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CHAPTER NINETEEN

Determination of Host RNA Helicases Activity in Viral Replication

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Abstract

RNA helicases are encoded by all eukaryotic and prokaryotic cells and a minority of viruses. Activity of RNA helicases is necessary for all steps in the expression of cells and viruses and the host innate response to virus infection. Their vast functional repertoire is attributable to the core ATP-dependent helicase domain in conjunction with flanking domains that are interchangeable and engage viral and cellular cofactors. Here, we address the important issue of host RNA helicases that are necessary for replication of a virus. This chapter covers approaches to identification and characterization of candidate helicases and methods to define the biochemical and biophysical parameters of specificity and functional activity of the enzymes. We discuss the context of cellular RNA helicase activity and virion-associated RNA helicases. The methodology and choice of controls fosters the assessment of the virologic scope of RNA helicases across divergent cell lineages and viral replication cycles.

1. Introduction

1.1. RNA helicases are ubiquitously active at the virus–host interface

Viruses are intracellular parasites that require many of the same essential processes as their host. All eukaryotic cells require the function of RNA helicases in all processes involving RNA (Jankowsky and Jankowsky, 2000; Linder, 2006; Linder and Jankowsky, 2011). To date, the activity of 13 host-encoded helicases and three virus-encoded RNA helicases in virus biology has been documented (Table 19.1). The scope of activities in all steps of viral replication and the interface with the antiviral host response demonstrates their remarkable versatility and potential utility as antiviral targets (Jeang and Yedavalli, 2006; Linder and Jankowsky, 2011; Ranji and Boris-Lawrie, 2010).

RNA helicases provide malleable connections between a viral infection and the host innate response (Ranji and Boris-Lawrie, 2010). In some viral infections, RNA helicase activity benefits the virus by promoting viral gene expression and squelching the antiviral response. In others, RNA helicase activity benefits the host by sensing viral nucleic acid and triggering antiviral response. The mechanisms that explain the versatility of RNA helicases are important to define but poorly understood. Experiments to determine the role of RNA helicases in viral replication are the focus of this chapter and stand from the perspective of the virus. We address RNA helicase activity in the steps of viral replication: gene expression, morphogenesis, and replication of viral nucleic acid. The complete elucidation of the role of RNA helicases in viral replication and the pathogenesis of disease is a priority in biomedical research.

Table 19.1 Overview of the role of cellular RNA helicases in virus replication

Cellular RNA helicase superfamily member	Virus	References
DDX1	Human immunodeficiency virus type 1 (HIV-1)	Edgcomb *et al.* (2011), Fang *et al.* (2004, 2005), and Robertson-Anderson *et al.* (2011)
	John Cunningham virus (JCV)	Sunden *et al.* (2007)
	Infectious bronchitis virus	Xu *et al.* (2010)
DDX3	HIV-1	Garbelli *et al.* (2011), Ishaq *et al.* (2008), Liu *et al.* (2011), and Yedavalli *et al.* (2004)
	Vaccinia virus	Kalverda *et al.* (2009) and Schroder *et al.* (2008)
	Hepatitis B virus (HBV)	Wang and Ryu (2010), Wang *et al.* (2009), and Yu *et al.* (2010)
	Hepatitis C virus (HCV)	Angus *et al.* (2010), Ariumi *et al.* (2007), Chang *et al.* (2006), Oshiumi *et al.* (2010), and Owsianka and Patel (1999)
DDX5/p68	HCV	Goh *et al.* (2004)
	SARS coronavirus (SARS-CoV)	Chen *et al.* (2009)
DDX6/Rck/p54	HCV	Ariumi *et al.* (2011), Jangra *et al.* (2010), Miyaji *et al.* (2003), and Scheller *et al.* (2009)
	Dengue virus (DENV)	Ward *et al.* (2011)
	Adenovirus	Greer *et al.* (2011)
	Retroviruses: HIV-1, prototype foamy virus (PFV)	Chable-Bessia *et al.* (2009) and Yu *et al.* (2011)
DHX9/RHA	Retroviruses: HIV-1, human T-cell leukemia virus type 1, bovine leukemia virus, spleen necrosis virus, feline leukemia virus, Mason-Pfizer monkey virus (MPMV)	Bolinger *et al.* (2007, 2010), Fujii *et al.* (2001), Hartman *et al.* (2006), Li *et al.* (1999), Ranji *et al.* (2011), Roy *et al.* (2006), Sadler *et al.* (2009), Tang and Wong-Staal (2000), and Westberg *et al.* (2000)

(*Continued*)

Table 19.1 (*Continued*)

Cellular RNA helicase superfamily member	Virus	References
	Herpes simplex virus 1 (HSV-1)	Kim *et al.* (2010)
	Bovine viral diarrhea virus	Isken *et al.* (2003)
	HCV	He *et al.* (2008)
	Foot and mouth disease virus	Lawrence and Rieder (2009)
	Kaposi sarcoma-associated herpesvirus (KSHV)	Jong *et al.* (2010)
DDX24	HIV-1	Ma *et al.* (2008) and Roy *et al.* (2006)
DHX30	HIV-1	Zhou *et al.* (2008)
DDX41	HSV-1	Zhang *et al.* (2011)
DDX56	West Nile virus	Xu *et al.* (2011)
DDX60	Vesicular stomatitis virus (VSV), poliovirus, Sendai virus (SeV), HSV-1	Miyashita *et al.* (2011)
Mov10	Hepatitis delta virus (HDV)	Haussecker *et al.* (2008)
	Retroviruses: HIV-1, simian immunodeficiency virus, murine leukemia virus, feline immunodeficiency virus, equine infectious anemia virus	Abudu *et al.* (2011), Burdick *et al.* (2010), Furtak *et al.* (2010), and Wang *et al.* (2010)
RH116	HIV-1	Cocude *et al.* (2003)
UAP56	Influenza A virus	Kawaguchi *et al.* (2011), Momose *et al.* (2001), and Wisskirchen *et al.* (2011)
	KSHV	Majerciak *et al.* (2010)

1.2. RNA helicases exhibit enzymatic and nonenzymatic functions

Viral and cellular RNA helicases uniformly display modular, genetically separable, catalytic, and scaffold domains (Ranji and Boris-Lawrie, 2010). Some RNA helicases exhibit processive, ATP-dependent unwinding activity on nucleic acid, while others unwind RNA duplexes in a nonprocessive but also ATP-dependent fashion (Linder and Jankowsky, 2011). RNA helicases have also been shown to remodel ribonucleoprotein complexes (RNPs; Fuller-Pace, 2006; Jankowsky and Bowers, 2006; Linder, 2006; Linder *et al.*, 2001). RNP remodeling can be accomplished independent of

duplex unwinding (Jankowsky and Bowers, 2006). In addition, RNA helicases can function as binding partners of other proteins, and these interactions are not always dependent on catalytic helicase or ATPase activities (see Chapter 16).

Therefore, it is important to devise experiments that aim to distinguish functions of RNA helicases that require enzymatic capacity (e.g., unwinding, RNP remodeling, ATPase) from nonenzymatic functions (e.g., protein-binding partners).

1.3. The experimental design to characterize the role of an RNA helicase in viral replication is focused on four issues

Elucidation of RNA helicase activity in replication of viruses has the potential to produce important fundamental information broadly significant to cell biology and unveil newly appreciated targets for therapeutic drugs (Ranji and Boris-Lawrie, 2010). Four critical issues are:

(1) To determine whether the candidate RNA helicase is necessary for viral replication,
(2) To define features of helicase that are necessary for selective and specific recognition of viral RNA and viral protein,
(3) To measure the biochemical and biophysical requirements for productive interaction,
(4) To document whether the candidate RNA helicase is a component of viral particle and potential to antagonize induction of the antiviral state in target cells.

These issues enumerate the scope of this chapter. The experimental methods discussed have been utilized to demonstrate RNA helicase A (RHA) is an important host factor in viruses that infect humans and animals. RHA activity has been demonstrated in retroviruses, hepatitis C virus, foot and mouth disease virus, and bovine viral diarrhea virus (Bolinger *et al.*, 2007, 2010; Hartman *et al.*, 2006; He *et al.*, 2008; Isken *et al.*, 2003; Lawrence and Rieder, 2009).

