Short Hairpin RNAs Delivered by Lentiviral Vector Transduction Trigger RIG-I-Mediated IFN Activation and Isolation of HCV with Enhanced Kinetics and Viral Assembly

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SHORT HAIRPIN RNAs DELIVERED BY LENTIVIRAL VECTOR TRANSDUCTION
TRIGGER RIG-I-MEDIATED IFN ACTIVATION AND ISOLATION OF HCV WITH
ENHANCED KINETICS AND VIRAL ASSEMBLY

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This is dedicated to Kathy (Howard) Weatherhead and Dr. Nathan Griggs
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ABSTRACT

Activation of the type I interferon (IFN) pathway by small interfering RNA (siRNA) is a major contributor to the off-target effects of RNA interference in mammalian cells. While IFN induction complicates gene function studies, immunostimulation by siRNAs may be beneficial in certain therapeutic settings. Various forms of siRNA, meeting different compositional and structural requirements, have been reported to trigger IFN activation. The consensus is that intracellularly expressed short-hairpin RNAs (shRNAs) are less prone to IFN activation because they are not detected by the cell-surface receptors. In particular, lentiviral vector-mediated transduction of shRNAs has been reported to avoid IFN response. Here we identify an shRNA that potently activates the IFN pathway in human cells in a sequence- and 5’-triphosphate-dependent manner. In addition to suppressing its intended mRNA target, expression of the shRNA results in dimerization of interferon regulatory factor-3, activation of IFN promoters and secretion of biologically active IFNs into the extracellular medium. Delivery by lentiviral vector transduction did not avoid IFN activation by this and another, unrelated shRNA. We also demonstrated that retinoic-acid-inducible gene I, and not melanoma differentiation associated gene 5 or toll-like receptor 3, is the cytoplasmic sensor for intracellularly expressed shRNAs that trigger IFN activation.

Hepatitis C virus (HCV) is a leading cause of liver disease and much about its life cycle is still poorly understood. We have isolated a mutant virus, named SAV III, though serially passaging JFH-1 through a cell line expressing siRNA against SRBI, a known HCV receptor. We have shown that this virus spreads faster in cell culture and produces more infectious viral particles as compared to wild type HCV. In addition, we have also shown that the HCV core protein is present in higher concentrations despite other viral proteins having similar levels of expression between WT and SAV III. SAV III also has 10 fold more intracellular and extracellular infectious virus as compared to WT, suggesting enhanced viral assembly. We propose that more SAV III core is assembled into viral particles, resulting in the core protein being stabilized. Several mutations have been identified and we are working to determine what mutation, or groups of mutations are responsible for the mutant phenotype.
CHAPTER ONE

INNATE IMMUNE RESPONSE TO EXOGENOUS RNA

INNATE IMMUNE SYSTEM

The innate immune system fights against invading pathogens by recognizing and responding to pathogen associated molecular patterns (PAMPs) such as components of bacterial cell walls and double stranded RNA (dsRNA). The RNA normally present in the cytoplasm of mammalian cells is capped or modified in some way and dsRNA is not present. Upon viral infection dsRNA and RNAs lacking caps or other modifications may be present and cells can recognize this as foreign and activate an antiviral response (Fig. 1.1). Cells express pattern recognition receptors (PRR) that detect these foreign molecular structures.

Figure 1.1- Overview of IFN-β activation by viral RNA
Toll-like receptors (TLRs) 3,7,8, and 9 are the main PRRs involved in recognizing viral nucleic acids. These TLRs can activate signaling cascades, which result in the induction of type I interferons (IFN) (reviewed by (1)). Double-stranded RNA is recognized by TLR3, single stranded RNA (ssRNA) by TLR7 and TLR8, and un-methylated CpG DNA by TLR9. These receptors are located in endosomes and phagosomes. It has been suggested that part of how host RNAs evade detection and immune activation by TLRs is the compartmentalization that keeps TLRs from being able to bind cytosolic RNA since the TLRs never “see” the cytoplasm. Some groups have shown that the immune response to synthetic siRNAs involves TLR7 and TLR8. There is cell type specificity in the expression profiles of TLRs and some cell types may have defective TLR signaling pathways (1).

Retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) have been identified as cytosolic receptors for RNA. RIG-I and MDA5 both respond to intracellular RNA although each responds to a different set of viruses and types of RNA (2). RIG-I has been found to recognize RNA viruses such as flaviviruses, paramyxoviruses, orthomyxoviruses, and rhabdoviruses. RIG-I induces IFN in a TLR independent manner and has two tandem caspase recruitment domains (CARDs) which are involved in signal transduction. RIG-I was shown to be important in IFN induction in response to RNA virus via RNAi studies (3). While the TLR pathway has been shown to be important in dendritic cells, the RIG-I pathways seems to be essential for most other cell types.

Mitochondrial antiviral signaling protein (MAVS), also known as IFN-β promoter stimulator (IPS-1), virus induced signaling adaptor (VISA) and CARD adaptor inducing IFN-β (CARDIF) has recently been identified (4-7). MAVS has been shown to function downstream of RIG-I and upstream of IκB kinase (IKK) and TANK-binding kinase 1 (TBK1). RIG-I interacts with MAVS via its CARD domain. Over expression of the MAVS CARD domain was not able to induce interferon however deletion studies have shown it to be essential in the antiviral signaling pathway. The mitochondrial localization of MAVS is also essential to its functioning (6,8).

Once the cell has recognized the pathogen, IRF-3 is phosphorylated, dimerizes, and the signal ultimately activates NF-kB, ATF-2/c-Jun and IRF-3, which together form the
enhanceosome. The enhanceosome assembles at the IFN-β promoter, which results in initiation of transcription. IRF-7 is also involved in induction of the IFN-β promoter. IRF7 is expressed at low levels in most cells until viral challenge when transcription of IRF-7 is induced (9). This is maintained through signaling of IFN-α/β, which is spontaneously expressed at low levels. IRF-3 seems to be more important for IFN-β production and contributes little to IFN-α production while IRF7 is able to induce both IFN-α and IFN-β (10).

**RIG-I AS A SENSOR OF EXOGENOUS RNA**

In several studies, RIG-I has been shown to be a mediator in recognizing transfected RNA. In the case of viral RNA, the different structure of the RNA as compared to host RNA is part of how foreign RNA is recognized. RIG-I has been shown to recognize long dsRNA as well as synthetic short dsRNA with blunt ends. It has also been shown that in vitro-transcribed or viral ssRNA can induce IFN-α production (11,12). It was found that a 5’ triphosphate in either ssRNA or dsRNA conferred IFN-α inducing activity. When the in vitro-transcribed RNA was capped, the IFN induction was reduced.

RNA modifications can greatly affect the immunostimulatory effects of RNA. For example, cholesterol conjugated siRNA, as well as nuclease resistant RNA also does not activate the immune system. Uridine content also seems to play an important role in immune activation. 2’-ribose modification of uridine was able to abolish the immune response (1). A reduction in IFN-α production was also seen when either a pseudouridine or 2-thiouridine was used in place of uridine or if 2’-O-methylated uridine 5’-triphosphate was incorporated in place of UTP (11). Neither endosomal maturation nor TLR7 were required for IFN induction. The triphosphate RNA when endogenously transcribed and not capped also induced IFN in a RIG-I dependent manner.

It was also found that RIG-I is required for IFN induction in response to rabies virus and the IFN response was abrogated when dephosphorylated RNA was used. RIG-I was also shown to directly bind to the 5’-triphosphate and is the direct receptor responsible for recognizing the 5’-triphosphate RNA. Host RNA such as 7SL RNA, a component of the signal recognition particle, however, has a 5’-triphosphate and is present at high copy numbers in the cytosol, thus
the 5’-triphosphate may not be the only feature responsible for distinguishing self and viral RNA (11,12). RIG-I is expressed in almost all cell types.

**siRNA AS AN ACTIVATOR OF INNATE IMMUNITY**

siRNA has recently become a popular method of studying the function of proteins. RNA interference (RNAi) was originally discovered in plants and has anti-viral functions in plants. In animals, micro RNAs (miRNA) are the main form of RNAi and only have partial complementarity to their targets which results in translational repression (13). miRNA is expressed as a long transcript of 10’s to 100’s of nucleotides long (Fig. 1.2). This primary miRNA (pri-miRNA) and is cleaved in the nucleus by Drosha, resulting in a hairpin-loop of 60-70 nucleotides. This pre-miRNA is exported to the cytoplasm where it is further processed by Dicer to form a 20-22nt double stranded miRNA:miRNA* duplex with a 5’ phosphate and a 2nt 3’ overhang (14-17). siRNA has perfect complementarity to it’s target mRNA, resulting in cleavage of the mRNA, is usually expressed as a short RNA which folds into a hairpin-loop and the loop is then cleaved by dicer resulting in a short dsRNA. The miRNA or siRNA fragments are then loaded into the RNA-induced silencing complex (RISC). The RISC-RNA complexes then target complementary mRNA leading to shutdown of gene expression of the targeted mRNA. In some studies these 21-mer RNAs were shown to be small enough to bypass immune recognition of long dsRNA (11). Protein kinase R (PKR) requires at least 30bp to be activated. Several studies later studies have since shown varying immune response to synthetic or *in vitro* transcribed RNA (1).
One study by Judge et al. (18) showed a sequence dependence of siRNA stimulation of an innate immune response. ssRNAs containing poly(U) or GU-rich sequences tend to be immunostimulatory. In this study it was found that the sequence 5’-UGUGU-3’ appeared to be an immunostimulatory motif. This was supported by data showing that a single U to C base substitution significantly reduced the siRNA mediated induction of IFN-α. When new siRNA were designed to target the same protein but at a different locus and avoided GUGU or poly(U)
motifs, there was little to no IFN induction (18). It was also shown that knockdown was independent of immune stimulation by the siRNA.

siRNA induction of IFN was also studied by Iggo and Pebernard (19). 2',5'-oligoadenylate synthetase 1 (OAS1) is a gene commonly used to measure interferon response. They found that OAS1 induction was dependent on the amount of vector used, as measured through OAS1 induction. Transcription termination usually occurs when RNA polymerase-III encounters a run of 4-5 Ts. However around 15% of transcripts continue past the first 4T signal to a second string of T’s further down and transcribed a 35 nt transcript. However, when extra Ts or the down stream sequence was mutated OAS1 expression was still induced (19). In addition, a mutation resulting in a smaller dsRNA stem rendered the siRNA unable to induce interferon. This study also showed no correlation between OAS1 induction and target gene silencing (19).

It has been shown that siRNAs synthesized by the T7 polymerase system induce IFN. However, this method can be modified to incorporate two 3’-adenosines, which prevents base pairing with the initiating guanines. RNase T1 and calf intestine alkaline phosphotase (CIAP) can then remove the 5’-triposphates. It was found that blunt end siRNAs induce interferon while siRNA with 3’ overhangs do not. The presence of a guanine residue on T7-transcribed RNAs prevented the activation of IFN and the residual amount of guanine associated with the 5’ end of the transcript was proportional to the IFN response (20).

The method by which RNA enters cells also seems to be of importance in determining if there will be an IFN response. Neither naked RNA nor empty cationic lipids were able to induce interferon. In a study by Ma (21), DOTAP liposomes complexed with siRNA were able to activate signal transducer and activator of transcription 1 (STAT1). DOTAP liposomes alone were able to induce some activation of STAT1 but to a lesser degree than the siRNA complexed liposomes. DOTAP complexed with ssRNA corresponding to the ds siRNA was also able to induce the immune stimulated STAT1. The sequences of immunostimulatory RNA in this study did not contain the previously described motif, which may suggest there are other sequences effective in inducing IFN responses (21).

One method to avoid IFN activation is to transfected a plasmid, which codes for the desired siRNA. In a study by Robbins (22), the UGUG motif also seemed to contribute to stronger induction of innate immunity. In this study, immunostimulatory effects of stable expression of
shRNA were compared to transfection of synthetic siRNA. Synthetic siRNAs that were tested showed strong IFN induction while only slight, sporadic induction of IFN was seen when shRNAs of the same sequence were expressed by the cells (22).

Recently another class of small RNAs called Piwi-interacting RNA (piRNA), have been discovered. This class of RNA seems to be important in controlling transposon activity. These RNAs are usually 25-27 bp long and have not been shown to interact with mRNA. Also, no immunostimulatory effects of this RNA have been described (23). miRNA has been shown to have some antiviral activity against HCV. These miRNA, which were induced by IFN, had sequence specificity for the HCV RNA and transfection of synthetic miRNA resulted in partial knockdown of HCV (24). miRNAs thus far have not been described much as being involved in IFN induction or response.
CHAPTER TWO

SHORT HAIRPIN RNAs DELIVERED BY LENTIVIRAL VECTOR TRANSDUCTION TRIGGER RIG-I-MEDIATED IFN ACTIVATION

INTRODUCTION

RNA interference can be a very useful technique in studying the importance of a specific protein. While investigating the importance of cyclophilins, our lab discovered an shRNA which, upon transduction, was capable of significantly knocking down hepatitis C virus (HCV) despite having only a moderate effect on its target protein. Moreover, other shRNAs that targeted the same protein with more efficiency had little to no effect on HCV expression. The knockdown of HCV also seemed to be much more rapid then is typically expected with an RNAi effect. This led us to suspect an innate immune response to the RNA. We then investigated which pathway and proteins of the innate immune system may be involved.

MATERIALS AND METHODS

Cells, antibodies, and RNAs

GS5 and LH86 cells have been described previously (25,26). Huh-7 and 293FT cells were maintained in DMEM supplemented with 10% FBS. We used the following antibodies: anti-CyPA (Biomol, Plymouth Meeting, PA); anti-CyPB (Affinity BioReagents, Rockford, IL); anti-Ku80, anti-Flag, and anti-actin (Sigma-Aldrich, St. Louis, MO); anti-IFN stimulate gene (ISG)15 (Rockland Immunochemicals, Gilbertsville, PA); anti-NS5A (Virogen, Watertown, MA); and anti-NS3 (in-house). GSB1 and H801 cells have been described previously (27). Poly I:C was purchased from Sigma-Aldrich, and synthetic hairpin RNA was purchased from Integrated DNA
Technologies (Coralville, IA). Synthetic siRNA was purchased from Ambion (Austin, TX).

**Gel electrophoresis and western blotting**

Protein contents of cell lysate were quantified with the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA), and an equal amount of total protein was loaded in each lane. Samples for IRF-3 dimerization assay were run on a polyacrylamide gel under nondenaturing conditions (28). Other samples were denatured and separated by SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane and stained with the appropriate antibodies with the SNAP i.d.™ system (Millipore, Worcester, MA) according to the manufacturer’s instructions.

**Transfections**

For luciferase assays, cells were seeded to a confluency of 50%, and for all other assays, cells were seeded to a confluency of 30%. The next day, transfections of DNA plasmids and synthetic RNAs were performed with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Plasmids**

Plasmids pGL3-IFNA1, pGL3-IFNB, pRL-TK, pCMV-Flag-IRF-3, and pCR3.1-IRF-7A have been described previously (29). Short hairpin RNAs (shRNAs) were expressed from a human immunodeficiency virus (HIV)–based lentiviral vector (25,30), and sh-PCAF was constructed on the basis of a previously reported sequence (19). Plasmid sh-B971/H1 was constructed by cloning of the DNA fragment encoding the sh-B971 RNA into pSilencer 3.0-H1 (Ambion, Austin, TX) according to the manufacturer’s instructions. The RIG-I and TLR3 constructs have been described (31,32). The RIG-I C construct encodes Flag-tagged, C-terminal 707 aa of human RIG-I cloned into a bicistronic expression vector modified from pBICEP-CMV-1 (Sigma-Aldrich, St. Louis, MO), in which the CMV promoter was replaced with the elongation-factor-1 promoter. The MDA5, MDA5-C constructs were kindly provided by T. Fujita (3). HCV genotype 2a NS3-4A protease was expressed from the pCMV-3Tag-1a plasmid (Stratagene, La Jolla, CA).

