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Title:
Nucleotide sequences and modifications that determine RIG-I/RNA binding and signaling activities

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Abbreviations:
RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated protein 5; HCV, hepatitis C virus; UTR, untranslated region; IFN, interferon; IRF3, interferon regulatory factor 3; CARD, caspase activation and recruitment domain; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; 5’ppp, 5’ triphosphate; MEFs, mouse embryo fibroblasts; CTD, C-terminal domain; RD, repressor domain; nt, nucleotide
Abstract

Cytoplasmic viral RNAs with 5’ triphosphates (5’ppp) are detected by the RNA helicase RIG-I, initiating downstream signaling and interferon (IFN)-α/β expression that establish an anti-viral state. We demonstrate here that the hepatitis C virus (HCV) 3’ untranslated region (UTR) RNA has greater activity as an immune stimulator than several flavivirus UTR RNAs. We confirmed that the HCV 3’UTR polyU/UC region is the determinant for robust activation of RIG-I-mediated innate immune signaling, and that its antisense sequence, polyAG/A, is an equivalent RIG-I activator. The polyU/UC region of the fulminant HCV JFH-1 strain was a relatively weak activator, while the antisense JFH-1 strain polyAG/A RNA was very potent. PolyU/UC activity does not require primary nucleotide sequence adjacency to the 5’ppp, suggesting that RIG-I recognizes two independent RNA domains. Whereas polyU-50 or polyA-50 sequences were minimally active, inserting a single C or G nucleotide into these respective RNAs increased IFN-β expression. PolyU/UC RNAs transcribed in vitro using modified uridine 2’ fluoro or pseudouridine ribonucleotides lacked signaling activity while functioning as competitive inhibitors of RIG-I binding and IFN-β expression. Nucleotide base and ribose modifications that convert activator RNAs into competitive inhibitors of RIG-I signaling may be useful as modulators of RIG-I mediated innate immune responses and as tools to dissect the RNA binding and conformational events associated with signaling.
Introduction

The initial reaction of mammalian cells to invading viral pathogens is an innate immune response mediated by host pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) (3). PAMPs presented during a viral infection include DNA and double-stranded (ds) or single-stranded (ss) RNA. dsRNA PAMPs can exist in the form of the viral genome or as a replication intermediate (24, 31). Viral dsRNA is recognized in the late endosome by Toll-like receptor (TLR) 3 (2), while uridine-rich short ssRNA is recognized by TLR7 and TLR8 in the endosomal compartments of plasmacytoid dendritic cells (8). Cytoplasmic 5’ triphosphorylated ssRNA and short dsRNA are recognized by the RNA helicase retinoic acid inducible gene I (RIG-I) (13, 14, 22). Long cytoplasmic dsRNA, such as polyI:C, is recognized by the related helicase melanoma differentiation associated antigen 5 (MDA5) (14, 15). Studies with RIG-I knock-out mice show that RIG-I is required for IFN production in response to several paramyxoviruses, the orthomyxovirus influenza virus, and the flavivirus Japanese encephalitis virus, whereas MDA5 is required for responding to picornaviruses (15, 20). RIG-I and MDA5 are individually dispensable for signaling in response to reovirus or dengue virus infection (20). RIG-I and MDA5 cooperate to trigger an innate immune response to West Nile virus (10).

Cytosolic 5’ triphosphates (5’ppp) generated during viral RNA transcription or replication are required for RIG-I activation, suggesting that the 5’ppp may be a structural feature that distinguishes viral RNA from self RNA in virally infected cells (13, 22). Capping/removing the 5’ppp or inserting modified nucleotides into the RNA abrogate RIG-I signaling (13). While capping or the absence of a 5’ppp likely prevents RNA binding to the RIG-I C-terminus (7, 30),
the mechanisms underlying the effects of nucleotide modifications on RIG-I activity have not
been elucidated. In particular, it is not known how nucleotide modifications affect RIG-I binding.

RIG-I and MDA5 contain two N-terminal caspase activation and recruitment domains (CARDs) in addition to a helicase/ATPase domain (35). Recent data suggest that the helicase activity of RIG-I is inversely correlated to its downstream signaling activity (30), whereas RIG-I’s ATPase activity appears to be directly correlated with its downstream signaling activity (6, 11, 30, 35). RIG-I also contains a C-terminal domain (CTD) that recognizes the 5’ppp of ssRNA and coincides with a repressor domain (RD) that regulates RIG-I signaling (7, 25, 30). After RIG-I binds to viral RNA, it undergoes conformational changes that promote self-association (25). Subsequently, RIG-I binds to the mitochondrial membrane-associated IPS1 protein via CARD-CARD interactions, thereby activating IPS1 (16). This sets off a signaling cascade resulting in activation of the transcription factors IRF3 and NF-κB, which then induce IFN-α/β production and the subsequent induction of IFN-stimulated genes (ISGs). IFN-α/β production leads to a cytotoxic response, promotes an antiviral state in neighboring uninfected cells, and helps stimulate the subsequent adaptive immune response (4).

Hepatitis C virus (HCV) is a positive sense, ssRNA virus in the Flaviviridae family. RIG-I recognizes the HCV 5’ and 3’ untranslated regions (UTRs) in TLR3-deficient human hepatoma (Huh7) cells (18, 29). RIG-I activation by single-stranded 5’ppp-containing HCV RNAs was recently linked to sequence composition and length, specifically homopolyuridine and homopolyriboadenine motifs longer than 50 nucleotides (26). Although RIG-I signaling is activated during infection by other members of the viral family Flaviviridae (e.g. dengue and West Nile viruses) (6, 9, 10, 20), the relative activities of Flaviviridae RNAs as RIG-I activators have not been compared. We show here that the HCV 3’UTR RNA is a significantly more potent
RIG-I activator than 5’ppp-containing dengue, West Nile, or yellow fever virus UTR RNAs. Similar to the results of recent experiments using the HCV Con1 strain 3’ UTR RNAs (26), our data confirm that the uridine-rich polyU/UC region of the HCV J4L6 strain 3’UTR is the determinant for this robust immunostimulation, and that its antisense sequence, polyAG/A, is an equivalent RIG-I activator. Surprisingly, the polyU/UC sequence from the 3’UTR of the fulminant HCV JFH-1 strain was a relatively weak activator, while the antisense JFH-1 strain polyAG/A RNA was very potent.

