7B). RA9/mNLS cells did not show this significant change with adox treatment, demonstrating that the effect of adox is specific to the mNLS mutant (Table 2 and Figure 3.7B). This result suggests that RG methylation may be a requirement for sequestration of RHA to stress granules during oxidative stress. PABP staining did not change. In the cell, the effect of adox is not specific as it is a general methylation inhibitor. However, the fact that the RA9/mNLS mutant did not show a significantly different number of non-granular cells provides a good indication that we are looking at a specific effect. Further, we tested the effect of adox followed by arsenite treatment on K417R/mNLS, K236E/mNLS and K54A/55A/236E/mNLS. Results show that these mutants that were primarily granular in the absence of oxidative stress, are still primarily granular and, further, there was no significant increase in the number of non-granular cells (Table 2 and Figure 3.7B) upon adox pretreatment as compared to the no adox control. Therefore, it appears that mutants of RHA that are segregated in stress granules in the absence of oxidative stress are unaffected by oxidative stress or by adox treatment.

**PCE RNA requires intact N-terminal RHA residues for association in the cell**

We have determined that RHA mutants with a defect in translation initiation are sequestered to stress granules. RNA co-precipitation was used to determine if the various RHA mutants are still able to bind the PCE RNA. Here we utilized an endogenous cellular PCE: the junD PCE RNA (36). HEK293 cells were transfected with plasmids that express FLAG-tagged RHA mutants in three independent experiments. Western blot verified equivalent expression of FLAG-tagged RHA mutants and also that the proteins
were immunoprecipitated (Figures 3.8A and 3.8B). Total RNA and RNA isolated from IPs with FLAG antibody were harvested and evaluated by RT-PCR with primers specific for junD. As expected, total RNA preparations displayed junD (Figures 3.8C and 3.8D). mNLS, RA9/mNLS and K417R/mNLS mutants all co-immunoprecipitate junD PCE RNA (Figures 3.8C and 3.8D). The K236E/mNLS and the K54A/55A/236E/mNLS mutants are unable to immuno-precipitate the junD RNA (Figures 3.8C and 3.8D). These results demonstrate that the RHA mutants that localize into stress granules do so independent of their ability to bind to PCE RNA and further that the N-terminal residues K54,K55 and K236 are required for this interaction in the context of the full length protein. Previous data from our lab has shown that these residues are required for the \textit{in vitro} and \textit{ex vivo} interaction of the isolated N-terminal domain of RHA with PCE RNA \cite{136}.

\textbf{Mutations in conserved RHA domains eliminates HTLV-1 translation in complementation assays}

We next tested if the various RHA mutants were able to facilitate PCE translation in complementation assays, where endogenous RHA was downregulated. For suitability in complementation assays, site-directed mutagenesis was performed on parental Flag-tagged RHA to introduce silent mutations at RHA siRNA target sites. The plasmids were transfected into Cos7 cells with or without RHA downregulation by siRNA (Figure 3.9A). Representative immunoblots demonstrate that each mutant was expressed and resistant to downregulation by siRNA (Figure 3.9B). Immunofluorescent staining with
Flag antisera indicated that RHA mutation had no observable effect on RHA subcellular localization (Figure 3.10A, 3.10B and 3.10C).

For complementation assays, endogenous RHA was downregulated with siRNAs targeted to two regions of the RHA sequence. Treatment with these siRNAs has previously been shown to have no significant effect on global RNA or protein synthesis (250). To identify possible deleterious effects of short-term RHA overexpression on protein synthesis or cell growth, metabolic labelling and cell proliferation assays were performed, respectively. The results demonstrated that overexpression of RHA wild type or representative RHA mutants did not produce any measurable effects on either de novo translation or cell viability compared to empty vector transfected cells (Figure 3.11).

**Conserved N-terminal amino acids are required for translation of PCE-containing RNA in complementation assays**

In complementation assays where endogenous RHA was depleted with siRNA, expression of Fl-RHA rescued PCE activity (Table 3). Similar to the overexpression assays, ΔdsRBDI, K236E, or K54A/K55A failed to rescue PCE activity (Table 3, p-values for all mutants were < 0.05 in comparison to Fl-RHA). Taken together, these results implicate conserved N-terminal residues that are important for binding and polysome loading are critical for PCE activity.

**mNLS RHA mutants efficiently translate PCE RNA in complementation assays**

Previous results demonstrated that RHA co-precipitates with PCE RNA in both nuclear and cytoplasmic fractions and that nuclear education is required for efficient translation of PCE-containing mRNAs (251;252). To determine whether or not nuclear
interaction of PCE and RHA are required for PCE activity, the activity of mNLS was investigated (253). As compared to the overexpression assay, mNLS gain-of-function was even more pronounced in the complementation assay, where mNLS rescued PCE activity at a magnitude 7-fold higher than WT (Table 3). This data is consistent with the association of mNLS with heavy polysomes. These results indicate that nuclear interaction between RHA and PCE mRNA is not required for robust translation.

Explanation for the nuclear education requirement of PCE includes interaction with another nuclear protein, or that interaction with residual endogenous RHA in the nucleus is sufficient. We also tested a representative combination mutant K236E/mNLS for PCE activity. Down-regulation of RHA and transfection of K236E/mNLS rescued PCE activity to a similar magnitude of WT RHA in complementation assays. Comparison between mNLS and K236E/mNLS indicated that addition of K236E reduced PCE activity (P-value <0.05 ) (Table 3). Taken together, these results support the hypothesis that K236E/mNLS is associated with stalled translation complexes in cytoplasmic granules.

**RHA catalytic domain is necessary for PCE activity**

Expression of either K417R or K417A did not rescue PCE activity compared to Fl-RHA upon downregulation of endogenous RHA (Table 3, p-values < 0.05 comparing Fl-RHA with K417R or K417A). Overall, these results support a role for RHA ATPase activity in facilitating translation of PCE-containing mRNA. Because RHA has been reported to be relatively inefficient at unwinding short RNA duplexes, we hypothesize
that RHA utilizes its conserved catalytic core to induce mRNP remodeling that favors translation of complex PCE-containing RNA (254).

**RHA C-terminal residues are required for PCE activity**

RA9 failed to rescue PCE activity in RHA siRNA-transfected Cos7 cells (Table 3, p = 0.001 in comparison to Fl-RHA). These results indicate that RHA amino acids 1168-1269, specifically the RG motifs, are required for PCE activity. However the C-terminal mutants were found to be associated with polysomes indicating they are able to initiate. The results suggest either a block downstream of initiation, or that the mRNP is sequestered in a translationally-repressed high molecular weight complex, commonly referred to as “pseudo-polysome” (255). To distinguish between these possibilities, cells transfected with RA9 were treated with 400 μM puromycin to induce pre-mature polypeptide chain termination prior to sucrose gradient fractionation (256). Puromycin treatment shifted RA9 protein from heavy polysome fractions to mid-weight fractions corresponding to 80S and light polysome fractions (Figure 3.12). Because the RA9 complex is associated with puromycin-sensitive polysomes, we speculate that disruption of the RG motifs blocks or reduces the efficiency of a post-initiation step of translation.

**RHA RG-rich residues are important for a post-initiation step in translation**

Mutation of RHA RG residues results in a loss of PCE activity, but the RA9 mutant is still on polysomes and not sequestered to stress granules. We hypothesized that the mutant loses activity due to a post-initiation defect in translation of PCE RNA, possibly in elongation or in peptide release. To monitor for post-initiation defects in the RA9/mNLS mutant, sucrose gradient analysis was repeated with the omission of
cycloheximide. Cycloheximide treatment blocks translation elongation and prevents run off of translating ribosomes (15). Omission of cycloheximide is expected to allow ribosomes to run-off the RNA, producing a shift to lower molecular weight fractions across the profile (257). However, if mutation of RHA causes a defect in translation elongation or termination, ribosomes are expected to run off the RNA at a slower rate and sediment in heavier fractions compared to RHA samples with no defect. mNLS and RA9/mNLS transfected cells were harvested in the presence (Figure 3.13A) or absence (Figure 3.13B) of cycloheximide treatment and the lysates were separated on linear sucrose gradients. We used the mNLS combination mutant to obtain more cytoplasmic protein making for easier analysis of predominantly cytoplasmic lysates. Results show that omission of cycloheximide causes the majority of mNLS protein to shift from the polysome fractions to the 80s fractions, while the majority of the RA9/mNLS protein is distributed in the 80s and light polysome fractions (Figure 3.13B). Tubulin was used as a control to show equivalent processing of the samples. To better visually quantify the results we repeated the experiments but this time pooled fractions into mRNP, 40s, 60/80s, light polysome (LP) and heavy polysome fractions (HP) (Figure 3.13C). Here the differences between the samples in the absence of cycloheximide are apparent (Figure 3.13C lower two panels). The differences in polysome association for RA9/mNLS is indicative of reduced ribosome run-off compared to mNLS. This observation suggests a role for RHA’s RG-rich domain in facilitating a post-initiation step in translation. Possible mechanisms include changes in cofactor or miRNA binding that affects efficient translation.
Discussion

This study defined roles for the functional domains of RHA in translation of PCE RNAs. Herein, the N-terminal lysine residues that are known to convey interaction with the RNA, a conserved central lysine important for catalytic activity, and C-terminal arginine residues are shown to be essential for PCE activity in both overexpression (Table 1) and complementation assays (Table 3). The results also indicate that PCE does not require nuclear interaction with RHA and suggest the role of essential nuclear education for PCE activity is attributable to a separate yet-to-be-identified factor. Our studies with sucrose density gradients show roles for RHA in two stages of translation: pre-initiation and post-initiation.

Mutation of the N-terminal or catalytic helicase residues reduces RHA’s polysome association indicating a defect in initiation. Moreover, these mutants are sequestered to stress granules in an eIF2α-independent manner. These results suggest that RNA unwinding or RNP remodeling is a key component to RHA-PCE activity. We hypothesize that the inability of RHA to recognize and bind to the target RNA, or to hydrolyze ATP, locks RHA in translationally-stalled complexes which localize to stress granules. Abortive translation initiation is a well known trigger for stress granule formation (19). Drugs that inhibit translation initiation by targeting the helicase eIF4A, such as hippuristanol and pateamine A, induce stress granules in the absence of eIF2α phosphorylation (26-28). Hippuristanol treatment resulted in sequestration of the mNLS and RA9/mNLS mutants into stress granules. Therefore it is possible that RHA augments the activity of eIF4A in translation initiation, and inhibition of either of their translation
initiation activities results in stress granule formation. Our data is supported by previous work showing that mutations of certain RNA binding proteins result in stress granule formation. A recent study showed that Sam68 cytoplasmic mutants lacking the NLS result in stress granule formation, and these mutants were unaffected by arsenite treatment (258). Mutations in fused in sarcoma (FUS) protein induce cytoplasmic stress granules (259). Both these proteins contain RG rich or RGG domains.