2. Methods Used To Study Cell-Associated RNA Helicase in Cultured Mammalian Cells

2.1. Downregulation with siRNA and rescue by exogenous expression of the RNA helicase

This method was employed to evaluate whether RHA is necessary for efficient translation of retroviruses (Bolinger *et al.*, 2007, 2010; Hartman *et al.*, 2006). The first component of the experiment is transfection with siRNA complementary to the RHA mRNA or a nonsilencing control

siRNA that is a scrambled (Sc) sequence lacking any match in Genbank (sequences for siRNAs are delineated below). Transfections were performed in HEK293, COS7, or HeLa cells. The second component of the experiment is evaluation of rescue by exogenously expressed siRNA-resistant epitope-tagged RNA helicase (e.g., FLAG-RHA). Taken together, these components test whether a cellular factor is necessary for viral activity. Furthermore, the evaluation of mutant siRNA-resistant epitope-tagged RHA reveals alleles that are sufficient for activity. RHA downregulation studies in HEK293 cells demonstrated that RHA is necessary for efficient translation of HIV-1 mRNA. The rescue observed by siRNA-resistant FLAG-RHA but not a mutant allele (K417R) deficient in the ATP binding determined the necessary role of ATP-dependent helicase activity in viral mRNA translation (Bolinger *et al.*, 2010).

The advantage of siRNA/shRNA downregulation is the ability to investigate the necessary and sufficient role of an RNA helicase. The major caveat to siRNA downregulation is the potential for off-target effects. Two sets of controls are requisite to address this issue. First, evaluation of two or more distinct siRNA that target different sequences is necessary. Likewise, at least two different shRNA vectors are standard in experiments that select cells with long-term downregulated helicase. In either case, similar results of downregulation and rescue are evidence of targeted downregulation of the gene of interest. In either case, the loss-of-function phenotype is rescued by exogenously expressed RNA helicase.

Second, it is important to determine whether the siRNA effect is selective and not secondary to collateral damage to cell viability, steady state mRNA, global protein synthesis, or another biological process. An approach to evaluate the potential effect of helicase downregulation within the time frame of siRNA treatment on cellular proliferation is a colorimetric MTT assay. Metabolic labeling is useful to assess global mRNA synthesis and stability or global protein synthesis. Briefly, cells are incubated with [^{3}H]-uridine or [^{35}S]-cysteine/methionine to label newly synthesized RNA or proteins, respectively. Levels are determined by precipitation with trichloroacetic acid and scintillation. These assays determined that cellular proliferation, mRNA levels, or translation are not significantly increased or decreased upon downregulation of RHA for 48 h (Hartman *et al.*, 2006).

2.1.1. Method for RHA silencing with siRNA and rescue

1. Incubate 1×10^{6} HEK293 or COS7 cells in a 100-mm dish.
2. Incubate overnight and then perform siRNA treatment:

siRNA preparation: For 1 plate,

- Combine 120 µl OptiMEM media (Gibco) + 20 µ*M* siRNA in a sterile microfuge tube.
  i. Tube 1: RHA target #1 and #2; 20 µ*M* combined

ii. Tube 2: Sc siRNA

siRNA sequences:
RHA target #1: UAGAAUGGGUGGAGAAGAAUU
RHA target #2: GGCUAUAUCCAUCGAAAUUUU
Sc siRNA: UAGACUAGCUGACGAGAAAUU

- Combine 1.8 ml OptiMEM media and 50 μl oligofectamine (Invitrogen) in 15-ml sterile tube.
- Incubate at room temperature for 15 min.
- Add the siRNA–OptiMEM mix to the 15-ml tube and incubate at room temperature for 25 min. During this incubation, remove media from the cultured cells and wash twice with DMEM that is not supplemented with FBS or antibiotics. Add 5 ml DMEM.
- After the 25-min period, add the siRNA mix to cultured cells; Incubate for 3–5 h at 37 °C.

3. Aspirate the siRNA-containing medium and add 10 ml of DMEM with 10% FBS and 1% antibiotic. Incubate at 37 °C for 48 h.

   Note: adequate downregulation of helicases may require a sequential siRNA treatment. In this case, incubate the treated culture for 48 h and then replate cells at the density of 1×10^6 cells/100-mm dish. Incubate overnight and repeat the siRNA treatment as described in step 2. Cotransfection of rescue plasmid is advised at 6–18 h after the final siRNA treatment.
4. After overnight incubation, transfect with 10 μg of HIV-1 provirus and 30 μl FuGene6 (Roche). Perform replicate transfections with and without 5 μg of siRNA-resistant pcDNA-FLAG-RHA or pcDNA-FLAG.
5. Incubate at 37 °C for 48 h. Harvest cells from each 100-mm plate in 1 ml of 1 × PBS. Resuspend cells in 250–500 μl of RIPA cell lysis buffer (50 m*M* Tris, pH 8.0, 0.1% SDS, 1% Triton-X, 150 m*M* NaCl, 1% deoxycholic acid, 2 m*M* PMSF).

An approach complementary to downregulation of the cellular RNA helicase is exogenous expression of mutated alleles of the RNA helicase. In the case of RHA, expression of the amino-terminal RNA-binding domain dominantly interferes with translation of retrovirus mRNA (Ranji *et al.*, 2011).

2.2. Coprecipitation of RNA helicase with target RNA or protein cofactors

RHA is a ubiquitous RNA-binding protein that modulates posttranscriptional expression of retroviruses (Ranji and Boris-Lawrie, 2010). RHA recognizes structural features of a 5′ terminal posttranscriptional control element (PCE) within the complex 5′ UTR to facilitate polyribosome loading and efficient virion protein synthesis. Evidence for convergent cellular adaptation of the PCE/RHA RNA switch has been presented for the *junD* proto-oncogene.

junD is representative of cellular transcripts that contain a complex 5′ UTR, yet are reliant on cap-dependent translation initiation.

To assess the scope of RHA translational control in cells, microarray screens evaluated RNAs that coprecipitate with FLAG epitope-tagged RHA (Marcela Hernandez and Kathleen Boris-Lawrie, submitted). In parallel, transcripts that were depleted from polyribosomes upon RHA down-regulation were also identified. These microarrays are applicable to any helicase of interest and the outcome candidate genes are validated by complementary RT-PCR and/or quantitative real-time PCR. These screens are useful to identify distinct RNA helicase–mRNP complexes, including those in viral particles.

2.2.1. Methods for RNA and protein immunoprecipitation

2.2.1.1. Epitope immunoprecipitation

1. Typically four 150-mm plates of HEK293 or COS7 cells are transfected per IP. Four 150-mm plates of 2×10^6 COS7 cells are incubated overnight. As above, transfect with 15 μg of pcDNA-FLAG-RHA or pcDNA-FLAG and 45 μl FuGene6 (Roche) for 48 h.
2. Harvest cells from each plate in 2 ml of 1 × PBS.
3. Pool the cells from the four plates into a 15-ml sterile tube. *Remove 1 ml aliquot for western blot analysis to verify expression of FLAG-RHA.*
4. Pellet by centrifugation at $500 \times g$ for 5 min at 4 °C, washing three times with 4 ml of ice-cold 1 × PBS.
5. Resuspend washed cell pellet in 1 ml of 1 × PBS and move to a 1.5-ml microcentrifuge tube. Pellet and add an approximate equal volume of polysome lysis buffer (100 m*M* KCl, 5 m*M* $MgCl_2$, 10 m*M* HEPES (pH 7.0), 0.5% NP40, 1 m*M* DTT). Supplement with RNase inhibitors and protease inhibitors (10 μl of 100 U/ml RNaseOUT, 10 μl of protease inhibitor cocktail and 2 μl of 200 m*M* vanadyl ribonucleoside complexes per milliliter of polysome lysis buffer). Pipette the mixture gently to resolve clumps of cells.
6. Incubate mRNP preparation on ice for 5 min and freeze promptly at −180 °C (liquid nitrogen). *Keep frozen until FLAG-RHA expression is verified by immunoblotting.*
7. Distribute 1 ml of FLAG beads (2 ml of 50% slurry) in a 15-ml sterile tube. Centrifuge at $350 \times g$ for 2 min at 4 °C. Remove supernatant.
8. Wash beads three times with 4 ml ice-cold NT2 buffer (50 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 1 m*M* $MgCl_2$, 0.05% NP40). To wash, centrifuge $350 \times g$ for 2 min at 4 °C, remove liquid with aspirator and resuspend in ice-cold NT2 buffer and invert the tube several times.
9. Thaw mRNP lysate on ice and centrifuge at 13,000 rpm for 15 min. If the preparation is cloudy, then the volume should be increased.