To gain a deeper understanding of RNA features recognized by RIG-I, we tested RNAs of varying lengths and sequence composition. Although polyU-50 or polyA-50 RNAs were inactive, the insertion of a single C or G nucleotide, respectively, increased RIG-I mediated signaling significantly, suggesting that both sequence and length influence signaling activity. Nucleoside modifications in the base or at the 2’ ribose position have been reported to abrogate innate immune signaling through both TLR 7/8 and RIG-I pathways (8, 27). To probe the mechanism of the effect on RIG-I signaling, we substituted pseudouridine for uridine or replaced uridine 2’ hydroxyls with fluoro groups in HCV 3’UTR RNAs. The data demonstrate that, although stimulation of IFN-β is abrogated, the RIG-I-RNA binding interaction was not diminished significantly. Furthermore, the modified polyU/UC RNAs behave as competitive inhibitors of RIG-I binding and IFN-β induction. The data extend the range of known RNA features associated with RIG-I mediated activation of innate immune signaling, provide a possible correlation between HCV 3’UTR sequence identity and virulence, and describe modified RNAs that could modulate innate immune stimulation or be used as tools to dissect specific steps in the activation process.
Materials and Methods

Cells and viruses. Huh7 cells were provided by Dr. P. Yang (Harvard Medical School) and Dr. S. Behrens (Fox Chase Cancer Center). Huh7 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 1% of a standard antibiotic-antimycotic solution (Invitrogen). Wild-type and RIG-1−/− murine embryonic fibroblasts (MEFs) were a gift from Dr. J. Jung (University of Southern California). MEFs were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 1% of a standard antibiotic-antimycotic solution (Invitrogen), and 200 µg ml⁻¹ of neomycin. Sendai virus (SenV) Cantel strain was obtained from Charles River SPAFAS (Wilmington, MA). The vesicular stomatitis reporter virus (VSV-luc) was provided by Dr. S. Whelan (Harvard Medical School) and will be described in detail elsewhere.

DNA methods. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen). pIFN-β-luc plasmid was a gift from Dr. J. Jung (University of Southern California). pCMV-luc plasmid was purchased from Promega. T7-HCV 3’ UTR (genotype 1b strain J4L6) and T7-HCV X in vitro transcription plasmids were a gift from Dr. R. Chung (Massachusetts General Hospital). pNS3/4A (ss1 RNA) and pEFBos FLAG-RIG-I plasmids were gifts from Dr. M. Gale (University of Washington-Seattle).

RNA methods. Dengue virus 5’ and 3’ untranslated region (UTR) RNAs and 3’ stemloop (SL) RNA were derived from serotype 4 virus strain 814669 (AF326573.1). The West Nile virus 3’SL RNA was derived from strain NY99 (FJ411043.1). The yellow fever virus 3’SL RNA was derived from vaccine strain 17D (X03700.1). PolyU/UC RNA was transcribed from a T7 PCR product generated using T7-HCV 3’UTR plasmid, a forward primer with a flanking T7 promoter sequence, and a reverse primer (21). PolyU/UC-ss1 and ss1-polyU/UC chimeric RNAs were
generated by PCR using pNS3/4A and T7-HCV 3’UTR plasmids as templates, one pair of external primers (with forward primer containing T7 promoter sequence), and one pair of internal primers (both chimeric oligonucleotides). The recombinant PCR products were cloned in T vector (Promega) and sequenced. Correct clones were amplified in E. coli and plasmid DNA was harvested using QiaFilter Plasmid Maxi kit (Qiagen). The plasmids were digested to release their inserts, the insert fragments were gel purified, and the purified inserts were then used as templates for transcription using the T7 Megashortscript kit (Ambion). J4L6 polyU/UC-80 nucleotide (nt), -70nt, and -60nt deletion RNAs were generated using the Milligan transcription method (21) whereby a T7 promoter primer was annealed to an oligonucleotide with reverse complementary sequence to generate a partially double-stranded template. The Milligan transcription method was also used to generate J4L6 polyAG/A-100nt, J4L6 polyAG/A-60nt, polyG/GC-60, polyU-50, polyU-35, polyA-50, U33CU16, A33GA16, JFH-1 polyU/UC, and JFH-1 polyAG/A RNAs. All of these in vitro transcribed RNAs contain three guanines at the extreme 5’ end to facilitate transcription by the T7 polymerase. All other unmodified RNAs were transcribed from linearized plasmid DNA templates using the T7 Megashortscript kit (Ambion). Biotinylated RNAs were transcribed using the T7 Megashortscript kit and biotin-11-cytidine-5’-triphosphate (Roche Diagnostics). 2’-fluoro-uridine and 2’-fluoro-cytidine substituted transcripts were made using the DuraScribe T7 transcription kit (Epicenter) according to the manufacturer’s protocol. Pseudouridine-substituted RNAs were made using the T7 Megashortscript kit and pseudouridine-5’-triphosphate (Trilink). After transcription, the DNA template was hydrolyzed with DNase, proteins were separated from transcribed RNA by phenol-chloroform extraction, nucleotides were removed by quick-spin gel filtration column chromatography, and then the RNA was by precipitated with ethanol and ammonium acetate or sodium acetate. RNA was
sedimented by centrifugation, washed with 70% ethanol, dried briefly, and resuspended in RNase-free water. RNA concentration was determined by absorbance in a spectrophotometer.