RHA RG mutants are polysome associated and possibly stalled or slowed down due to a post-initiation defect. Data from the cycloheximide-deficient gradients shows that RA9/mNLS is still on polysomes, as compared with mNLS, that shifts predominantly into the 80s fractions. Possible explanations are 1) mutation of the arginine residues disrupts binding with a factor that enhances translation elongation/release. 2) Mutation of the arginine residues allows binding of a miRNA that causes repression of protein translation by inhibiting elongation or causing co-translational degradation of synthesized protein. Preliminary unpublished data from our lab using mass spectrometry to identify RHA binding partners, suggests that RHA interacts with PABP and elongation factors eEF1A and eEF1B (Jing and Boris-Lawrie). It is possible that RHA brings in another factor to interact with the elongation factors thereby enhancing elongation. PABP interacts with various translation factors including the translation release factor RF3 (260;261). It is possible that mutation of the RG residues abolishes or reduces the interaction of RHA with PABP or RF3 or both thereby affecting peptide release. miRNAs are known, in some cases, to cause repression of translation in a post-initiation manner (262). The let-7a miRNA represses the translation of lin–41 mRNAs by causing
degradation of the nascent polypeptide as it is released from translation (263). Mutation of the RHA arginine residues might allow disruption of cofactor binding that then allows a repressive miRNA to bind the mRNA. RHA has previously been shown to be associated with the RISC complex components (68).

Exposure to oxidative stress triggers the sequestration of RHA to stress granules, but mutation of the RG-rich domain reduces sequestration into stress granules under conditions of oxidative stress. Stress granules formation is favored by inhibition of translation initiation, and repressed by inhibition of translation elongation (20). Therefore, the decreased stress granule formation phenotype displayed by the RG mutant is consistent with a post-initiation defect. Prior data supports our findings. Li et al showed that knockdown of eIF5A promotes translation elongation in stressed cells, and a knockdown of this protein results in impaired polysome disassembly and impaired stress granule assembly (248). A point to note is that mutation of the RG-residues does not affect localization of RHA to stress granules during hippuristanol treatment. Therefore recruitment of RHA to stress granules during eIF2α-dependent stress granule formation requires intact arginine residues, which differs from the mechanism of recruitment of RHA to stress granules during eIF2α-independent stress granule formation.

The RG-rich region has previously been shown to be important for stress granule localization (247;258). Studies have also shown that inhibition of methylation of the RGG residues of FMRP and FXR1P results in a change in the localization of the proteins upon arsenite treatment (249). Our studies with adox indicate that methylation of RG residues is a partial requirement for localization of RHA to stress granules, under
conditions of stress. The RHA RG-rich domain facilitates interaction with poly-A binding protein (PABP) (Jing and Boris-Lawrie, unpublished data) that may facilitate RNA circularization and ribosome recycling to augment protein output. Consequently, circularization or ribosome recycling might be affected by mutation of RHA arginine residues and could add to the post-initiation defect we observed. A similar model was proposed for bovine diarrheal virus RNA (264). Work is in progress to identify the co-factors that bind to the RG-rich domain and are required for PCE translation. It will be interesting to test if the same co-factor is also required for the sequestration of RHA to stress granules during stressful conditions. Furthermore, we have shown that the sequestration of RHA mutants in granules is independent of the ability of each of these mutants to bind to PCE RNA. Additionally, although RHA cycles to stress granules in response to oxidative stress, it is not a core component required for stress granule formation.

Translation enhancement by RHA is specific to PCE RNA and does not affect general translation (36). Therefore it is likely distinct from the activity observed for DHX29 or Ded1 (yeast homolog of mammalian DDX3) (265;266). DXH29 seems to be a more general enhancer of complex mRNA translation, given that it binds to ribosomal subunits and induces conformational changes in the ribosome RNA binding cleft to accommodate complex structured mRNAs (267). Similarly DDX3 binds to several translation initiation factors, including the cap binding protein, eIF4E (268), as well as eIF4A, eIF2α, and PABP (269). Furthermore, induction of cell stress by sodium arsenite treatment leads to the co-localization of these factors in cytoplasmic stress granules. In
line with these observations, overexpression of DDX3 reduced cap-dependent, but not HCV IRES-dependent translation from a bicistronic reporter RNA (270) and increased translation of RNAs containing highly structured 5' UTRs (271). Our current understanding is that DDX3, like DHX29, is a general translation regulator for RNA containing a structured 5' UTR, with unknown target specificity. By contrast, RHA activity on PCE templates fills a unique niche to specifically regulate translation of PCE-containing mRNAs.
Figure 3.1. Description of RHA mutants. Depiction of RHA domain structure with substituted amino acids indicated. Deletion constructs are illustrated below. Dotted line indicates deleted regions.
Figure 3.2. RHA loss of function mutants exhibit differences in polysome association
HEK293 cells were transfected with Fl-RHA mutants and separated on a 15-47.5% sucrose density gradient. Lysates from each fraction were subjected to immunoblotting with antisera to RHA or Flag as indicated. Representative A$_{254}$ ribosomal RNA profile is indicated by purple line above blots for (A) Select N-terminal mutants (B) K54A/55A/236E and K417R mutants and (C) C-terminal mutants. Immunoblotting in Figure 3.2B was carried out with rabbit antisera to FLAG that shows the presence of a non-specific band (indicated by the red triangle) below the specific FLAG band (indicated by a black line). All other immunoblotting was carried out with mouse antisera to FLAG.

Figures A and C were performed by Cheryl Bolinger (Phd) in a collaborative manuscript.
Figure 3.2. RHA loss of function mutants exhibit differences in polysome association
Figure 3.3. Certain cytoplasmic RHA mutants localize in granules (A) Immunofluorescence staining of Fl-RHA mutants in Cos7 cells. Flag-RHA is indicated in green. DAPI was used as a nuclear stain and is indicated in blue. Merged images indicate that Fl-RHA mNLS mutants are restricted to the cytoplasm. (B) Cos7 cells were transfected with Fl-RHA mutants and separated on a 15-47.5% sucrose density gradient. Lysates from each fraction were subjected to immunoblotting with Flag antisera. (C) Cos7 cells were transfected with indicated RHA cytoplasmic mutant plasmids. Where indicated, cells were treated with hippuristanol for 30 mins prior to fixing. Forty-eight hours post transfection, cells were subjected to immunofluorescence staining with Flag antisera and AlexaFluor594 (pseudo-colored red) as well as PABP antisera and AlexaFluor488 (pseudo-colored green). DAPI was used as a nuclear stain (blue). Merged images show colocalization of granular RHA mutants with endogenous PABP. (D) The percentage of granular and non-granular cells for each mutant is graphed. Counts were repeated in triplicate and averaged. Error bars are indicated and one-sided t-tests were performed to assess statistical significance of each RHA mutant compared to mNLS. Dunnet’s method was used and p <0.01 is considered statistically different from mNLS. Asterisks indicate statistically significant differences in samples compared to mNLS and p-values are shown above each sample.

Figures A and B in this figure were performed by Cheryl Bolinger (PhD) in a collaborative manuscript.
Figure 3.3. Certain cytoplasmic RHA mutants localize in granules
Figure 3.4. RHA mutants localize to cytoplasmic stress granules but not P bodies (A) Cos7 cells were transfected with RHA K236E/mNLS expression plasmid. Forty-eight hours post transfection, cells were subjected to immunofluorescence staining with rabbit Flag antisera and AlexaFluor594 (pseudo-colored red) as well as TIA-1 antisera and AlexaFluor488 (pseudo-colored red). DAPI was used as a nuclear stain (blue). (B) Cos7 cells were transfected with indicated RHA mutant plus TIA-1-YFP expression plasmids. Forty-eight hours post transfection, cells were subjected to immunofluorescence staining with rabbit Flag antisera and AlexaFluor594 (pseudo-colored green). DAPI was used as a nuclear stain (blue), and TIA-YFP is pseudo-colored red. Where indicated, cells were treated with cycloheximide for 1h prior to fixation. (C) Cos7 cells were transfected with indicated RHA mutant and GFP-G3BP-1 expression plasmids. Forty-eight hours post transfection, cells were subjected to immunofluorescence staining with rabbit Flag antisera and AlexaFluor594 (pseudo-colored green). DAPI was used as a nuclear stain (blue), and GFP-G3BP-1 is shown in green. (D) Co-expression of indicated RHA mutant with P body marker RFP-Dcp1a. Forty-eight hours post transfection, cells were subjected to immunofluorescence staining with mouse Flag antisera and AlexaFluor488 (pseudo-colored green). DAPI was used as a nuclear stain (blue), and RFP-Dcp1a is shown in red. (E) Immunoblot showing that the RHA cytoplasmic mutants do not trigger eIF2α phosphorylation. The upper panel shows immunoblotting with mouse FLAG antisera demonstrating equivalent transfection of mutants. The middle panel shows immunoblotting with phospho-eIF2α antisera and the lower panel shows immunoblotting with eIF2α antisera. Arsenite treated cell lysate was used as a positive control to show eIF2α phosphorylation.

Certain images (Figure A, Figure B upper two panels, Figure D first panel) in this figure were performed by Cheryl Bolinger (PhD) in a collaborative manuscript.
Figure 3.4. RHA mutants localize to cytoplasmic stress granules but not P bodies
Figure 3.5. RA9 mutation diminishes the recruitment of RHA to stress granules upon oxidative stress Cos7 cells were transfected with RHA mNLS or RA9/mNLS expression plasmids. Forty-eight hours post transfection, cells were treated with arsenite for 30 mins and subjected to immunofluorescence staining with Flag antisera and AlexaFluor594 (pseudo-colored red) as well as PABP antisera and AlexaFLuor488 (pseudo-colored green). DAPI was used as a nuclear stain (blue). The percentage of granular and non-granular cells for each mutant is indicated. Counts were repeated in triplicate and averaged. One-sided t-tests were performed to assess statistical significance of RA9/mNLS compared to mNLS. Dunnet’s method was used and p <0.01 is considered statistically different from mNLS. Asterisks indicate statistically significant differences in samples compared to mNLS and p-values are shown.
Figure 3.6. RHA is not an essential component for stress granule formation Cos7 cells were treated as indicated in Figure 3.9. TIA-YFP plasmid was transfected where indicated. Forty-eight hours post-transfection cells were treated with or without arsenite to induce oxidative stress. (A) A fraction of the cells were lysed and lysates were subjected to immunoblotting with antisera to RHA to confirm efficient downregulation of RHA. Tubulin serves as a loading control. SCI indicates scrambled siRNA and RHAI indicates RHA specific siRNA. (B) The number and size of stress granules as represented by TIA-YFP fluorescence does not change with RHA or sc siRNA treatment. DAPI was used as a nuclear stain (blue), and TIA-YFP is pseudo-colored green. (C) Cells were subjected to immunofluorescent staining with PABP antisera and AlexaFluor488 (pseudo-colored green). DAPI was used as a nuclear stain (blue). The number and size of stress granules as represented by PABP staining did not change with RHA or scrambled siRNA treatment.
Figure 3.6. RHA is not an essential component for stress granule formation
**Figure 3.7. Effect of Adox pre-treatment on RHA mutants** (A) Cos7 cells were left untransfected and treated with indicated amounts of Adox after 48 h. Cells were subjected to immunofluorescence staining 48 h post-transfection with PABP antisera and AlexaFluor488 (pseudo colored green) as well as FLAG antisera and AlexaFluor594 (pseudo-colored red) where indicated. DAPI was used as a nuclear stain. (B) Cos7 cells were transfected with indicated RHA mutants and treated with indicated amounts of Adox after 24 h. Cells were subjected to arsenite treatment 48 h post-transfection for 30 min followed by immunofluorescence staining with PABP antisera and AlexaFluor488 (pseudo colored green) as well as FLAG antisera and AlexaFluor594 (pseudo-colored red). DAPI was used as a nuclear stain.
Figure 3.7. Effect of Adox pre-treatment on RHA mutants
Figure 3.8. PCE RNA requires intact N-terminal RHA residues for association in the cell Representative immunoprecipitation (IP) assay to test if RHA mutants bind to endogenous junD PCE RNA. Empty FLAG plasmid or indicated RHA mutant expression plasmids were transfected into HEK 293 cells and FL-tagged proteins were subject to IP with FLAG antibody (mouse) conjugated to agarose beads. Immunoblot of equivalent aliquots of input lysate and equivalent aliquots of agarose beads determined the IP efficiency was similar for each protein. RNA was harvested from the remaining beads and subjected to RT-PCR with junD primers. (A and B) Western blot with Flag-specific antiserum (rabbit) detected equivalent expression of RHA mutants (first panel). The FLAG (rabbit) antibody detects a non-specific band (indicated by the red triangle) just below the specific FLAG band (indicated by the black line). Tubulin was used as a loading control (second panel). Western blot with Flag-specific antiserum (rabbit) detected IP was similar for all the mutants (third panel). (C and D) PCR amplification products from indicated RNA preparation incubated with or without reverse transcriptase (RT+ or -). Representative samples of total RNA and RNA isolated from IP samples were evaluated with junD-primers. The junD amplification product is 232 bp (indicated by a black line) and the lower band is primer dimer.
Figure 3.8. PCE RNA requires intact N-terminal RHA residues for association in the cell
Figure 3.9. RHA mutant plasmids express protein resistant to siRNA downregulation. (A) Protocol time line. (B) Representative immunoblots from three independent assays. Cos7 cells were treated as indicated in A and cell lysate subjected to immunoblotting with antisera to Flag epitope tag. Actin serves as a loading control and RHA immunoblot confirms downregulation of endogenous RHA.