**Luciferase assay for Interferon promoter activity**
293FT cells were seeded in 24-well plates and were transfected 16 h later with 400 ng of a shRNA expression vector, 40 ng of pGL3-IFNA1 or pGL3-IFNB, 20 ng of pRL-TK, and 50 ng of pCR3.1-IRF-7A. Cells were collected 48 h after transfection. Luciferase assays were performed with the Dual-Glo® Luciferase Assay system reagents (Promega, Madison, WI) and luminescence quantified with a Modulus Microplate reader (Turner BioSystems, Sunnyvale, CA). Ratios of firefly luciferase (from the pGL3 vectors) to Renilla luciferase (from the pRL-TK vector) were calculated, and that of the sh-B971 sample was normalized to 100%.

**Lentiviral vectors**
Sequences of shRNA are shown in Table 1. Lentiviral vector production and transduction were performed as described previously (30). Viral vectors were pelleted by ultracentrifugation at 50,000 × g at 4°C for 3 hours and resuspended in a volume of PBS that was 1% of the original medium volume. The titers of the concentrated vectors were then measured with a p24 ELISA kit (ZeptoMetrix, Buffalo, NY).

**Real-time reverse transcription-PCR**
Real time reverse transcription–PCR (RT-PCR) was performed as described previously (25). The primers used were OAS1 forward, 5’-AGG TGG TAA AGG GTG GCT CC-3’ and OAS1 reverse 5’- ACA ACC AGG TCA GCG TCA GAT-3’; RIG-I forward 5’-GAG GCA GAG GAA GAG CAA GAG G-3’ and RIG-I reverse 5’-CGC CTT CAG ACA TGG GAC GAA G-3’; GAPDH forward 5’-TCA CTG CCA CCC AGA AGA CTG-3’ and GAPDH reverse 5’-GGA TGA CCT TGC CCA CAG C-3’. The primers for HCV detection were 5’-CGC TCA ATG CCT GGA GAT TTG-3’ and 5’-GCA CTC GCA AGC ACC CTA TC-3’.

**Flow cytometry**
For flow cytometry, GS5 cells were fixed 48 h after treatment in a solution of 2% paraformaldehyde and analyzed with a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). Mean GFP intensity was plotted, and that of the sh-NTC sample was normalized to 100%.

**RNA extraction and northern blots**
Total RNA from transiently transfected 293FT cells was extracted with RNA STAT-60 (Tel-Test, Friendswood, TX) and separated on a 7.5% urea polyacrylamide gel. The transfer of RNA onto nitrocellulose membrane and hybridization were performed according to standard molecular biology protocols. The probe for detecting the expression of sh-B971 and its variants was a synthetic DNA oligomer corresponding to the bottom strand of sh-B971. Radioactive labeling of the probe was performed with an end-labeling protocol with T7 polynucleotide kinase (Ambion, Austin, TX). The exposure and detection of the radioactive signal was performed with a Typhoon Imager (GE Healthcare, Piscataway, NJ) with Quantity One software (Bio-Rad, Hercules, CA).

**RESULTS**

**A short-hairpin RNA directed at CyPB induces IFN production in human embryonic kidney cells**

To investigate the potential role of the cyclophilins (CyPs) in HCV replication (33), we delivered several shRNAs directed at mRNAs of three CyPs into HCV replicon cells by means of a lentiviral vector, using a murine U6 promoter to drive the expression of the shRNA (Figure 2.1 A) (30). We observed a discrepancy between two anti-CyPB shRNAs (B971 and B710) in their relative efficiency in knocking down CyPB expression and in suppressing HCV. Lentiviral vector sh-B971 was less efficient in knocking down CyPB expression but potently inhibited HCV NS5A expression in a human hepatoma cell line containing replicating HCV RNA (Figure 2.1 B, left). Viral inhibition was independent of CyPB knockdown, as control medium from transfected 293FT cells that did not contain any lentiviral vector particles, generated by omission of the packaging plasmids during transfection, also inhibited HCV replication (Figure 2.1 B, right) without affecting CyPB expression. The fast kinetics of viral inhibition (complete inhibition with 48 h, data not shown) was also more consistent with IFN- than with RNAi-based inhibition. The presence of IFN in the lentiviral vector preparation of sh-B971 was confirmed by strong induction of 2’-5’-oligoadenylate synthetase 1 (OAS1), a classic IFN-induced gene, in both naïve Huh-7 and the HCV replicon cell line (GS5) treated with the medium (Figure 2.1C). In addition, HCV replication in an IFN-resistant HCV replicon cell line (H801), in contrast to that in a wild-type replicon cell line (GSB1) (27), was not inhibited by the sh-B971 medium
(Figure 2.1 D), suggesting the lack of additional viral inhibiting agents in the sh-B971 medium. Expression of sh-B971 in 293FT cells also induced dimerization of IRF-3, confirming the activation of the IFN production pathway in these transfected cells (Figure 2.1 E). Finally, sh-B971 was able to activate both IFN-α and IFN-β promoters, although the activation of the IFN-α promoter required coexpression of IRF-7, which is normally expressed at very low levels in 293-based cells (Figure 2.1 F). These results demonstrate that sh-B971 is a potent activator of IRF-3 and IRF-7, master regulators of IFN expression in human cells.
Figure 2.1- A small-hairpin RNA directed at CyPB induces IFN production in human embryonic kidney cells. (A) Sequence of sh-B971, which was expressed from a self-inactivating human immunodeficiency virus (HIV) vector with a murine U6 promoter (59). (B) Inhibition of HCV expression by culture media of sh-B971-transfected 293FT cells. GS5 cells were treated with
culture supernatant taken from 293FT cells transfected with various shRNA plasmids with (left) or without (right) the packaging plasmids overnight. Cells were then cultured in fresh media for an additional 6 days before being lysed for western blotting. (C) OAS1 induction by culture supernatant from 293FT cells transfected with sh-B971. Huh 7 and GS5 cells were treated with culture supernatant from 293FT cells transfected with either sh-Luc or sh-B971 for 24 h before RNA extraction and real-time RT–PCR analysis. OAS1 RNA level was normalized to that of GAPDH RNA. (D) Transfected culture media failed to suppress HCV replication in an IFN-resistant cell line. HCV replicon cells were cultured as described earlier (34) and then treated with the indicated culture medium from transfected 293FT cells. HCV RNA was analyzed with real-time RT–PCR. (E) IRF-3 dimerization in response to sh-B971 expression. Flag-IRF-3 was cotransfected with a shRNA into 293FT cells. Cells were lysed 24 h after transfection, and total cell lysate was separated on a polyacrylamide gel under non-denaturing conditions, transferred and stained with an anti-flag antibody. (F) IFN-α and IFN-β promoter activation by sh-B971 expression. Sh-NTC, sh-C454 (an shRNA directed at CyPC), or sh-B971 was cotransfected along with luciferase reporter plasmids with or without IRF-7. The ratios of firefly luciferase readings to Renilla luciferase readings were plotted.

**RIG-I mediates the IFN induction by sh-B971**

We next investigated the role of the different viral/exogenous RNA sensors, RIG-I, MDA5, and TLR3, in sh-B971–triggered IFN production. Mammalian expression plasmids encoding each of these proteins, as well as the dominant negative (DN) mutants of RIG-I and MDA5, were transfected into 293FT cells with shRNAs and an IFN-β promoter reporter construct. The signaling to IFN-β promoter and the expression of the PRR proteins were then examined 48 h after transfection. In the absence of sensor proteins, the sh-B971 increased activation of the IFN-β promoter by 2.6-fold (Figure 2.2 A). Coexpression of MDA5 or TLR3 did not increase or decrease sh-B971’s ability to activate IFN-β promoter relatively to the negative control shRNA (sh-NTC), but in the presence of RIG-I coexpression, the induction of IFN-β promoter by sh-B971 was increased to ~30-fold. Moreover, ectopic expression of a DN mutant of RIG-I (RIG-I C), but not that of MDA5 (MDA5-C), completely abrogated IFN promoter activation by sh-B971. With the exception of TLR3, which required prolonged exposure of the western blot to be detected, the cytoplasmic sensors and their mutants were expressed at comparable levels (Figure 2.2 B). Moreover, activation of IRF-3 (Figure 2.1 E) and IFN promoters (Figure 2.1 F) in 293FT cells, which do not contain a functional TLR3 signaling pathway (34), indicates that TLR3 plays a negligible role, if any, in IFN induction by sh-B971. The combination of sh-B971 and RIG-I produced the highest level of IFN-β promoter activity, which were confirmed by western blotting showing that endogenous ISG15 induction was only
detectable in cells cotransfected with sh-B971 and wild-type RIG-I (Figure 2.2 B). To confirm further that biologically active IFN was released from these cells, we applied the culture medium of the transfected 293FT cells to an HCV replicon cell line (GS5) in which NS5A-GFP expression is used for monitoring viral RNA replication (35). HCV replication in this cell line is extremely sensitive to IFN, and the effect of the cytokine can be readily measured as the change in the mean GFP intensity of the treated cells. As shown in Figure 2.2 C, culture medium from sh-B971 efficiently suppressed HCV replication, resulting in a decrease in the NS5A-GFP intensity within 48 h of treatment. Cotransfecting wild-type RIG-I produced a medium with stronger inhibition, whereas the RIG-C drastically suppressed the antiviral effect of the medium. Finally, real-time RT-PCR analysis revealed that sh-B971, but not the negative control shRNA, strongly activated expression of endogenous RIG-I, a well-characterized ISG whose induction requires paracrine/autocrine action of IFN (36,37). As expected, poly I:C activated RIG-I expression in the same assay (Figure 2.2 D). These results, taken together, show that RIG-I is the cellular sensor that mediates the IFN induction by sh-B971.
Figure 2.2- Sh-B971 acts through the RIG-I pathway to trigger IFN activation. (A) RIG-I, and not melanoma differentiation associated gene 5 (MDA5) or toll-like receptor 3 (TLR3), mediated IFN induction by sh-B971. Various PRRs and their mutant proteins were coexpressed with either sh-NTC or sh-B971 along with the luciferase reporters. The firefly luciferase readings were normalized to Renilla luciferase readings, and the value of sh-B971 was set to 100. (B) Proper expression of the transfected PRR proteins and induction of ISG 15 expression when sh-B971 was coexpressed with RIG-I. RIG-I, RIG-I C, MDA5 and MDA5 C are all tagged with two tandem copies of Flag epitope. TLR3 is tagged with single Flag epitope and required prolonged exposure for detection. (C) A dominant negative mutant of RIG-I blocked sh-B971-triggered IFN production. ShRNAs were cotransfected with either RIG-I or a dominant negative form of RIG-I (RIG-I C) into 293FT cells. The culture supernatant was then tested for its ability to suppress NS5A-GFP expression in GS5 cells with an overnight treatment. (D) Upregulation of RIG-I RNA level by sh-B971 expression. 293FT cells were transfected with sh-NTC, sh-B971 or
polyinosinic-polycytidylic acid. RNA was extracted 24 h after transfection and analyzed by real-time RT PCR. The RIG-I RNA levels were normalized to those of GAPDH RNA.

**Structure and sequence determinants of shRNA-mediated IFN activation**

The majority of the shRNAs that we use in the lab do not activate RIG-I expression and IFN signaling despite having essentially the same structure as sh-B971, so we wanted to determine whether the sequence of sh-B971 is distinctive enough to trigger the production of IFN. We first tested a synthetic siRNA duplex with the same target sequence as sh-B971. This siRNA (si-B971-syn) should resemble the final Dicer product of sh-B971 except for the 5’ ends. The synthetic siRNA contains 5’-OH groups, whereas the Dicer products probably contain a 5’ monophosphate on one strand and a 5’ triphosphate on the other. Si-B971-syn knocked down CyPB expression as efficiently as sh-B971 (Figure 2.3 A) while failing to activate IFN production, as measured by the GFP-HCV assay (Figure 2.3 B). To determine whether the sequence of the intact hairpin RNA before Dicer cleavage is sufficient to trigger IFN, we tested a synthetic shRNA (sh-B971-syn) that had exactly the same sequence as the predicted intracellular sh-B971 transcript generated by the U6 promoter. Again, the 5’ end of the synthetic sh-B971 had a 5’-OH group instead of any phosphate. Sh-B971-syn behaved similarly to si-B971-syn in that it knocked down CyPB expression without activating IFN response (Figure 2.3). These results suggest that the 5’-end status of sh-B971 is important for IFN activation, consistent with the previously finding that a 5’-triphosphate is required for RIG-I activation (11,12)
Figure 2.3- Structural determinants of IFN activation by sh-B971. 293FT cells were transfected with shRNA-expressing lentiviral plasmids or synthetic RNAs. (A) Knockdown of CyPB expression by various forms of B971 siRNA. Cells were collected 5 days after transfection and analyzed by western blot for detection of CyPB. (B) Lack of HCV inhibition by synthetic forms of sh-B971. Culture supernatant from transfected 293FT cells was collected 48 h after transfection and used to treat GS5 cells overnight; the GS5 cells were then analyzed by flow cytometry.