**Transfection and luciferase reporter assays.** 3 x 10⁴ Huh7 cells were plated per well of a 24-well plate. After 24 hours, the cells were transfected with 100 ng of pIFN-β-luc (firefly luciferase) and 1 ng of pCMV-luc (*Renilla* luciferase) using Lipofectamine 2000 (Invitrogen). After a 24-hour incubation, equal moles of each viral RNA fragment were denatured for 3 min at 90°C then renatured by slow cooling in renaturation buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 3 mM MgCl₂, 0.1 mM EDTA) and transfected using Lipofectamine 2000. 24 hours after RNA transfection, cells were lysed in 100 µl of passive lysis buffer (Promega), and an aliquot was analyzed using the dual-luciferase reporter assay system (Promega).

**ISG56 immunoblot.** 28 hours after Huh7 cells were transfected with the viral RNAs, the cells were lysed (10 mM Tris pH 8, 200 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM DTT, 1X phosphatase inhibitor cocktail 1 (Sigma), 1X phosphatase inhibitor cocktail 2 (Sigma), 1x protease inhibitor cocktail (Sigma)). Cell debris was removed by centrifugation (200g for 3 min), and a Bradford assay was used to determine the protein concentration. 15µg of total protein were electrophoresed into a 9% Tris-glycine SDS-polyacrylamide gel. Immunoblot analysis was performed using polyclonal anti-ISG56 antibody (provided by G. Sen, Cleveland Clinic) and monoclonal anti-beta actin (AC-15) antibody (AbCam). Proteins were detected with a horseradish peroxidase-conjugated secondary antibody and were visualized by chemiluminescence.

**ELISA.** Wild-type or RIG-I⁻/⁻ MEFs were mock-transfected or transfected with equal moles of renatured *in vitro*-transcribed HCV 3’UTR, polyU/UC, or X RNA. After 24 hours, cell culture supernatants were collected and analyzed for IFN-β production using an enzyme-linked
immunosorbent assay (PBL Biomedical Laboratories). The levels of IFN-β expression were
determined by comparison to the linear portion of a standard curve.

**Preparation of RIG-I cell extract.** Cell extracts were prepared essentially as described by
Chang *et al.* (6, 11, 30, 35). Briefly, subconfluent Huh7 cells were transfected with pEFBos
FLAG-RIG-I plasmid and then incubated for about 48 hours. Cells were scraped into cold
phosphate buffered saline, sedimented by centrifugation, and then resuspended in two volumes
of hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 1X protease cocktail
inhibitor (Sigma), 1 mM DTT). The cells were allowed to swell for 15 minutes on ice and were
then broken using a Dounce homogenizer. The extract was clarified by centrifugation (15
minutes at 14,000 rpm at 4°C), and the protein concentration of the supernatants was determined
using a Bradford assay. Aliquots were stored at -80°C.

**Competitive RIG-I pull-down assay.** We standardized our RIG-I binding analyses across
the experiments by using competitive pull-down assays using biotinylated polyU/UC RNAs.
This was important in order to rule out the possibility that biotinylated RNAs with other
sequences may not have interacted equivalently with the streptavidin particles, thereby
preventing binding comparisons. 1 µg of biotinylated HCV J4L6 strain polyU/UC RNA was
incubated for 1 hour at 25°C with or without excess nonbiotinylated competitor RNA and 30 µg
of FLAG-RIG-I cell extract. Following the incubation, the mixture was transferred into 400 µl of
wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40) containing 50 µl
streptavidin MagneSphere paramagnetic particles (Promega), and rocked at 4°C for 2 hr. The
RNA-protein complexes were collected by magnetic separation, washed three times with wash
buffer, resuspended in SDS-PAGE sample buffer, boiled for 5 min, and electrophoresed into a
Tris-glycine 7.5% SDS-polyacrylamide gel. FLAG-tagged protein within the pull-down fraction was analyzed by immunoblotting using M2 anti-FLAG antibody (Sigma).
Results

Comparative analysis of activation of TLR3-independent signaling by viral UTR RNAs.

To determine if flavivirus UTR RNAs activate TLR3-independent signaling, we transfected equal moles of renatured 5’ triphosphorylated full-length dengue virus (DEN) 5’ or 3’ UTR RNAs or the conserved 3’ terminal stemloop RNAs from DEN, West Nile virus (WNV), or yellow fever virus (YFV) into TLR3-deficient Huh7 cells (18), and measured expression from an IFN-β promoter-luciferase reporter as an indication of early signaling events. The HCV 3’UTR RNA served as a positive control, while the HCV ss1 RNA and no RNA (mock) were used as negative controls (29). The full-length 5’ and 3’ DEN UTR RNAs (105nt and 383nt respectively) activated IFN-β to moderate levels compared to the negative controls, but the DEN, WNV, and YFV stemloop (SL) RNAs (110nt) were minimally active (Fig. 1A). As expected, the HCV 3’UTR RNA was a strong activator (Fig. 1A) (29). To confirm the luciferase reporter data using a direct assay for IFN production in response to the viral RNAs, we measured downstream IFN stimulated gene (ISG) 56 expression by immunoblot. The ISG56 expression pattern mirrored that of the IFN reporter assay (Fig. 1B). These data suggest that endogenous IFN-β was produced and released in response to the transfected RNAs, leading to downstream ISG expression (1). Activation of interferon expression by the HCV 3’UTR was also compared with that induced by Sendai virus infection. As shown in Fig. 1C, the HCV 3’UTR was significantly better as an activator of IFN-β compared to Sendai virus infection. These results demonstrate that the HCV 3’UTR RNA is a considerably more potent activator of TLR3-independent innate immune signaling than flavivirus UTR RNAs and Sendai virus. We assessed the role of the 5’ppp in activation by treating the \textit{in vitro}-transcribed HCV 3’UTR, DEN 5’UTR, and DEN 3’UTR RNAs with a phosphatase, or by adding a m7GpppG cap (13). Both phosphatase treatment and
capping the 5’ end reduced activation of the IFN-β reporter to background levels (data not shown). These results confirm that the 5’ppp has a critical role in activation of IFN-β expression. We note, however, that despite the fact that all of the viral RNAs tested in Figure 1 contained a 5’ppp, we observed significant variability in the extent of IFN-β activation. We conclude that the 5’ppp is necessary, but not sufficient for IFN-β activation.