I would like to acknowledge Cheryl Bolinger (PhD) who kindly agreed to let me use this figure in my dissertation.
Figure 3.10 RHA mutation does not affect nuclear distribution. Immunofluorescent staining of Flag-RHA mutants in Cos7 cells. Flag-RHA is indicated in green. DAPI was used as a nuclear stain and is indicated in blue. (A) N-terminal mutants; (B) ATPase mutant; (C) C-terminal mutants.

Certain images (Figure A panels 1, 2 and 4, Figure B and Figure C panels 1 and 2) in this figure were provided by Cheryl Bolinger (PhD) in a collaborative manuscript.
Figure 3.11. RHA overexpression does not alter global cellular translation or viability Cos7 cells were transfected with indicated RHA expression plasmids in three independent experiments. (A) *De novo* protein synthesis was measured by $^{35}$S-Met/Cys labeling and TCA precipitation as described in materials and methods. (B) Cell viability was measured by MTS assay. Error bars indicate standard deviations.

I would like to acknowledge Cheryl Bolinger (PhD) who kindly agreed to let me use this figure in my dissertation.
Figure 3.12. RHA RA9 mutant is associated with puromycin sensitive polysomes
Cos7 cells were transfected with the RA9 expression plasmid followed by treatment with 400 µM puromycin where indicated for 1h prior to 20min incubation with cycloheximide before harvesting. Lysates were separated on a 15-47.5% sucrose density gradient and subjected to immunoblotting with Flag (mouse) antisera. Representative $A_{254}$ ribosomal RNA profile is indicated above immunoblots.

I would like to acknowledge Cheryl Bolinger (PhD) who kindly agreed to let me use this figure in my dissertation.
**Figure 3.13. RA9 mutant RHA shows a defect in elongation** HEK 293 cells were transfected with mNLS or RA9/mNLS expression plasmids. Cells were treated with cycloheximide (CHX) for 20 min prior to harvesting where indicated. Lysates were separated on a 15-47.5% sucrose density gradient and subjected to immunoblotting with Flag (rabbit) or Tubulin antisera. The FLAG (rabbit) antibody detects a non-specific band (indicated by the red triangle) just below the specific FLAG band (indicated by the black line). Every second fraction is combined (A and B) or fractions were pooled together (C) as mRNP, 40s, 60/80s, Light polysome (LP) and heavy polysome (HP).
Figure 3.13. RA9 mutant RHA shows a defect in elongation.
Figure 3.14. Effect of mutation of RHA functional domains. The hypothetical model displaying RHA (shown in green) as part of the translation initiation complex that is required for remodeling of the mRNP to initiate translation of PCE RNAs. Mutation of the N-terminal domain of RHA results in a loss of binding of RHA to PCE RNA. This gives rise to defective initiation complexes that are shuttled to stress granules (panel A). Similarly, mutation of the helicase residue of RHA impairs the ability of RHA to help eIF4A to remodel the mRNP and also results in defective initiation complexes that shuttle to the stress granules (panel A). The RHA RGG residues are required for a post-initiation step in translation of PCE RNAs and mutation of these residues could interfere with cofactor binding, or miRNA binding, or both leading to a loss of PCE RNA translation.
Figure 3.14. Effect of mutation of RHA functional domains
Table 3.1. Select RHA mutant overexpression does not facilitate PCE activity. Cos7 cells were transfected with RHA mutant expression plasmid and ACHneo HTLV-1 provirus as indicated in Figure 3.9. Values are derived from at least 3 independent assays.

Mean fold change is relative to Cos7 cells transfected with empty vector (value = 1.0). Gag protein was measured by ELISA and gag RNA quantified by real-time RT-PCR. One-sided t-tests were performed on log transformed values to assess statistical significance of each RHA mutant. Holm’s method was used to adjust for multiple comparisons. P <0.05 is considered statistically different from expression of vector only control. NA, not applicable.

I would like to acknowledge Cheryl Bolinger (PhD) who kindly agreed to let me use this table in my dissertation.
Table 3.2. Adox pre-treatment affects mNLS sequestration to stress granules upon oxidative stress. Cos7 cells were transfected with indicated RHA mutant expression plasmids and treated with Adox (where indicated) twenty-four h post transfection. Cells were treated with arsenite forty-eight h post transfection before fixing and labeling. The percentage non-granular cells for each mutant was calculated. Counts were repeated in triplicate and averaged. Standard deviations are indicated.

The p-value is calculated using one-sided t-tests and Dunnet’s method to assess statistically significant differences. The number of non-granular cells at 0 μM Adox treatment is used as the standard for each sample. Asterisks indicate significant change.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>% non-granular cells</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 μM Adox</td>
<td>50 μM Adox</td>
</tr>
<tr>
<td>mNLS</td>
<td>15.47±6.38</td>
<td>34.15±3.68</td>
</tr>
<tr>
<td>RA9/mNLS</td>
<td>54.17±8.75</td>
<td>64.57±2.39</td>
</tr>
<tr>
<td>K417R/mNLS</td>
<td>4.43±2.61</td>
<td>6.79±4.34</td>
</tr>
<tr>
<td>K236E/mNLS</td>
<td>4.29±0.86</td>
<td>2.32±0.67</td>
</tr>
<tr>
<td>K54/55/236/mNLS</td>
<td>2.89±2.41</td>
<td>2.74±2.17</td>
</tr>
<tr>
<td>Mutant</td>
<td>Mean fold change&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P-value&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Empty vector</td>
<td>0.3 ± 0.2</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Fl-RHA</td>
<td>1.0 ± 0.0</td>
<td>NA</td>
</tr>
<tr>
<td>ΔdsRBDI</td>
<td>0.1 ± 0.2</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>K236E</td>
<td>0.1 ± 0.4</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>K54A/K55A</td>
<td>0.2 ± 0.4</td>
<td>0.02*</td>
</tr>
<tr>
<td>RA9</td>
<td>0.3 ± 0.2</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>mNLS</td>
<td>7.6 ± 0.1</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>K236E/mNLS</td>
<td>1.2 ± 0.2</td>
<td>0.16</td>
</tr>
<tr>
<td>K417R</td>
<td>0.1 ± 0.7</td>
<td>0.02*</td>
</tr>
<tr>
<td>K417A</td>
<td>0.2 ± 0.6</td>
<td>&lt;0.05*</td>
</tr>
</tbody>
</table>

**Table 3.3. Select RHA mutants fail to complement PCE activity after downregulation of endogenous RHA.** Cos7 cells were transfected with siRNA, RHA mutant expression plasmids and ACH<sup>neo</sup> HTLV-1 provirus as indicated in figure 3.9. Values are derived from at least 3 independent assays.

<sup>b</sup> Mean fold change is relative to Gag protein/gag RNA value for Fl-RHA samples (value = 1.0). Gag protein was measured by ELISA and gag RNA quantified by real-time RT-PCR.

<sup>c</sup> One-sided t-tests were performed on log transformed values to assess statistical significance of each RHA mutant. Holm’s method was used to adjust for multiple comparisons. *P <0.05 is considered statistically different compared to expression of Fl-RHA. NA, not applicable

I would like to acknowledge Cheryl Bolinger (PhD) who kindly agreed to let me use this table in my dissertation.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB1218</td>
<td>gcctgggatgagctcggatggggggcgaagagcagatacgtttt</td>
</tr>
<tr>
<td>KB1219</td>
<td>aaacgtactctgccttgcgcccccatccgacagcatccagcgc</td>
</tr>
<tr>
<td>KB1216</td>
<td>gaaagcggctgggtgtacatattcagaggaactttgctggaaacagattttc</td>
</tr>
<tr>
<td>KB1217</td>
<td>gaaaacctttcagcaaaagtcttgtaaatatagccgccagcgccttc</td>
</tr>
<tr>
<td>KB1088</td>
<td>cacccgggtacactggggtacctttgggtgatggtgtaagcttactcc</td>
</tr>
<tr>
<td>KB1089</td>
<td>ggaagtaagcttcacacactttcagacatccaggttgggtggtt</td>
</tr>
<tr>
<td>KB1676</td>
<td>ccacccacccgcgttacatgatcagatcacttacttactgatactctgacac</td>
</tr>
<tr>
<td>KB1677</td>
<td>gatcaggagactagtaagtgggatctactgtacccgggtggtggtg</td>
</tr>
<tr>
<td>KB1658</td>
<td>ccacagatggcccgagactaaaccactttggttgggtctagtcctgt</td>
</tr>
<tr>
<td>KB1659</td>
<td>cagggactcagacaaaccagtttttagctgtcgggccatctggg</td>
</tr>
<tr>
<td>KB1440</td>
<td>ggatcaataaggaataggcagcagacgtcc</td>
</tr>
<tr>
<td>KB1441</td>
<td>gaagtgcgtgcacacttctatttgatcc</td>
</tr>
<tr>
<td>KB 1676</td>
<td>cccaccccggttacatgctacacctatcactgatactctgcacac</td>
</tr>
<tr>
<td>KB 1677</td>
<td>gatcaggagactagtaagtgggatctactgtacccgggtggtggtg</td>
</tr>
<tr>
<td>KB 1673</td>
<td>gatgtagggagacacacaggtt</td>
</tr>
<tr>
<td>KB 1674</td>
<td>aaccttggtggtcctccgacac</td>
</tr>
<tr>
<td>KB 1678</td>
<td>gatgtagggagacacacaggtt</td>
</tr>
<tr>
<td>KB 1679</td>
<td>aaccttggtggtcctccgacac</td>
</tr>
<tr>
<td>KB 1077</td>
<td>cgcctcctcatggtgcagacataag</td>
</tr>
<tr>
<td>KB 1078</td>
<td>cattgctgtgagcccaatggagacg</td>
</tr>
<tr>
<td>KB 1393</td>
<td>gagcgtatcgagagttgctggtgacttgagcaggaac</td>
</tr>
<tr>
<td>KB 1394</td>
<td>gttgcctgcaacacttgcggaaactttctctgatagcct</td>
</tr>
<tr>
<td>KB 1395</td>
<td>gagactacgcaggggctagtgtgagcaggtgcag</td>
</tr>
<tr>
<td>KB 1396</td>
<td>cgcatactgctgcactactagcagtcgtctgt</td>
</tr>
<tr>
<td>KB 1418</td>
<td>gatgtagggagacacacaggtt</td>
</tr>
<tr>
<td>KB 1413</td>
<td>actgaatacctctccgcttatatcc</td>
</tr>
<tr>
<td>KB 1414</td>
<td>gatcaggagagctggtgctggttgctgctgctggacatcc</td>
</tr>
<tr>
<td>KB 1415</td>
<td>aatccatagggccccgcacacttgcgtctggaatcc</td>
</tr>
<tr>
<td>KB 1416</td>
<td>ttgcacaggagcgagagttgctgcttgatct</td>
</tr>
<tr>
<td>KB 1417</td>
<td>atacgccccacctctgtcctccgtctgagca</td>
</tr>
<tr>
<td>KB 1303</td>
<td>ttcgccttggactgatggtggtgag</td>
</tr>
<tr>
<td>KB 1304</td>
<td>ggcgctttgtgcgttgactagcag</td>
</tr>
</tbody>
</table>