To determine the contribution of the individual residues of the sh-B971 sequence, we introduced a series of point mutations into the shRNA and tested them for IFN induction. We changed the first nucleotide from A to G, C, or T while maintaining base-pairing between nucleotides +1 and +47. These mutant shRNAs lacked the ability to activate IFN production (Table 2.1). Changing the +1 nucleotide to G while leaving the +47 nucleotide intact also abolished IFN activation by the shRNA (A1/G), as did the reciprocal mutation U47/C. The importance of the first nucleotide was further confirmed by the inability of sh-B971+1 to activate IFN. The target of sh-B971+1 was shifted one nucleotide downstream on the CyPB mRNA, producing a shRNA starting with a G at the +1 position. The presence of an A at the +1 position was not, however, sufficient to render a shRNA competent for IFN activation, as replacing the first nucleotide of the sh-NTC with an A did not generate an IFN-inducing shRNA (NTC-A and NTC+1). These results indicate that a protruding/unpaired A at the end of the hairpin or the RNA duplex, a potential result of “breathing” at the end of the dsRNA, is not sufficient to trigger IFN induction as previously suggested (19).
Table 2.1- Sequence of the siRNA duplexes

<table>
<thead>
<tr>
<th>Name</th>
<th>siRNA Sequence</th>
<th>Interferon Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTC</td>
<td>5'-GACUGAACAGGUGUGUCUGAUU-3'</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3'-UUGACUUGUAACACGCACAU-5'</td>
<td></td>
</tr>
<tr>
<td>NTC-A</td>
<td>5'-AACUGAACAGGUGUGUCUGAUU-3'</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3'-UUGACUUGUAACACGCACAU-5'</td>
<td></td>
</tr>
<tr>
<td>NTC-1</td>
<td>5'-ACUGAACAGGUGUGUCUGAUU-3'</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3'-UUGACUUGUAACACGCACAU-5'</td>
<td></td>
</tr>
<tr>
<td>B971</td>
<td>5'-ACUCUACCAACACUGACCUCU-3'</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
<td></td>
</tr>
<tr>
<td>B971-G</td>
<td>5'-GCUCUACCAACACUGACCUCU-3'</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
<td></td>
</tr>
<tr>
<td>B971-C</td>
<td>5'-CUCCUACCAACACUGACCUCU-3'</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
<td></td>
</tr>
<tr>
<td>B971-T</td>
<td>5'-UCUCUACCAACACUGACCUCU-3'</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
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<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
<td></td>
</tr>
<tr>
<td>A1/G</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>U47/C</td>
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</tr>
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<td></td>
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<td>B15A4</td>
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<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
<td></td>
</tr>
<tr>
<td>B18A1</td>
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<td></td>
</tr>
<tr>
<td>Loop A</td>
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</tr>
<tr>
<td></td>
<td>3'-UUGAGAUGUUGUGUAGUGACUG-5'</td>
<td></td>
</tr>
<tr>
<td>PCAF</td>
<td>5'-AAGAAUUAUCUAGUGACAGCUU-3'</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>3'-UUCUUAAUAAUAAUGUACGUCUG-5'</td>
<td></td>
</tr>
</tbody>
</table>
Two point mutations located farther into the stem structure of the shRNA (9G9 and B18A1) also reduced its ability to induce IFN even though the base-pairing was perfectly maintained in these mutants. Finally, replacing the 9-nt hairpin loop with a 7-nt loop that had been previously shown to abolish shRNA-mediated RNAi (loop A mutant) (38) eliminated sh-B971’s ability to induce IFN, suggesting the importance of RNA processing in the induction. To determine whether the inability of the mutant shRNAs to induce IFN was due to lower expression levels, we performed northern blotting analysis of the shRNA expression on the wild-type and two mutants. The mutants A1/G and Loop A were chosen because their final siRNA products have exactly the same sequence as that of the wild-type sh-B971 and can thus be detected with the same efficiency by the same probe. Although sh-A1/G and sh-Loop A were clearly unable to activate IFN-β promoter (Figure 2.4 A), they were both expressed at levels comparable to those of the wild-type sh-B971 product (Figure 2.4 B). Interestingly, the final siRNA product of sh-Loop A was slightly smaller than those of sh-B971 and sh-A1/G, suggesting that cleavage did occur and perhaps occurred one or two nucleotides into the stem to compensate for the shorter loop.
Figure 2.4-Comparably expressed mutant forms of sh-B971 do not induce IFN. (A) IFN activation by select sh-B971 mutants. The shRNA expression plasmids were transfected into 293FT cells with the luciferase reporters to measure IFN-β promoter activation. The firefly luciferase readings were normalized to Renilla luciferase readings, and the value of sh-B971 was set to 100. (B) Intracellular levels of siRNA products of sh-B971 and mutants. RNA was extracted from transfected 293FT cells and analyzed by northern blotting with a DNA oligonucleotide probe that is complementary to all three forms of sh-B971 (wt, sh-A1/G and Loop A).

RIG-I-mediated IFN induction by sh-B971 is independent of a blunt end of the dsRNA

Blunt-ended siRNA has been previously reported to be stronger inducers of IFN than the siRNAs with overhangs (39). Indeed, a previously reported IFN-inducing shRNA, sh-PCAF (p300/CREB-binding protein–associated factor), contains a blunt end (19) and was more potent in activating IFN than sh-B971 (Figure 5 A), which is predicted to form an overhang of 2–3 Ts at
each end of the final siRNA. We therefore constructed a version of the sh-B971 that would be
blunt at the end that is not processed by Dicer by adding two extra As to the 5’-end of the
shRNA. This modification (Blunt sh-B971) did not increase the ability of sh-B971 to activate
IFN-β promoter (Figure 2.5 A). We confirmed, in two independent experiments, that IFN
induction by sh-PCAF was also mediated by RIG-I. First, cotransfection of DN RIG-I resulted a
50- to 100-fold inhibition of IFN induction by sh-PCAF (Figure 2.5 B), whereas wild-type RIG-I
increased IFN induction by several fold in the same assay. Second, when HCV NS3-4A
protease, which cleaves MAVS, thereby blocking the RIG-I pathway, was coexpressed with
either sh-B971 or sh-PCAF, IFN induction by these shRNAs were severely compromised (Figure
2.5 C), further substantiating a role of the RIG-I and MAVS pathway in mediating IFN induction
by both the blunt-ended sh-PCAF and the sh-B971 with overhang. The proper expression of
NS3-4A protease was confirmed by western blotting (Figure 2.5 D).
Figure 2.5- RIG-I mediated IFN induction by shRNA is independent of a blunt end of the dsRNA. (A) IFN-β activation in response to blunt-ended shRNAs. Both sh-PCAF and Blunt sh-B971 contained two extra As at the 5'-end of the shRNA, making the non-hairpin end of the shRNA blunt rather than having an overhang of two TTs. (B) RIG-I dependency of IFN-β activation by sh-PCAF. (C) Blockade of shRNAs-triggered IFN activation by HCV NS3-4A. A mammalian expression plasmid encoding the NS3-4A protease from HCV isolate JFH-1 was cotransfected with the shRNA and luciferase reporter plasmids. (D) Western blot showing expression of HCV NS3-4A in transfected 293FT cells. Cell lysate from (C) was separated by SDS–PAGE and probed with an anti-NS3 antibody.

Sh-B971 expressed from an H1 promoter triggers IFN induction

To assess the contribution of the promoter choice in IFN activation by intracellular expressed shRNA, we expressed sh-B971 from another commonly used pol III promoter, the
human H1 promoter. Both the original, mU6-driven sh-B971 and the H1-driven sh-B971 activated IFN-β promoter (Figure 2.6 A) and resulted in secretion of IFN into the transfected cell-culture media, which in turn suppressed HCV replication (Figure 2.6 B). Proper expression of the siRNA (Figure 2.6 C) and the subsequent knockdown of CyPB expression (Figure 6 D) all appeared normal for sh-B971 expressed from the H1 promoter plasmid, which has a backbone different from that of our lentiviral vector carrying the mU6 promoter. These data suggest that IFN induction by sh-B971 is not restricted to a particular promoter or expression construct. Further supporting this conclusion was the observation that the expression cassette by itself, removed and isolated from the lentiviral plasmid by restriction digestion, could also activate IFN production in transfected 293FT cells (data not shown).

Figure 2.6- Sh-B971 expressed from an H1 promoter triggers IFN activation. Sh-B971 expressed from an H1 promoter was capable of (A) activating IFN-β promoter and (B) triggering IFN production to inhibit HCV replication in GS5 cells. (C) Intracellular levels of U6- and H1-
driven sh-B971 products. RNA extraction and northern blotting were performed as described in Figure 4B. (D) Knockdown of CyPB expression by sh-B971 expressed from an H1 promoter.

**ShRNAs delivered via lentiviral transduction trigger IFN activation in vitro**

To this point, all the IFN induction experiments were done with transient transfection of DNA vectors and it was possible that certain features of the double-stranded plasmid DNA are responsible for IFN induction. We first tried to address this point by transfecting just the shRNA-expressing cassette, generated either by PCR or restriction enzyme digestion, into 293FT cells and confirming that these fragments of ~200bp were sufficient to trigger IFN induction (Figure 2.7). To definitively rule out any contribution by dsDNA, we used a lentiviral transduction system which has been suggested to express shRNAs that can escape detection by PRRs and IFN activation (22). We produced lentiviral particles containing shRNAs from 293FT cells using standard methods, centrifuged them to separate the vectors from the IFN-containing media, and then used them to infect naïve 293FT cells (Figure 2.8 A). Both sh-B971 and sh-PCAF vectors induced IFN production when delivered as concentrated lentiviral particles, measured both by HCV suppression (Figure 2.8 B) and by OAS induction (Figure 2.8 C) in Huh-7 cells. To rule out the possibility that residual IFN in the concentrated viral particles was responsible for these results, we added 100 units/ml IFN to the negative control vector sample before the concentration step. This preparation, designated sh-NTC*, was not able to trigger IFN production in naïve 293FT cells, suggesting that the concentration step effectively removed the soluble IFN from the viral particle pellet. Proper knockdown of the siRNA target of sh-B971 was confirmed by this route of shRNA delivery (Figure 2.8 D). To prove definitively that IFN induction by the shRNAs was mediated by the lentiviral infection route, we tested the effect of an inhibitor of HIV reverse transcriptase, Nevirapine, on IFN induction by sh-B971 and sh-PCAF. As shown in Figure 2.8 E, inclusion of Nevirapine at the time of transduction effectively blocked the ability of both shRNAs to induce IFN in the transduced cells, suggesting the importance of the reverse transcription step in the expression of the shRNAs delivered by the lentiviruses. To determine whether lentiviral vector-delivered shRNA can trigger IFN induction in cells other than 293FT cells, we transduced a human hepatoma cell line, LH86, which has been reported to produce IFN upon viral infection (26), and examined IFN induction in these cells. Culture medium from LH86 cells transduced with sh-PCAF contained biologically active
IFN, which suppressed HCV replication in GS5 cells (Figure 2.8 F), indicating that the ability of shRNAs delivered by lentivirus to induce IFN response was not limited to 293FT cells.

Figure 2.7- ShRNA-expression cassette is sufficient to trigger IFN induction. The expression cassette, which contains the mU6 promoter, followed by sh-B971-encoding RNA and a pol III termination signal, was generated by either PCR or BamHI digestion and then transfected into 293FT cells along with the lentiviral vectors expressing either sh-luc or sh-B971. A different transfection reagent, LT-1, was also tested. The supernatant of the transfected cells was then applied to the GFP-HCV reporter cell line to monitor the presence of functional IFN in the media.
Figure 2.8- ShRNAs delivered by lentiviral transduction trigger IFN activation in vitro. (A) Diagram of the experimental setup. Lentiviral vectors were concentrated by ultracentrifugation for removal of soluble IFN proteins in the vectors prepared from transfected 293FT cells. (B) Transduction of 293FT cells with the concentrated shRNA vectors triggered IFN production.
Culture supernatant was collected 48 h after transduction and was used to treat GS5 cells overnight; the GS5 cells were then analyzed 48 h after treatment by flow cytometry. (C) OAS induction in Huh 7 cells treated with culture supernatant from lentivirally transduced cells. Huh 7 cells were treated with culture supernatant for 24 h before RNA extraction and RT-PCR analysis. OAS1 expression level was normalized to that of GAPDH RNA. (D) Knockdown of CyPB expression in transduced cells, which were collected 5 days after transduction for western blotting. (E) Blockade of lentivirus-triggered IFN activation by a HIV reverse transcriptase inhibitor. 293FT cells were transduced in the presence of 80 nM of Nevirapine; culture supernatant was collected 48 h after transduction and used to treat GS5 cells. (F) Culture medium from LH86 cells transduced with sh-PCAF suppressed HCV replication. This experiment was done as depicted in (A) with the LH86 cells in the place of 293FT cells in the transduction step.

**DISCUSSION**

It has been reported that certain chemically synthesized and phage polymerase-in vitro transcribed siRNAs can nonspecifically induce IFN responses and produce off-target effects via various PRRs, including TLRs. However, the induction of IFN response by shRNAs and its underlying mechanisms have not been as well studied. The actual number of shRNAs that are capable of triggering IFN response will certainly be larger than the few that have been reported in the literature, yet very little is known about the unique characteristics of the select shRNAs and the pathway that they use to activate IFN production. The present study identifies RIG-I, but not MDA5 or TLR3, as the mediator for activation of IFN responses by two shRNAs that are distinct in sequence and structure but both capable of IFN induction in human cells. This was demonstrated by induction of IRF-3 dimerization, activation of IFN promoters, induction of endogenous ISGs (ISG15, OAS and RIG-I), and secretion of IFN, all of which depended on RIG-I and its downstream adaptor, MAVS. In addition, we show that delivery of these shRNAs via lentiviral transduction does not reduce their IFN-inducing capacity, indicating that the ability of lentiviral vector transduction to avoid IFN induction by shRNAs, as reported previously (22), may not be universally applicable to all the shRNAs.

Specific recognition of dsRNAs or ssRNAs bearing 5’ triphosphates by RIG-I is presumably determined mostly by structural features other than the nucleotide sequence of the RNA. Yet IFN activation by sh-B971 exhibited a stringent dependence on specific nucleotides at multiple positions of the shRNA. An AA dinucleotide at the beginning of the U6 transcript has previously been suggested to result in aberrant transcription, and preserving a C/G sequence
at positions –1/+1 suggested to avert IFN induction (19). We indeed observed a strict requirement for an adenylate at the +1 position of sh-B971 for RIG-I recognition and IFN activation, but we observed no difference in expression levels or the apparent sizes of the sh-B971 RNAs bearing either an A or a G at the +1 position. Furthermore, mutations introduced elsewhere in the shRNA also abolished or diminished sh-B971’s ability to activate IFN, suggesting additional sequence requirement for efficient RIG-I recognition and IFN triggering. Despite these results, because we were not successfully in cloning and sequencing the vector-expressed siRNA, we cannot exclude the possibility that the adenylate at the +1 position interferes with transcription and that the resultant abnormal transcript contributes to IFN induction.

Interestingly, the loop A mutant, which contains a predicted loop of 7 nucleotides, generated a siRNA duplex inside the cells that is slightly smaller than that of the shRNAs with a wild-type hairpin loop, suggesting the processing by Dicer into the stem, perhaps fulfilling the requirement of a length of 9 nucleotides for the hairpin loop (38). This mutant form of sh-B971 was not, however, able to trigger IFN activation.

Despite the abilities of both sh-B971 and sh-PCAF to activate the RIG-I pathway, the two shRNAs are unrelated in sequence. Two short stretches of siRNA sequences, GUCCUUCCCAA and UUGUGU, that have been previously defined as IFN- or cytokine-activating motifs (18,40) are not found in either sh-B971 or sh-PCAF. Any common sequence motifs of IFN-activating shRNAs, if any, remain to be defined. The two shRNAs also differ in that one is predicted to contain one blunt end and the other two ends with overhangs. These results suggest that, although blunt ends may increase siRNA’s ability to be recognized by RIG-I (39), they are not required for IFN activation by an endogenously expressed shRNA. The best-characterized RNA structure motif recognized by RIG-I is the 5’-ppp, which is absent from virtually all the cellular RNAs as a result of either 5’ capping or internal cleavage before their appearance in the cytoplasm. A synthetic short hairpin RNA that has the same sequence as sh-B971 but lacks the 5’-ppp failed to induce IFN, suggesting the 5’-end status of the intracellularly expressed sh-B971 contributes to IFN activation. Whether or not the 5’-end of a shRNA is capped has not been investigated. Murine U6 RNA does not contain the trimethylguanosine cap that is present on mRNAs and other U small nuclear RNAs; instead it contains a γ-monomethyl phosphate cap at its 5’-end (41). Capping of heterologous transcripts produced from the mU6 promoter, however,
requires a stem loop at the 5’-end of the transcript and an AUAAUC sequence immediately after (42). Most shRNAs, including sh-B971 and sh-PCAF, would not meet these requirements and thus should contain unmodified 5’-ppp. Similarly, no evidence of a cap structure for H1 transcripts could be found in the literature. We attempted to express sh-B971 using a miRNA expression cassette and the pol II promoter (43). The primary transcript generated with this construct would be capped at 5’-end by a trimethylguanosine cap and the final siRNA duplex would bear a monophosphate at the 5’-ends of both strands because of Drosha and Dicer cleavage. This version of the sh-B971 vector was much weaker in its ability to trigger IFN activation. Unfortunately the intracellular expression of the RNA duplex was also much weaker and barely detectable by northern blotting. In addition, no knockdown of the target CyPB mRNA was seen with this miRNA-based sh-B971 (data not shown). As a result, whether sh-B971, if expressed at higher level from this construct, could effectively activate IFN remains unclear.

