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Regulation of PKR by HCV IRES RNA: Importance of Domain II and NS5A

Rebecca Toroney¹, Subba Rao Nallagatla¹, Joshua A. Boyer¹, Craig E. Cameron², and Philip C. Bevilacqua^{1,*}

¹Department of Chemistry, Pennsylvania State University, University Park, PA 16802, USA

²Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA

Abstract

The protein kinase PKR is an essential component of the innate immune response. In the presence of dsRNA, PKR is autophosphorylated, which enables it to phosphorylate its substrate, eIF2 α , leading to translation cessation. Typical activators of PKR are long dsRNAs produced during viral infection, although certain other RNAs can also activate. A recent study indicated that full-length internal ribosome entry site (IRES), present in the 5'-UTR of hepatitis C virus (HCV) RNA, inhibits PKR, while another showed that it activates. We show here that both activation and inhibition by full-length IRES are possible. The HCV IRES has a complex secondary structure comprising four domains. While it has been demonstrated that domains III-IV activate PKR, we report here that domain II of the IRES also potently activates. Structure mapping and mutational analysis of domain II indicate that while the double-stranded regions of the RNA are important for activation, loop regions contribute as well. Structural comparison reveals that domain II has multiple, non-Watson-Crick features that mimic A-form dsRNA. The canonical and non-canonical features of domain II cumulate to a total of ~33 unbranched base pairs, the minimum length of dsRNA required for PKR activation. These results provide further insight into the structural basis of PKR activation by a diverse array of RNA structural motifs that deviate from the long helical stretches found in traditional PKR activators. Activation of PKR by domain II of the HCV IRES has implications for the innate immune response when the other domains of the IRES may be inaccessible. We also study the ability of the HCV non-structural protein NS5A to bind various domains of the IRES and alter activation. A model is presented for how domain II of the IRES and NS5A operate to control host and viral translation during HCV infection.

Keywords

HCV IRES; PKR; NS5A; innate immunity; domain II

Introduction

The protein kinase PKR is an integral part of the human innate immune response mechanism, which is the cell's first line of defense against viral infection.^{1,2} Upregulated by

*Corresponding author. pcb@chem.psu.edu; Phone: (814) 863-3812; Fax: (814) 865-2927.

Postal address: 104 Chemistry Building, University Park, PA 16802.

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interferon induction, PKR becomes activated by binding long stretches of dsRNA (≥ 33 bp), leading to homodimerization and autophosphorylation.³⁻⁵ The C-terminal kinase domain of phosphorylated PKR then phosphorylates its substrate, translation initiation factor eIF2 α , leading to shut-down of cellular translation and inhibition of viral proliferation.⁶ One function of PKR as part of innate immunity is to discriminate between self and non-self, which it accomplishes by recognition of molecular patterns in RNA.⁷ This recognition is mediated in large part by two tandem N-terminal dsRNA binding motifs (dsRBMs) known as the dsRNA binding domain (dsRBD), which sense RNA in a non-sequence-specific fashion through contacts to the phosphate backbone in the major groove and 2'-hydroxyls in the minor groove.⁸⁻¹⁰

It has been clearly established that PKR recognizes and is regulated by viral RNAs.⁶ During viral infection, PKR is thought to be primarily activated by viral genomic dsRNA, although transient dsRNA from replicative intermediates of RNA viruses or transcription intermediates of DNA viruses can also activate PKR.⁶ Typically, viral RNAs activate PKR through long contiguously base-paired regions, a feature that is rare in cellular RNAs. However, RNAs with more complex secondary structures also activate PKR, mimicking dsRNA via RNA dimerization or coaxial stacking of shorter helices.¹¹⁻¹³ Some of these RNAs also contain certain helical imperfections, such as bulges and internal loops.^{7,14} On the other hand, certain viruses, such as Epstein-Barr and adenovirus, produce non-coding RNAs with multi-helix junctions that function to *inhibit* PKR activity.¹⁵⁻¹⁷ In this study we seek to elucidate the structural basis of PKR regulation by a particularly complex non-coding viral regulator of PKR, the internal ribosome entry site (IRES) of the hepatitis C virus. We also illustrate the importance of non-Watson-Crick secondary structure and structural mimicry in PKR recognition of HCV IRES RNA.