**Table 3.4.** Sequence of DNA oligonucleotides used in indicated Experimental Procedure
Chapter 4. Protein footprinting of RNA helicase A reveals a lysine residue required for stress granule localization during oxidative stress

Abstract

RNA Helicase A (RHA) is an abundant nucleo-cytoplasmic DEIH RNA helicase that is required for translation of select viral and cellular RNAs. RHA recognizes a structured RNA element in the 5’ untranslated region of these RNAs termed the post-transcriptional control element (PCE). Here we describe protein footprinting of epitope-tagged full-length RHA to identify residues involved in interaction of RHA with spleen necrosis virus PCE RNA. In distinction from previous studies with recombinant protein, we used mammalian RHA that was immunoprecipitated from cells and incubated it with \textit{in vitro} synthesized RNA. Free surface exposed lysine residues are modified using a selective lysine modifying reagent, whereas nucleic acid-bound lysine residues are shielded from modification. Subsequent mass spectrometry distinguishes between unmodified and modified lysine residues. Analysis of protected residues revealed that K806 of RHA is important for the sequestration of the protein to stress granules during oxidative stress. Further, our analysis reveals that the lysine modification patterns between full length RHA and the recombinant N-terminal domain are identical. In conclusion, we have identified previously uncharacterized RHA residues that may be important for PCE translation.
Introduction

The DEIH Superfamily2 (SF2) helicase member RNA Helicase A (RHA) is an abundant nucleo-cytoplasmic shuttling protein that has known roles in transcriptional and post-transcriptional regulation of a diverse subset of genes (226). Although RHA is mainly a nuclear protein (61), about 5% is present in the cytoplasm and plays a role in translational regulation of selected viral and cellular RNAs (36;45;46). RHA ensures efficient polyribosome loading and thereby enables productive translation of these RNAs (36).

RHA translation target RNAs are recognized by a specific feature which is designated the post-transcriptional control element (PCE). PCE is a highly structured RNA element present in the 5' untranslated region of these RNAs (42;48). Specific interaction of RHA with PCE is essential for translation of these PCE RNAs (36).

Mutation of RHA amino acid residues in the N-terminal domain (K54A, K55A, and K236E), the DEIH box (K417R), and the C-terminal domain (mutation of 9 RG residues to AG) results in a loss of PCE translation (Bolinger, Ranji and Boris-Lawrie in prep). Mutations that disrupt the RNA structure of PCE also block translation of the downstream open reading frame (36;48). Viral PCE containing RNAs include the spleen necrosis virus (SNV), human immunodeficiency virus 1 (HIV-1) and human T-cell lymphotropic virus type 1 (HTLV-1) gag RNAs amongst others (36;45;46).

Overexpression of RHA has been shown to increase translation of these viral RNAs and downregulation has been shown to decrease protein production (36;45;46). Furthermore, the cellular AP1 transcription factor junD, a known growth control gene, also requires
RHA to bind to PCE in its 5' untranslated region (UTR) for its productive translation (36).

RHA is a 142 kD multi-domain protein (Figure 1; NCBI conserved domain database). As with all RNA helicases, RHA contains a conserved ATPase-dependent helicase core that is flanked by domains conserved amongst other RNA binding proteins. Two double-stranded RNA binding domains (dsRBDs) at the N-terminus are highly conserved in proteins such as protein kinase R (PKR), *Xenopus laevis* XlrbpA (PKR homologue) and Dicer (58). In a recent study we demonstrated that features of the N-terminal dsRBDs are required for specific recognition of PCE target RNAs by RHA. Mutagenesis confirmed that 3 conserved lysine residues (K54, K55 and K236) are contact points to PCE RNA. Mutation of these residues abrogates binding of the N-terminal or full length RHA to PCE RNA (136) (Bolinger, Ranji and Boris-Lawrie in prep). Expression of the N-terminal domain squelches PCE translation, but expression of the mutant N-terminal domain eliminates the squelching effect on translation of PCE containing RNAs (136).

The central region of the protein contains a DEIH helicase core that is conserved amongst other SF2 RNA helicases (136). The C-terminal region of RHA has three annotated domains; the helicase-associated domain 2 (HA2) that is present in select RNA helicases, the domain of unknown function (DUF), which is conserved in Ago2 and GW182 proteins (NCBI conserved domain database) and the carboxyl-terminal arginine and glycine-rich (RG-rich) domain that is methylated by the PRMT1 methylase (Figure 1) (61;123). Domain analysis determined that the bacterially purified N-terminal dsRBDs
of RHA were sufficient for binding to PCE RNA, whereas the isolated DEIH helicase core and the C-terminal domain were unable to efficiently interact with PCE RNA (136). However it is possible that amino acid residues outside of these domains may contribute to PCE recognition or regulation by the full length protein.

We hypothesize that the N-term domains of RHA binds specifically to PCE RNAs and the helicase remodels the RNP to facilitate efficient ribosome scanning to the starting AUG codon. Our goal was to identify additional residues of mammalian full length RHA that are important for interaction with PCE RNA. To this end, we developed a protein footprinting method derived from the work of Kvaratskhelia et al to map the PCE RNA interacting amino acids (272-274). Amino acid residues bound to PCE RNA are protected from modification by the lysine-specific modifier NHS (N-hydroxysulfosuccinimide)-biotin. In this study we focused on lysine residues given their positive charge, which makes them good candidates for protein-protein and protein-RNA interactions, as well as the role we identified for K54, K55 and K236 in the N-terminal domain (136).

Differential accessibility of lysine residues to biotin in free versus RNA-bound protein can be readily detected by subsequent mass spectrometric analysis of trypsin digested peptides. This technique has been used successfully to identify amino acids involved in protein-protein and protein-RNA interactions (274;275).

Our results reveal that the pattern of surface exposed lysine residues of the N-terminal domain is identical in both the recombinant purified N-terminal RHA and the mammalian purified full-length RHA. This is an indication that the conformation of the domain in the full length protein is not changed drastically in the recombinant purified
isolated domain. Further, protein footprinting of RHA with PCE RNA identified two lysine residues (K806 and K1048) that are protected. Mutation of these residues did not affect PCE binding but mutation of K806 did affect the recruitment of RHA to stress granules during oxidative stress.

**Materials and Methods**

**Cloning**

All primer sequences are described in supplementary table 1. Fl-RHA and the N-term expression plasmid pGex2TN300 were described previously in Hartman 2006 and Ranji 2010 respectively. FLRHA\(^{\text{\Delta NLS}}\), FLRHA\(^{\text{K1048E}}\), FLRHA\(^{\text{K806E}}\), FLRHA\(^{\text{\Delta NLSK1048E}}\) and FLRHA\(^{\text{\Delta NLSK806E}}\) were created using SDM with the following primer pairs and template combinations. KB1077 and KB1078 were used to create the \(\text{\Delta NLS}\) mutation in FLRHA, FLRHA\(^{\text{K806E}}\) and FLRHA\(^{\text{1048E}}\) expression plasmids. KB2048 and KB2049 were used to create the K1048E mutation in the FLRHA. KB2050 and KB2051 were used to create the K806E mutation in the FLRHA expression plasmid.

**In vitro transcription of PCE RNA**

T7 RNA polymerase was used to synthesize PCE RNA (Promega). Template DNA was generated by PCR with primers KB588 and KB664 on plasmid pYW100 (42). RNA was phenol chloroform extracted, ethanol precipitated and passed through a G25 column (Roche) to remove unincorporated nucleotides.

**Immunoprecipitation and footprinting of epitope tagged protein**

HEK 293 cells were transfected with FLAG tagged RHA expression plasmid. Cells were harvested forty-eight hours post-transfection and pelleted by centrifugation at 3500rpm.
for 3 min. Cells were lysed in Cell M lytic buffer (Sigma) supplemented with protease inhibitors for 20 min on ice, then centrifuged at 12,000 rpm for 10 min at 4°C to collect proteins. Lysates were incubated with FLAG conjugated agarose beads (Sigma) overnight at 4°C. The beads were washed twice with high salt RIPA buffer (50 mM tris pH8, 0.1% SDS, 1% tritonX, 1 M NaCl, 1% deoxycholic acid, 2mM PMSF), twice with low salt RIPA buffer (50 mM tris pH8, 0.1% SDS, 1% tritonX, 150 mM NaCl, 1% deoxycholic acid, 2mM PMSF) and twice with low salt 1X FLAG wash buffer (50 mM Hepes, 150 mM NaCl). FLAG beads were resuspended in low salt 1X FLAG wash buffer and PCE RNA (1 µM) was added to samples, and the beads were rotated for 2 h at 4°C to allow binding of PCE RNA to RHA. The beads were washed with low salt 1X FLAG wash buffer and resuspended again in low salt 1X FLAG wash buffer. 200 µM NHS-biotin (Pierce) was added where indicated and the beads were rotated for 1 h at 37°C to allow for biotin modification of exposed lysine residues. The beads were then washed and boiled in 2X SDS dye and run on a 4-15% SDS acrylamide gel. Controls without RNA or without NHS-biotin were included. RHA was visualized by microwave blue staining, excised from the gel and destained overnight in 50% acetonitrile. The gel pieces were washed three times with distilled water and vortexed in 100 % acetonitrile until gel pieces were white and shrunken. The gel slices were lyophilized in a Speed Vac at 45°C for 15 min. 0.2 µg/µl Trypsin stock was prepared by adding 10 mM HCl to modified sequencing grade trypsin (Roche). Stock solution was diluted 20-fold with 50 mM ammonium bicarbonate pH 8.0 and 50 µl of diluted trypsin was added to the gel pieces. In gel digestion of samples was carried out overnight with shaking at 250 rpm at
room temp. 100% acetonitrile was added to the samples followed by vortexing for 10 min. After spinning down the samples the solution was transferred to a new tube and the peptide mixture was dried completely using vacuum desiccation at medium heat. The samples were resuspended in 10 μl HPLC grade water and subjected to mass spec analysis.