So far as we know, ours is the first report of IFN activation in the target cells by shRNAs delivered by lentiviral transduction. A previous report of IFN induction by lentiviral vector-expressed shRNA only examined the IFN generated in the vector-producing cells, which then up-regulated IFN-stimulated genes in the transduced cells (44). The distinction is important as lentiviral vectors used in a gene-therapy setting will likely be purified and free of any IFN that has been generated during the vector preparation step, but IFN activation in the target cells would pose a more serious concern. Our data suggest the importance of screening shRNAs for IFN induction in the transduced cells in vitro before large-scale studies. An HIV reverse transcriptase inhibitor efficiently blocked IFN production by both sh-B971 and sh-PCAF when delivered by transduction, indicating the virion-encapsulated RNA was not able to trigger IFN activation. In this respect, it is interesting to note that positive-stranded RNA viruses, which produce dsRNA intermediates in the cytoplasm during replication (12,45-47), often replicate in membrane enclosed vesicles (48), This sequestration of viral dsRNA in membranous structures may shield the RNA from the cytoplasmic PRRs and contribute to a successful infection.

IFN-induction and RNAi by shRNAs appear to be independent functions of the same RNA (11). Our results also showed that IFN-induction by sh-B971 is independent of its ability to suppress target mRNA expression through RNAi. On the other hand, it might be possible to screen for duel functional siRNAs that confer therapeutic benefits by both RNAi and
immunostimulation (49). For example, siRNAs that target either viral genomes or cellular
cofactors of the viruses can be screened for their ability to trigger IFN activation in hopes of find
“super siRNAs” with increased efficacy against IFN-sensitive viruses.
Hepatitis C virus is an enveloped positive-stranded RNA virus in the Flaviviridae family. It is a major cause of liver disease and about 3% of the world’s population is chronically infected. Current therapy is a combination of pegylated interferon and ribavirin, however treatment is only successful in approximately 50% of patients. Hepatitis C virus often leads to liver cirrhosis or hepatocellular carcinoma and is a leading cause of liver transplantation (50).

The HCV genome is approximately 9.6 kb and encodes a polyprotein, which is cleaved co- and post-translationally by host and viral proteases to generate at least 10 mature proteins (Figure 3.1). The resulting proteins are core, E1 (envelope), E2, p7 (ion channel), nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The virus has one open reading frame (ORF) and both a 3’ untranslated region (3’ UTR) and a 5’ untranslated region (5’ UTR). There are 6 genotypes designated 1-6 and each genotype has multiple subtypes designated by letters. The most prevalent genotype in patients is 1, which is also the genotype that is most resistant to treatment. The 5’ and 3’ UTR are highly conserved between genotypes (51,52).
Figure 3.1. Map of HCV genome and brief description of protein function

The core protein is 191 amino acids and is processed to yield a 173aa protein. Core localizes to lipid droplets in the cytoplasm, which are a unique morphological characteristic of cells expressing HCV core (53,54). The core protein makes up the HCV capsid and has been shown to dimerize in cells and assemble in certain cell free conditions (55-57).

E1 and E2 are envelope proteins and are glycosylated. These glycoproteins have a C-terminal hydrophobic membrane anchor and N-terminal ectodomains. The N-terminal is directed to the endoplasmic reticulum (ER) lumen where it is modified by N-linked glycosylation. E1 and E2 are mostly retained in the ER and form a non-covalent heterodimer (58). This heterodimer interacts with cell surface receptors to mediate viral entry. E2 contains two hypervariable regions, which together with the relatively high mutation rate of RNA viruses contributes to immune evasion (59).

P7 is a 63 aa ion channel and is essential for virus infectivity and is genotype specific (60,61). P7 is important for assembly (61-63). A protein may also be made from an alternative reading frame (ARF) in the core region. Antibodies to this ARF protein have been found in patient sera (64) but it is not required for HCV replication (65).

NS2 is a 250aa protease that works in conjunction with NS3 to cleave the NS2/3 junction. It is not required for NS3 function or subgenomic viral replication (66-68). However, NS2, and particularly the C terminal region is required for infectious virus production and may be important for protein-protein interactions (69).
NS3 has an N-terminal serine protease domain that works with NS2 and NS4A and a C-terminal helicase/NTPase domain. The NS2/3 protease mediates cleavage at the 2/3 site and the NS3/4A protease cleaves the downstream HCV proteins (70). It is also involved in ablating RIG-I triggered immune response by cleaving the mitochondrial antiviral signaling protein (MAVS), a protein in the RIG-I pathway (4-7).

NS4A is a 54aa protein and is an essential cofactor for NS3’s protease function as well as an important cofactor for NS3’s helicase function (71). It is also responsible for the ER targeting of NS3 (72).

NS4B is a 266 aa membrane associated protein involved in membrane rearrangement and organization of the HCV replication complex (RC) (73) and has also been shown to oligomerize which may be a mechanism for membranous web formation (74).

NS5A is a phosphoprotein which may be either basal- or hyperphosphorylated and is involved in viral replication as well as viral assembly. A switch between the two phosphorylation states may be involved in a switch between NS5A’s involvement in replication to viral assembly (75). NS5A interacts with viral proteins as well as many host proteins and many cell culture adaptations have been mapped to this protein (76-80).

NS5B is an RNA dependent RNA polymerase which has a relatively high error rate resulting in high mutations rates. NS5B has the common polymerase motif of 3 domains consisting of a thumb, palm and fingers domain (81).

In addition to the protein coded by HCV, the 5’ and 3’ UTRs are also very important for viral replication. Both the 5’ and 3’ regions are highly conserved, form extensive secondary structure and are necessary for replication (52,82). The 5’ UTR contains and internal ribosomal entry site (IRES) which is responsible for cap-independent translation initiation of the HCV RNA (82,83).

The hepatitis C virus is a relatively recently discovered virus. It was first described in 1975 (84) but was not isolated until 1989 (85,86). Without a small animal model and inability to propagate the virus in cell culture it was very difficult to study the virus. Studies relied on either expression vectors or chimpanzee studies. In 1999 a subgenomic replicon system was developed which allowed replication of the viral RNA and proteins although infectious viral particles were not produced (87). This allowed much to be discovered about the viral replication and function of many of the proteins. The most recent advance was the discovery of a clone that is able to
efficiently replicate in cell culture (88-91). A clone termed JFH-1 was isolated from a Japanese patient with fulminate hepatitis and this clone is unique in that it can produce infectious virus in cell culture without adaptive mutations.

**VIRAL ENTRY**

HCV uses several cellular receptors to attach to the surface of cells and it is then internalized via a pH-dependent clathrin mediated endocytosis (92,93). Glycosaminoglycans (94,95) and low density lipoprotein receptors (LDLR) (96-99) may be involved in initial binding of the virus. The cellular receptors CD81 (98,100-104), scavenger receptor class B member 1 (SRBI) (101,105-107), claudin-1 (CLDN1) (108) and occludin (OCLN) (109-111) have been characterized recently as being important for viral entry in cell culture. CD81 is a member of the tetraspanin protein family that has 4 transmembrane domains and 2 extracellular loops and has been shown to bind HCV E2 (103). This interaction can be blocked by anti-CD81 antibodies, soluble CD81 peptides, or soluble E2. Nevertheless, cell to cell transmission of the virus may or may not depend on CD81 (98,112-116). E2 binding to CD81 is species specific, which partially accounts for the tropism of HCV.

SRBI is a receptor that interacts with both low and high-density lipoproteins (LDL/HDL) (117) as well as HCV E2 (101,105). CD81 and SRBI however are not sufficient for HCV infection. Expression of these receptors, either endogenous or exogenous, did not allow all cell lines to be permissive to HCV infection (101). It was then discovered that cell line, which were non-permissive to HCV infection, became permissive upon expression of exogenous claudin-1 (CLDN1) which was found through a cDNA library screen (108). However, some cell lines remained non-permissive to HCV infection despite expressing these 3 known receptors, suggesting additional receptors. Through a cDNA screen it was found that occludin (OCLN) plus the three previously identified receptors rendered mouse cells permissive to HCV infection. CD81 and occludin are responsible for the species specificity of HCV entry between mouse and human while mouse CLDN-1 and SRBI work equally well as human in mediating entry (110).
REPLICATION

Like other RNA viruses, HCV replicates on intracellular membranes (118). HCV modifies the ER membrane to set up replication complexes that include the NS proteins as well as viral RNA and host cofactors (73,77). A large portion of the NS proteins are not actually included in the RC (119,120) which can be determined by their resistance to protease digestion. These NS proteins not in the RC may be involved in viral assembly or blocking innate immune response.

VIRAL ASSEMBLY AND RELEASE

HCV assembles on lipid droplets adjacent to the ER (121). Many of the nonstructural proteins have been found to be involved in viral assembly as well. NS5A has been shown to be associated with the lipid droplets (121). NS2 has been shown to interact with E1, E2, p7, NS3 and NS5A (122) and mutations in NS2 which do not affect RNA replication can either restore infectious particle production in intergenotypic chimeras or block production of infectious virus (62,63,123). Disruption of these protein interactions through mutations can inhibit viral assembly. Moreover, some mutations in nonstructural proteins may still allow assembly of core containing particles that are non-infectious despite having similar sedimentation properties (123). Thus the HCV nonstructural proteins may play a roll in viral maturation as well as assembly. Viral maturation is important for the stability of intracellular HCV particles as even infectious intracellular viral particles seem be targeted to degradation if not further processed to its lower density form (124). The high-density form of the virus likely assembles on the lipid droplet which is associated with the ER and then moves through the Golgi system where it undergoes maturation.

ADAPTIVE MUTATIONS

Although a strain of wild-type HCV, JFH-1, is able to replicate in cell culture without adaptive mutations, many groups have found mutations through long term passaging or selective pressure (62,78,125-128). In 2006 Zhong et al. reported a mutant virus isolated in cell culture
though long term culturing of infected cells (128). Isolated virus had increased expansion kinetics, higher peak titers, and increased buoyant densities. A single mutation in E2 (G451R) was found to be largely responsible for the mutant phenotype. It was also found that the cells changed over time to express lower levels of CD81. HCV with the G451R mutation appeared to be less dependent on CD81, purportedly through an alteration in CD81 binding by the virus. Another E2 mutation was also found through long term passaging of JFH-1, which was reported to be involved in CD81 binding (129). The E2 mutation N415D increased affinity of E2 for CD81 and also rendered the virus less sensitive to neutralization of another entry cofactor, SRBI.

Many other studies have implicated non-structural proteins in production of more infectious virus. This is most likely due to enhanced assembly as many non-structural proteins have been implicated in assembly. JFH-1 infected Huh 7.5 cells were passaged as well as supernatant passaged over a period of time and several mutations developed which lead to higher replication and higher viral titers (79). A mutation in NS5A, V2440L was found to increase infectious viral titers by 10 fold despite having similar levels of RNA replication as compared to wild type. Viral kinetics were also increased. A separate experiment in the same report found a mutation in p7 that lead to 100 fold increases in viral production and increased kinetics and also had no effect on RNA replication. Mutations other non-structural proteins have also been reported to increase virus production and kinetics. Serial passaging reveled mutations in p7, NS2, and NS3, which alone or in combination increased viral titers more than 2 logs over wild type (62,127). Thus mutations in either structural or non-structural proteins may enhance HCV replication, virus production, or kinetics.

**LIVER OVERVIEW**

The liver is the largest internal organ and is responsible for many important functions including metabolism, and exocrine and endocrine functions. The liver produces bile which is excreted into the duodenum or stored in the gall bladder and is necessary emulsifying fats. It is also involved in metabolism. It is involved in both the breakdown and building of carbohydrates, proteins, and fats as well as detoxification. The liver regulates glucose levels through storage of glucose as glycogen. It also produces clotting factors and serum proteins.
The liver is comprised mostly of hepatocytes which account for 70% of the mass of the liver. Biliary epithelial cells (BEC), stromal cells, stellate cells, kupffer cells, and blood vessels are also part of the liver. The interhepatic bile ducts (IHBD), portal vein and hepatic artery are referred to as the “portal triad” and run in parade thought the liver. This is surrounded by a single sheet of hepatocytes known as hepatic plates. Sinusoid spaces in between these sheets are connected to a system of blood vessels and capillaries. The portal vein delivers blood plasma, which moves through the sinusoidal space and delivers metabolites and toxins through contact with the basal side of the hepatocytes. Adjoining hepatocytes secrete bile through their apical surface which flows though the bile caniculi (grooves on the cell surface) which then transports it through the IHBD and into the extrahepatic bile ducts (EHBD) and into the gall bladder. Here it can be stored and then released into the duodenum.

Figure 3.2- Cellular Architecture of the liver. A. The liver is shown in red, the stomach in yellow, and the gall bladder and extrahepatic ducts in green. B. Sheets of hepatocytes (pink) (hepatic plates) are separated by sinusoid spaces surrounding a central vein. Bile caniculi on the surface of adjacent hepatocytes drain bile into the bile ducts (green), which run parallel to portal veins (blue) and hepatic arteries (red) to form the “portal triad”. Figure reproduced with permission (130).
LIVER DEVELOPMENT

The endoderm layer gives rise to hepatocytes and BECs while the mesoderm is the source for stromal cells, stellate cells, kupffer cells, and blood vessels. The endoderm is established during gastrulation and develops into a primitive gut tube that is subdivided into the foregut, midgut, and hindgut. The embryonic liver generates from the ventral foregut endoderm. A potion of the ventral foregut epithelium near the developing heart thickens and forms an out-pocket that is known as the hepatic diverticulum. The liver and interhepatic biliary tree develop from the anterior portion of the hepatic diverticulum and the gall bladder and extrahepatic biliary ducts arise from the posterior portion. The hepatic endoderm, known as hepatoblasts, delaminates from the epithelium and move into the nearby septum transversum mesenchyme (STM) to form the liver bud. The stellate and fibroblasts of the liver arise from the STM (131,132). Next, the liver is vascularized and colonized by hematopoietic cells and thus grows rapidly. Hepatoblasts which are near the portal veins become BECs that will line the lumen of the intrahepatic bile ducts (IHBD). However, most of the hepatoblasts will develop into hepatocytes (130).

Adult liver tissue is able to regenerate when up to 70% of its mass is removed (133,134). Mature hepatocytes are responsible for most regeneration, although, hepatic progenitor cells are also present. These are activated when hepatocytes’ proliferation is inhibited such as in severe cirrhosis. These cells are believed to reside in the small terminal bile ducts. These cells, when activated, can form either hepatocytes or BECs. However, disease impairs the ability of the liver to regenerate and transplantation is still often necessary.