For instance, add 200 μl of polysome lysis buffer and freeze at −80 °C for 15 min. Thaw in 37 °C water bath and centrifuge at 13,000 rpm for 15 min.

10. Reserve aliquots for control treatments: one-tenth of the preparation for RNA analysis and one-tenth for protein analysis at −80 °C. Dilute remainder in 4 ml of ice-cold NT2 buffer. Supplement with 1000 units of an RNase inhibitor (25 μl RNaseOUT), 400 μ*M* vanadyl ribonucleoside complexes (8 μl), and 20 m*M* EDTA.
11. Incubate the bead/lysate slurry overnight at 4 °C tumbling end over end.
12. Centrifuge 350 × *g* for 2 min at 4 °C and reserve supernatant at −20 °C for subsequent analysis.
13. Wash beads three times with 3 ml of ice-cold NT2 buffer (supplemented with 1000 units RNAseOUT, 10 μl 200 m*M* VRC, and EDTA to 20 m*M*) by tumbling end over end at 4 °C for 2–5 min. Centrifuge at 350 × *g* for 2 min at 4 °C. Aspirate the supernatant.
14. Prepare 3× FLAG elution buffer (Sigma FLAG Tagged Protein Immunoprecipitation Kit, Product Code FLAGIPT-1) by combining 60 μl of 5 μg/μl 3 × FLAG peptide solution to 2 ml of 1 × wash buffer. Add 200 units of RNAseOUT.
15. Add 2 ml of 3× FLAG elution buffer to each IP reaction. Incubate with gentle shaking for 30 min at 4 °C.
16. Centrifuge 350 × *g* for 2 min at 4 °C. Decant the supernatant to a fresh tube.

2.2.1.2. RNA extraction for genome-wide or candidate target mRNA identification TRIzol (Invitrogen) may be used to extract the RNA from the beads independent of elution from the FLAG beads for a small-scale RNA IP or in cases where target RNA is being validated simply by RT-PCR. However, elution is useful for RNA preparation for selected applications, including microarray. In this case, we recommend following the RNA isolation procedure from Qiagen's RNeasy MinElute Cleanup Kit (Cat. #74204) after following the steps:

1. Precipitate the RNA by adding 5 ml 100% EtOH and 200 μl of 3 *M* NaOAc to the supernatants from step 16 (above). Incubate on ice for 10 min followed by centrifugation at 10,000 rpm for 15 min.
2. Resuspend each pellet in 200 μl water.
3. Add 150 μl of water to 50 μl of lysate saved for total RNA.

We recommend final elution volumes to be 20 μl for IP samples and 50 μl for total RNA samples. Eluates may be assessed for concentration by nanospectrometer. Freeze samples at −80 °C.

All of the above solutions and buffers are prepared in RNase–DNase-free H_2O.

2.2.1.3. Protein extraction for proteomic analysis or candidate target protein identification The approach of RNA-epitope coprecipitation described above (Section 2.2.1.1) is useful to prepare samples for mass spectrometry. The outcomes have identified cofactors that are coprecipitated with epitope-tagged helicase (Wei Jing, Mamuka Kvaratskehlia, and Kathleen Boris-Lawrie, unpublished). We have characterized the process to generate FLAG-RHA complexes for mass spectrometry. Comparison of four preparative approaches are summarized in Fig. 19.1.

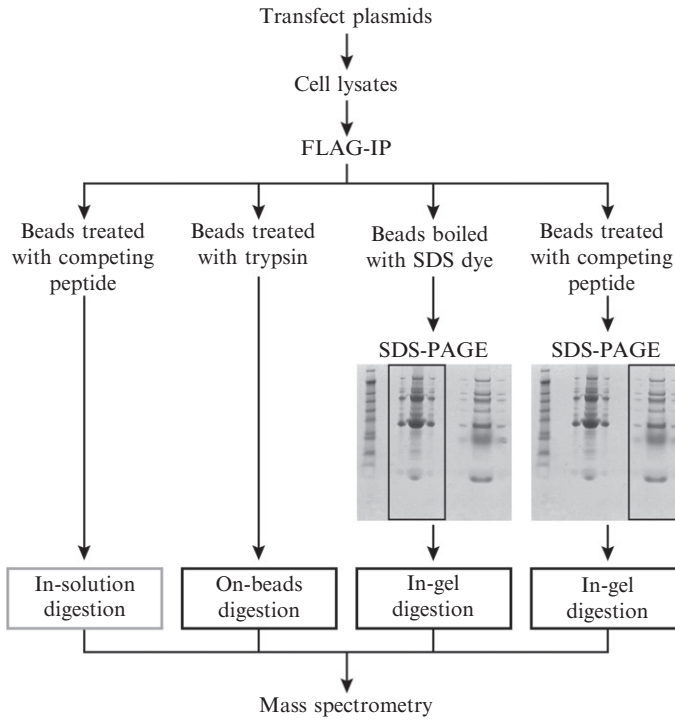


Figure 19.1 Scheme to elute and process samples following epitope immunoprecipitation for mass spectrometry. The approach of epitope immunoprecipitation is coupled with mass spectrometry to identify cofactors that coprecipitate with FLAG-tagged RHA. Immunoprecipitated protein can be trypsin-digested directly on sepharose-conjugated beads (On-beads digestion) or digested postelution with a competing peptide (In-Solution Digestion). Alternatively, beads can be treated with competing peptide or directly lysed in SDS buffer followed by SDS-PAGE. Specific band of interest or entire lane can then be trypsin digested.

2.3. Polysome association of RNA helicase with target RNA or protein cofactors

It is well appreciated that RNA–nuclear protein interaction induces RNP rearrangements that are necessary to produce a translation-competent mRNA template in the cytoplasm. RNA-binding proteins and *cis*-acting viral RNA sequences have the potential to modulate the function and fate of export and translation RNP.

In a typical ribosomal profile analysis, nontranslating, free mRNPs are separated from higher molecular mass of 40S and 60S ribosomal subunits, 80S monosomes and polyribosomes. The analysis of protein and RNA complexes in each gradient fraction is a potent technique to characterize cellular RNA helicase and viral protein(s) and RNAs that interact with the translation apparatus of the cell. The 5′ UTR of all retroviruses and selected complex cellular mRNAs contain a *cis*-acting PCE that is necessary for polysome loading and efficient translation of viral mRNA. The technique of ribosomal profile analysis demonstrated the specific interaction of PCE–RNA with host cofactor RHA as absolutely necessary for polysome association and translation of the PCE-containing viral and cellular mRNAs (Bolinger *et al.*, 2007; Hartman *et al.*, 2006).

2.3.1. Ribosomal profile analysis

1. Typically two 150-mm plates of HEK293 or COS7 cells are utilized to generate one profile. Incubate overnight four 150-mm plates of 5×10^6 COS7 cells and transfect with siRNA and/or expression plasmids of interest (15 μg total plasmid amount per plate) as described in Section 2.1.1 for 48 h.
2. Prior to harvesting, add cycloheximide at a final concentration of 0.1 μg/ml to the culture medium and incubate at 37 °C for 20 min.
3. Harvest cells from each plate in 2 ml of 1 × PBS. Centrifuge at $500 \times g$ for 5 min at 4 °C.
4. Resuspend the cells in 450 μl gradient buffer (10 m*M* HEPES, 10 m*M* NaCl, 3 m*M* $CaCl_2$, 7 m*M* $MgCl_2$, 0.5% NP40, 1 μl 1 *M* DTT, 80 U RNaseOUT (Invitrogen) and 1 μl/ml cycloheximide). Incubate on ice for 15 min; vortex gently at 5 min intervals.
5. Centrifuge at $3000 \times g$ for 5 min at 4 °C.
6. Layer the clarified lysate on a 15–47.5% sucrose gradient. Centrifuge at 36,000 rpm at 4 °C for 2.25 h (Beckman L-80 ultracentrifuge, SW41 rotor).
7. Decant gradient fractionates and measure A_{254} absorbance to generate the ribosomal RNA profile. Fractions can be combined as mRNPs, 40S and 60S, 80S, light polyribosomes, and heavy polyribosomes, respectively.