**Analysis of N-term RHA lysine residues**

RHA N-term was expressed as purified as described in Ranji 2010. Purified N-term protein was incubated in the presence or absence of 400 μM NHS-biotin (Pierce) in 50 mM Hepes pH 8.0 at 37°C for 30 min (total volume 100 μl). The reactions were quenched using 10 μl lysine quenching solution (0.5 M sodium acetate, 0.5 M lysine) for 15 min at room temp. Ten μl of 20% SDS and 1 μl of 1M DTT (dithiothreitol) were added and the samples were incubated at 70°C for 20 min. Ten μl of 1M iodoacetamide was added and the samples were incubated at room temp for 45 min. The reaction was concentrated using a speedvac at medium heat (45°C) and loaded onto an SDS acrylamide gel. Bands were visualized by microwave blue staining, excised and processed as described above for RHA bands.

**Mass spectrometry analysis**

As discussed in McKee et al 2009, matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid in 75% acetonitrile to a final concentration of 5 mg/ml. Each sample was mixed with matrix solution and pipetted up and down to mix followed by air drying. The samples were analyzed in a MALDI-TOF instrument equipped with a curved field reflectron feature in the reflectron mode to analyze samples (Kratos Analytical
Instruments, Manchester, U.K.). Peak assignment was carried out by comparing the monoisotopic mass/charge values for the detected peaks with the theoretical profile of RHA tryptic fragments that was generated by the MS-digest program of the ProteinProspector search engine (http://prospector.ucsf.edu/prospector/mshome.htm). A mass increment of 226 Da was used to assign NHS-biotin modified peptides.

**Transfection and cell culture**

Cos7 or HEK 293 cells were grown in Dulbecco’s Modified Eagle Media (Gibco) supplemented with 10% FBS (Gibco) and 1% antibiotic (Gibco) prior to transfection. For RNA immunoprecipitation assays 2 x 10^6 cells were plated into 10 mm dishes the day prior to transfection. Five µg of RHA expression plasmids were transfected into each 10 mm dish using FuGENE 6 at a ratio of 4:1 FuGENE 6:DNA (w/v) following manufacturer’s protocol (Roche) and incubated at 37°C in 5% CO₂ for 48 h. For immunofluorescence assays, 1 x 10^5 Cos7 cells were seeded on glass coverslips into 6 well plates. After 24 h, cells were transfected with 1 µg RHA expression plasmids using FuGENE 6 as described above. For arsenite treatment 0.1 M sodium arsenite (Ricca chemical company) was added dropwise to cells to a final concentration of 1 mM 30 min prior to harvesting the cells.

**Immunofluorescence**

Twenty-four to forty-eight hours post transfection, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X100, then blocked in 10% normal goat serum (Vector Labs) for 45 min at room temperature. Cells were washed 3 times for 5 min in PBS then incubated in primary antibody solution prepared in normal antibody
dilution buffer (Scytek) for 2 h at 37°C (1:200 rabbit FLAG antisera from Sigma, 1:100 mouse anti PABP antibody from Abcam). Washes were repeated 3 times for 5 min in PBS + 0.05% Tween (Fisher Scientific) and followed by incubation in anti-mouse Alexafluor488 or anti-rabbit Alexafluor594 secondary antibody (Molecular probes) prepared in normal antibody dilution buffer for 1 h at 37°C. Cells were again washed and mounted onto glass slides on a drop of Vectashield mounting medium (Vector Labs) containing DAPI and then sealed. Cells were visualized on a Leica confocal microscope. All images are representative fields from at least two independent experiments.

**RNA coprecipitation**

Cells from two 10 mm plates were harvested per sample in PBS, resuspended in 100 µl of polysome lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES pH 7.0, 0.5% NP40, 1 mM DTT) supplemented with RNAse out (Invitrogen) and protease inhibitors (Sigma). The soluble lysate was incubated with anti-FLAG M2 monoclonal antibody-conjugated agarose beads (Sigma) equilibrated in NT2 buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM MgCl₂, 0.05% NP40) overnight at 4°C. The mRNP complexes were washed with NT2 buffer and RNA was extracted with Trizol reagent (Invitrogen) following manufacturer’s protocol. As described (36) reverse transcription (RT) reactions used random hexamer and PCR utilized junD gene-specific primers KB1303 and KB1304.

**Results**

RHA is a multidomain protein (Figure 4.1A) that stimulates the translation of PCE containing RNAs (36). Domain studies identified that features of the bacterially purified N-terminal domain of RHA are required for PCE RNA interaction. We first
compared the lysine modification profile of the isolated bacterially purified N-terminal domain of RHA (Figure 4.1B) to the lysine modification profile of the purified mammalian full-length protein (Figure 4.1C). These experiments were carried out without the addition of PCE RNA, in an attempt to discern if the N-terminal domain has a similar conformation whether expressed alone or in the context of the full-length protein. A second approach to determine the residues of full-length RHA involved in PCE binding was carried out using a protein footprinting method that identified HIV-1 integrase residues that interact with the LEDGF protein (McKee 2009) as well as Human replication protein A residues that interact with single-stranded DNA (shell 2005). Solvent-accessible lysine residues are modified with primary amine specific NHS-biotin unless they are bound to their cognate RNA, in which case these lysines are shielded from modification.

In order to identify residues of full length RHA that change their accessibility to the modifier NHS-biotin upon binding of SNV PCE RNA, we purified RHA from HEK293 cells as described (see materials and methods) and subsequently incubated the samples for 2 h at 4°C with or without SNV PCE RNA prior to NHS-biotin modification. We have previously validated these conditions for equilibrium binding between the RHA N-terminal domain and SNV PCE RNA (136). Initial optimization to determine the correct concentration of NHS-biotin was carried out by performing footprinting analysis on purified RHA N-terminal domain protein with increasing concentrations of NHS-biotin (0-1mM). Finding optimal concentrations of NHS-biotin is a requirement to avoid modification of all lysine residues, irrespective of surface exposure, which would lead to
a change in the tryptic digestion pattern from the unmodified samples. The optimal concentration of NHS-biotin was 200 µM, which was selected for all of the following experiments (data not shown).

**Lysine modification pattern is identical between RHA N-terminal and full-length RHA**

The N-terminal domain of RHA was purified from bacteria as described (136) and subjected to lysine modification to identify surface exposed lysine residues. N-terminal RHA was incubated in the absence and presence of NHS-biotin prior to processing samples as described in the methods section. The samples were separated by electrophoresis on an SDS-acrylamide gel and the N-terminal RHA bands were excised and processed for mass spectrometry as indicated in the methods section (Figure 4.1B). N-terminal RHA lysine residues modified by biotin were readily detected by mass spectrometric analysis of tryptic peptide fragments in 2 independent experiments. Figure 4.2A shows a representative MALDI-TOF spectrum of unmodified and modified N-terminal RHA tryptic fragments with mass/charge (m/z) peaks assigned. The peak with a mass of 984.6 is a biotin modified peak corresponding to residues 25-31 of RHA (K29 is biotin modified). This peak is absent in the unmodified RHA sample. There are 22 lysines in the N-terminal domain of RHA out of which 21 were detectable in peptide fragments identified by mass spectrometry (Figure 4.2B). Of these, 11 were modified with NHS-biotin (highlighted in yellow) indicating that they are surface exposed residues. RNA binding residues K54 and K55 were modified by NHS-biotin, whereas, K236 was not modified. The crystal structure for the N-terminal domain of RHA complexed with RHA
is not yet available, however the crystal structure of the second dsRBD of the *Xenopus laevis* RNA-binding protein A with double-stranded RNA is available (201). The dsRBDs are conserved in general structure amongst species. Therefore we used CN3D software (NCBI) to model the structure of the RHA dsRBDs using xlrbpA as a reference structure and located the possible positions of the conserved lysines 54 and 55 on the first dsRBD of RHA (Figure 4.2C left panel). They were predicted to be surface exposed, matching the footprinting data. Residue K236 was similarly predicted to be surface exposed on the second dsRBD of RHA (Figure 4.2C right panel), in contradiction to the footprinting data. We do not expect this residue to be surface exposed, but K236 might be exposed to the RNA after a confirmation change occurs upon the first dsRBD binding to the RNA.

Epitope tagged RHA expressed in mammalian cells was isolated by immunoprecipitation as described in the methods section. This protein is functional and binds to PCE RNA (Bolinger, Ranji and Boris-Lawrie in prep). Full length RHA was left unmodified or subjected to NHS-biotin modification as described in the methods section. The samples were separated by electrophoresis on an SDS-acrylamide gel to ensure separation of RHA from other co-purified proteins (Figure 4.1C). Biotin modified Fl-RHA runs slightly higher than unmodified Fl-RHA due to the increase in size caused by multiple lysine biotin modifications. RHA bands were excised (indicated by red triangles in Figure 4.1C) and processed for mass spectrometry as described in the methods section. The lysine modification patterns of the first 300 amino acids of the full length RHA and that of the recombinant N-terminal RHA were identical, which indicates that the
structure of the N-terminal domain does not experience detectable changes when expressed by itself (Table 1). However, it is important to note that some lysine residues present in peptide fragments from full length RHA were not detected. This strongly indicates that the conformation of N-terminal domain is similar in both the context of the full length protein and the isolated domain.

**Protein footprinting of RHA detects protected residues**

Unmodified protein, NHS-biotin modified protein and modified PCE RNA-complexed protein were subjected to SDS-PAGE and trypsin proteolysis (Figure 4.1C). SDS-PAGE ensured that the RHA fragments were well separated from co-purified proteins and mass spectrometry analysis revealed only RHA tryptic peptides. NHS-biotin modification adds 226 Da for every modified lysine present in the detected peptide. Lysine residues that are bound by RNA are protected from modification by NHS-biotin. In the samples with bound PCE RNA, this modified peak disappears or decreases in intensity. Therefore we focused on lysines that were biotinylated on free RHA but shielded from modification in the RHA-PCE complex. We identified protected lysine residues based on the appearance of peaks in the mass spectrum of biotin modified RHA that are absent in the modified RHA-PCE complex as well as the unmodified RHA sample. These peaks correspond to the mass of the tryptically digested fragment plus the mass of the biotin molecule(s) attached to the fragment. An example of a lysine protection is illustrated in figure 4.3A. The peaks with masses of 1812 Da and 1830 Da correspond to fragments expanding residues 1038-1050 (containing biotin modified K1048) and 796-809 (containing biotin modified K806) respectively in the RHA.
modified sample. These peaks are absent in the unmodified RHA and the modified RHA-PCE complex samples indicating protections. The intensity of the surrounding peaks remains similar. Figure 4.3B shows an example of a modified lysine residue that is not shielded. The peak corresponding to 1143 Da is not present in the unmodified RHA sample but appears in the modified RHA sample and is also present in the RHA-PCE complex sample. This peak represents a fragment expanding RHA residues 185-193, which contains both K191 and K193. In total, we detected 14 biotin modified residues (Table 1 and Figure 4.3C highlighted in yellow). Out of the 14 residues, 4 possible protected lysine residues (K199, K392, K806 and K1048) were detected (Figure 4.3C residues in bold and highlighted in yellow). The data was reproducible for only K806 and K1048, which led us to speculate that these other residues might be involved in transient interactions and hence are not always detected as protected residues. We detected less than 14 biotin modified lysine residues in the N-terminal RHA samples, but we had far less coverage of the full length RHA (as indicated by residues in red) compared with the coverage of N-terminal RHA (Table 1). This coverage difference is due to the limited amount of Fl-RHA we could purify from HEK293 cells. K54, K55 and K236 were not included in any of the detectable fragments (Table 1) and hence we were unable to determine their modification or protection status.
Mutation of protected lysine residues does not affect binding of RHA to junD PCE RNA