HEPATOCELLULAR CARCINOMA DEVELOPMENT

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer and the third leading cause of cancer deaths worldwide (135). Most HCC is caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) (136) and the risk of HCC is increased by 11.5 to 17 fold in HCV infected patients(137,138). Antiviral therapy is only effective in preventing HCC in small proportion of patients and viral clearance is difficult in many patients despite treatment (139,140).
Development of HCC is a very complicated issue. It is a multistep process with both genetic and epigenetic changes, alteration of the expression of oncogenes and tumor suppressor genes, and deregulation of many cell signaling pathways. Genes and pathways affected include Wnt/β-catenin, p53, pRb, Ras, mitogen activated kinase (MAPK), Janus kinase (JAK), signal transducer and activator of transcription (STAT), phosphotidylinositol 3 kinase (PI3K)/Akt, Hedgehog, as well as growth factors such as epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) (Figure 3.3). Alteration of these genes often reverts the cells back to a more stem cell like state (130).

Cirrhosis of the liver is scarring and disease in liver function as a result of injury or chronic liver disease. Scarred tissue cannot function as a normal liver and causes health problems on its own, but can also develop into HCC. Patients with a cirrhotic liver have a 20-
30% chance of developing liver cancer if they have 20-30 years of chronic liver infection and 80-90% of all cases of HCC develop in a cirrhotic liver (141). Liver injury causes hepatic stellate cells (HSC) to become activated which results in transformation into myofibroblast-like cells which in turn produce extracellular matrix. This can eventually lead to liver fibrosis and, ultimately, permanent cirrhosis. Activated HSCs are responsive to platelet-derived growth factor (PDGF) and TGF-β which are cytokines that are involved in proliferation and fibrogenesis (142-144). Cytokines are upregulated during fibrogenesis and modulate inflammatory signaling from immune cells, which infiltrate the liver. PDGF can activate both MAPK and PI3K/Akt signaling cascades, which in turn leads to fibrogenesis and ultimately, HCC. The damaged liver may result in an increase in toxic metabolites, which can then cause more damage. Stellate cells express many stem cell markers such as CD133, nestin, c-kit, and p57 neurotrophin receptor (145-148). Pathways involved in stem cell differentiation and cancer formation such as Hedgehog and Wnt signaling pathways are also found in stellate cells and contributes to cell cycle control (149-151).

**HEPATITIS C VIRUS ASSOCIATED CARCINOGENESIS**

Hepatitis C virus establishes a chronic infection in as many as 80% of infected individuals. Current treatments are only successful in clearing the infection in about half of patients. These patients are at an increased risk as long-term infection is associated with hepatic fibrosis, cirrhosis, or HCC. Unlike HBV, which is a DNA virus, HCV is an RNA virus that replicates in the cytoplasm and does not integrate into the host genome. This means viral proteins are directly involved in carcinogenesis. Chronic hepatic inflammation and cirrhosis are associated with oxidative stress and may result in damage to cellular DNA (152-154). HCV may have a more direct role in carcinogenesis. HCC in cirrhotic livers develops more frequently in individuals infected with HCV than those with autoimmune hepatitis (155,156). Transplant is often necessary for HCV patients with HCC although the transplanted liver usually becomes infected as well thus continuing the cycle.

HCV core protein has been shown to interact with many cellular proteins including many tumor suppressors such as p53, p73, and pRb (157,158). HCV core prevents α-dependent cell growth arrest through the binding of p73 and translocation to the nucleus. HCV can also alter
the expression of p21WAF1, which is a major target of p53, and regulates cyclin/cyclin dependent kinase complexes involved in cell-cycle control and tumor formation. Many genes with functions involved in cell growth or oncogenic signaling were identified in a study where 372 genes were found to have altered transcription in Huh 7 cells expressing HCV core. Wnt and it’s downstream target, WISP-2 were both upregulated in core expressing cells and also accelerated cell growth (159). The Wnt pathway is very important in development. Wnt ligands activate a signal transduction cascade though interaction with their receptor. Parts of this pathway are often mutated in liver cancer, which allows β-catenin to be stabilized. Inhibition of the β-catenin degradation complex allows it to interact with proteins in the nucleus and disrupt transcriptional control (160).

TGF-β is a tumor suppressor gene, although in cancer cells, the pathway can be disrupted and secretion of TGF-β can cause immune suppression in surrounding cells that may detect the tumor, thus allowing the cancer to spread more. Samd3 is a member of the TGF-β pathway and is involved in fibrogenesis. TGF-β is upregulated in HCV core expressing cells (161,162). An increase in fibrogenic and necroinflammatory genes in the liver suggests the TGF-β shifts from tumor suppression to fibrogenesis which results in acceleration of liver fibrosis and the risk of HCC (144). Smad3 activation thresholds dictate TGF-β responses in hepatocytes and in HCV infected cells core protein decreases Smad3 activation. This may be responsible for switching TGF-β from a tumor suppressing function to tumor-promoting activities (163).

HCV NS3 has been shown to inhibit the activity of the p21WAF1 promoter in a dose dependent manner and is synergistic with core’s effect on p21 (164). NS3 also inhibits p53 function (165). Enhanced cell growth, JNK activation and DNA binding of the transcription factors AP-1 and ATF-2 are all seen in cells expressing NS3 (166).

HCV NS5A has been shown to be involved in a vast array of cellular functions including apoptosis, signal transduction, transcription, transformation, and reactive oxygen species (ROS) production. HCV NS5A inhibits transcription of p12WAF1 through binding and sequestering of p53. NS5A also binds the coactivator of p53 and hTAF(II)32, a component of the transcription factor TFID (167). NS5A also binds TBP and prevents binding of consensus DNA sequences by the TBP-p53 complex, inhibits excision repair cross complementing factor 3 protein-protein interactions, and activates NF-κB leading to prevention of apoptosis (168,169). NS5A also interacts with p85 PI3K to promote cell survival though the PI3K/Akt signaling pathway.
Forkhead transcription factor, an Akt substrate, is bound by NS5A in the context of the HCV polyprotein. This stimulates the phosphorylation of glycogen synthase kinase-3β which results in stabilization of cellular β-catenin and stimulation of β-catenin-responsive transcription factors (170). NS5A also interacts with TGF-β (171).

In addition to direct interaction with HCV proteins, oxidative stress due to immune response, mitochondrial dysfunction and ER stress contribute to disease. Immune related oxidative bursts contribute to HCV associated oxidative stress and both structural and nonstructural HCV proteins are associated with the generation of ROS. ROS generation is associated with NF-κB and STAT-3 activation (172,173). Expression of HCV core increases ROS. ROS may increase oxidative DNA damage, contributing to transformation. ROS may also activate MAPK, NF-κB, PI3K, p53, β-catenin/Wnt, and angiogenesis (174).

Another disease associated with chronic HCV infection is steatosis, or fatty liver. In this disease, abnormal amounts of lipid accumulate within the cell and can disrupt normal cell function. Steatosis occurs in 30-70% of patients with chronic HCV infection (175). Steatosis may trigger inflammation, ROS, and DNA damage and is associated with increase in HCC risk, independent of cirrhosis (174).

Through interaction with the above-mentioned proteins, HCV can disrupt the normal pathways that regulate cell growth and induce apoptosis when there is DNA damage or though response to the virus. Additionally, may of these studies were done expressing the HCV proteins alone, limiting somewhat, the biological relevance. Physical damage to the liver, oxidative stress, inflammation, steatosis, and other factors may also play an important role in carcinogenesis and work in favor of cancer development with the viral proteins. Still, not all patients with chronic hepatitis C develop HCC, demonstrating how complex the issue is. Given the poor prognosis of patients infected with HCV, much remains to better understood and better treatments to be developed.
CHAPTER FOUR

ISOLATION OF HCV WITH ENHANCED KINETICS AND VIRAL ASSEMBLY

INTRODUCTION

HCV uses many cellular proteins to gain entry and replicate in host cells. Much about these interactions is still poorly understood. Only recently has a virus been isolated that can be cultivated in the lab that allows research into the whole viral life cycle. We sought to isolate a mutant virus capable of entering cells lacking a known viral receptor in order to characterize the interaction of HCV with various receptors. Through passaging HCV in a cell line lacking SRBI we selected a virus capable of establishing an infection in that cell line and we then characterized that virus which we named serially adapted virus (SAV).

MATERIALS AND METHODS

Virus and Cell Culture
Huh-7.5 cells were provided by Charles M. Rice. LH86 cells were provided by Chen Liu (26) JFH-1 constructs were provided by Takaji Wakita. A human immunodeficiency virus (HIV)-based lentiviral vector was used to express the short hairpin RNAs (shRNAs). Lentiviral vector production and transduction were performed as described previously (30). Targets for shRNAs were as follows: CD81-369 aatctggagctgggagacaag, CLDN I-92 aacattaggaccttagaattt, OCLN-1846 aagtgaagagtacatggctgc, SRBI aagcagcaggtccttaagaac (176). L-NTC has been previously described (177). Stable cells expressing shRNAs were obtained by selection with 1.2 μg/ml of puromycin (MP Biomedicals, Solon, OH) for 3 weeks.

Western Blots, Immunostaining, and Antibodies
Protein contents of cell lysate were quantified with the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA), and an equal amount of total protein was loaded in each lane on SDS-PAGE gels. Primary antibodies used were anti-core (Affinity BioReagents, Golden, CO), anti-NS3 clone 2E3, anti-NS5A clone 7B5, NS5B clone 4B8 (BioFront Technologies, Tallahassee, FL), NS2, NS4B (generous gift from Ralf Bartenschlager), and anti-actin (Sigma-Aldrich). Secondary antibodies used were goat anti-mouse-HRP and goat anti-rabbit-HRP (Santa Cruz Biotechnology) and goat anti-mouse-FITC (Sigma-Aldrich).

Core ELISA

HCV core ELISA was performed according to the manufacturer's instructions for the HCV Antigen ELISA kit (Ortho-Clinical Diagnostics, Japan).

Intracellular Virus Collection

Intracellular virus was collected by 5 cycles of freeze-thaw lysis of infected cells in 100ul of media per million cells. Lysate was centrifuged and supernatant collected and used in subsequent assays.

TCID$_{50}$ Assay

The TCID$_{50}$ assay was modified from a procedure by Lindenbach (178). HCV L-NTC cells were seeded at 6x10$^3$ per well of a 96 well plate in 100ul and allowed to attach overnight. Culture media or intracellular lysate from infected cells was serially diluted at 1:10 with 4 wells per dilution. Cells were washed and fixed with paraformaldehyde after 3-4 days after infection. Cells were incubated in 3% hydrogen peroxide in PBS for 1 hour then washed and incubated with anti-NS3 clone 2E3 at a dilution of 1:1000 for 1 hour to overnight, washed 3 times, then incubated with goat anti-mouse HRP conjugated antibody (Santa Cruz Biotechnologies) for 1 hour. Cells were washed and then incubated with DAB substrate for 30 min and TCID$_{50}$ calculated using the Reed and & Muench calculator provided by Lindenbach (www.med.yale.edu/micropath/pdf/Infectivity%20calculator.xls).

Structural Protein Cloning and Sequencing

RNA was extracted from SAV III infected cells using the RNeasy Plus Kit (Quiagen) and RT PCR was performed using SuperScript III (Invitrogen) and random hexamer primers. Primers used to clone structural proteins were JFH-1 Age I f (gcctagccatggcgttagta) and Afl II r (agcggcccttaagaggtaag). Age I and Afl II sites were used to clone E1-p7 into the wild type
pJFH-1 pUC plasmid. RNA was invitro transcribes using Megascript (Ambion) and electroporated into L-NTC cells. Culture supernatant was collected and titered by TCID$_{50}$.

**Agarose focus formation assay**

Huh 7.5 L-NTC cells were seeded into chamber slides (Nalgene) to reach a confluency of 50% the next day. Cells were infected overnight. DMEM (2X) complete media was added to 1.2% agarose that had been heated and then cooled to about 50°C to give a final solution of 0.6% agarose. This was overlaid with a small volume of DMEM to keep the agarose from drying out. Two to three days post infection the agarose was carefully removed using a combination of aspiration and tweezers. Cells were then fixed and stained as described above.

**RESULTS**

**Isolation of a mutant virus through serial passaging**

Our lab previously isolated a virus able to replicate in cells lacking cyclophilin A (CyPA), an essential HCV cofactor (33). This helped elucidate the interaction and importance of CyPA in HCV replication. We termed this method of isolating cofactor independent mutations CoFIM in HCV. Seeking to use this approach with other cofactors we created several cell lines in which the entry cofactors CD81, OCLN-1, CLDN-1, and SRBI were knocked down by RNAi. A cell line in which SRBI expression was knocked down via shRNA was infected by wild type HCV and the percent infected monitored every 3-4 days. When control cells were infected (L-NTC) infection reached 100% by day 14, while L-SRBI cells remained less than 5 percent infected for over 40 days, after which, the infection level climbed to 65% by day 45 (Figure 4.1 A). Virus produced by these cells was termed serially adapted virus (SAV). Culture media was collected from day 43 to day 65, combined, and used to infect naive L-SRBI cells to generate SAV II. This virus, SAV II, was passaged in L-SRBI cells once again which produced SAV III (Figure 4.1 B). When L-SRBI cells were infected with SAV II, infection levels reached 35% by day 22 while cells infected with wild type virus remained close to 1% infected (Figure 4.1 C). SAV III had an even more enhanced infection rate, reaching 65% by day 19. A fourth generation, SAV IV, did not show significantly different infection rates in L-SRBI cells (data not shown).
Figure 4.1 - A mutant virus selection from SRBI knock-down cell line. (A) L-NTC and L-SRBI cells were infected with WT HCV. Cells were stained for NS3 at the indicated time points and percent infected calculated. After culturing for 45 days HCV infection increased significantly in L-SRBI cells. (B) Schematic of viral passaging. Culture media from d45-d60 was collected and used to infect naive L-SRBI cells. Culture media from the second round of infection was
collected and passaged through L-SRBI cells one again. This media was collected and the virus named SAV III. (C) Comparison of WT SAV II and SAV III in L-SRBI cells. WT, SAV II, and SAV III was used to infect L-NTC and L-SRBI cells. Cells were stained at the indicated time points and percent infected calculated.

**Characterization of SAV III kinetics**

Considering the enhanced spread of the virus in L-SRBI cells one possibility was that the virus had adapted to spread through cell-cell contact, which might be less dependent on SRBI. L-NTC cells were infected and then overlaid with agarose to reduce diffusion in culture media. Three days post infection the agar was removed, cells stained, and the number of cells per focus were counted. The foci in wild type infected cells averaged 2.75 while the SAV III averaged 8.85, although which much more variance. While foci of just 2 cells could be found in the SAV III infected cells, similar to the wild type, there were also foci of up to 30 cells found (Figure 4.2). This increase in spreading, nevertheless, does not rule out the possibility that the cells are simply making more virus. To further investigate the contribution of cell-cell spread L-NTC cells were infected and maintained at 10-20% confluency, thus limiting cell-cell contact. However, despite an overall decrease in kinetics, SAV-III still had enhanced infection rates over wild-type.
Figure 4.2- Viral spreading in WT and SAV III. Cells were plated onto chamber slides, infected, and overlaid with agarose. Three days post infection the agarose was removed and the size of foci counted.