2.3.2. Efficient isolation of protein from gradient fractions

1. Incubate 1 ml of each fraction with 50 μg BSA in TCA (final concentration 20%). Mix by inverting several times and incubate on ice for 30–60 min. Alternatively, fractions can be stored at −20 °C overnight.
2. Centrifuge at 12,000 rpm at 4 °C for 20 min. Discard supernatant and resuspend pellet in 500 μl acetone (combine selected fractions at this point).
3. Centrifuge at 12,000 rpm at 4 °C for 5 min. Discard supernatant and repeat acetone washes twice.
4. Spin the pellets in a SpeedVac for 20–30 min. *Note:* At this point, pellets may be stored at −20 °C for analysis later.
5. Resuspend pellets in 35 μl water and 35 μl 2 × SDS loading dye, boil for 5 min, and subject to SDS-PAGE and/or immunoblot analysis. Some samples may be chunky or yellowish in color but will resolve once loaded onto gel.

2.3.3. Isolating RNA from gradient fractions

1. Decant 1 ml of each fraction to a fresh tube and add equivalent volume of 100% ethanol, 10 μg glycogen, or 1 μg tRNA; incubate overnight at −80 °C.
2. Centrifuge at 12,000 rpm for 15–20 min at 4 °C.
3. Extract RNA from the pellet using TRIzol (Invitrogen) or RNeasy MinElute Kit (Qiagen) according to manufacturer's instructions.

RNA prepared from the fractions is suitable for several analyses, including native gel and Northern blotting, RT-PCR, quantitative real-time PCR, RNase protection assay, and analysis of polyA tail length.

2.3.3.1. Approach to distinguish ribosomes from nonribosomal RNPs In order to confirm that the gradient fractions represent polysome, EDTA supplementation will dissociate polysomes and generate an upward shift of ribosomal RNA in the A_{254} profile. Any polysome-associated protein and/or RNA will also shift toward the left of the gradient. For EDTA treatment, we recommend supplementation of 30 m*M* EDTA to the PBS used to harvest the cells and the cell lysis buffer.

A complementary approach to EDTA is inhibition of translation puromycin, a chain terminator. Puromycin (Sigma) supplementation of culture medium (400 μ*M*) is initiated 40 min prior to addition of cycloheximide for a total incubation time of 1 h. Puromycin treatment eliminates polysomes. By contrast, nontranslating RNP complexes or virus particles will remain intact in the heavy sucrose fractions.

2.3.3.2. Approach to distinguish ribosomes from intracellular virus-like particles The EDTA-mediated dissociation of ribosomes will produce a shift in polysome-associated proteins and RNAs. The failure to shift indicates that the RNPs in question do not represent polysomes. In particular, a lack of shift may be due to virus particles that cosediment with light and heavy polysomes. This differential provides a tool to differentiate between polysome-associated and virion-associated cellular and viral proteins and RNAs, respectively. Puromycin treatment will not disrupt the sedimentation of virus particles in the sucrose gradient.

3. Biochemical and Biophysical Methods to Study RNA Helicase

3.1. RNA-affinity chromatography for cofactor identification

RNA-affinity chromatography coupled with proteomic analysis is a powerful tool to identify host factors that specifically interact with the subject RNA bait. The differential between functional and nonfunctional RNAs has robustly identified host RNA helicase as a necessary effector protein of proto-oncogene junD PCE and retroviral PCE (spleen necrosis virus, human T-cell leukemia type 1 virus, HIV-1). The proteomic analysis was followed by RNA–protein coimmunoprecipitation that demonstrated selective interaction of RHA with structural features of PCE. RHA coprecipitate with HIV-1 RU5 more abundantly when compared to equimolar amounts of HIV-1 R or U5 RNA alone. The combination of RNA-affinity chromatography, RHA immunoblot analysis, and PCE activity assays determined HIV-1 R and U5 RNA elements interact synergistically with RHA to facilitate efficient gag mRNA translation (Bolinger *et al.*, 2010).

3.1.1. Biotinylation of RNA

1. Generate DNA templates for *in vitro* transcription by standard PCR with primers containing T7 promoter sequence.
2. Generate biotinylated *in vitro* transcripts using the MEGAshortscript™ Kit (Ambion) in the presence of 15 m*M* biotinylated UTP or CTP (ENZO) and 135 m*M* UTP (Promega), using T7 RNA polymerase.
3. Treat biotinylated transcripts with 1 unit DNase (Promega) at 37 °C for 30 min. Stop the reaction by bringing up the volume to 500 μl with DEPC-treated water and then add 500 μl of acid phenol. Shake and spin at 12,000 rpm for 2–3 min.
4. Take upper layer carefully into a new tube and add equal volume of chloroform. Shake and then vortex at 12,000 rpm for 2–3 min. Take upper layer into a new tube and add 1 μl glycogen, 2½ times the volume

ice-cold ethanol and 10% 3 *M* NaOAc. Shake and keep at −80 °C for at least 15 min.
5. Centrifuge 12,000 rpm for 10 min. Wash pellet with 500 µl of 70% ethanol. Spin at 12,000 rpm for 10 min. Dry pellet for 2 min and resuspend in 30 µl DEPC-treated water.
6. Separate 1 µl RNA on a 2% agarose gel and stain with ethidium bromide to verify the presence of the expected transcription product. Subject the RNA to the Quick Spin Sephadex G25 exclusion column (Roche) to remove nonincorporated dNTPs using the manufacturer protocol.

3.1.2. Isolation of cofactors associated with biotinylated RNA

1. Mix the slurry of streptavidin-coated beads by inverting several times. The 100 µl aliquot of beads is suitable for analysis of a candidate protein by immunoblot. The 200 µl aliquot of beads is suitable for reactions to identify RNA-interactive proteins by mass spectrometry.
2. Supplement the beads with 5 volumes of binding buffer (10 m*M* HEPES, pH 7.6, 5 m*M* EDTA, pH 8.0, 3 m*M* $MgCl_2$, 40 m*M* KCl, 5% glycerol, 1% NP40, 2 m*M* DTT). Gently mix by inverting 3–5 times. Centrifuge at 1200 × *g* for 1 min. Repeat these washes twice.
3. Supplement the reaction with biotinylated RNA. For candidate analysis by immunoblot, use 15 µ*M* of RNA. For detection of RNA-interactive proteins by mass spectrometry use ~8 µg.
4. Incubate for 1 h at 4 °C with gentle rocking. Do not vortex. Centrifuge at 1200 × *g* for 1 min and discard supernatant.
5. Add 1 ml of 2 m*M* Biotin blocking solution. Invert to mix, incubate at room temperature for 5 min. Centrifuge at 1200 × *g* for 1 min and discard supernatant. Add 1 ml binding buffer, spin, and discard. Repeat this blocking step once more.
6. Add HeLa nuclear or total cellular extract. Use 100–500 µg for the small-scale candidate analysis, and ~3 mg for the larger scale mass spectrometry-based screen. Supplement with 200 µl of binding buffer and incubate for 2 h at 4 °C with gentle rocking. Do not vortex. Incubation with lysate may be repeated to enrich isolation of low-abundance proteins.
7. Centrifuge at 1200 × *g* for 1 min and discard supernatant.
8. Add 1 ml of binding buffer, mix well by inverting, and incubate at room temperature for 1 min. Centrifuge at 1200 × *g* for 1 min and discard supernatant. Repeat washing for three additional times.
9. Wash four times with 200 µl elution buffer (10 m*M* HEPES, pH 7.6, 3 m*M* $MgCl_2$, 5 m*M* EDTA, pH 8.0, 0.2% glycerol, 2 m*M* DTT) with 40 m*M*, 100 m*M*, 200 m*M*, and 2 *M* KCl, respectively. Mix well by inverting; incubate at room temperature for 5 min, centrifuge at 1200 × *g* for 1 min and save supernatants in separate prechilled tubes.