While K806 is not located within any of the annotated domains, K1048 is in the DUF domain that is usually found at the C-terminus of helicases (NCBI conserved domain database). It is possible that K806 and K1048 are contact points for the RNA once the RNA is recognized by the N-terminal domain. Hence we created mutations in RHA at these residues to see if RNA binding was affected. Lysine 806 was mutated to glutamic acid and the corresponding mutant was named FLRHA$^{K806E}$. Lysine 1048 was also mutated to glutamic acid and the corresponding mutant was named FLRHA$^{K1048E}$. Since the cellular gene junD contains a PCE at the 5’ UTR (36), we tested the junD PCE binding ability of K806E and K1048E mutants. We have previously used endogenous junD RNA for RNA immunoprecipitation assays with a mutant RHA that lacks the nuclear localization signal (NLS). This mutation changes the RHA localization from mainly nuclear to cytoplasmic. We have demonstrated that deletion of the NLS (FLRHA$^{mNLS}$) does not affect the function of RHA, while increasing the amount of cytoplasmic RHA allowing for better immunoprecipitation of the RHA-PCE RNP (Bolinger, Ranji and Boris-Lawrie in prep). While FLRHA$^{mNLS}$ can bind junD RNA, FLRHA$^{K236mNLS}$ and FLRHA$^{K54/K55/K236mNLS}$ lose the ability to bind to junD RNA (Bolinger, Ranji and Boris-Lawrie in prep). We tested the FLRHA$^{K806E\; mNLS}$ and FLRHA$^{K1048E\; mNLS}$ mutants to see if these mutants could interact with junD PCE RNA. HEK293 cells were transfected with plasmids that express FLAG-tagged RHA mutants in two independent experiments. FLRHA$^{mNLS}$ was used as a positive control and
FLRHA$^{K54/K55/K236mNLS}$ was used as a negative control. Western blot verified equivalent immunoprecipitation of FLAG-tagged RHA mutants (Figure 4.4A). Total RNA and RNA isolated from IPs with FLAG antibody were harvested and evaluated by RT-PCR with primers specific for junD. As expected, total RNA preparations verified the presence of junD mRNA (Figure 4.4B). All RHA mutants co-immunoprecipitated junD RNA, except FLRHA$^{K54/K55/K236mNLS}$. As expected the FLAG alone control did not bind junD mRNA. These data indicate that these residues are not involved in RNA binding. The more likely explanation is that they require RNA binding to alter their position or orientation for subsequent events that facilitate the translation of the bound RNA.

**Intact K806 is required for segregation of RHA to stress granules during oxidative stress**

Previous work demonstrated that mutagenesis of lysine residues of RHA involved in RNA binding (K54, K55 and K236) or ATP binding (K417) in combination with the NLS mutations results in loss of RHA activity and sequestration of RHA to stress granules (Bolinger, Ranji and Boris-Lawrie in prep). The NLS mutations were made to ensure better visualization of epitope tagged (FLAG) cytoplasmic RHA. We hypothesized that the RNA binding and ATP binding residues are required for efficient initiation of translation by RHA, and that mutations in these residues result in defective initiation complexes, which are sequestered in stress granules. Mutations in the RG-rich domain of RHA also result in a loss of RHA activity, but these mutants are not sequestered in stress granules. We determined the cellular localization of the FLRHA$^{K806E}$ and FLRHA$^{K1048E}$ mutants as well as the FLRHA$^{K806E\,mNLS}$ and
FLRHA<sub>K1048E mNLS</sub> mutants to see if they had comparable localization to wildtype RHA or FLRHA<sub>mNLS</sub>. Results indicate that localization of FLRHA<sub>K806E</sub> and FLRHA<sub>K1048E</sub> mutants is comparable to wildtype RHA (data not shown). Additionally FLRHA<sub>K806E mNLS</sub> and FLRHA<sub>K1048E mNLS</sub> cytoplasmic mutants are not segregated into stress granules, similar to FLRHA<sub>mNLS</sub> (Figure 4.5A ).

Next, we determined the cellular localization under arsenite treatment conditions, which causes oxidative stress and results in efficient stress granule localization of FLRHA<sub>mNLS</sub> but not for mutants in the RG-rich domain. We tested the effect of arsenite treatment on the FLRHA<sub>K806E mNLS</sub> and FLRHA<sub>K1048E mNLS</sub> cytoplasmic mutants in 3 replicate experiments. While FLRHA<sub>K1048E mNLS</sub> is sequestered in stress granules upon arsenite treatment, our data demonstrates that FLRHA<sub>K806E mNLS</sub> shows inefficient localization to stress granules. Approximately 50% of the cells display a non-granular phenotype (Figure 4.5B).

**Discussion**

This study aimed to identify residues of RHA that are involved in interaction with PCE RNA by protein footprinting. In distinction to previous studies using recombinant protein, our study included experiments with immunoprecipitated mammalian protein. This method is advantageous as post-translational modifications found only in the mammalian purified protein may be required for RNA interaction. In addition, we compared the lysine modification pattern of a recombinant domain of RHA with that of the full length protein. The modification pattern between the two was identical, which indicates that post-translational modifications do not seem to affect the conformation of
the recombinant domain. These important data indicate that results garnered from analysis of the interaction between the N-terminal domain and PCE RNA are applicable to full length RHA (136). In fact, preliminary unpublished data suggests that the N-terminal residues are required for binding to PCE RNA in the context of the full length protein (Bolinger, Ranji and Boris-Lawrie in prep).

Footprinting of RHA revealed two protected lysine residues previously unknown to have a function in the RHA-PCE translational regulon. While mutation of these residues did not have a discernable effect on RNA binding, we observed a difference in the cellular localization of one of the lysine mutants (K806E) upon arsenite treatment. Arsenite treatment induces oxidative stress resulting in the formation of cytoplasmic RNA granules termed stress granules (19). Several RNA-binding proteins, RHA included (Bolinger, Ranji and Boris-Lawrie in prep), are segregated to stress granules upon arsenite treatment (19;20). Previous work has demonstrated that RG residues are required for segregation of RHA to stress granules (Bolinger, Ranji and Boris-Lawrie in prep). Preliminary data suggests the RG-rich domain has interactions with Poly A binding protein (PABP) and its mutation disrupts the interaction with PABP (Jing and Boris-Lawrie unpublished). In a similar fashion K806 might be a residue critical to co-factor or PABP interaction. We hypothesize that binding of RHA to PCE via the N-terminal domain changes the conformation of the protein thus positioning K806 for interaction with a cellular co-factor. Residue K806 is located in a helix between the helicase core and the HA2 domain of RHA and is conserved in RHA orthologs across different species (APSSP2 secondary structure prediction, NCBI conserved domains database and NCBI
Blast database). Future studies will focus on identifying the function of this lysine residue in RHA-PCE interaction.

The second lysine residue identified in this study, K1048, is located in a β-sheet in the conserved DUF domain of RHA (APSSP2 secondary structure prediction, NCBI conserved domains database). This domain is conserved in RHA orthologs across different species, as well as other RNA interacting proteins, such as DHX34 and Ago2. As the name indicates, the DUF domain is not currently associated with a definitive function (NCBI conserved domains database), however it shares sequence and structural similarities with the PAM2 motif in PABP interacting proteins that is required for interaction with PABP (276;277). The GW182 proteins involved in miRNA mediated translational repression also contain a DUF domain at the C-terminus. A recent study showed that a minimal GW182 protein comprising the C-terminal DUF and RRM domains was able to mediate translational repression of an mRNA when tethered to it (278). Therefore the DUF domain of RHA might be required for interaction with PABP or other cofactors to help facilitate translation of RHA target RNAs. While we could not detect a loss of RNA interaction due to mutation of K1048, it is possible that this mutation affects the PCE translation activity of RHA. Future studies will test the effect of mutations at this position on PCE RNA translation.

This study focused on lysine residues of RHA involved in interaction with PCE RNA due to their known role in the interaction of the N-terminal domain of RHA with PCE RNA. However, other amino acid residues such as arginines may also be important RNA contact points. Future studies using arginine specific amino-acid modifiers are
warranted to identify other critical residues of RHA required for PCE translation.

Identification of residues required for RHA-PCE interaction is an important stepping stone towards understanding the mechanism of the RHA-PCE translation regulation axis. Given that RHA regulates the translation of cellular mRNAs involved in growth control, as well as retroviral RNAs, there is a need to develop targeted drugs that are effective at selectively disrupting this interaction when needed.
Figure 4.1. RHA domain structure and isolation of recombinant or immunoprecipitated RHA. (A) RHA domains are illustrated and amino acids discussed in this study are marked. (B) SDS-PAGE of recombinant N-terminal RHA (amino acids 1-300) visualized by coomassie staining. Excised band is indicated with a red triangle. (C) SDS-PAGE of immunoprecipitated RNA visualized by coomassie staining. Excised bands are indicated with red triangles.
Figure 4.2. Identification of surface exposed lysine residues of recombinant N-terminal RHA. (A) Mass spectrometric data showing a biotin modified peak (indicated by the red arrow) in the RHA + Biotin sample. (B) Amino acid sequence of the N-terminal domain with red lettering indicating amino acids that were in detected peptides and highlighted in yellow are amino acids that are biotin modified. (C) Model of the N-terminal dsRBD1 (left panel) and dsRBD2 (right panel) obtained using NCBI cn3D software and the crystal structure of the *Xenopus laevis* RNA-binding protein A as a reference structure. The positions of K54, K55 and K236 in this model are indicated.
Figure 4.2. Identification of surface exposed lysine residues of recombinant N-terminal RHA.
Figure 4.3. Mass spectrometry of biotin modified full length RHA in complex with PCE reveals protected residues. (A) Mass spectrometric data showing two protected peaks (indicated by red arrows) in the biotin + RHA samples that are absent in the unmodified RHA or modified RHA+ PCE complexes. (B) Mass spectrometric data showing a biotin modified peak that is not protected by the addition of RNA (indicated in red) (C) Amino acid sequence of full length RHA with red lettering indicating amino acids that were in detected peptides and highlighted in yellow amino acids that are biotin modified. Protected residues are highlighted in yellow and bolded
Figure 4.3. Mass spectrometry of biotin modified full length RHA in complex with PCE reveals protected residues.
Figure 4.3. continued