We next wanted to determine if the adaptations in this virus enhanced infection of other cells lacking entry cofactors or if the enhancing mutations specifically improved infection of L-SRBI cells. When L-NTC cells were infected with wild type or the SAV III virus the mutant virus reached 100% infected faster and also produced more virus as measured by both titer and core ELISA (Figure 4.3 A-C). Even once the wild type infection reached 100%, the SAV III produced more than 10 fold more infectious virus (Figure 4.3 C). Although the rate of SAVIII infection in L-CyPA was not significantly higher than wild type (Figure 4.3 D), the amount of infectious virus produced was still 10 fold or more higher (Figure 3 E). SAV III also showed enhanced infection rates in cell lines expressing shRNA against other cell surface receptors known to be used by HCV (Figure 4.3 F-K).
Figure 4.3 - Kinetics of infection and viral production of SAV III in knockdown cells. Cells stably expressing control shRNA shRNA against viral cofactors were infected with WT HCV (---) or SAV III (---). (A-K) Cells were stained for NS3 and percent infected determined by microscopy. Media collected at 4 day intervals. Viral titers were quantified via TCID$_{50}$ assay. HCV core was quantified in media from (A) by ELISA.
SAV III appeared to create more virus and spread faster but we wanted to further characterize this phenotype. We wanted to examine if control cells were infected with lower titers of virus, would the SAV III would still spread faster. First we infected L-NTC cells with wild type or SAV III virus at an MOI of 0.005 and 2 or 10 fold dilutions of the stock. Despite starting out with 10 fold less virus, the SAV III was able to infect 100% of cells before the undiluted wild type. All SAV III infected cells showed sharper increase in percent infected as compared to the wild type (Figure 4.4 A). To ensure that this enhanced infection rate was not limited to the Huh 7.5 based cell lines, we also infected LH86 cells which is another hepatocellular carcinoma cell line capable of supporting HCV infection(26). The kinetics of infection in LH86 cells was very similar to L-NTC showing that the enhanced infection rate of SAV III is not limited to Huh 7.5 based cell lines (Figure 4.4 B).
Figure 4.4- Kinetics of infection with diluted virus in two cell lines. (A) Huh 7.5 based L-NTC and (B) LH86 cells were infected with HCV at a MOI of 0.005 or a 1:2 or 1:10 dilution (0.0025 or 0.0005 MOI respectively). Cells were stained for NS3 every 2 days to monitor what percent was infected.

Considering more virus is produced in SAV III infected cells, we wanted to investigate if the virus had higher replication, better assembly, release, or some other step in the viral life cycle was enhanced. Protein levels for NS2, NS3, NS4B, NS5A, and NS5B showed no significant difference between wild type and SAV III (Figure 4.5 A). However, core levels were 3-4 fold higher in SAV III samples as shown by western blot (Figure 5 A) as well as core ELISA on intracellular virus and over 6 fold higher in culture media (Figure 4.5 B). The ratio of extra-
intracellular core for wild type is 0.06 and for SAV III is 0.11. The ratio of extra- to intracellular infectivity is 0.46 for wild type and 0.68 for SAV III. Cells were also analyzed by flow cytometry, which confirmed the relative protein expression levels. Both intra- and extracellular infectivity levels were about 10 fold higher (Figure 4.5 C). Core localization in SAV III was normal (Figure 4.6).
Figure 4.5- Analysis of HCV core and infectivity in relation to other HCV proteins. L-NTC cells were infected with WT HCV or SAV III and cultured until > 95% of cells were infected. Media and cell lysate were collected and analyzed by (A) western blot, (B) ELISA, and (C) TCID 50 assay.

Figure 4.6- Localization of core protein in WT and SAV III.

Identification of mutations in SAV III and determination of the contribution of structural protein mutations

Since SAV III was isolated in a cell line lacking an entry receptor, we suspected that the structural proteins might have mutations that confer the phenotype. To investigate this we sequenced the structural proteins of SAV III. Four mutations were found. One mutation was found in core (K74T), two were found in E1 (I333V and H353Y), and one in E2 (I414T).
K47T mutation was reported previously (128) and found and reported to not significantly enhance viral infectivity. Mutations were also found in SAV III E1 and E2. These mutations were put into the wild-type JFH-1 construct. This virus was termed SAV structural mutant (SAV-S). Virus produced from this construct did not have enhanced viral kinetics (Figure 4.7). After examining the mutations in the structural proteins, the nonstructural proteins were sequenced. The nonstructural proteins were found to have mutations in NS2, NS3, NS5A, and NS5B (Table 4.1). Determining which mutation or mutations confer the enhanced viral phenotype will be investigated in future work.

![Figure 4.7- SAV-S kinetics. WT and SAV-S supernatant were diluted as indicated and used to infect L-NTC cells. Cells were stained and examined at the indicated time points and percent infected determined.](image)
We have isolated a virus with enhanced viral kinetics through serial passaging in a cell line with SRBI knocked down. This virus, SAV III, has increased kinetics in Huh 7.5 based cell lines with entry factors knocked down as well as a separate cell line, LH86. Considering SAV III was isolated from cells lacking SRBI but still has increased kinetics in cell lines lacking other entry factors as well as control cells, the selective pressure of reduced SRBI may have selected for an adaptation that was able to overcome the reduced infection efficiency of L-SRBI. We determined that SAV III infected cells contain and release more infectious virus than do wild

<table>
<thead>
<tr>
<th>HCV Protein</th>
<th>Mutation found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>K74T</td>
</tr>
<tr>
<td>E1</td>
<td>I333V</td>
</tr>
<tr>
<td></td>
<td>H352Y</td>
</tr>
<tr>
<td>E2</td>
<td>I414T</td>
</tr>
<tr>
<td>p7</td>
<td>none</td>
</tr>
<tr>
<td>NS2</td>
<td>Q862R</td>
</tr>
<tr>
<td>NS3</td>
<td>R1287G</td>
</tr>
<tr>
<td>NS4A/B</td>
<td>none</td>
</tr>
<tr>
<td>NS5A</td>
<td>V2208E</td>
</tr>
<tr>
<td></td>
<td>E2264G</td>
</tr>
<tr>
<td></td>
<td>L2264P</td>
</tr>
<tr>
<td></td>
<td>R2304M</td>
</tr>
<tr>
<td></td>
<td>S2385P</td>
</tr>
<tr>
<td>NS5B</td>
<td>I2848V</td>
</tr>
</tbody>
</table>
type infected cells. Producing more virus may allow SAV III to spread in cells lacking entry factors by increasing the number of virus-cell interactions. Additionally, cells infected with SAV III contain more core protein despite having similar levels of the non-structural proteins NS2, NS3, NS4B, NS5A, NS5B. It has been reported that assembled, mature virus particles have increased stability (179). This provides a possible explanation for the elevated amounts of core protein despite the lack of elevated amounts of non-structural proteins. As HCV is produced as a polyprotein, the same amounts of all proteins should be produced, however differences in stability can account for differences in observed protein levels at a given time.

Considering the cells were isolated out of a cell line lacking the entry receptor SRBI, the structural protein mutations were investigated, as they may be responsible for the increase in viral kinetics. A study in which JFH-1 was propagated long term in cell culture gave rise cells which had reduced levels of CD81 along with a mutation in E2 (G451R) that allowed the virus to better infect these cells (128). In our study, we also found a mutation in E2 as well, I414T. This mutation has been reported to increase infectivity and determined the mutation lead to increased secretion of the virus, although our SAV-A, which contains this mutation, did not seem to have enhanced infectivity. We found the difference between intra- and extracellular viral titers for wild type and SAV III respectively were more comparable than in the above mentioned study suggesting that increased secretion was not the main mechanism responsible, or only contributed a small amount to the increased kinetics. The ratio of extra- to intracellular core was 0.06 for wild type and 0.11 for SAV III which is only a 2 fold difference between wild type and SAV III although the difference in total core is greater, 3.5 fold for intracellular and 6.6 fold for extracellular. In comparing the extra- to intracellular infectivity, there is even a smaller difference in the ratio for the two viruses. Extra- to intracellular ratios for infectivity are 0.46 vs. 0.68 for the wild type and SAV III respectively. Despite this small difference in viral release, there is a 10 fold difference in intracellular infectivity and a 15 fold difference in extracellular infectivity between wild type and SAV III. This suggests that the SAV III produces more infectious viral partials rather than simply having improved release.

Combined mutations in core, NS2, and NS4B have been reported to increase infectious viral production as well as kinetics without having a significant effect on RNA replication (180), similar to the phenotype observed in SAV III. Infected cells had increase specific infectivity and
viral particles were found to be more stable. Stability of SAV III viral particles has not been investigated. Combinations of mutations will be tested in further studies.

Mutations in NS5A and NS2 have been reported in multiple studies to increase viral production with some having little effect on RNA replication (62,76,79,127,180,181). Given NS5A’s role in assembly and previously reported adaptive mutations, it is likely that one of the NS5A mutations in SAV III are at least partially responsible for the observed phenotype. An adapted virus reported by Kaul et al. (79) displayed a phenotype similar to SAV III. In this study V2440L was found to increase infectivity and kinetics either alone or in combination with other mutations identified in that study. Also in that study, mutations in structural proteins were found to have no significant impact on viral kinetics of infectivity. RNA replication was not altered by the V2440L mutation and it was determined that the mutation lead to increased assembly rather than release of infectious virus. This mutation is near the NS5A-NS5B cleavage site and altered cleavage efficiency may affect viral assembly. When immunodeficient transgenic mice xenografted with primary human hepatocytes were infected, the mutants reverted back to wild type in some mice when analyzed after 2 weeks. Protein levels for each viral protein in the V2440L mutant was not reported although it was shown to have more core protein. Unfortunately, this study did not compare expression of multiple viral proteins so it is not clear if core stability is specifically enhanced in the V2440L mutant as in SAV III.

Another study by Mishima et al. (182) also found mutations in NS5A and NS5B which lead to an increase in viral titer but also an increase in cytopathicity. Increased cytopathicity was also seen with SAV III (data not shown). The mutations found by Mishima were C2441S, P2938S, and R2985P. Many of these mutations reverted back to wild-type after long term infection in human hepatocyte chimeric mice (21 to 56 days), perhaps due to selective pressure to be less cytopathic or simply different selective pressures in the humanized mouse liver. Since SAV III was under selective pressure in the L-SRBI cells, that may have fostered the maintenance of titer enhancing mutations. SAV III was cultured up to 22 days in control cells and did not show any revision to wild type. SAV III was not cultured longer than 22 days so it is not clear if a longer passaging would lead to revertants.
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  Cloning of shB971 into pSHAG-MAGIC2

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LENTIVIRAL TRANSDUCTION OF shRNA

Design and Cloning

Lentiviral vectors are useful for creating cell lines that stable express exogenous transcripts, such as shRNA. We use a human immunodeficiency virus (HIV) based vector with a 4-plasmid system\(^\text{(25,30)}\). Viral genes are expressed from 4 plasmids which results in the production of viral vectors which can deliver the gene of interest but do not carry viral genes necessary to replicate, thus it is a single round infection. The 4-plasmid system is safer than a three-plasmid system as the viral genes are separated onto four plasmids making it almost impossible for them to recombine into a complete viral genome. The vesicular stomatitis virus glycoprotein (VSV-G) is used as the envelope protein rather than the HIV envelope proteins as it has a wider tropism and is able to infect a wide variety of cells commonly cultured. The lentiviral plasmid pHIV7-puro plasmids with are self-inactivating to ensure there is no further replication in target cells. The lentiviral plasmids that we work with contain the integration sequence, puromycin resistance gene under control of the SV40 promoter, and the shRNA of interest under control of the murine U6 promoter or for L-Luc, the human U6 promoter.
**shRNA Target design**

Target sequences for shRNA were found by using the cDNA sequence for the gene of interest and the target finder provided by Ambion ([http://www.ambion.com/techlib/misc/siRNA_finder.html](http://www.ambion.com/techlib/misc/siRNA_finder.html)). Targets were selected which had 47.6% G/C content. Sequences with four or more T's were avoided, as these would cause Pol III to terminate transcription.

**shRNA production**

Target sequences were engineered into the template 3’ primer shown below. The underline represents the target sequence followed by its complement. The bold text is the loop. To design the primer, replace first underlined text with the target sequence and the second with its complement with the exception of the capitalized AA in place of the second underlined text. The loop sequence is taken from a Brummelkamp publication (38) which demonstrated effective target knockdown.

Target for B971

5’ AAactctaccaacactgace 3’
3’ Primer for B971
5’ TgctGGATCCAAAAAAactcataaactgactgctACTCTTGAAAggtcagtgttgtaggagt
AAACAAAGGCTTTTCTCCAAGGG 3’

5’ Primer for Lentiviral vector
5’ GAACTAGTGGATCCGACGCC 3’

**Cloning of shRNA into pHIV-7**

PCR was carried out using pHIV-7-Puro sh-CyPA vector as the template. The PCR product of about 320 bp was cloned into pCR2.1 using TOPO cloning (Invitrogen). The insert was sequenced with m13f or m13r (provided with TOPO kit). The insert and L-Luc plasmid were then cut using BamH I and the sh-Luc cassette replaced with the insert from pCR2.1. The PCR product contains the entire expression cassette so the shRNA will express not matter which orientation it inserts into the vector.

**Cloning of B971 with H1 promoter**

Cloning was done using the pSilencer 3.1 H1 cloning kit from Ambion according to manufacturer’s instructions. Briefly, linerized plasmids are provided with the kit into which can be used to ligate annealed oligonucleotides corresponding to the desired shRNA. Oligos were designed using the insert design tool provided by Ambion (http://www.ambion.com/techlib/misc/psilencer_converter.html). Oligos were diluted to 1 ug/ul in TE (10 mM Tris, 1 mM EDTA). The two complementary oligos were then combined and diluted in the provided annealing buffer, heated to 90 °C for 3 min, incubated at 37°C for 1 hour to anneal. Annealed oligos were ligated into the provided vector and transformed into DH5α cells. Individual colonies were screened by diagnostic digest with BamH I and Hind III to identify colonies with the insert.

Oligos used are listed below. The underline sequence corresponds to the target and it’s complement. Bolded sequence represents the loop. Overhangs for BamH I and Hind III are designed into the oligo.
H1-Top Oligo B971
5'-GATCC ACTCCTACCAACACTGACC TTCAAGAGA GGTCAGTTGCTGGTAGGAGTTT
TTTTGGAAA-3'

H1-Bottom Oligo B971
5'-AGCTTTTCCAAAA AAACTCCTACCAACACTGACC TCTCTTGAA
GGTCAGTTGCTGGTAGGAGTTTG
TTTTGGAAA-3'

H1-Top Oligo NTC
5'-GATCC GACTGAAGGTGTGCTGGTA TTCAAGAGA TACCAGCACACCTTCAGTCTT
TTTTGGAAA-3'

H1 Bottom Oligo NTC
5'-AGCTTTTCCAAAA AAGACTGAAGGTGTGCTGGTA TCTCTTGAA
TACCAGCACACCTTCAGTC G-3'

Cloning of shB971 into pSHAG-MAGIC2

The plasmid pSHAG-MAGIC2 is used to express shRNA as a microRNA, which is cleaved in the nucleus by Drosha, exported to the cytoplasm, and then cleaved by Dicer. It is designed to work with a retroviral expression system so it can be transfected alone or used to produce retroviral vectors. Propagation of the plasmid requires the pir1 gene so PIR1 competent bacteria are required. Cloning was carried out as described by Paddison et al. (183). The oligos used to clone pSM2 B971 are shown below.

pSM2 B971 Temp
5’tgcttgagagtcgacagcg aaaaactcctaccaacactgacc tagtgaagccacagatgtta ggtcagtgttggtaggagtttg
tgctactgcctcgga

5'miR30xho
5'-'cag aag get cga gaa ggt ata ttg cttg aca gtg agc g -3’
3' miR30eco
5’- cta aag tag ccc ctt gaa ttc cga ggc agt agg ca -3’

U6 Sequencing primer
5’-gta act tga aag tat ttc g-3’

PRODUCTION OF LENTIVIRAL VECTORS IN 293-FT CELLS

T75 protocol

1. One day before transfection, plate 6x10^6 293-FT cells in 12ml of DMEM. The cells should be 90-95% confluent at the time of transfection (next day). Adjust the cells numbers plated so you can get around 90% confluency the next day.