10. Dialyze the 2 *M* KCl elutants against binding buffer overnight at 4 °C. Concentrate on Millipore concentrator columns or by SpeedVac; Analyze by SDS-PAGE.

Downstream processing analyses are: staining with Coomassie or silver stain; in-gel trypsin digestion and mass spectrometry; or immunoblotting with antisera against candidate proteins. If cellular helicases are identified to interact with a specific RNA bait, additional experiments are warranted to confirm specificity of interaction. Electrophoretic mobility shift assays (EMSAs) and RNA-immunoprecipitation assays can be used to examine the specificity of interaction of the identified helicase with the RNA. The RNA–protein complexes isolated by RNA-affinity chromatography will be selectively competed by excess RNA in an RNA EMSA. A specificity control is necessary for RNA-affinity chromatography and typical controls are c-myc, gapdh, and nonfunctional mutant RNAs. An additional control of streptavidin beads without RNA bait is required to measure nonspecific enrichment of protein of interest. The RNA-affinity chromatography with total, nuclear, and/or cytoplasmic extracts provides interference to the subcellular compartment of an RNA–host protein interaction.

3.2. Determination of RNA-binding activity using EMSA

This approach can be used to evaluate the binding activity of an RNA helicase to viral and/or cellular RNA elements. The component of the experiment can include full-length-purified or individual domains of the helicase. An important consideration is to establish the specificity of interaction by comparing functional and nonfunctional RNA elements with generic control-like double-stranded RNAs (dsRNAs). EMSAs are useful for initial screening of RNA-binding activity of helicases. The quantitative assessment of RNA binding by fluorescence anisotropy (FA) is warranted to measure the binding affinity.

The results of EMSAs and FA experiments showed that the N-terminal domain of RHA exhibits higher binding affinity for SNV and junD PCE than for nonfunctional mutant PCE RNA or nonspecific control RNAs (Ranji *et al.*, 2011). By comparison, the isolated DEIH domain lacks detectable binding to the SNV and junD PCE RNAs, and the C-terminal RG-rich domain bound nonspecifically, as designated by interaction with nonspecific control RNAs.

3.2.1. Methods for measuring RNA-binding affinity using EMSA

3.2.1.1. In vitro *transcription*

1. Generate *in vitro* transcripts using the RiboMAX^{TM} large-scale RNA production system (Promega) in the presence of [α-^{32}P]UTP/[α-^{32}P] CTP (PerkinElmer Life Sciences), using T7 RNA polymerase.

2. Treat transcription reactions with 1 unit DNase (Promega) at 37 °C for 30 min, separate on 8% denaturing urea gels, and elute in probe gel elution buffer (0.5 *M* NH_4OAc, 1 m*M* EDTA, 0.1% SDS) for 30 min at 37 °C and then overnight at 4 °C.
3. Precipitate RNAs in 95% ethanol and 0.3 *M* NaOAc at −80 °C for 20 min in the presence of glycogen (20 μg), collect by centrifugation at 12,000 rpm for 10 min, wash in 500 μl of 75% ethanol. Resuspend in DEPC-treated water and measure the scintillation counts per microliter of RNA.
4. Treat RNA for 2 min in boiling water bath and move to room temperature water bath for 2 to yield native RNA conformation. Dilute the RNA to 100,000 cpm in DEPC-treated water.

3.2.1.2. Expression and purification of recombinant proteins The preparation of expression plasmids for the RHA N-terminal (N-term), DEIH, and C-terminal (C-term) domains are described in Ranji *et al.* (2011). Proteins were expressed in BL21-CodonPlus optimized cells (Stratagene). The cells were treated with 1 m*M* isopropyl-β-D-thiogalactopyranoside (United States Biochemical Corp.) for 2.5 h at 33 °C. Cell pellets were resuspended in 1 × PBS with 10 μl/ml protease inhibitor mixture (Sigma) and 1 μl/ml 1 *M* dithiothreitol, and subjected to 5000 units of pressure in an Aminco French pressure cell. Soluble proteins were harvested by centrifugation in a Sorvall RC5C SS-34 rotor at 12,000 rpm for 20 min at 4 °C.

1. Recombinant RNA helicase domains are purified from the soluble protein lysate on glutathione-Sepharose beads (Pierce). Incubate the lysate and beads overnight, wash four times with 50 m*M* HEPES and 150 m*M* NaCl (pH 7.0), wash once with 50 m*M* HEPES and 500 m*M* NaCl (pH 7.0), and once with thrombin cleavage buffer (20 m*M* HEPES, pH 8, 0.15 *M* NaCl, and 2.5 *M* $CaCl_2$). Biotinylated thrombin (10 units) is useful to release N-term RHA from the GST tag and can be further removed from the solution by incubation with streptavidin.
2. For purification of the DEIH domain of RHA, incubate soluble protein lysate with 2 ml of glutathione-Sepharose (GE Healthcare) for 30 min at room temperature. Wash the beads with 20 ml of buffer containing 50 m*M* HEPES, pH 7.3, 500 m*M* NaCl, 5 m*M* DTT, and 1 m*M* EDTA. Elute the DEIH domain from the beads using buffer containing 50 m*M* HEPES, pH 7.3, 25 m*M* reduced glutathione, 500 m*M* NaCl, 5 m*M* DTT, and 1 m*M* EDTA. Dialysis of protein fractions in the same buffer without glutathione is important to remove glutathione.
3. His-tagged RNA helicase was purified from the soluble protein lysate on nickel-Sepharose (GE Healthcare). Incubate the lysate with 2 ml of nickel beads at room temperature for 1 h. Remove nonspecifically bound proteins by extensive washing of the beads with 20 ml of buffer

containing 50 m*M* HEPES, pH 7.4, 500 m*M* NaCl, 7.5 m*M* CHAPS, 20 m*M* imidazole, and 4 m*M* β-mercaptoethanol. Elute the C-term domain in 50 m*M* HEPES, pH 7.4, 500 m*M* NaCl, 7.5 m*M* CHAPS, 500 m*M* imidazole, 5 m*M* EDTA, and 4 m*M* β-mercaptoethanol and dialyze against the same buffer without imidazole.

4. Evaluation of each protein preparation for size and purity is necessary. The results of SDS-PAGE are followed by assessment of the concentration of recombinant proteins by Bio-Rad DC protein assay.

3.2.1.3. Gel electrophoresis

1. Incubate recombinant protein and 100,000 cpm of *in vitro* transcribed α-^{32}P-labeled RNA (above) in EMSA buffer (2% glycerol, 0.8 m*M* EGTA, 0.2 m*M* EDTA, 2 m*M* Tris, pH 7.6, 14 m*M* KCl, and 0.2 m*M* $Mg(OAc)_2$) for 30 min on ice.
2. Electrophoresis is performed at 4 °C and using 5% native Tris borate/EDTA-acrylamide gels. Fix, dry, and expose overnight in a PhosphorImager cassette.

3.3. Determination of RNA-binding activity using FA

FA assays using synthetic 5′-fluorescein-tagged SNV PCE RNAs verify EMSA RNA-binding trends and determine the RNA-binding affinity of the RNA helicase domains (a related approach is described in this volume in Chapter 11). The length limit of chemical RNA synthesis is <100 nt. For RHA-SNV-PCE studies 96- and 98-nt RNAs were labeled. The specificity and selectivity of functional PCE RNA was determined relative to nonfunctional controls. The SNV-PCE^{AC} and SNV-PCE^{AB}, which are necessary for RHA translation activity and for precipitation of epitope-tagged RHA in cells, were chosen for FP analysis and are described in Ranji *et al.* (2011).