Hepatitis C is a single-stranded positive-sense RNA virus that encodes a single polyprotein flanked by 5'- and 3'-UTRs.^{18,19} The IRES element, located in the 5'-UTR, functions in recruiting and positioning translation initiation complexes and the ribosome at the start codon.^{20,21} The secondary structure of the HCV IRES is highly conserved, consisting of four distinct independently folded domains that form an overall extended and innately flexible structure (Fig. 1).²²⁻²⁵ These domains perform essential functions during viral replication and translation: domains I and II are indispensable for genome replication, while domains II, III, and IV are critical for translation initiation.²⁶⁻²⁸ In particular, cryo-EM studies indicate that domain II and elements of domain III make direct contacts with the 80S ribosome.^{29,30} Domain II induces conformational changes in the 40S subunit, potentially mediating the joining of the 40S and 60S subunits, while domain III positions the start codon, located in domain IV, within the mRNA binding site. Domain II is of particular interest as it is the only domain that functions in both replication and translation. Overall, the HCV IRES represents a highly structured, non-self RNA with domains that serve disparate roles within the virus lifecycle, suggesting that it may represent an ideal target for RNA sensors in the innate immune system. Indeed, previous studies show that full length IRES can inhibit³¹ or activate PKR³², with the latter study assigning this function to domains III-IV.

Herein we analyze full-length HCV IRES from HCV-1b and its isolated domains as regulators of PKR's phosphorylation activity. Our results indicate that full-length IRES can serve both as an activator and an inhibitor of PKR, with the particular function depending on the concentration of the IRES. Surprisingly, we find that while a construct consisting of domains III-IV does activate PKR as reported,³² domain II, which contains fewer base pairs and is interrupted by multiple imperfections, is the more potent activator. We employ footprinting and mutational analysis to map the interaction of PKR within domain II and observe that the apical and internal loops of domain II are particularly important. Overlays

with reference model RNA structures suggest that the structural features of these regions of domain II may in fact mimic dsRNA. Lastly, we investigate the role of NS5A as an antagonist of PKR activation and present an overall model of PKR function that accommodates these data.

Results

PKR can be both activated and inhibited by full-length HCV IRES

An early study³¹ indicated that the HCV IRES functions similarly to viral RNA from Epstein-Barr¹⁵ and adenovirus^{17,33} in that it inhibits PKR activation. Typically, such inhibition occurs through competition between viral RNA and dsRNA for binding to the dsRBD. While activating dsRNA promotes dimerization of PKR on a single RNA molecule, inhibitor RNA binds PKR in a manner that prevents functional dimerization, locking PKR in an inactive complex.⁵ In this earlier study,³¹ activation assays were conducted at HCV IRES RNA concentrations below ~80 nM, which turns out to be too low to promote functional dimerization and activation of PKR (see below). A more recent study has shown that at IRES concentrations above ~50 nM, PKR undergoes activation.³² Thus, one prior study showed that the full-length IRES inhibits PKR, while another showed that it activates PKR.

In an effort to reconcile these studies, we first tested full-length HCV IRES (nucleotides 1-388) for inhibition of PKR (Fig. 2a). Dephosphorylated PKR (0.6 μ M) and poly I:C (1 μ g/mL), a known activator of PKR, were incubated with increasing concentrations of HCV IRES (Fig. 2a).¹ As expected from the earlier study,³¹ inhibition was observed. Inhibition of poly I:C-mediated activation of PKR was observed starting at 1.25 μ M HCV IRES, with inhibition increasing with IRES concentration. The slight increase in activation with lower concentrations of HCV IRES (Fig. 2a) suggested that the IRES might also function as an activator.