The polyU/UC sequence is responsible for the potent activation of IFN-β by the HCV 3’UTR. To understand why the HCV 3’UTR is significantly more potent than the other viral RNAs tested, we tested each half of the 3’UTR RNA independently. While this work was in preparation for publication (33), Saito et al. reported a similar approach for analyzing the RNAs of HCV 1b Con1 strain (26). The 5’ half of the 3’UTR is a polyU/UC sequence that is unlikely to form stable secondary structure because it is very pyrimidine-rich and forms few canonical Watson-Crick basepairs. The 3’ half of the 3’UTR has the potential to fold into three stemloops and is referred to as the HCV X RNA (32). When transfected into Huh7 cells, the polyU/UC region activated the IFN-β reporter to levels comparable to the full-length 3’UTR; however, the HCV X RNA was minimally active (Fig. 2A). To verify that the polyU/UC RNA activates innate immune signaling through RIG-I, we compared the ability of the polyU/UC RNA to induce IFN-β in wild-type (wt) and RIG-I knock-out mouse embryo fibroblasts (MEFs). The polyU/UC RNA was a potent inducer of IFN-β in the wt MEFs, but this activation was completely abrogated in RIG-I knock-out MEFs, demonstrating that RIG-I is necessary for signaling in response to the polyU/UC RNA (Fig. 2B). We also performed a competitive RIG-I pull-down assay using extract from Huh7 cells overexpressing FLAG-RIG-I (25) to determine if polyU/UC RNA can bind to RIG-I (Fig. 2C). Biotinylated polyU/UC RNA pulled down RIG-I from the extract (lane 1), and non-biotinylated polyU/UC and HCV 3’UTR RNAs were able to compete
for 83% and 73% of this binding, respectively (Fig. 2C, lanes 2 and 3). Non-biotinylated ss1 and X RNAs were less effective competitors of RIG-I binding (0% and 27% reduction, respectively) (Fig. 2C, lanes 4 and 5). To verify that the X RNA was not less stable than the HCV 3’UTR or polyU/UC RNAs, we measured stability of radiolabeled HCV 3’UTR, polyU/UC, and X RNAs over time in RIG-I cell extract and found that the X RNA is not less stable than the HCV 3’UTR or polyU/UC RNAs (data not shown).

Activation does not require the 5’ppp and activating RNA domain to be immediately adjacent. To determine if potent induction of IFN-β expression required that the 5’ppp be immediately adjacent to the activating nucleotide sequence, we created chimeric RNAs containing the polyU/UC sequence fused to the 5’ or 3’ terminus of the 350nt HCV ss1 non-activating sequence. Schematic representations of these constructs are presented in Figure 3A. If the 5’ppp needed to be immediately adjacent to the polyU/UC sequence for potent activation, then the ss1-polyU/UC RNA would be expected to have less activation potential than the polyU/UC-ss1 RNA. RNAs were transcribed and tested for activation of IFN-β reporter expression. The results (Fig. 3B) suggest that both chimeric RNAs are signaling activators. These data are evidence that the polyU/UC region is a functional motif that can be separated from the 5’ppp by over 300 nucleotides while retaining potent activity.

Interrupted short homopolymeric uridine and adenine RNAs activate signaling. To determine if there is a minimal length or motif required for potent activation by the polyU/UC RNA, we transcribed RNAs with 3’ terminal deletions (Fig. 4A) and, following transfection into Huh7 cells, measured luciferase expression from the IFN-β-luc plasmid. As shown in Fig. 4B, polyU/UC-80nt retained full activity compared to full-length polyU/UC-106nt. However, further 3’ deletions resulted in gradual reductions in activity (Fig. 4B). 5’ terminal deletions of
polyU/UC were also tested and deletion of 30nt or more from the 5’ end (that shortened or deleted the 28U tract) completed abrogated activity (data not shown). These data suggest that the intact 28U region is critical for activation. Since the polyU/UC RNA is 70% uridine, we decided to test whether potent RIG-I stimulation is limited to uridine-rich sequences. We tested the activity of the complement of polyU/UC, the polyAG/A RNA, which is relevant to HCV infection because it is generated during minus-strand synthesis. Interestingly, the polyAG/A RNA had activity comparable to the full-length polyU/UC RNA (Fig. 4C), suggesting that potent RIG-I activation is not limited to uridine-rich sequences. The polyAG/A-60nt deletion RNA (containing 28A and 11A tracts) also exhibited full activity, while the corresponding polyU/UC-60nt RNA (containing 28U and 11U tracts) showed reduced activity, and the polyG/GC-60nt RNA (containing 28G and 11G tracts) showed minimal activity (Fig. 4C). These data suggest that length requirements for potent RIG-I activation vary with the nucleotide sequence. Since uridine- and adenine-rich sequences were both potent RIG-I activators, we determined if 50-nucleotide RNAs containing a homopolymeric tract of uridine or adenine of 50 nucleotides are also strong immunostimulators. Both polyU-50 and polyA-50 (26) activated IFN-β expression poorly (Fig. 4D). To determine whether their lack of activity was due to insufficient length (50 nucleotides), or because homopolymeric composition is not adequate for potent RIG-I signaling, we tested the activity of 50-nucleotide polyU and polyA RNAs with a cytidine or guanine nucleotide inserted after the 33rd U or A, respectively. The rationale for this approach is based on the observation that in the J4L6 polyU/UC RNA, runs of uridine are not continuous, but are interrupted by cytidine (Fig. 4A). Interestingly, both U_{33}CU_{16} and A_{33}GA_{16} RNAs exhibited higher activity than homopolymeric polyU-50 and polyA-50 RNAs (15- and 52-fold higher, respectively) (Fig. 4E), suggesting that interrupting the homopolymeric sequence with a single C
or G nucleotide enhances activation potential. To confirm that the activities of the most immunostimulatory adenine-rich RNAs correlated with RIG-I/RNA binding, competitive pull-down experiments were performed. The data (Fig. 4F) demonstrate that the polyAG/A-100nt (lane 2, 86% reduction), polyAG/A-60nt (lane 3, 88% reduction), and A_{33}GA_{16} (lane 4, 89% reduction) RNAs were effective competitors of RIG-I binding to the biotinylated polyU/UC RNA. X RNA (lane 5, 15% increase) did not compete for binding. Importantly, these results suggest that activation of RIG-I is a function of both RNA sequence and length.