2. For each transfection sample, prepare complexes as follows:
   a. Dilute DNA (16ug total premixed 4 plasmids, see below) in 1mL of DMEM or OPTI-mem ® without FBS.
      i. Plasmid 1 (6ug): lentiviral vector (e.g. pHIV-7/GFP or LGR-1 etc.)
   b. Mix Lipofectamine 2000 gently before use. Dilute 40ul of Lipofectamine 2000 in 1 mL of DMEM or OPTI-mem (R) without FBS. Incubate for 5 minutes at room temperature.
   c. Combine the diluted DNA with diluted Lipofectamine 2000 (total volume should be around 500ul). Incubate for 20 minutes at room temperature.

3. Add the 2 mL of complexes into each T-75 containing cells and medium. Mix by rocking the plate back and forth.

4. Replace the transfection media between 4-6 hours post-transfection.

5. 48 hours post-transfection, collect the culture medium (now containing lentiviral vectors) and add 14ml fresh growth media back to each well. Filter the collected medium through 0.45 micron filter into a Falcon 15-ml tube, label with the vector name (e.g. pHIV-1/GFP,
LGR-1 etc.), time of collection (e.g. 48-hour collection) and date. Store at minus 80 degree or use immediately for transduction.

6. Repeat step 5 at 72 and 96 hours post-transfection.

Note: You can get an idea of whether the transfection worked or not by examine the cells transfected cells under a microscope. The expression of VSV-G causes “synthecia” formation, which means many cells fused together to form giant cells. A good amount of synthecia would indicate a good transfection, which is a prerequisite for high titer vector production. In addition, if the lentiviral vector used contains a fluorescent marker (e.g. GFP, DsRed), you can examine the expression of the marker using an inverted fluorescence microscope before vector collection to verify that at least the transfection worked properly.

### CONCENTRATION OF LENTIVIRAL VECTORS

1. Autoclave appropriate tubes such as 32 mL, 25 x 89 mm, thick wall, polycarbonate tunes.
2. Fill tubes with up to 25 mL of supernatant containing lentiviral vectors. Balance all tubes, adding PBS if necessary.
3. Using the SW-32 Ti rotor, centrifuge at 50,000xg for 3 hours.
4. Very carefully, decant the supernatant and without tilting the tube back, aspirate off the residual liquid near the lip of the tube. Add 1:100 volumes of PBS and place tubes in 4°C overnight.
5. Resuspend pellet by pipetting and store at -80°C or use immediately.

### QUANTIFICATION OF LENTIVIRAL VECTORS

Lentiviral vectors were quantified using the p24 antigen ELISA from ZeptoMetrix. Media should be diluted 1:100 to 1:10,000. Concentrated vectors should be diluted 1:10,000 to 1:1,000,000. The dilution needed may vary from sample to sample. Prepare all dilutions as a serial dilution of 1:10 or 1:100 in PBS. Follow manufacture’s instruction for ELISA.
TRANSDUCTION AND SELECTION OF STABLE CELL LINES EXPRESSING SHRNA

1. Seed 2E5 cells in 6 well plate. Seed the number of wells needed for the shRNA vectors plus one control. Allow to attach overnight.
2. Remove media from cells and replace with 0.5 to 1 volume of media containing lentiviral vectors. Incubate overnight.
3. Replace media.
4. Add media containing puromysin 48-72 hours post transduction.
5. If cells are >80% confluent, split or expand to larger culture dish. For Huh 7.5 based cells use 1.2ug/mL puromysin.
6. Continue to replace media once to twice a week or as needed once a large amount of dead cells accumulate. If cells become confluent, trypsinize and move to larger culture dish or discard some cells to maintain them below 80% confluency. Continue until all control cells are dead. This typically takes 3 weeks.
7. Once all control cells are dead, maintain cells in 0.5X puromysin media. Cells may now be used in subsequent assays. Real-time RT PCR or western blotting may be used to check for target knockdown.

TRANSFECTION OF SYNTHETIC RNA AND POLY IC

RNA transfections were done using Lipofectamine 2000® according to the recommended RNAi protocol. All synthetic RNA was diluted in the annealing buffer provided and for the siRNA, was annealed according to the manufacture’s instructions (Ambion). Poly IC was diluted to 1ug/ul in annealing buffer and the amount transfected matched to the recommended amount of recommended DNA for transfection.

CLONING OF EXPRESSED SHRNA

To determine if the shRNA was expressed as expected we attempted to clone the shRNA from the cells using the miRCat micro RNA Cloning kit from Integrated DNA Technologies.
(IDT). The cloning is based on attaching linkers to the extracted small RNA, reverse transcribing, amplifying by PCR, then digest, concatamerize, and sequence. Total RNA was extracted from 293FT cells which had been transfected with shB971 or shNTC. RNA was extracted using the PureLink miRNA extraction kit (Invitrogen). Manufacturer’s instructions were followed and GelStar was used to visualize RNA on the gel. The kit did not indicate a starting amount of RNA. I used the RNA that was recovered from one column using the pure link kit (approximately 1E6 cells). If this is repeated, using more starting RNA may help.

NORTHERN BLOTTING

Labeling the oligo

The RNA oligo was radiolabeled using the KinaseMax kit from Ambion (Cat#1520). After labeling with P-32, the resulting reaction was run on a 15% acrylamide/urea gel. The probes were gel purified following the protocol provided through Technical Bulletin #171 on the Ambion/ Applied Biosystems website (http://www.ambion.com/techlib/tb/tb_171.html). After “probe elution” the probe was purified using a NucAway Spin Column (Ambion; AM10070).

RNA gel and transfer

The RNA was loaded onto 7.5% urea acrylamide gels and transferred to a nitrocellulose membrane. Transfer was done according to standard protocols.

1. Rinse gel in DEPC water
2. Soak in 0.05 N NaOH
3. Soak in 20X SSC for 40 min (10 gel volumes)
4. Soak membrane in 20X SSC for 15 minutes
5. Transfer

Prehybridization/hybridization Solution:

6X SSC
5X Denhardt’s Reagent
0.5% (w/v) SDS
1 ug/mL poly (A)
100ug/mL salmon sperm DNA
DEPC Treated water

Hybridization and washes

Hybridization of the membrane was done in the radiation room using a rocker. After hybridization of membrane with radioactive probe, the membrane was washed following the steps in the attached document.

Table A.1

Charged Membrane Northern Washes

<table>
<thead>
<tr>
<th>Wash</th>
<th>Solution</th>
<th>Temperature</th>
<th>Time</th>
<th>Volume</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1x SCC + 1% SDS</td>
<td>23°C (RT)</td>
<td>10 min</td>
<td>50 mL</td>
<td>Rocker</td>
</tr>
<tr>
<td>2</td>
<td>2X SCC</td>
<td>RT</td>
<td>15 min.</td>
<td>50 mL</td>
<td>Rocker</td>
</tr>
<tr>
<td>3</td>
<td>2X SCC</td>
<td>RT</td>
<td>15 min.</td>
<td>50 mL</td>
<td>Rocker</td>
</tr>
<tr>
<td>4</td>
<td>0.5X SCC + 0.1% SDS</td>
<td>RT</td>
<td>15 min.</td>
<td>50 mL</td>
<td>Rocker</td>
</tr>
<tr>
<td>5</td>
<td>0.1X SCC + 0.1% SDS</td>
<td>RT</td>
<td>15 min.</td>
<td>50 mL</td>
<td>Rocker</td>
</tr>
<tr>
<td>6</td>
<td>0.1X SCC + 1% SDS</td>
<td>50°C</td>
<td>15 min.</td>
<td>50 mL</td>
<td>Chamber</td>
</tr>
<tr>
<td>7</td>
<td>0.5X SCC + 0.1% SDS</td>
<td>68°C</td>
<td>10 min.</td>
<td>40 mL</td>
<td>Chamber</td>
</tr>
<tr>
<td>8</td>
<td>0.5X SCC +0.1% SDS</td>
<td>68°C</td>
<td>10 min.</td>
<td>40 mL</td>
<td>Chamber</td>
</tr>
<tr>
<td>9</td>
<td>0.5X SCC + 0.1% SDS</td>
<td>68°C</td>
<td>10 min.</td>
<td>40 mL</td>
<td>Chamber</td>
</tr>
</tbody>
</table>

Exposure and detection of radioactive signal

A phosphorimaging screen and cassette was used to detect radioactivity of the membrane. The image was developed on the Typhoon Imager using Quantity One software.
LUCIFERASE ASSAY FOR IFN ACTIVATION

To amplify signal, pCR3.1-IRF7A may be co-transfected although it is not required. It is preferable to set up the transfection in the morning, as the media will need to be changed 4-6 hours post transfection. 293-FT cells do not adhere well so be vary careful during media changes. This system uses firefly luciferase as the reporter for interferon and a Renilla luciferase as an internal control for transfection efficiency and sample concentration. Relative luciferase units are calculated by comparing the firefly reading to Renilla readings. For this study, we used reporters for IFN-α and IFN-β. The Renilla luciferase is under control of a thymidine kinase promoter.

![Figure A.2 Diagram of Luciferase reporter constructs](image)

Procedure:
1. Seed 5E4 293FT or other cells in 24 well plate in triplicate for each sample to be tested. Allow to attach overnight.
2. Prepare a supermix of plasmids to be transfected.
   Per sample (for 3 wells)
   - 1.2 ug shRNA vector
   - 120 ng pGL3-IFNA1 or pGL3-IFNB
   - 60 ng pRL-TK
   - 150 ng pCR3.1-IRF-7A or pcDNA 3.1 vector
3. According to the recommended procedure for Lipofectamine 2000®, add appropriate amount of DNA mix to OPTI-mem®. In a separate tube add the appropriate amount of Lipofectamine® to OPTI-mem®. Incubate for 5 minutes.
4. Combine DNA and Lipofectamine mixture. Incubate for 20 min. Add carefully to wells and rock plate to mix.
5. Change media 4-6 hours post transfection. If transfection reagent is left on cells for too long they will die.
6. Forty-eight hours post transfection, remove media and very carefully wash cells with PBS.
7. Add 100 uL of diluted passive lysis buffer (PLB), included in Dual-Glo luciferase assay kit (Promega).
8. Place plate on rocker for 15 min.
9. Collect lysate and put into labeled 0.5 or 1.5 mL tube. Samples may be stored at 4°C for up to 1 month or at -20°C for up to one year. Clearing of the lysate by centrifugation is unnecessary but may be done if they lysate will be used for protein determination.
10. Turn on plate reader and activate PMT. It takes 5 min for the PMT to warm up.
11. In a 96 well plate on ice, add 5 ul of lysate to each well in triplicate for each replicate. (One shRNA will have 9 wells, 3 replicates and 3 wells of the lysate for each sample). Up to 20 ul of lysate may be used but we found for this system 5 ul is sufficient.
12. Using a multichannel pipette if possible, load 100 ul of prepared LAR II (from kit) and measure luminescence. Try to minimize time between the addition of the reagent to each well.
13. Add 100 ul of Stop-and-Glo to each well and measure luminescence.

**PREPARATION OF GS5 CELLS FOR ANALYSIS BY FLOW CYTOMETRY**

1. Trypsinize treated GS5 cells. Spin at 1300 RPM for 10 min.
2. Carefully aspirate off all media.
3. Resuspend cells in FACS Fix buffer (2% paraformaldehyde in PBS).
4. Apply cell solution to 2 cm square of nylon mesh held over the mouth of a FACS tube (12x75 mm) to remove any clumps of cells.
5. Cells are ready to be analyzed. If they will not be analyzed right away, store on ice or at 4°C and protect from light. Samples may be stored up to one day without significant problems.
The following procedure may be used for either analysis or sorting. If the cells are to be sorted, they of course must not be fixed but for analysis the cells may be fixed. Fixed cells may be worked with outside of the hood. If analyzing a smaller number of cells are needed (1E6). For sorting, use at least 3E7 cells. The CD81 antibody (BD biosciences) is only reported to be useful for FACS and the recommended antibody concentrations are based on cell number, not dilution volume. This protocol is written with recommended volumes for sorting the L-CD81 cells and then smaller volumes for the accompanying controls.

1. Aspirate media from cells and wash with PBS.
2. Add trypsin. Incubate for about 3 min or until the cells detach. Gently tap the flask to loosen the cells.
3. Add appropriate amount of media to the flask. Pipette cells to wash from flask and break up any clumps. Pipette at least 1E6 (1/10 of a T-75) of the CD81 cells into a 15mL conical and 1E5 into a separate conical tube for the negative/unstained control. Also, prepare a tube of CD81 positive cells such as L-NTC.
4. Spin cells for 10 min at 1300 RPM (Pellet Cells program). If you wish to fix the cells, resuspend in FACS FIX (PBS + 2% paraformaldehyde) and spin again. During this time, make up the primary antibody. Be sure to wipe the antibody tube with ethanol before putting it into the hood. Add 1ul per million cells of anti-CD81 to 1.25 mL of media.
5. Aspirate off media being careful to not disturb the pellet. Add 1mL of antibody to the tube with most of the CD81 cells and resuspend pellet. Add 250ul of the diluted antibody to the positive control. (Add 1 mL of plain media to the negative control cells and set aside.) Incubate cells with antibody for 10 min on rocker.
6. Add 6 mL of media to the L-CD81 cells and the positive control (L-NTC) and spin down. During this time prepare the secondary antibody. Add 2.5ul of secondary to 1.25 mL of media.
7. Aspirate off the media and resuspend the CD81 cells in 1 mL of diluted secondary and use 250 uL to resuspend the positive control cells. Incubate for 10 min on rocker, covered.
8. Add 6 mL to each tube, including the negative control. Spin down. While the cells are centrifuging you may want to get out the cell strainers and FACS tubes and label them.

9. Aspirate the media off being careful to get all the media but not disturbing the cells.

10. Resuspend the stained L-CD81 cells in 1 mL of FACS buffer. Use PBS for fixed cells or FACS sorting buffer (PBS + 5% FBS) for live cells to be sorted. Resuspend the control cells in 0.5 mL of buffer.

11. Pipette resuspended cells through a strainer into a FACS tube.

**INTRACELLULAR LYSATE COLLECTION OF HCV**

Infectious HCV may be isolated from inside cells. This lysate can be used for titering, to infect cells, in a Core ELISA, and in experiments comparing released (extracellular) to intracellular virus.

1. Trypsinize cells, count and spin down at 1300 RPM for 10 minutes.
2. Resuspend cells in 100ul of media per 1 million cells. Transfer to 1.5 mL tubes.
3. Store at -80°C or go directly to freeze-thaw lysis.
4. Lyse cells by 5 freeze-thaw cycles using a dry ice and ethanol bath and 37°C waterbath.
5. Pellet debris by centrifugation at 13,000 RPM for 10 min.
6. Collect supernatant and store at -80°C or use right away.