Next, we tested full-length HCV IRES for activation of PKR in the absence of poly I:C. Activation was tested over full-length IRES concentrations ranging from 0.02 to 5 μ M (Fig. 2b). We confirmed that full-length HCV IRES activates PKR, starting at ~160 nM IRES. As in the presence of perfect dsRNA,^{4,34} PKR activation displays a bell-shaped dependence on HCV IRES concentration with maximal activation near 0.64 μ M HCV IRES. Relative to a 79 bp standard activator, full-length IRES activates up to ~125% the level of activation by 79 bp. We also note that the concentration of HCV IRES required to inhibit PKR is similar in panels (a) and (b) of Figure 2, as expected. The inhibitory arm is typical for PKR activators and is attributed to titration of active PKR dimers to monomers on separate RNA molecules.^{5,35} In summary, PKR is both activated and inhibited by the IRES, with the particular activity depending on the reaction conditions.

PKR is activated by multiple IRES domains, but most potently by domain II

The HCV IRES folds into a highly conserved, stable secondary structure with long dsRNA-like stems containing numerous defects (Fig. 1).^{24,36,37} Within the IRES are four independently-folding domains, numbered I-IV. These domains include domain I, a GC-rich hairpin; domain II, a stem-loop structure interrupted by two bulges and three internal loops, L1-L3, and an apical loop, L4 (Fig. 1, inset); Domain III, a large structure divided into five subdomains, IIIa-IIIe, that include two four-way junctions and a three-way junction,³⁸ numerous internal loops and bulges, and a pseudoknot (Fig. 1); and domain IV, a small hairpin at the base of an internal loop that contains the start codon.

¹In the inhibition study, the concentration of IRES is kept at or above 10 μ g/mL, while the concentration of poly I:C is constant at 1 μ g/mL. This set up assures at least 10 times more nucleotides of the inhibitor.

Given the highly complex secondary structure of the HCV IRES, we sought to determine which IRES domains are responsible for activation of PKR. The RNA constructs tested for activation of PKR consisted of HCV 1-130, which includes domains I and II of the IRES (Fig. 1, 5'-end to the asterisk), 131-388, which comprises domains III-IV (Fig. 1, asterisk to 3'-end), and 39-119, which contains just domain II (Fig. 1, inset). A previous study indicated that domains III-IV of the IRES are critical for PKR activation, but that domain II is dispensable and unable to activate PKR independent of the other IRES elements.³² We also find that domains III-IV activate PKR, with activation increasing through 5 μ M RNA (Fig. 3a, c). However, we also find that domains I-II activate in a similar fashion to domains III-IV (Fig. 3a, c). In fact, at 5 μ M RNA, domains I-II activate PKR to a greater extent, at ~80% the activation level of the 79 bp standard, than domains III-IV, which activate only up to ~50%. Additionally, over all the RNA concentrations tested, domains I-II display 1.5-4.5-fold greater activation than domains III-IV. These observations suggest that in full-length IRES, domains I-II make a major contribution to activation.

Within domain I, and downstream of domain II, there are stretches of multiple G's and C's that could potentially lead to multimerization of this RNA and significantly increase the total number of base pairs. In this scenario, it is possible that PKR activation observed for domains I-II is actually due to the presence of an RNA dimer, as was recently found for HIV TAR RNA.¹¹ To investigate this possibility, we renatured domains I-II at a high RNA concentration of 20 μ M and then analyzed for RNA dimers on a native gel at an RNA concentration of 10 μ M. A slower mobility species was observed on both native and denaturing gels (data not shown) that indicated possible dimer formation.

To eliminate the possibility of RNA dimers, domain II alone was prepared. We chose a construct that begins at 39, which eliminates domain I, and ends at 119, which eliminates the C-rich 3'-end. This 39-119 construct, referred to as 'domain II' (Fig. 1, inset), therefore lacks both GC-rich potential dimerization regions. We also note that our domain II construct has the same number of base pairs as the native domain II (see below).² On a native gel, in the presence of high concentration of domain II (10 μ M), only one major species was observed, which migrated at the expected mobility for a monomer relative to RNA markers, and no species were observed at the expected mobility for a dimer (data not shown). These observations suggest that domain II exists as a monomer in solution at or below 10 μ M RNA.