3.3.1. Method for fluorescence anisotropy measurements

1. Synthetic RNA oligonucleotides labeled at the 5′-nucleotide with fluorescein (Dharmacon) are resuspended in DEPC-treated water (20 μ*M* RNA).
2. Treat RNA at 80 °C for 2 min and then 60 °C for 2 min to facilitate native conformation. Incubate 3 μl of 20 μ*M* RNA in 5.25 μl of DEPC-treated water, 3.75 μl of 100 m*M* HEPES, pH 7.5, and 1.5 μl of 1 *M* NaCl. Complete the incubation by addition of 1.5 μl of 100 m*M* $MgCl_2$.
3. Add DEPC-treated water to bring final volume of RNA solution to 300 μl. *Note:* For folded RNA, add 285 μl DEPC-treated water; for

RNA that does not require folding, add 297 μl DEPC-treated water. This final working solution is 200 n*M*.

4. Begin mixing reactions according to Table 19.2. Add DEPC water to the tubes first, then the 5 × FP buffer (2% glycerol, 0.8 m*M* EGTA, 0.2 m*M* EDTA, 2 m*M* Tris, pH 7.6, 14 m*M* KCl, and 0.2 m*M* Mg $(OAc)_2$), then the protein and finally, the RNA. Vortex each tube briefly.
5. Incubate reactions for 30 min at room temperature in the dark to allow samples to reach equilibrium.
6. Perform FA measurements in triplicate by loading wells in Corning 3676 low volume 384-well black nonbinding surface polystyrene plates.
7. Scan plate in MD SpectraMax M5 fluorimeter (Molecular Devices). For fluorescein, use excitation wavelength of 485 nm and emission wavelength of 525 nm. Measure both anisotropy and intensity.

Table 19.2 Template for FA measurements is constructed to calculate of equilibrium dissociation constant by using variable amounts of recombinant N-terminal RHA domain with constant amount of 5′-fluorescein-labeled RNA

RHA N-term (n*M*)	DEPC water (μl)	5 × FP buffer (μl)	RHA N-term 2 μ*M* (μl)	RHA N-term 10 μ*M* (μl)	RNA 200 n*M* (μl)
Blank	80.00	20	0.00	0	0
0	70.00	20	0.00	0	10
25	68.75	20	1.25	0	10
50	67.50	20	2.50	0	10
75	66.25	20	3.75	0	10
100	65.00	20	5.00	0	10
125	63.75	20	6.25	0	10
150	62.50	20	7.50	0	10
175	61.25	20	8.75	0	10
200	60.00	20	10.00	0	10
250	57.50	20	12.50	0	10
300	55.00	20	15.00	0	10
350	52.50	20	17.50	0	10
400	50.00	20	20.00	0	10
450	47.50	20	22.50	0	10
500	45.00	20	25.00	0	10
600	64.00	20	0.00	6	10
800	62.00	20	0.00	8	10
1000	60.00	20	0.00	10	10
1400	56.00	20	0.00	14	10
1600	54.00	20	0.00	16	10
2000	50.00	20	0.00	20	10

8. Plot anisotropy as a function of increasing protein concentration. Obtain the equilibrium dissociation constants (K_d) by fitting the binding curves to a single-binding site model on KaleidaGraph as described (Stewart-Maynard *et al.*, 2008). Calculate weighted averages and SD as described (Taylor, 1997).

Equilibrium binding conditions should be determined by varying the incubation temperature and time. For RHA and SNV or junD PCE RNA, incubation for 30 or 60 min produces similar binding patterns indicating equilibrium binding by 30 min. Binding temperature and time should be empirically established for each helicase and its corresponding nucleic acid.

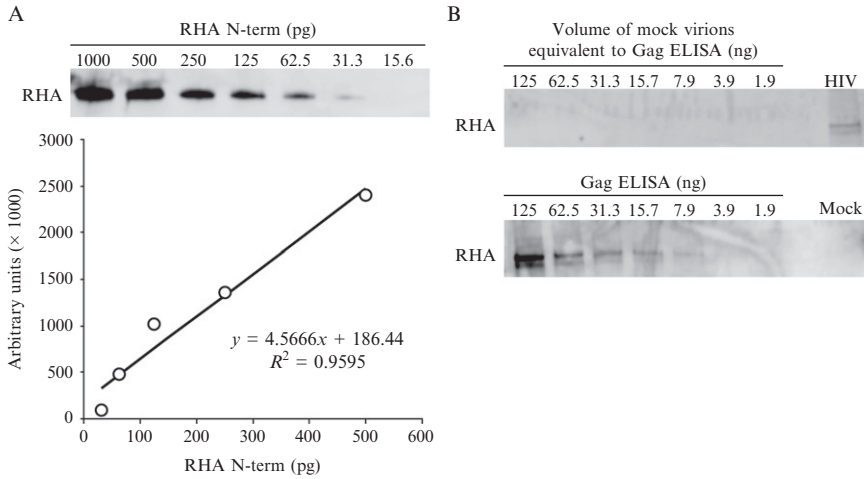
In both EMSA- and FA-based approaches, control dsRNAs should be used to compare RNA binding and establish a baseline for RNA-binding activity. Nonfunctional SNV-PCE dsRNA stem loops (termed mutAC which is a structural mutant of SNV PCE^{AC}), which lacks translation activity and does not coprecipitate RHA in cells, was used as control to establish a baseline of RHA-binding affinity. The $minihelix^{Lys}$ 35-nt RNA derived from the acceptor-TΨC stem of human $tRNA_3Lys$ and the human 7SL 27-nt hairpin RNA further provided generic dsRNA controls that lack RHA translation activity. In order to generate maximum power for statistical analysis for binding affinities, we recommend that EMSAs and FA measurements should be repeated with at least three independent preparations of protein and RNA.

4. Methods Used to Study Virion-Associated RNA Helicase in Cultured Mammalian Cells

4.1. Detection of RNA helicase in virion preparations

Viruses package host proteins in the virus particle that promote replication of virus in the subsequent generation. The analysis of whether an RNA helicase is incorporated into progeny virus particles is limited by sensitivity of immunoblotting and the purity of virus particle preparation. Given these caveats, the analysis distinguishes the role of the helicase in the producer cell (in which the virus particles are produced) and the target cells (in which the nascent virus infects and replicates).

RHA is a necessary cellular cofactor for HIV-1 replication and infectivity on primary lymphocytes. Early in the HIV-1 lifecycle, cell-associated RHA is necessary for translation of viral mRNA. The molecular basis of RHA translational stimulation involves the specific and selective interaction with structural features of the 5′ UTR via the amino-terminal residues of RHA and tethering of the ATPase-dependent helicase activity that facilitates ribosome access to the open reading frame. A minority of cell-intrinsic RHA is assembled into virus particles and the virion-associated RHA (~2 mol/particle)



C Calculations

62.5 ng of p24 = 862 (× 1000) arbitrary units = 148 pg of RHA (based of N-term RHA standard curve)

148 pg of RHA/62.5 ng of Gag

148 pg of RHA = ? molecules

Molecular weight of RHA = 142 kDa

$= 6.27 \times 10^{8}$ mol/62.5 ng of Gag

62.5 ng of Gag (p24) = ? molecules

Molecular weight of Gag (p24) = 24 kDa

$= 1.56 \times 10^{12}$ mol

6.27×10^{8} molecules of RHA/1.56×10^{12} mol of Gag

4.01×10^{-4} molecules of RHA/mol of Gag

1 virion = 5000 mol of Gag

$4.01 \times 10^{-4} \times 5000 = 2$ mol of RHA/virion

Figure 19.2 RHA is incorporated into HIV-1 particles. (A) Indicated mass (pg) of purified N-terminal domain (aa 1–300) of RHA was evaluated by RHA immunoblot and a standard curve was generated. Graph summarizes densitometry of N-term RHA. (B) HEK 293 cells were transfected with HIV-1^{NL4-3} or left nontransfected for 48 h. Cell-free medium from indicated cells was isolated on 25% sucrose pad and particles were lysed in RIPA buffer. Gag p24 ELISA on cell-free medium was performed indicated ng of particles was evaluated by RHA immunoblot. (C) Based on the N-term RHA standard curve from (A), amount of RHA for 62.5 ng of Gag p24 was calculated. Number of RHA molecule per virion was calculated assuming 1 virion equals 5000 Gag p24 molecules (Briggs *et al.*, 2004).