C

<table>
<thead>
<tr>
<th></th>
<th>mgdvknflya wcgkrtmtps yeiravgnkn rqkfmcvevqv</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>egnytgmgn stnkdaqsn aardfynylv rineikseev</td>
</tr>
<tr>
<td>81</td>
<td>pafgvasppp ltdtpdttae aegdlpttmg gplpphlalk</td>
</tr>
<tr>
<td>121</td>
<td>aennsevgas ggyvpptwdr ganlddyys rkeeqevqat</td>
</tr>
<tr>
<td>161</td>
<td>leseevdlna ghgnwtlen akarlqnyfq kekiqgeyky</td>
</tr>
<tr>
<td>201</td>
<td>tqvpgdhnrs fiaemtiyik qlgrrifer hgsnkkaaq</td>
</tr>
<tr>
<td>241</td>
<td>scalslvqgl yhlqveays gltckkeget vepykvlnsq</td>
</tr>
<tr>
<td>281</td>
<td>dlehqlqnii qelnleilpp pedpsvval nigklaqfep</td>
</tr>
<tr>
<td>321</td>
<td>sqrstqyvqyv pwsppqswhn pwtsnideq pfafatpeqi</td>
</tr>
<tr>
<td>361</td>
<td>smdlknelmy qleqdhdlqa ilqerekkpv kkesfesilea</td>
</tr>
<tr>
<td>401</td>
<td>isqnsnvviir gatcggktcq vphqliddfi qndraaecli</td>
</tr>
<tr>
<td>441</td>
<td>vvtqprrisa vsvaerveafe rgeepkscg ysvrfesilp</td>
</tr>
<tr>
<td>481</td>
<td>rphasimfct vgrlrlkeaa gigrshivv ideherdint</td>
</tr>
<tr>
<td>521</td>
<td>dfllvvlrdv vqaypevriv lmsatidtsm fceyffncpi</td>
</tr>
<tr>
<td>561</td>
<td>ieveygtypv qeyfledcig mthfvpdppkk kkkkkdckddg</td>
</tr>
<tr>
<td>601</td>
<td>geddancn1 igcdeygprr rsmsqlneq etpfeileal</td>
</tr>
<tr>
<td>641</td>
<td>lkyietlnvp gavlrvlpww nliytmqkhl emnpfghsr</td>
</tr>
<tr>
<td>681</td>
<td>yqilplhsqi preeqrvkvd pvpvgtvki lstniaetsi</td>
</tr>
<tr>
<td>721</td>
<td>tindvvyvid sckqvkrlfi ahnnmtnyat wwashtnleq</td>
</tr>
<tr>
<td>761</td>
<td>rgraqrvrp gfcflhcsra rferlehtmt pemfrtple</td>
</tr>
<tr>
<td>801</td>
<td>ialsikllrl ggiggflaka iepppldavi eaehtrleld</td>
</tr>
<tr>
<td>841</td>
<td>alddandeltp lgrilalpli eprfgkmmim gcifyvgdai</td>
</tr>
<tr>
<td>881</td>
<td>ctiaaatcfp enefigkrl giyhrnfagn rphshvalls</td>
</tr>
<tr>
<td>921</td>
<td>vfqawddarm ggseeirfcr ehkrlnmatal rmtweaqvkl</td>
</tr>
<tr>
<td>961</td>
<td>keilingsgfp edclltqvft ntgpdnhldv visllafgy</td>
</tr>
<tr>
<td>1001</td>
<td>pnvzychkekr kittegra lihkssvncp fssqdmkyps</td>
</tr>
<tr>
<td>1041</td>
<td>pfvfgekirc traisamt gvtplqliff askkvqsgdgq</td>
</tr>
<tr>
<td>1081</td>
<td>ivlvdwidk qisheaaaci tglraameal vvevtkqpai</td>
</tr>
<tr>
<td>1121</td>
<td>isqldpyvenr mlmrrqisr psaaglnmli gstrygddgor</td>
</tr>
<tr>
<td>1161</td>
<td>ppkmroydng sgyrggssy sggygggygys sggygsagy</td>
</tr>
<tr>
<td>1201</td>
<td>gsansfragy gagvggyrg vrsrgfrqns ggdyrgpsq</td>
</tr>
<tr>
<td>1241</td>
<td>yrsggfgqrg gggrggtgy fggqrggggyy</td>
</tr>
</tbody>
</table>

167
Figure 4.4. Mutation of protected residues does not affect binding of RHA to PCE RNA

Representative immunoprecipitation (IP) assay to test if RHA mutants bind to endogenous junD PCE RNA. Empty FLAG plasmid or indicated RHA mutant expression plasmids were transfected into HEK 293 cells and FL-tagged proteins were subject to IP with FLAG antibody (mouse) conjugated to agarose beads. Immunoblot of equivalent aliquots of agarose beads determined the IP efficiency was similar for each protein. RNA was harvested from the remaining beads and subjected to RT-PCR with junD primers. (A) Western blot with Flag-specific antiserum (rabbit) detected IP was similar for all the mutants. (B) PCR amplification products from indicated RNA preparation incubated with or without reverse transcriptase (RT+ or -). Representative samples of total RNA and RNA isolated from IP samples were evaluated with junD-primers. The junD amplification product is 232 bp.
Figure 4.4. Mutation of protected residues does not affect binding of RHA to PCE RNA
Figure 4.5. K806 mutation diminishes the recruitment of RHA to stress granules upon oxidative stress. Cos7 cells were transfected with indicated RHA mutant plasmids. Forty-eight hours post transfection, cells were treated with arsenite for 30 mins where indicated. Cells were subjected to immunofluorescence staining with Flag antisera and AlexaFluor594 (pseudo-colored red) as well as PABP antisera and AlexaFLuor488 (pseudo-colored green). DAPI was used as a nuclear stain (blue). (A) Confocal images of FLRHA<sup>mNLS</sup>, FLRHA<sup>K806EmNLS</sup> and FLRHA<sup>K1048EmNLS</sup> mutants in the absence of arsenite treatment. (B) Confocal images of FLRHA<sup>mNLS</sup>, FLRHA<sup>K806EmNLS</sup> and FLRHA<sup>K1048EmNLS</sup> mutants with arsenite treatment.
Figure 4.5. K806 mutation diminishes the recruitment of RHA to stress granules upon oxidative stress.
<table>
<thead>
<tr>
<th>Lysine(K) amino acid number</th>
<th>Lysine in a detected peptide (N-term analysis)</th>
<th>Lysine in a detected peptide (Full length analysis)</th>
<th>Biotin modified (N-term analysis)</th>
<th>Biotin modified (Full length analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>29</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>33</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>54</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>76</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>120</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>146</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>152</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>182</td>
<td>√</td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>191</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>193</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>199</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>220</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>235</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>236</td>
<td>√</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>264</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>265</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>266</td>
<td>√</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>275</td>
<td>-</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.1. Comparison of the modified lysine residues of the RHA N-terminal recombinant domain and the mammalian purified full-length Fl-RHA. All N-terminal lysines residues are indicated by the amino acid number. √; indicates these amino acids are detected in peptide fragments (in columns 2 and 3) or are modified by NHS-Biotin (in columns 4 and 5). - ; indicates that these amino acids are not detected in peptide fragments (in columns 2 and 3) or are not modified by NHS-Biotin (in columns 4 and 5). Tan shading indicates similar results from the RHA N-term and RHA full length analysis.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB1077</td>
<td>CGTCCTCCCATGGCCTACGACAATG</td>
</tr>
<tr>
<td>KB1078</td>
<td>CATTGTCGTAGGCCATGGGAGGACG</td>
</tr>
<tr>
<td>KB 2048</td>
<td>GTATTTGGTGAAGAGATTGCAACTCG</td>
</tr>
<tr>
<td>KB 2049</td>
<td>CGAGTTCGAATCTTCACCAAATAC</td>
</tr>
<tr>
<td>KB 2050</td>
<td>GCTCTTAGCATAAGAATTCTGCGGC</td>
</tr>
<tr>
<td>KB 2051</td>
<td>GACGCAGAAGTTCTATGCTAGAGC</td>
</tr>
<tr>
<td>KB 588</td>
<td>GTACTACGGATTCAGTCCGG</td>
</tr>
<tr>
<td>KB 664</td>
<td>AAATCTAGACGATAGAATTCTAATACGACTCACTATAGGGGTCGGCCGTCCTACACATTGTGTT</td>
</tr>
<tr>
<td>KB1303</td>
<td>GTCGCCCCATCGACATGGACACG</td>
</tr>
<tr>
<td>KB1304</td>
<td>GCCGCTGTGACGTTGAGGCTGAGG</td>
</tr>
</tbody>
</table>

Table 4.2. Sequence of DNA oligonucleotides used in indicated experimental procedure
Chapter 5. Perspectives

Molecular basis of RHA recognition and translation of selected complex mRNAs

This dissertation contributes new understanding of the mechanism of translational modulation of PCE-containing complex mRNAs by the DEIH box helicase RHA. The outcomes open new issues and perspectives to fully understand the regulatory potential of the 5’ untranslated region in expression of the proteome. We have proven that the DExD/H box helicases play a strategic role in regulating complex mRNAs, including pathogenic viruses and cellular proto-oncogenes.

Domain studies showed that the N-terminal domain of RHA specifically recognizes PCE RNA of both viral and cellular origin, in preference to other non-specific double stranded RNAs (136). The conserved lysine residues present in both domains are contact points with the RNA and mutation of these residues destroys interaction of the N-terminal domain with PCE RNA (136). These residues are necessary, but may not be sufficient for selective interaction with PCE RNAs. Therefore, other residues in the N-terminal domain that are not conserved amongst other dsRBDSs may also contribute to specific recognition of PCE. Protein kinase R (PKR) also contains two dsRBDSs at the N-terminus, which bind RNA (206;279;280); this interaction induces dimerization and activation of the kinase domain. Thus, the dsRBDSs of PKR cooperate upon interaction
This arrangement is similar in principle to cooperative binding of two dsRBDs of RHA with PCE RNA (136). Unlike RHA, PKR can be strongly activated by poly(I) poly(c) dsRNA of particular length, and does so without structural or sequence constraints (190;281). Further, PKR dsRNA binding depends on the length of the dsRNA (282). I postulate that, substitution of one, or both dsRBDs of RHA with the dsRBD of PKR would eliminate RHA specificity for PCE RNAs and broaden mRNAs subject to RHA translational control. An alternative outcome is sustained specificity for PCEs, which is due to other residues of RHA, and which contributes to PCE target specificity. In this situation, specific binding to PCE target mRNA would be retained and future mutagenesis would define the biophysical basis for the RNA-Protein interaction.

Our overarching hypothesis is that RHA is required to supplement the helicase activity of eIF4A during formation of the translation initiation complex. Attributable to the complex RNA structural features, RHA and eIF4A partner to facilitate remodeling of the 5’ UTR for efficient ribosome scanning. Both the RNA recognition and ATP-dependent helicase activity of RHA are required and their mutation produces a stalled translation initiation complex, which culminates in RHA localization to stress granules. Further evidence that RHA interacts with eIF4A in the initiation complex is that treatment with the eIF4A inhibitor hippocristanol localizes RHA to stress granules (Ranji and Boris-Lawrie, unpublished). Future co-precipitation experiments will assess the composition of the initiation complex assembled on the RHA RNP and confirm RHAs interaction with eIF4A and possibly other initiation factors. Preliminary mass spectrometry analysis of RHA binding partners in our lab has identified relevant
translation initiation factors and also post-initiation factors (Jing and Boris-Lawrie, unpublished).

Our studies indicate that RHA N-term and helicase mutants are sequestered in stress granules, and may be independent of interaction with PCE-mRNAs. Therefore, the location of PCE RNAs during the stalling still remains an open question. Possibilities are that more sensitive assays are needed to detect PCE RNAs that remain associated with RHA during localization to stress granules; alternatively, PCE RNAs remain in post-initiation complexes that are polysome associated, but produce unstable polypeptides. Two complementary approaches to address these possibilities are: 1. Fluorescence in-situ hybridization to detect PCE RNAs localized with stress granule markers. 2) Sucrose density gradient fractionation and RT-quantitative PCR for endogenous PCE RNAs upon transfection with selected RHA mutants.