Activation assays of domain II were then performed at RNA concentrations ranging from 0.15 to 10 μ M (Fig. 3b). Within this concentration range, domain II potently activated PKR, with maximal activation at 5 μ M RNA. Additionally, the level of activation by domain II at 5 μ M was ~1.4 fold greater than activation for the 79 bp standard at its maximum, and greater than the maximal activation for domains I-II and domains III-IV. Domain II also displayed the bell-shaped dependence on RNA concentration typical of PKR activators, with activation decreasing slightly at 10 μ M domain II (Fig. 3c).³ We previously reported that a 5'-triphosphate, which is naturally at the end of T7 transcripts, can contribute to activation of PKR by certain largely ssRNAs but not by dsRNAs.⁷ To test whether the 5'-triphosphate of the domain II transcript contributes to activation of PKR, we removed the 5'-triphosphate by treatment with calf intestinal phosphatase (CIP). The CIP-treated RNA activated PKR to the same levels as untreated domain II (Fig. S1), indicating that this 5'-triphosphate does not contribute to activation of PKR. This result is as expected given that domain II normally is

²Two G residues were inserted upstream of nucleotide 39 to aid with T7 transcription (see Fig. 1, inset). To eliminate potential interference with RNA folding, these G's were separated from the base of domain II by five native single stranded nucleotides. As shown below, these nucleotides do not interfere with the fold of the RNA or contribute to dimerization.

³Activation assays were not conducted above 10 μ M to ensure the RNA remained as a monomer.

found within the context of the full-length IRES and so does not have a 5'-triphosphate, and also supports a contribution of domain II to activation of PKR that is similar to that of a double-stranded RNA (see below). To summarize, domain II is a monomeric RNA that potently activates PKR in the absence of domain I and the 3'-flanking region of domain II. That domain II alone activates PKR is surprising because it has only 24 Watson-Crick base pairs and GU wobbles. This observation suggested the possibility of an important role for non-Watson-Crick motifs in activation, as will be discussed further. Next, we compared eIF2 α phosphorylation and PKR binding by the various HCV domains.

PKR activation by IRES elements leads to potent phosphorylation of eIF2 α

During viral infection, PKR activation via autophosphorylation leads to downstream regulation of translation initiation through phosphorylation of eIF2 α , a cellular substrate of PKR.⁴ We thus sought to determine the potential biological relevance of PKR activation by IRES elements. Activation assays were performed in the presence of eIF2 α and indicated that all IRES elements facilitate potent phosphorylation of eIF2 α (Fig. 4, lower bands). For instance, 0.625 μ M full-length IRES led to 77% PKR activation and 110% eIF2 α phosphorylation relative to 79 bp control (Fig. 4a). Similarly, domains III-IV phosphorylated eIF2 α up to 112% (Fig. 4b), while domain II led to 125% eIF2 α phosphorylation (Fig. 4c). Thus, eIF2 α phosphorylation mirrors PKR activation: domain II is a stronger activator, which leads to slightly higher eIF2 α phosphorylation as compared to domains III-IV. Notably, both domain II and full-length IRES lead to comparable levels of eIF2 α phosphorylation; for example, 93 and 110% phosphorylation at 0.625 μ M RNA was observed, suggesting that domain II alone may be nearly as effective as full-length IRES in activating PKR.

Binding affinity of IRES elements for the PKR dsRBD p20

Based on our activation assays, we expected that extent of activation for various IRES elements might correlate with extent of binding to the dsRBD, p20. Native mobility gel-shift assays of full-length IRES, domains III-IV, and domain II were therefore performed (Fig. S2). We observe that p20 binds both full-length IRES and domains III-IV tightly and in multiple complexes, with complex formation starting at $\sim$ 0.25 μ M p20 for the full-length IRES and at $\sim$ 0.5 μ M for domains III-IV (Fig. S2a, b). These similarities suggested that the other domains in the IRES might not contribute substantially to binding. Indeed, domain II bound considerably more weakly, with very weak complex formation occurring only at $\sim$ 2.5 μ M p20. Additionally, domain II gave only a single, low mobility complex, which required a 20% gel to resolve (Fig. S2c). Thus, domain II appears to bind p20 considerably more weakly than domains III-IV. Despite relatively poor gel-shifting by domain II, in-line probing assays revealed protection of domain II by p20 as low as 0.6 μ M (see below), supporting dynamic binding that gel shifts resolve only poorly.