**Modified RNAs are competitive inhibitors of signaling activity and RIG-I binding.** The immunostimulatory activity of 5’ppp RNA was shown to be inhibited by capping or removing the 5’ppp or by introducing nucleoside base and ribose modifications (13). We tested the effect of ribose 2’ hydroxyl modification on the immunostimulatory potential of the HCV 3’UTR and polyU/UC RNAs. Consistent with previous reports (13), replacement of uridine 2’ hydroxyls with fluoro groups in the HCV 3’UTR and polyU/UC RNAs reduced IFN-β activity to background levels (Fig. 5A). Less dramatic reductions in activity were observed when cytidine 2’ hydroxyls were replaced with fluoro groups (Fig. 5A), likely because there are fewer cytidines in both RNAs as compared to uridines. Next we tested how incorporation of a modified base affects IFN-β activation. 100% substitution of uridine with pseudouridine, which has the same chemical formula as uridine but a shifted glycosidic bond, also abrogated activity (Fig. 5B). To determine whether the inhibition of IFN-β induction by incorporation of 2’fluoro (F)-dUTP or pseudouridine was due to reduction in binding to RIG-I, we performed a competitive RIG-I pull-down assay. In the presence of three-fold molar excess of 2’F-dUTP- or pseudouridine-modified polyU/UC RNAs, the amount of RIG-I pulled down by biotinylated polyU/UC RNA was reduced 99% and 74 %, respectively (Fig. 5C, lanes 2 and 3). X RNA was less effective as a
competitor (Fig. 5C, lane 4, 18% reduction). These results suggest that RIG-I signaling is diminished by 2’ hydroxyl and base modifications, but the modifications do not affect RIG-I binding to the RNA. To determine if these modified RNAs function as competitive inhibitors of IFN-β induction, we co-transfected unmodified polyU/UC RNA with equal or excess molar quantities of 2’F-dUTP- or pseudouridine-modified polyU/UC RNAs (Fig. 5D). Both 2’F-dUTP- and pseudouridine-modified polyU/UC RNAs functioned as competitive inhibitors of IFN-β induction, while the X RNA was not inhibitory (Fig. 5D). These data suggest that the modified RNAs trap RIG-I in an inactive complex, thereby decreasing innate immune signaling. To begin to understand why the modified RNAs failed to activate signaling, we asked whether base and ribose modifications affected the conformation of the polyU/UC RNA as measured by native gel electrophoresis. When pseudouridine- and 2’F-dUTP-modified polyU/UC RNAs were electrophoresed into a native polyacrylamide gel, we found that the pseudouridine-modified polyU/UC RNA displayed slower migration than unmodified RNA, suggesting a larger Stokes radius. Conversely, the 2’ hydroxyl substitutions did not affect RNA mobility under these conditions (data not shown).

**JFH-1 polyAG/A RNA, but not JFH-1 polyU/UC RNA, is highly immunostimulatory.**

The polyU/UC and polyAG/A sequences tested thus far in this study were from the HCV genotype 1b J4L6 strain. The length and composition of the polyU/UC region varies somewhat among different HCV strains (17), and we considered the possibility that these variations could result in strain-specific innate immune stimulation. Saito et al. reported that full-length genomic HCV RNA (Con1 strain) lacking the 3’UTR had much lower immunostimulatory potential than full-length genomic RNA, suggesting that the polyU/UC sequence is a critical determinant for RIG-I-mediated signaling in response to the full-length viral genomic RNA (26). The HCV
genotype 2a JFH-1 strain, which was isolated from a patient with fulminant hepatitis in Japan, is unique in that it replicates efficiently and produces infectious particles in cell culture (19, 34, 37). We reasoned that the enhanced replicative properties of the JFH-1 virus might correlate with diminished activation of innate immune signaling in comparison with other HCV strains. Therefore, we compared the immunostimulatory activities of the JFH-1 polyU/UC and polyAG/A RNAs to those of the J4L6 RNAs (Fig. 6A). Interestingly, the JFH-1 polyU/UC RNA had half the immunostimulatory activity of the J4L6 polyU/UC and polyAG/A RNAs (Fig. 6A), a finding that correlates with the higher replicative potential of the JFH-1 strain. In contrast, the JFH-1 polyAG/A RNA was more stimulatory than the J4L6 polyU/UC and polyAG/A RNAs (Fig. 6A). To determine if the observed activity in the IFN-β reporter assay (Fig. 6A) correlated with a virus infection model, we transfected Huh7 cells with J4L6 or JFH-1 polyU/UC or polyAG/A RNAs, waited 24 hours, and then infected with vesicular stomatitis virus expressing firefly luciferase (VSV-luc) at increasing multiplicities of infection (MOI). Activation of innate immune signaling by the RNAs was expected to establish an anti-viral state that would limit VSV replication. The results (Fig. 6B) demonstrate that inhibition of VSV-luc replication correlates well with IFN-β reporter induction by the different RNAs. Taken together, the results in Figure 6 strongly suggest that innate immune signaling potential is defined in part by specific sequences present in the HCV 3’UTR polyU/UC and polyAG/A domains, which vary among the different strains.
Discussion

In this paper, we have examined sequence and structural elements required for potent RIG-I-mediated innate immune signaling that manifests as IFN-β expression and the establishment of an anti-viral state. In addition to confirming 5’ triphosphorylated uridine- and adenine-rich ssRNAs as potent activators of RIG-I signaling (26), the results presented here suggest that 1) the 3’UTR RNAs or the structurally conserved 3’-terminal stem loop RNAs from several flaviviruses are comparatively weak signaling activators when evaluated side-by-side with HCV 3’UTR RNA; 2) immediate adjacency of the 5’ppp and the HCV polyU/UC sequence is not essential (i.e. separating the two determinants by over 300 nucleotides did not reduce signaling significantly); 3) RIG-I/RNA binding and signaling activity are determined by both RNA length and nucleotide sequence (e.g. inactive short homopolymeric uridine and adenine RNA sequences can be converted to signaling activators by interrupting the homopolymer with single C or G nucleotides, respectively); 4) nucleoside base and ribose 2’ hydroxyl modifications that block signaling activity do not inhibit the binding interaction between RNA and RIG-I; and 5) polyU/UC and polyAG/A sequences from two different HCV strains have distinct immunostimulatory potentials that may be factors in defining their different replicative capacities.