(Fig. 19.2) promotes infectivity by a mechanism that remains poorly elucidated. Downregulation of cell-intrinsic RHA using the approach described in Section 2.1 impairs the infectivity of progeny virions on primary human lymphocytes and reporter cell lines.

4.1.1. Method for quantitative detection of virion-associated RNA helicase

1. Incubate 1×10^6 HEK293 cells in a 100-mm dish overnight, transfect with 10 μg of pNL4-3 HIV-1 provirus and 30 μl FuGene6 (Roche) for 48 h.
2. Collect the virus-containing tissue culture supernatant in a Falcon tube. Pellet the debris and broken cells by centrifugation at 2000 rpm for 5 min at room temperature. Collect the supernatant through a 0.45 μm filter.
3. Ultracentrifuge the filtered supernatant over 0.5–1 ml of 25% sucrose pad at 35,000 rpm (100,000 × *g*) for 2 h at 4 °C in a Beckman ultracentrifuge using SW-41 rotor or comparable ultracentrifuge and rotor (e.g., Sorvall).
4. Remove supernatant by decanting, then wipe the inside wall of the tube with rolled paper towel to remove as much supernatant as possible (do not touch the bottom of the tube).
5. Add 100 μl of RIPA cell lysis buffer (50 m*M* Tris, pH 8.0, 0.1% SDS, 1% Triton-X, 150 m*M* NaCl, 1% deoxycholic acid, 2 m*M* PMSF). Leave the tube at 37 °C for 15 min and pipette several times to resuspend the viral pellet. Briefly vortex the pellet and collect in an eppendorf.
6. In parallel, harvest cells from plate in 1 ml of 1 × PBS, resuspend in 500 μl of RIPA cell lysis buffer on ice for 15 min with an intermittent vortex.
7. Use 25 μl of virion lysate to perform Gag p24 ELISA (Zeptometrix) with appropriate range of standard curve (7.8–125 pg/ml Gag).
8. Quantify the Gag units in the viral lysate and load a range of 2–125 ng/ml Gag units for SDS-PAGE. Immunoblot with RHA and HIV-1 Gag antisera.
9. In parallel, immunoblot serial dilutions of the recombinant RHA (15.7–1000 ng). Quantify the signal intensity of the bands; generate a standard curve and compare the abundance of RNA helicase from the virion immunoblot (Fig. 19.2).

A potential caveat is the association during ultracentrifugation of culture medium of virions with extracellular microvesicle contaminants. To overcome this pitfall, use conditioned medium from equivalent cultures of noninfected cells. The immunoblot will ascertain possible contaminants. An additional step is to perform a second ultracentrifugation (100,000 × *g*) through a 15%/65% sucrose step gradient. Purified virus particles are collected at the 15%/65% sucrose interface and may be concentrated at

100,000 × *g*. Herein, the virus preparation is placed at the top of a 20–70% continuous sucrose gradient, centrifuged at 100,000 × *g* at 4 °C for 16 h. Fractions are then collected and subjected to immunoblotting with antisera against viral core and the RNA helicase under investigation.

The viral RNP is disrupted and the envelope solubilized in 1% Triton X-100 at room temperature for 5 min in a wash buffer (10 m*M* Tris–HCl, pH 8.0, and 100 m*M* NaCl). The treated samples are centrifuged at 10,000 × *g* for 10 min and the pellet is washed three times with wash buffer. This approach will ensure that the cellular helicase is incorporated into viral cores and is not merely sticking to the outside of viral envelope. The majority of the HIV-1 matrix (MA) and capsid (CA) proteins are removed following the treatment with 1% Triton X-100, whereas substantial amounts of reverse transcriptase and nucleocapsid proteins, together with viral genomic RNA, are recovered. This preparation is designated the viral RNP complex.

To probe whether the RNA helicase requires specific interaction with the viral RNA for incorporation in the assembling HIV-1 virions, mutant proviruses are a suitable tool. The transfected provirus is used to provide mutant virion precursor RNA. A negative control is expressed by a provirus with nonfunctional mutation of the viral RNA packaging signal (Ψ).

4.2. Detection of virion-associated protein and RNA: Host and viral factors

RNA helicases are composed of domains that are interchangeable among RNA-interactive proteins: C-terminal arginine and glycine-rich domain; central DEIH helicase domain; and N-terminal double-stranded RNA-binding domains (dsRBDs) with conserved α-β-β-β-α topology. These domains provide a modular structure for multiple protein–protein and protein–RNA interactions. Such interactions can result in selective incorporation of certain host factors into assembling virions. Furthermore, interactions of RNA helicase with viral core proteins and viral nucleic acids can contribute to productive morphogenesis of the virions.

Using the approaches described in Sections 2.1.1 and 4.1.1, HIV-1 virions produced from cells treated with RHA siRNA or nonsilencing control siRNA were examined for packaging of viral and cellular factors in virions. Equivalent quantities of virions were subjected to immunoblot or RNA extraction to examine virion-associated RNA. Typically, 5–100 ng of HIV-1 Gag (as measured by Gag ELISA) is sufficient for immunoblotting and 25–500 ng HIV-1 Gag is sufficient for RNA extraction. Using the methods described below, it was shown that virions produced from cells treated with RHA siRNA are deficient in RHA but not deficient in another cellular cofactor LysRS. The results of RT-real-time PCR with

HIV-1 gag primers (described below) demonstrated viral RNA packaging efficiency is not reduced by RHA downregulation.

4.2.1. Method to screen candidate virion-associated protein(s) and RNA

1. Incubate 1×10^6 HEK293 cells/100-mm dish and culture overnight.
2. Treat with 20 μ*M* RHA siRNAs or nonsilencing Sc RNAs as described in Section 2.1.1.
3. After second siRNA treatment, transfect cells with 10 μg pNL4-3 HIV-1 provirus in 1:3 DNA to FuGene6 (Roche) ratio.
4. Harvest cells from plate in 1 ml of 1 × PBS and lyse in 250–500 μl of RIPA cell lysis buffer. Perform immunoblotting to verify downregulation of RHA from siRNA treatment. Also, immunoblotting with viral protein antisera measures the effect of RHA downregulation on viral gene expression.
5. Collect the virus-containing tissue culture supernatant in a sterile tube. Pellet the debris and broken cells by centrifugation at 2000 rpm for 5 min at room temperature. Collect the supernatant and pass it through 0.45 μm filter.
6. Ultracentrifuge the filtered supernatant over 0.5–1 ml of 25% sucrose pad at 35,000 rpm (100,000 × *g*) for 2 h at 4 °C in a Beckman ultracentrifuge using SW-41 rotor or use a comparable ultracentrifuge and rotor (e.g., Sorvall).
7. Remove supernatant by decanting, then wipe the inside wall of the tube with rolled paper towel to remove as much supernatant as possible (do not touch the bottom of the tube).
8. Add 100 μl of RIPA cell lysis buffer (50 m*M* Tris, pH 8.0, 0.1% SDS, 1% Triton-X, 150 m*M* NaCl, 1% deoxycholic acid, 2 m*M* PMSF). Leave the tube at 37 °C for 15 min and pipette several times to resuspend the viral pellet. Briefly vortex the pellet and collect in an eppendorf. Use 25 μl of virion lysate to perform Gag p24 ELISA (Zeptometrix) with appropriate range of standard curve (7.8–125 pg/ml p24). Use equivalent p24 units for Sci/RHAi virions for immunoblotting.
9. Mix 50 μl virion preparation in 0.5 ml of TRIzol LS (Invitrogen) and isolate the RNA, treat with DNaseI (Ambion), extract with acid phenol (Ambion), and precipitate in 100% ethanol and 0.3 *M* NaOAc. Resuspend in DEPC-treated water.
10. Treat total virion RNA with Omniscript reverse transcriptase (RT, Qiagen) and random hexamer primer for 1 h at 37 °C. Use 10% of the cDNA preparation for *gag* and *β-actin* real-time PCR, respectively with HIV-1 *gag* primers KB1614 (GTAAGAAAAAGGCACAGCAAGCAGC) and KB1615 (CATTTGCCCCTGGAGGTTCTG) or

β-actin primers KB1252 (TCACCCACACTGTGCCCATCTACGA) and KB1253 (CAGCGGAACCGCTCATTGCCAATGG) and Lightcycler480 SYBR Green master mix (Roche) in a Lightcycler480 (Roche, Germany). Generate standard curves to determine RNA copy numbers on pHIV-1^{NL4-3} or β-*actin* plasmid in the range of 10^2 to 10^8 copies.