Notably, the inhibitory arm for PKR activation by full IRES begins at $\sim$ 1.2 μ M (Fig. 2b), while it begins at $>$ 5 μ M (Fig. 3c) for domains III-IV despite similar gel shifting by p20. This may be because of tight, non-productive binding modes for domains III-IV. In addition, activation by domains III-IV is $\sim$ 3.3-4-fold less potent than by domain II, despite tighter binding of p20 to domains III-IV. These observations also suggest that perhaps some of the binding modes of domains III-IV are non-productive, or even inhibitory, for activation, perhaps due to spacing of binding sites (see Discussion). These data further suggest that much of the activation by full-length IRES may be due to domain II alone (see Discussion). Because it contributes significantly to PKR activation, we focused our remaining studies on domain II.

Experimental determination of domain II secondary structure

As mentioned, domain II of the HCV IRES is atypical of PKR activators in that it has fewer than 33 canonical base pairs and contains several loop and bulge imperfections.^{22,24} Structures of domain II have been solved and these reveal an overall bent, or hook-like, shape of the domain, induced by an asymmetric internal loop (L1) (Fig. 1, inset).³⁹⁻⁴¹ NMR studies have identified a number of notable features of domain II: stacking in L1, numerous noncanonical base pairs in L2 and L3, and stacking and hydrogen bonding within L4.³⁹ Thus, although domain II contains a number of structural motifs that initially appear atypical of a PKR activator, noncanonical motifs are present that have the potential to act as mimics of dsRNA.

Ribonuclease structure mapping experiments were performed to test the structure of our domain II construct (Fig. 5). We used ribonucleases T1 and A, which cleave after single-stranded G and single-stranded C and U, respectively, as well as ribonuclease V1, which cleaves before double-stranded and stacked nucleotides.⁴²⁻⁴⁴ Ribonuclease T1 cleaved after two G's present in single-stranded loop regions as well as after G's in the single-stranded 5'-end. Also, there was absence of T1 cleavage after all but one of the G's predicted to be involved in base-pairing, and this G (G60) is adjacent to a G•U wobble, which may induce breathing of the helix. As expected, RNase A cleaved primarily after C's and U's in loop regions, especially at U56 in L1 and U80, C83 and C84 in L4. In addition, RNase A cleavage is largely absent from pairing regions, with the exception of some moderate cleavage near bulges or the ends of helical regions, indicative of breathing. Lastly, RNase A did not cleave appreciably after any of the U's and C's in L2, consistent with U•U and U•C base pairs from NMR.

The dsRNA-specific ribonuclease RNase V1 cleaved strongly before nucleotides on the 5'-strand of pairing regions P2, P3, and P4; strongly on both strands of P1; and weakly in the 3'-strand of P3 (Fig. 5a, b). Cleavage by RNase V1 was also observed before residues A53 and A54 of L1, which is consistent with NMR structures that show stacking of these nucleotides.³⁹ Thus, according to RNA structure mapping, the overall secondary structure of the domain II construct used herein is largely consistent with predicted and NMR secondary structures.

Mapping of p20 binding to domain II

To further characterize PKR's interaction with domain II, nuclease and in-line probing footprinting experiments were conducted in the presence of p20. Nuclease footprinting was performed by pre-incubating trace amounts of radiolabeled domain II with 10 μ M p20, which according to Fig. S2c (bottom panel) should be saturating, and then treating with either RNase A or RNase V1 (Fig. 6a, c). We observed variable extents of p20-dependent protection from nuclease digestion throughout domain II (see Fig. S3a for quantitation). For example, nucleotides in the 3'-strand of L3 (L3_{3'}) were 75% protected from RNase A cleavage, while C42, which is located in the 5'-single-stranded tail, was only 7% protected. Similarly, nucleotides in the 5'-strand of P3 (P3_{5'}) were just 30% protected from RNase V1 cleavage, while C62 of P2_{5'} was 50% protected.