**IMMUNOFLOURESCENCE STAINING**

1. Wash cells in chamber slides with 1x PBS (1ml per chamber of the 6 well)
2. Remove 1x PBS by aspiration, fix cells in 500 μL of FIX solution (250μL for 12 well) for 10 minutes.
3. Remove FIX solution and discard in formaldehyde waste, wash cells in 1 mL PBT 3 times, 10 minutes each time.
4. Remove PBT by aspiration, block cells for 30 min. to 1 hour in 0.5 mL PBTG at room temperature or overnight at 4°C.
5. Remove PBTG by aspiration, add 250 μL pre-diluted primary antibody, make sure the antibody solution covers all the cells in the chamber. Incubate for 1 hr. or overnight in cold room.

6. Remove primary antibody and return to a new vial (mark the vial with the number of times the antibody has been used), wash cells in 0.5 mL PBT or PBS 4 times, 10 minutes each time.

7. Remove PBS by aspiration, add 250 μL of pre-diluted secondary antibody to cells, make sure the antibody solution covers all the cells in the chamber.

8. Incubate at 4° C overnight or room temperature for 1 hr.

9. Remove secondary antibody and return to a new vial (mark the vial with the number of times the antibody has been used), wash cells in 0.5 mL PBT or PBS 3 times, 10 minutes each time. Mount slides with mounting media containing DAPI such as Vectashield with DAPI (5 ul for small slides, 10 ul for large) or continue on to next step and stain with DAPI.

10. Remove PBS by aspiration, add 300 μL 1:100 DAPI solution, make sure the DAPI solution covers all the cells in the chamber. Incubate at room temperature for 15 minutes.

11. Remove DAPI and return to a new vial (mark the vial with the number of times the DAPI has been used), wash cells in 0.5 mL PBS for 10 minutes, and then 1 mL PBS for 10 minutes.

12. Remove the slides from the PBS and blot edge on a Kimwipe or remove PBS by aspiration and remove the uprights from the chamber slide, immediately add 10 uL mounting solution per slide and mount cells with a glass coverslip. Seal the edges of the coverslips with nail polish or rubber cement.

**Solutions**

**10x PBS (phosphate buffered saline)**

for 1 liter:
80 g NaCl
2 g KCl
17.8 g Na₂HPO₄.2H₂O
add all to 800 mL dH₂O
check pH and adjust to approximately 7.2
- autoclave to sterilize

**FIX Solution (4% paraformaldehyde/PBS)**
for 10 mL:
1.0 mL 10X PBS
2.5 mL 16% paraformaldehyde
6.5 mL dH₂O

**PBT (PBS/0.2% Triton X-100)**
for 500 mL:
50 mL 10X PBS
5 mL 20% Triton X-100
445 mL dH₂O

**PBTG (PBT, 0.2% BSA, 5% NGS)**
for 25 mL:
0.05 g BSA (bovine serum albumin)
1.25 mL NGS (normal goat serum)
23.75 mL PBT

**Mounting Solution**
for 50 mL:
1 g n-propyl gallate
5 mL 10X PBS
40 mL glycerol
5 mL dH₂O

**MEASURING HCV TITER BY TCID₅₀ ASSAY**

This method is easier to use with multiple samples and more replicates. It does not require counting foci but simply determining if a well is positive (containing at least one focus). If a more precise titer is desired, more replicates can be done. For most virus, such as WT JFH-1, the
5 dilutions are sufficient. However, for high titer virus it may be necessary to dilute the first sample 1:10 or alter the set up to go from top to bottom rather than left to right. This allows for two more dilutions but limits the capacity of the plate to three samples.

1. The day before the experiment, seed 6.0 x 10^3 /100ul Huh-7.5 cells/well in a 96-well plate.
2. Prepare serial dilutions of the HCV stock to be titered. Prepare dilutions in DMEM + 10% FBS. We used a 96-well plate to prepare dilutions. The first well contained undiluted virus if working with culture supernatant and a 1:10 dilution if working with cell lysate. Dilutions were prepared in a volume of 150ul (15 ul of virus + 135ul of media).

Figure A.3- Diagram of TCID<sub>50</sub> set up. The different colors represent different virus samples.

<table>
<thead>
<tr>
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<td>1E-3</td>
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<td>1E-5</td>
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</table>

3. Add 100 μL of virus dilutions to each well in a 4 X 6-well column. This allows testing of four viruses over a range of 6 dilutions on a single plate. Include at least one uninfected well on each plate as a negative control. 10^6 dilution is enough for HCV titering. For more accurate titers, use 8x6 pattern for 2 samples per plate.
4. Incubate for 3-4 d at 37°C in 5% CO₂. During this time, the cells will become confluent, and cytopathic effects may be visible at the lowest virus dilutions.
5. Gently aspirate the media and wash the cells with PBS, 100 μL/well.
6. Aspirate the PBS, add 100 μL/4% paraformaldehyde, and fix the cells for at least 10 min RT.
7. Remove the paraformadehyde (put in hazardous waste). The plates can now be safely worked with on the bench top.
8. Wash the cells twice with PBS.
2. Quench endogenous peroxidase by filling each well with 100 μL of PBS containing 3% H₂O₂ (1:10 dilution from 30%). Incubate for 30 min at room temperature. If bubbles are still forming, add fresh H₂O₂ and incubate again.
3. Wash the plate twice with PBS and fill each well with 40 μL of blocking buffer (PBS-T + 1% BSA (fraction V) + 0.2% milk). *filter the buffer if you want to save it for more than a couple days.
4. Place the plate on a rocker and block for 30 min at room temperature or overnight at 4°C.
5. Replace the blocking buffer with PBS-T containing anti-NS3 (BioFront Technologies, Tallahassee, FL) monoclonal antibody 1:1000. Incubate with gentle rocking at room temperature for 1 h or overnight at 4 C. (I prefer to dilute my antibodies in blocking buffer)
6. Wash twice with PBS and once with PBS-T.
7. Add 30 μL/well goat anti-mouse-HRP (Santa Cruz) diluted 1:1000 with PBS-T. Incubate with gentle rocking at room temperature for 30 min.
8. Wash twice with PBS and once with PBS-T.
9. Dilute DAB substrate (Thermo # 34065) 1:10 with accompanying buffer. Add 40 μL/well freshly prepared DAB Incubate at room temperature for 20 min, depending on the desired color intensity. Note that shorter incubation times help to minimize background staining.
10. Remove DAB. Discard as hazardous waste. Wash cells twice with PBS and once with distilled water.
12. (Optional) Counterstain cell nuclei for a few minutes with hematoxylin and wash them with distilled water.
13. Air dried plates can be stored indefinitely, but for better cell morphology, fill each well with 100 μL PBS + 0.03% sodium azide, replace the cover, and seal the edges with parafilm.

14. Read plates under microscope and score the virus titer as TCID$_{50}$/ml.
   A calculator for determining TCID$_{50}$ is available at:
   www.med.yale.edu/micropath/pdf/Infectivity%20calculator.xls

**TITERING PROTOCOL FOR HCV IN CHAMBER SLIDES**

This method requires you to count each focus. At low dilutions, it may be impossible to differentiate individual foci. Short incubation time is important to ensure that only primary foci are being counted, and none due to secondary infections.

1. Seed 1E5 cells per well of a 4 chamber slide the day before infection.
2. 16-24 hours post seeding, prepare viral dilutions. Use a total volume of 500 uL

<table>
<thead>
<tr>
<th>Vol Media</th>
<th>Vol. Virus</th>
</tr>
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<tr>
<td>1</td>
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<tr>
<td>1:10</td>
<td>450 uL</td>
</tr>
<tr>
<td>1:50</td>
<td>500 uL</td>
</tr>
<tr>
<td>1:100</td>
<td>500 uL</td>
</tr>
</tbody>
</table>

3. Change media 16-24 h post infection
4. Fix slides 2 days post infection.
5. Stain with NS3 Ab.

**FOCUS FORMING ASSAY**

Preformed as above with the following changes:
Infect cells late in the day. Approximately 16 hours later, replace media with agarose media as described below.
1. Prepare a 2X DMEM stock using powder DMEM, sterile ddH₂O, (7.4 g/L) sodium bicarbonate, 20% FBS, and 1:50 antibiotic solution (100X stock). Sterile filter.
2. Prepare a 1.2% agarose solution using sterile ddH₂O. Place in waterbath and allow to cool to approximately 50°C.
3. Carefully aspirate media from chambers.
4. Mix equal volumes of agarose and 2X media which is warmed to 37°C. For 1 4 chamber slide 6 mL is recommended (3 mL of agar, 3 mL of media).
5. Immediately after mixing, add 1 mL of agarose mixture to each chamber. Once cool add 250 ul of media on top of the agar to keep it from drying out.
6. Remove the agar media 2 days post infection. Lift the agar carefully by using a pipette tip on an aspirator and then use tweezers to remove the cube of media once you have it lifted. Be very gentle and remove it slowly to help prevent separating the cells from the slide.
7. Fix slides and stain as normal.

**TRANSFECTIONS WITH LIPOFECTAMINE 2000®**

1. Seed cells 16-24 hours prior to transfection. Cells are seeded as follows:

<table>
<thead>
<tr>
<th>293FT</th>
<th>IFN induction</th>
<th>6 well</th>
<th>2.5e5</th>
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<tr>
<td>293FT</td>
<td>Luc Assay</td>
<td>24 well</td>
<td>1E5</td>
</tr>
<tr>
<td>293FT</td>
<td>Vector Production</td>
<td>T-75</td>
<td>6E6 (5E5/mL)</td>
</tr>
<tr>
<td>Liver cells</td>
<td>General</td>
<td></td>
<td>1e5/mL</td>
</tr>
</tbody>
</table>

2. Dispense appropriate amount of OPTI-mem ® into tubes according to manufacturer’s protocol. Use 1 tube for each plasmid or set of plasmids adjusting volume for replicates. One tube with a larger volume can be used for the Lipofectamine needed for all transfections.
3. Add appropriate amount of Lipofectamine ® to OPTI-mem ® in tube.
4. Add appropriate amount of plasmid to all tubes.
5. After 5 min. incubation, add Lipofectamine- OPTI-mem ® mixture to tubes with plasmid.
6. Incubate for 20 min.
7. Carefully add mixture to cells and gently rock the plate to mix well.
8. Collect cells after 48-72 hours to analyze for transfectant.

**IRF-3 DIMERIZATION**

Cells were seeded to a confluency of 50%. IRF-3 plasmid or pcDNA 3.1+ was transfected using Lipofectamine 2000® according to manufacturer's instructions. Cell lysate was collected after 24 hours. IRF-3 dimerization is transient and is degraded after dimerization so the incubation time needs to be optimized to give ample expression of the flag-IRF-3 but before the dimers are degraded.

**NATIVE PAGE FOR IRF-3 DIMERIZATION**

Adapted From (28)

**2X Native PAGE sample buffer**

- 125 mM Tris-CL pH 6.8, 30% glycerol, BPB

**Gel**

- 7.5% Acrylamide/bis 29:1, 0.375M Tris-CL, pH8.8, (no SDS)

No stacking gel.

**Lysis Buffer**

- 50mM Tris-Cl pH 8.0, 1% NP40, 150 mM NaCl, 1:100 protease inhibitor cocktail

**Electrophoresis Buffer**

- Upper Chamber Buffer (Must be stored at 4°C): 25 mM Tris-Cl, pH 8.4, 192 nM glycine, 0.2% sodium deoxycholate
- Lower Chamber buffer: 25 mM Tris-Cl, pH 8.4, 192 mM glycine

**Extract Preparation**

Add lysis buffer (100ul/3 million cells) and vortex. Remove insoluble fraction by centrifugation (13,000 RPM for 10 min). Store extract at -80°C.

**Pre-run**

Pre-run is necessary. Run constant current, 40 mA for 30 minutes.

**Sample electrophoresis**
Mix the extract (2μL) and sample buffer (2μl) then load on the gel. Run at 25 mA for 50 minutes. Overloading may cause a disturbance of IRF-3 binding.

**Blotting**
Soak gel in SDS electrophoresis buffer for 30 min to improve transfer. Transfer the proteins to PVDF membrane. Using wet method, transfer overnight at 90 mA at 4°C.

**Western Blotting**
Stain the membrane as usual. Anti-Flag antibody was used.

**REVERSE TRANSCRIPTION**

RNA was extracted using the RNeasy Plus Kit from Qiagen according to manufacture’s instructions. SuperScript III ® (Invitrogen) was used to generate the cDNA. For all reactions the maximum volume of RNA (8μl) was used as template and random hexamers were used as primers. Reactions were carried out as specified in the product information.

**REALTIME RT PCR**

Total RNA was extracted using the RNeasy Plus kit. It is important that genomic DNA be removed before realtime PCR. The RNeasy plus kit includes a column that accomplishes this, but if using another RNA extraction method, be sure to DNase treat and then further purify the sample. The concentration was found using the Nanodrop and then the samples were taken to the Cloning Facility for analysis. GAPDH was used as an internal control.

**HCV SEQUENCING**

SAV III was sequenced by using cDNA isolated from infected cells and the following primers to run PCR reactions and then to sequence the PCR products.
Table A.4- HCV Sequencing Primers

<table>
<thead>
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<th>Primers</th>
<th>Sequences</th>
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<td>EcoR I F</td>
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</tr>
<tr>
<td>ClaI (1) R</td>
<td>GGTCCCCCACGTAGAGAGC</td>
</tr>
<tr>
<td>ClaI (1) F</td>
<td>GTTGCGTGCCAGTCTCGC</td>
</tr>
<tr>
<td>BsaB I R</td>
<td>CGACCAACTTCCTCAATGCTGC</td>
</tr>
<tr>
<td>BsaB I F</td>
<td>CGTTGGGCTCTTCCGAGC</td>
</tr>
<tr>
<td>ClaI (2) R</td>
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</tr>
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<tr>
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<tr>
<td>Xbal R</td>
<td>CCATGATTACGCAAGCTTGCG</td>
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**IN VITRO TRANSCRIPTION**

The plasmid pJFH-1 pUC or a mutated form was linearized with XbaI and checked by gel electrophoresis on a 0.7% agarose gel to ensure complete linearization. It was then phenol-chloroform extracted remove RNase and other contaminants. Invitro transcription was performed using the MegaScript T7 kit® (Ambion) according to manufacturer’s instructions. RNA was phenol-chloroform extracted and analyzed on a denaturing gel to check the quality of the RNA. RNA was then electroporated into Huh 7.5 based cells.

**RNA gel Protocol**

10X MOPS

For 200 mL
8.37 g MOPS
2.177 g NaAc
4mL EDTA (0.5 M)
Filter Sterilize

1. In a sterile flask combine:
   For 30 mL of gel:
   - 300 mg agarose
   - 3 mL 10X MOPS
   - 21.6 mL DEPC treated H$_2$O
2. Heat 45 seconds on high in the microwave then cool to 65°C.
3. In fume hood, add 5.4 mL formaldehyde and 1 uL ethidium bromide.
4. Pour gel.
5. Add 3 ug RNA to appropriate 5X running buffer (supplied in MegaScipt Kit).
6. Heat RNA at 65°C for 10 minutes followed by cooling on ice for 2 minutes.
7. Load and run sample in 1X MOPS for approximately 2 hours.
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BIOGRAPHICAL SKETCH

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Doctorate of Philosophy (enrolled Fall 2005, anticipated graduation Spring 2011), Biology, Florida State University, Tallahassee, FL
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PUBLICATIONS


PRESENTATIONS, AND POSTERS