Following the report that the 3’UTR of the HCV RNA activates TLR3-independent innate immune signaling (29), we carried out experiments to determine if corresponding regions from other Flaviviridae members had similar potentials. The results (Fig. 1) demonstrate that, although the 5’ and 3’UTRs of dengue virus (type 4) elicited measurable stimulation of innate immune signaling, the smaller, highly structurally-conserved 3’-terminal stemloop RNAs (5, 12) of dengue (type 4), yellow fever (17D vaccine strain), and West Nile (NY99 strain) viruses were
minimally active. The increased activity of the DEN 5' and 3' UTRs did not correlate with elevated uridine or adenine compositions compared to the stemloop RNAs (DEN 5'UTR 29% U, 29% A; DEN 3'UTR 17% U, 29% A; DEN 3'SL 23% U, 25% A; WNV 3'SL 19% U, 24% A; YFV 3'SL 21% U, 28% A; HCV 3'UTR 49% U, 14% A; ss1 19% U, 20% A). Removing the 5'ppp from the HCV 3’UTR, DEN 5’UTR, and DEN 3’UTR completely abrogated IFN-β stimulation, suggesting that activation was mediated through RIG-I and not MDA5 (data not shown) (7). After observing the large relative differences in RNA immunostimulation potentials (Fig. 1), we subsequently localized the robust activation of IFN-β expression to the HCV polyU/UC region (Fig. 2). While this work was in preparation for publication (33), Saito et al. reported similar results using 3’UTR sequences from a related HCV strain, Con1 (26). The precise sequences in the dengue 5’ and 3’UTR RNAs that activate signaling have not been mapped. However, these RNAs lack uridine or adenine repeats that are found in the HCV 3’UTR sense and antisense RNAs, suggesting that sequences other than polyU/UC or polyAG/A may have weak activating potential.

We considered the physiological relevance of testing activation potentials of short (60-350 nucleotide) RNA sequences, as compared to the full-length RNAs. It has been reported that both HCV replicon RNA (29) and full-length genomic RNA (26) activate signaling. HCV genomic RNA lacking the 3’UTR has much less immunostimulatory potential compared to full-length genomic RNA (26), suggesting that both the 5’ppp and the polyU/UC sequence can be sensed by RIG-I, despite the length of primary nucleotide sequence separating them. Here, we generated chimeric RNAs that allowed us to test the dependence of activation on immediate adjacency of the 5’ppp and the polyU/UC RNA sequence. The results (Fig. 3) revealed that potent activation does not require the immediate adjacency of the 5’ppp and the activating polyU/UC sequence.
Mechanistic models suggest that the 5’ppp is bound by the RIG-I C-terminal domain (CTD) (7, 30), and that the polyU/UC nucleotide sequence is bound elsewhere, presumably by the RNA helicase (DECH) domain. The fact that RIG-I can sense the 5’ppp and RNA nucleotide activation motifs, despite their separation by 350 nucleotides (Fig. 3), or by over 9000 nucleotides of primary sequence in the case of the HCV replicon and genomic RNAs (26, 29), suggests that RIG-I may have significant structural flexibility or may undergo co-folding events with the RNA in order to bridge the 5’ppp and nucleotide activation domain. It is possible that long range RNA-RNA or protein-RNA interactions bridge the 5’ and 3’ ends of the HCV RNA, thereby enhancing RIG-I sensing of both the 5’ppp and downstream nucleotide activation domains.

The data described here provide new insights into defining the RNA determinants required for innate immune signaling. Our data (Fig. 4) and those of others (26) suggest that activation of IFN-β reporter expression is proportional to the length of the polyU/UC region, with little activity observed using polyU/UC RNAs with fewer than 60 nucleotides. Although homopolymeric polyU-50 and polyA-50 RNAs were inactive in our assays (Fig. 4D and (26)), interrupting these homopolymers by inserting a single C or G nucleotide, respectively, increased IFN-β reporter expression significantly (Figure 4E). These experiments followed from the observation that the uridine tracts in the J4L6 polyU/UC sequence are interrupted by intermittent cytidines (Fig. 4A). We considered the possibility that introducing an additional interruption into the polyU/UC or polyAG/A RNA might further enhance activity. The results (data not shown) indicate that inserting a single C or G nucleotide to interrupt the 43U or 43A tracts of the JFH-1 polyU/UC or polyAG/A RNAs into 14U and 28U or 14A and 28A tracts, respectively, did not increase IFN-β reporter expression. We speculate that when starting with a homopolymer,
nucleotide interruptions may increase RIG-I activation until a plateau is reached, after which
further interruptions have no effect or may even reduce activation of RIG-I. Others have noted
that RNA-protein interactions are enhanced by discontinuities in RNA structure (e.g. bulged
nucleotides, non-canonical base pairs) (28); however, defining the molecular basis for the
enhanced activity of these short (50 nucleotide) interrupted homopolymer RNAs will require
further experimentation. RIG-I is activated by both the polyU/UC RNA and its antisense
sequence polyAG/A, but not by polyG/GC (Fig. 4) (26). The polyU/UC and polyAG/A binding
surfaces on RIG-I have not been defined, and it will be interesting to determine how RIG-I can
bind specifically and functionally to both polypyrimidine- and polypurine-rich RNA sequences.