RNA prepared from the virus particles is suitable for native gel electrophoresis, Northern blot, RNase protection assay, polyA length analysis.

HIV-1 virions package two copies of viral genomic RNA, which dimerize via noncovalent linkage. The approach of native gel electrophoresis and Northern blots on virion RNA preparations is useful to ascertain sustained HIV-1 RNA dimerization during downregulation of a candidate RNA helicase. A caveat is redundant functional activity of other RNA helicase superfamily members and viral RNA chaperones. In addition, the folding of viral RNA detected in this assay is not comprehensive and alternative folds may not recapitulate efficient viral replication.

4.3. Measurement of infectivity of RNA helicase-deficient virions

It is critical to investigate whether the candidate RNA helicase is important to sustain viral infectivity. If a cellular helicase is necessary for viral infection, downregulation of endogenous protein is expected to decrease virion-intrinsic RNA helicase. Herein, the outcome is production of progeny virions that are poorly infectious. Alternatively, the RNA helicase can act as a restriction factor. Herein, the downregulation may result in enhanced infectivity.

To examine the effect of RHA downregulation on HIV-1 infectivity, equivalent cell-free virion preparations were used for infection of human PBMCs or a HeLa-based Luciferase reporter cell line, TZM-bl. TZM-bl is a genetically engineered HeLa cell line that expresses high levels of HIV-1 receptor and coreceptors: CD4, CXCR4, and CCR5 (Platt *et al.*, 1998; Wei *et al.*, 2002). These cells contain reporter cassettes of Luciferase and β-galactosidase that are each expressed from Tat-inducible HIV-1 LTR. Expression of these reporter genes is directly dependent on production of HIV-1 Tat postinfection. HEK293 cells were used as producer cells and were treated with RHA siRNA or Sc siRNA as described in Section 2.1.1 (Fig. 19.3A). Infections of TZM-bl cells were performed with 2 ng Gag aliquots of virions deficient in RHA or containing RHA as described below. RHA-deficient virions are twofold less infectious on TZM-bl cells compared to virions containing RHA (Fig. 19.3B). Further, expression of siRNA-resistant RHA in the producer cells is sufficient to rescue HIV-1 infectivity on TZM-bl cells (Fig. 19.3B).

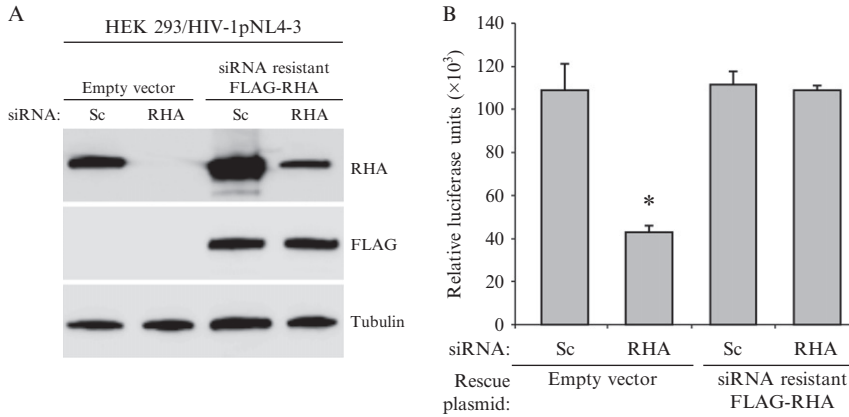


Figure 19.3 RHA downregulation in producer cells reduces HIV-1 infectivity on target cells. (A) HEK 293 cells were transfected with scrambled (Sc) or RHA (RHA) siRNAs, and then second dose of siRNA with either empty vector or siRNA-resistant FLAG-RHA and VSV-G pseudotyped HIV-$1^{NL4-3\Delta Env}$ for 48 h. Immunoblot of total cell protein with indicated antiserum verified RHA downregulation, expression of siRNA-resistant FLAG-RHA and equal protein loading, respectively. (B) Cell-free virus equivalent to 2 ng Gag was used to infect TZM-bl cells and Luciferase activity determined at 48 h ($n=3$). Asterisk indicates statistically significant difference from Sc siRNA control was observed ($P \leq 0.0005$).

4.3.1. Method for measuring infectivity of RHA-deficient HIV-1 virions

1. Incubate 1×10^6 HEK293 cells/100-mm dish and culture overnight.
2. Treat with 20 μ*M* RHA siRNAs or nonsilencing Sc RNAs as described in Section 2.1.1.
3. After second siRNA treatment, transfect cells with 10 μg pNL4-3 HIV-1 provirus in 1:3 DNA to FuGene6 (Roche) ratio. For rescue experiments, cotransfect 5 μg of siRNA-resistant pcDNA-FLAG-RHA or pcDNA-FLAG with 10 μg HIV-1 provirus.
4. Harvest cells from plate in 1 ml of 1 × PBS and lyse in 250–500 μl of RIPA cell lysis buffer. Perform immunoblotting to verify downregulation of RHA from siRNA treatment.
5. Collect the virus-containing tissue culture supernatant in a Falcon tube. Pellet the debris and broken cells by centrifugation at 2000 rpm for 5 min at room temperature. Collect the supernatant and pass it through 0.45 μm filter.
6. Quantify the HIV-1 Gag p24 units in the supernatant. Use 25 μl of supernatant to perform Gag p24 ELISA (Zeptometrix) with appropriate range of standard curve (7.8–125 pg/ml p24).

7. Incubate 2×10^5 HeLa TZM-bl cells per well in a 6-well plate and culture overnight.
8. Infect HeLa TZM-bl cells with 0.2, 2, and 20 ng HIV-1 p24 respectively in 6-well plates by spinoculation at $1500 \times g$ for 1 h at 32 °C. Adjust the volumes of HIV-1 containing supernatant to 500 μl with complete DMEM media.
9. Harvest the HeLa TZM-bl cultures 48 h postinfection and extract in 75 μl NP40 lysis buffer (20 m*M* Tris–HCL, pH7.4, 150 m*M* NaCl, 2 m*M* EDTA, 1% NP40, and 2 m*M* PMSF).
10. Perform Luciferase assay (Promega) on 10 μg cell lysate (determined in DCA Bradford assay). The mock-infected cell lysate is a requisite control for background luminescence, and is subtracted from the sample wells.

In order to further characterize the role of cell-associated RHA in promoting or restricting viral infectivity in target cells, equivalent amounts of RHA-deficient virions and virions containing RHA is used to infect target cells (like TZM-bl, MAGI, and PBMCs) that have experienced either control or RHA siRNA treatment. If cell-intrinsic RHA acts as an antiviral factor, then an increase in viral infectivity will be observed when RHA is downregulated in target cells. On the contrary, a reduction in viral infectivity is expected if cell-intrinsic RHA acts to promote viral infection in target cells.

For reporter assays, including the TZM-bl infectivity assay, the linear range of detectable infection is determined in advance. This parameter addresses pitfalls of infections with high virus input that results in cell killing. We recommend titration of the infection parameters using range of 0.2–20 ng HIV-1 Gag p24 ELISA units. Alternatively, the virus multiplicity of infection is utilized in the range of 0.1–10. Infection or transduction via spinoculation at $1500 \times g$ for 1 h at 32 °C results in uniform infection outcome. A further alternative to spinoculation is infection at 37 °C for 2 h in the presence of 8 μg/μl polybrene or 40 μg/ml DEAE-dextran.

5. Concluding Remarks

Besides their cellular functions, RNA helicases also perform roles that are beneficial to viruses that infect plant and animal cells. A complete understanding of targets, mechanisms, and redundancy among RNA helicases is critical for new avenues toward efficacious antiviral therapies. The methods described in this chapter focused on RHA, a necessary host cofactor for retrovirus replication. However, these approaches can readily be adapted for other cellular RNA helicases with pro- or antiviral roles.

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