The C-terminal (RG-rich) domain of RHA is emerging as a functionally distinct lynchpin in regulating RHA translational activity. In spite of the loss of RHA translational activity, FLRHA RA9 remains on polysomes. RG-rich domains are known to be involved in protein-protein interaction with hnRNPA1 and nucleolin (191-193). and we have identified another candidate cofactor, poly A binding protein (PABP). PABP protects the poly A tail of mRNAs from exonuclease degradation. In addition, PABP interacts with eIF4G bound to the 5’ cap of the mRNA. The eIF4G-PABP interaction tethers the RNA termini in a circular configuration that is prone for efficient re-initiation of the scanning ribosomes (13). In vitro and co-immunoprecipitation experiments determined that RHA interacts with PABP, and the RG-rich domain is required for this
interaction (Jing and Boris-Lawrie, unpublished). The interaction between RHA at the 5’ terminus and PABP at the 3’ terminus may bolster interaction with eIF4G to promote efficient re-initiation. RHA interaction with PABP was recently identified on the picornavirus genomic RNA in experiments with foot-and-mouth disease virus (FMDV) (134). RHA co-immunoprecipitates with PABP, FMDV 2C and 3A structural proteins and the FMDV 5’ UTR. These redundant interactions of FMDV RNA and proteins implicate an important role for RHA in the replication of this picornavirus (134). Of particular interest is the fact that FMDV infection results in relocalization of RHA from the nucleus to the cytoplasm. We speculate that FMDV requires RHA in the cytoplasm to bolster viral replication while at the same time reducing its nuclear presence as a transcription factor of cellular anti-viral genes RHA has also been proposed to circularize the 5’ and 3’ UTRs of the bovine viral diarrhea virus RNA; this activity may operate a switch between genome replication and translation (132).

Experimental evidence also points to a possible role for the C-terminal domain in a post-initiation step of translation of PCE RNAs by RHA. Mechanistically, our data posit that C-term mutations eliminate interaction with cofactors needed for post-initiation steps in PCE RNA translation. For instance disruption of interaction of RHA with an elongation factor would slow ribosome transit and decrease polypeptide synthesis. Furthermore, improper interaction with ribosome release factors is known to result in stop codon readthrough and results in non-sense protein production. Nonsense protein production triggers degradation of the polypeptide. A necessary first step is co-

177
immunoprecipitation approaches to verify the MS data that RHA binds to elongation factors 1A and 1B given reliable antibody reagents.

An additional possibility is that RHA C-terminal residues are necessary to counter the activity of inhibitory cofactors or miRNA. We posit that mutation of RHA C-terminal residues changes conformation features necessary to derepress the cofactor or miRNA. We draw on the literature that miRNAs inhibition of protein synthesis occurs at a post-initiation stage; mechanisms are inhibiting ribosome elongation or co-translational protein degradation (262). Prior unpublished data from the lab using PCE RNAs that contain a 3’ UTR that appears to lack miRNA target sites were resistant to C-terminal mutations (Bolinger and Boris-Lawrie, unpublished). Future work will test if miRNA binding or miRNA-RNP activity is disrupted by RHA binding to the RNA.

Protein footprinting tested residues of FL-tagged RHA that interaction with PCE RNA in cells. We defined two previously uncharacterized residues of RHA at K806 and K1048 that are involved in interaction with PCE RNA. Because K to E mutations did not eliminate co-immunoprecipitation of PCE RNA, we expect that these residues are not critical contact points. Instead, we speculate that initial PCE binding of RHA induces conformational changes in RHA that expose distal residues including K806 and K1048, that affect subsequent steps in RHA translational activity. PCE translation assays will be informative to this point. Finally, consistent with this working model K806A diminished stress induced relocation of RHA to stress granules, similar to the RA9 C-terminal mutant. Future studies to identify the relevant binding partners of RHA will consider the possible role of arginine methylation in modulating cofactor interactions. Follow up
protein footprinting studies will shed light on relevant arginine residues of RHA that contact PCE RNA.

**Translational modulation by RHA: Model of genetically separable initiation and post-initiation mechanisms**

Based on our experimental data, we propose a two component mechanistic model for RHA translational activity on PCE mRNAs (Figure 5.1A). Under steady state conditions, N-terminal residues of RHA recognize and bind to PCE in the 5’ UTR of target RNAs. RNA binding and proximity to eIF4A provides ATP-dependent helicase activity to remodel the PCE RNP complex. Sufficient helicase activity culminates in efficient polysome loading of PCE RNA in the cytoplasm. RHA affinity for PABP strengthens the circular polysome. If the N-terminal residues are mutated, RHA is unable to bind to its target RNA, but remains associated with eIF4A and PABP in an abortive initiation complex that is sequestrated to stress granules. Likewise, mutation of the ATP-binding residue results in abortive initiation. While PCE is bound to RHA, energy is unavailable to remodel the RNP and the defunct initiation complex is sequestered to stress granules.

By contrast, C-terminal residues manifest translation elongation phenotypes (Figure 5.1B). C-terminal R to A mutations or deletions result in stalled polysome complexes and a lack of detectable polypeptide. Oxidative stress induces stress granule sequestration of FL-RHA but not FL-RHA C-terminal mutants, or cells treated with an inhibitor of R methylation (adox). The latter suggests that PRMT1-mediated methylation of C-term residues or necessary co-factors of C-term are subject to methylation for their localization to stress granules. Under steady state conditions, RHAs N-terminal residues recognize
and bind to PCE in the 5’ UTR of target RNAs. Upon RNA binding, the helicase residues assist eIF4A to remodel the PCE RNP complex, which allows efficient polysome loading of PCE RNA in the cytoplasm and culminates in efficient mRNA circularization. If the N-terminal residues are mutated, RHA is unable to bind to its target RNA, but it may bind other translation initiation factors resulting in abortive initiation complexes. This in turn leads to reduced translation and sequestration to stress granules. Similarly, if the ATP-binding residue is mutated, RHA can bind to RNA, but is unable to remodel the RNP and therefore, like the N-terminal mutants, shows reduced translation and is sequestered in stress granules. The C-terminal residues are required for efficient translation elongation and/or efficient mRNA circularization. Mutation of the C-terminal RG residues results in a post-initiation defect, thereby leading to stalled polysome complexes, and reduced translation of the RNA. Addition of arsenite causes RHA to localize to stress granules, but this localization to stress granules is not efficient in mutants with changes in the RG-rich domain or if the cells are treated with adox. The latter suggests that PRMT1-mediated methylation of C-term residues or co-factors that bind the C-term are important for transport of RHA to stress granules.

**RHA is implicated as a target for disease treatment**

We speculate that RHA will be determined as a useful candidate to target anti-viral, anti-autoimmune and anti-cancer therapies. Our research to define the mechanism of RHA translational regulation is vital to inform execution of such therapies. RHA is involved in the replication cycles of retroviruses, picornaviruses and flaviviruses (226) (described in chapter 1) which are important targets for anti-viral therapy. RHA is
implicated in autoimmune disease because autoantibodies to RHA are a serological marker for systemic lupus erythematosus (SLE), and these antibodies correlate with higher incidence of SLE in young or non-African-American patients (119;283;284).

Multiple studies link RHA to cancer progression. 1) Our lab has shown that RHA translationally regulates the growth control gene junD; we will test if dysregulation by RHA contributes to cell transformation (36). 2) Two connections of RHA to breast cancer have been reported: the ability of RHA to interact with the breast cancer susceptibility gene BRCA1 (63), and the identification of six variants of the RHA gene in a cohort of French-Canadian families with familial breast cancer (209). Bioinformatics analysis of the mutations in the breast cancer cohort predicted that some of them are deleterious to RHA function (209). 3) RHA binds to the promoter of the multidrug resistance gene MDR1 and enhances expression of MDR1, which contributes to resistance of a broad range of cancer drugs in drug-resistant cells (66). Further, RHA is constitutively phosphorylated in drug-resistant cells by DNA-PK and this phosphorylation is required for MDR1 promoter activation (285). Inhibition of DNA-PK, by a small molecule termed DMNB, reduces the MDR1 promoter activity in drug-resistant leukemia cells (286). 4) Finally, RHA binds to the EWS-FLI1 oncprotein that is expressed in Ewing’s Sarcoma tumors. RHA interaction with the EWS-FLI1 gene promoter to enhances transcription of this oncogene (287). Downregulation of RHA and the inhibition of RHA interaction with EWS-FLI1 reduced the viability of Ewing’s sarcoma tumor cells, implicating RHA as a tool to counter the sarcoma (208).
RHA is involved in many processes in the cell

In addition to transcriptional regulation and translational regulation, a recent study implicated RHA in the innate immune response in plasmacytoid dendritic cells (121). RHA acted as a bacterial DNA sensor to trigger the production of interferons and interferon stimulated genes of the antiviral innate response (121). We speculate the relocalization of RHA to the cytoplasm during FMDV infection may invoke RHA activity in innate sensing. Because the DHX30 has a similar domain structure to RHA we speculate this related RNA helicase plays a redundant role in innate sensing in other cell types.

Mounting evidence is revealing the intricate involvement of RHA in well-controlled processes in the cell. Moving ahead, an important open issue is to define how and when RHA activity is regulated, based upon cell type and conditions in the cell. RHA may be affected by cellular compartmentalization, transcriptional control, posttranscriptional gene expression, and post-translational modifications. Prior studies have shown that RHA can be cleaved by caspase during apoptosis (119). We have defined that serum deprivation of cells increases active caspase, concomitant with a decrease in RHA and JunD protein levels (unpublished Sharma and Boris-Lawrie). The N-terminus of RHA is released by caspase cleavage (unpublished Picking and Boris-Lawrie). This outcome is downregulation of RHA activity on target RNA. Therefore, cleavage of RHA by caspase is one possible mechanism to regulate the activity of RHA under certain conditions. Another possible mechanism is the activity of activated PKR on RHA. PKR was shown to phosphorylate the N-terminus of RHA and was shown to
impair binding of N-term of RHA to dsRNA (288). In conclusion, RHA is a ubiquitous player in gene expression, innate response and viral replication and understanding the regulation of RHA is important to these broad areas of cell biology. The new understanding of translational regulation of PCE-containing genes by RHA in this dissertation is an essential contribution to the comprehensive understanding of how RHA co-ordinately modulates multiple processes in viruses and their host cells.
Figure 5.1. Overview of effects of mutations of RHA functional domains
Cytoplasmic RHA is polysome associated and results in robust PCE RNA translation. Mutation of the N-terminal RNA binding residues or the helicase ATP-binding residue results in a reduction in polysome association, loss of PCE RNA translation and sequestration in stress granules. Mutation in the RG-rich domain retains polysome association but results in a loss of PCE RNA translation. Arsenite treatment causes cytoplasmic RHA to shuttle to stress granules; localization to stress granules is reduced if mutations of the RG-rich domain are made in cytoplasmic RHA. Solid red lines indicate that these mutants shuttle to stress granules in the absence and presence of arsenite. The dashed red line indicates that this mutant shuttles to stress granules only in the presence of arsenite.
Reference List


187

188
Ref Type: Generic


192