It was reported previously that RNAs with ribose 2’ hydroxyl substitutions (e.g. 2’-O-methyl
and 2’fluoro) exhibit reduced signaling through TLR7 and RIG-I (8, 13, 27). Substituting uridine
with pseudouridine, which has a shifted glycosidic bond, is also known to abrogate RIG-I
signaling (13). Correspondingly, HCV 3’UTR and polyU/UC RNAs transcribed with 2’fluoro
(2’F)-dUTP and 2’F-dCTP showed diminished stimulatory activities (Fig. 5A), as did polyU/UC
RNA transcribed with pseudouridine (Fig. 5B). We demonstrate here that, despite these activity
losses, the binding interactions between RIG-I and 2’F-dUTP or pseudouridine-modified
polyU/UC RNAs were retained (Fig. 5C). The mechanisms underlying the activity losses are not
understood; however, the results reported here strongly indicate that the disruption of RIG-
I/RNA binding can be excluded. The in vitro binding data (Fig. 5C) were extended in a
functional assay, wherein modified RNAs were shown to diminish IFN-β induction when co-
transfected with unmodified activator polyU/UC RNA (Fig. 5D), likely because of competitive
RIG-I binding. These results suggest that RIG-I molecules that are bound to RNAs containing
modified nucleotides are trapped in an inactive intermediate form (Fig. 7). A recent report of
single molecule reconstructions of RIG-I bound to phosphorothioated oligodeoxynucleotides is consistent with this hypothesis (23).

The lengths and nucleotide sequences of the polyU/UC regions vary somewhat among different HCV isolates (17), and we considered the possibility that these variations might be associated with differences in innate immune responses. The HCV genotype 2a JFH-1 strain, which was isolated from a patient with fulminant hepatitis in Japan, is unusual in that it replicates efficiently and produces infectious particles in cell culture (19, 34, 37). We reasoned that the enhanced replicative properties of the JFH-1 virus might correlate with diminished activation of innate immune signaling in comparison with other HCV strains. Saito et al. reported that genomic HCV RNA (Con1 strain) lacking the 3’UTR was less active in innate immune signaling than full-length genomic RNA, suggesting that the polyU/UC sequence is a critical determinant for RIG-I-mediated signaling in response to full-length genomic RNA (26). The J4L6 polyU/UC sequence contains an uninterrupted 28-uridine tract, while the comparable JFH-1 sequence has a longer uninterrupted 43-uridine tract. Interestingly, the isolated JFH-1 polyU/UC RNA had half the immunostimulatory activity of the J4L6 polyU/UC and polyAG/A RNAs (Fig. 6). Alternatively, the JFH-1 polyAG/A RNA was nearly twice as stimulatory as the J4L6 polyU/UC and polyAG/A RNAs. Assuming that the activities of the isolated polyU/UC RNAs correlate with their behavior in the context of full-length genomic RNA (26), then the full-length JFH-1 strain genomic RNA would be predicted to have poor immunostimulatory activity. The corresponding polyAG/A-containing antisense RNA may not elicit a strong antiviral response because 1) for most positive strand RNA viruses, antisense strands are present in much lower amounts than sense strands; 2) the polyAG/A RNA may be shielded by RNA binding proteins, and/or 3) the accumulation of antisense strands may be accompanied by a parallel
increase in translated viral proteins, including NS3/4A, which is known to block RIG-I signaling by cleaving IPS1 from mitochondrial membranes. The presence of the polyU/UC region is highly conserved in HCV isolates, and is essential for viral RNA replication (36); therefore, its recognition by the RIG-I signaling pathway may illustrate the evolution of an elegant host antiviral response mechanism.

A model that extends previous summaries (25) and also indicates how modified RNAs may be used to dissect the steps in the signaling pathway is presented in Figure 7. In the absence of RNA ligand, RIG-I is a latent molecule whose activity is down-regulated by a C-terminal repressor domain (RD) (25). A positively charged groove, and specifically lysine 858, in the RD is likely the 5’ppp-binding site of RIG-I (7). However, a maximum of three nucleotides can fit in this groove, suggesting that nucleotide activating determinants such as the polyU/UC sequence bind to the RIG-I DECH box domain (7). The RIG-I RD activates the RIG-I ATPase by RNA-dependent dimerization (i.e. ATPase activity is stimulated by dimer formation) (7). Upon substitution of the 2’ ribose position or replacement of uridine with pseudouridine, RIG-I binding to the RNA is unaffected as compared to RNA with unmodified nucleotides; however, downstream signaling is abrogated. It is possible that the modified polyU/UC RNAs inhibit the subsequent RIG-I conformational changes, dimerization, or ATPase activation. Further experimentation will be required to address these questions. The competitive activity of the modified RNAs (Fig. 5) suggests that they may be useful as modulators of RIG-I mediated innate immune responses.
1 **Acknowledgments**

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**Figure Legends**

**Fig. 1.** IFN-β induction potential of HCV and flavivirus UTR RNAs. (A) 24 hours after plating, Huh7 cells were co-transfected with plasmids encoding firefly or Renilla luciferase under control of the IFN-β promoter or constitutive cytomegalovirus promoter, respectively. Following a 24-hour further incubation period, cells were mock-transfected or transfected in triplicate with equal moles of renatured *in vitro*-transcribed viral 5’ or 3’ UTR or 3’ stemloop RNAs. 24 hours later, the cells were lysed, and aliquots of the extracts were analyzed using a dual luciferase assay. The firefly luciferase light unit values were divided by the Renilla light units (transfection efficiency control) to generate the relative luciferase value. Bars show average relative luciferase values ± standard deviation. (B) 28 hours after viral RNA transfection, the cells were lysed and analyzed by SDS-PAGE and immunoblotting for ISG56 and actin. (C) Increasing amounts of HCV 3’UTR RNA (50 ng, 250 ng, 650 ng, 1 µg) or Sendai virus (50, 100, 250, 500 hemagglutinin units) were transfected or infected into Huh7 cells, and IFN-β reporter activation was measured 24 hours later as described in Figure 1A.

**Fig. 2.** Identification of the polyU/UC region of the HCV 3’UTR as the determinant of RIG-I activation. (A) HCV polyU/UC and X RNAs were transcribed *in vitro*, and equal moles of the RNAs were transfected into Huh7 cells. Their potency in activating the IFN-β reporter was determined as in Fig. 1A. Bars show average relative luciferase values ± standard deviation. (B) WT or RIG-I knock-out (KO) mouse embryo fibroblasts (MEFs) were mock-transfected or transfected in triplicate with equal moles of *in vitro*-transcribed HCV 3’UTR, polyU/UC, or X RNA. After a 24-hour incubation period, ELISA was used to measure IFN-β protein levels from cell culture media. Bars show average amounts of mouse IFN-β protein levels ± standard deviation.
deviation. (C) 1 µg of biotinylated polyU/UC RNA was incubated with or without 2.5-fold molar excess of nonbiotinylated competitor RNA and 30 µg of FLAG-RIG-I-containing Huh7 cell extract. RNA-protein complexes were recovered by pull-down assay using streptavidin magnetic particles. FLAG-tagged RIG-I protein within the pull-down fraction was analyzed by immunoblotting using M2 anti-FLAG antibody.

Fig. 3. Separating the 5’ppp and the polyU/UC region does not disrupt signaling. (A) Schematic representations of the chimeric RNAs, showing the activating polyU/UC region positioned upstream of ss1 and immediately adjacent to the 5’ppp, or downstream of ss1 and distant from the 5’ppp. (B) The chimeric RNAs were in vitro-transcribed and their ability to activate the IFN-β reporter was tested as in Fig. 1A. Bars show average relative luciferase values ± standard deviation.

Fig. 4. Examining the roles of RNA sequence composition and length in RIG-I activation. (A) Schematic representations of the different HCV J4L6 strain polyU/UC 3’ deletion RNAs tested. The black bars at the top indicate (left to right) 28 contiguous U residues, followed by 11 contiguous U residues, followed by a downstream U/C region. The gaps in between the bars correspond to single cytosine residues. (B-E) Activation of the IFN-β reporter by in vitro-transcribed RNAs. Bars show average relative luciferase values ± standard deviation (B) Activation by polyU/UC 3’ deletion RNAs. (C) Activation by polyAG/A-100nt (full-length), polyAG/A-60nt, and polyG/GC-60nt RNAs. (D) Activation by 50-nt and 35-nt homopolymeric uridine RNAs and 50-nt homopolymeric adenine RNA. (E) Activation by 50-nt homopolymeric uridine and adenine RNAs interrupted with a single C or G nucleotide, respectively. (F) 1 µg of
biotinylated polyU/UC RNA was incubated with or without 3-fold molar excess of nonbiotinylated competitor RNA and 30 µg of FLAG-RIG-I cell extract. The competitive binding assays were carried out as described in Fig. 2C.

**Fig. 5.** Ribose and base modifications affect RNA innate immune stimulation potential. (A-B) Activation of the IFN-β reporter by *in vitro*-transcribed RNAs. Bars show average relative luciferase values ± standard deviation. (A) Activation by HCV 3’UTR and polyU/UC RNAs transcribed with 2’fluoro-dUTP (2’F-dUTP) in place of UTP, or 2’fluoro-dCTP (2’F-dCTP) in place of CTP. (B) Activation by polyU/UC RNA transcribed with pseudouridine-5'-triphosphate in place of UTP. (C) 1 µg of biotinylated polyU/UC RNA was incubated with or without 3-fold molar excess of nonbiotinylated competitor RNA and 30 µg of FLAG-RIG-I cell extract. The competitive binding assays were carried out as described in Fig. 2C. (D) Unmodified polyU/UC RNA was transfected alone, or with 2’F-dUTP- or pseudouridine-modified polyU/UC RNA at 2:1 or 4:1 (modified:unmodified) molar excesses. IFN-β reporter activity was measured 24 hours post-transfection as described in Fig. 1A. Data are presented as a percentage of the control unmodified polyU/UC RNA activity (100%). Bars show average relative luciferase values ± standard deviation.

**Fig. 6.** Comparison of the HCV 1b J4L6 and HCV 2a JFH-1 strain polyU/UC and polyAG/A RNAs as activators of innate immune stimulation. (A) HCV J4L6 and JFH-1 RNAs were transcribed *in vitro* and transfected into Huh7 cells as described in Fig. 1A. Bars show average relative luciferase values ± standard deviation. (B) 24 hours after Huh7 cells were transfected with equal moles of J4L6 or JFH-1 polyU/UC, polyAG/A, or X RNAs, the cells were infected
with luciferase-encoding vesicular stomatitis virus (VSV-luc) at multiplicities of infection (MOI) of 0.05, 0.1, and 0.15. 4 hours post-infection, cells were lysed and luciferase activity as a measure of VSV replication was assayed. Bars show average luciferase values ± standard deviation.

Fig. 7. Model defining steps of RIG-I activation and where modified RNAs could block signaling. The actual order of steps may be different than what is described here. Ribose 2’ hydroxyl and base modifications do not affect binding to RIG-I, but may inhibit RIG-I activation at one of the downstream steps. Additional details are found in the text.
A

Relative luc activity

HCV 3'UTR  HCV polyU/UC  HCV X  HCV ss1  mock

B

[IFN-beta] (pg/ml)

HCV 3'UTR  HCV polyU/UC  HCV X  mock

C

competitor RNA

none  polyU/UC  3'UTR  ss1  X

RIG-I pull-down

lane 1 2 3 4 5
A

![Diagram of RNA structures: polyU/UC-ss1, ss1-polyU/UC]

B

![Bar graph showing relative luc activity: HCV 3'UTR, polyU/UC-ss1, ss1-polyU/UC, ss1, mock]
A

B

C

D

Relative luc activity (% polyU/UC)

Relative luc activity

Relative luc activity

Relative luc activity
**A**

Relative luc activity

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**B**

Luc activity

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RNA-binding, conformational change, and release of RD

Base- or ribose-modified polyU/UC RNA

Downstream innate immune signaling through IPS1