One concern was whether reduction in RNase cleavage activity reflected protection by p20 or was instead simply due to inhibition of ribonuclease activity. However, we note that certain domain II nucleotides are cleaved equally by ribonucleases in the absence and presence of p20. For example, P4_{5'} is cleaved by RNase V1 and is not appreciably protected by p20. Likewise, C55 of L1 is cleaved by RNase A similarly in the absence and presence of p20.

Because PKR is known to bind to RNA primarily through interactions with the 2'-hydroxyls,⁴⁵ we also performed in-line probing footprinting.⁴⁶ Any decreases in cleavage observed could be due to direct interaction of the RNA with p20, or to RNA conformational changes that lead to non-reactive alignments of the 2'-hydroxyls. In-line probing experiments were conducted analogously to nuclease footprinting, in that trace domain II was incubated with 10 μ M p20, but incubation was conducted for 40 h at slightly elevated pH (pH 8.3) in the presence of 20 mM MgCl₂ (Fig. 6b, c). In-line probing revealed p20-dependent protection at nucleotides between 64 to 80, which includes the 5'-strands of P3, L3, and P4, as well as at nucleotides flanking G94 on L3_{3'} (see Fig. S3b for quantitation).

Next, we mapped the nuclease and in-line cleavage protections onto the average minimized NMR structure of domain II (Fig. 6d). This revealed that p20 binds primarily above and below the L1 hinge bulge, while mostly avoiding L1 itself (Fig. 6d, yellow).³⁹ Within the protected regions, p20 interacts with the pairing elements P1-P4 of domain II, as expected. In addition, p20 protects portions of L2, L3, and L4, suggesting that these non-Watson Crick regions may mimic dsRNA, thereby facilitating binding of p20 (see Discussion).

Effects of mutations to domain II on PKR activation

In order to further explore how domain II activates PKR, we conducted a mutational analysis of domain II. Mutations in the apical loop, L4, and the pairing region, P4, of domain II were initially prepared. In particular, the L4 mutant 'L4-U5' exchanges the AGCCA nucleotides of the structured apical loop for U5, which should be largely unstructured, while the P4 mutants, '+2bp' and ' Δ 2bp', add and delete two base pairs within P4, respectively (Fig. 7a). We find that the addition of two GC base pairs in +2bp, which lengthens and stabilizes P4, enhances PKR activation relative to WT (Fig. 7b, c). For example, at 0.3 μ M RNA, activation by is 2-fold greater than WT activation. Conversely, deletion of two GU base pairs, which shortens the helix, somewhat diminishes activation, such that Δ 2bp does not reach the level of WT maximal activation at even the highest RNA concentration tested (Fig. 7b, c). Removing structure from the apical loop, in L4-U5, decreases PKR activation to a similar degree as Δ 2bp, consistent with interaction with PKR (Fig. 7b, c). Overall, this set of mutants suggests that both double-stranded and loops regions contribute to activation of PKR by domain II.

Next, mutations in the three internal loops of domain II were investigated. In particular, 'G71A/G94A' and 'C104U' potentially make L3 and L2 less structured, and ' Δ L1' eliminates the bend in domain II (Fig. 7a). As compared to the P4 and L4 mutants, the internal loop mutants had more subtle effects on activation, (Fig. S4). In particular, the percent activation of all three mutants at 10 μ M RNA was ~95%, as compared to 112% for WT, and at 2.5 μ M RNA, activation by all three mutants equaled WT. To examine possible effects of these mutants in more detail, time trials were performed, in which 1.25 or 5 μ M RNA was incubated with PKR for 3, 5, and 10 minutes (Fig. 7d, e). At 1.25 μ M RNA, activation by C104U and G71A/G94A was essentially equal to WT at every time point, while Δ L1 activation was 2 to 3-fold lower (Fig. 7d). At higher concentrations of RNA (5 μ M), Δ L1 activation was as much as 4-fold lower than WT, while the other mutants were now 1.7-fold lower (Fig. 7d, e). The decrease in activation for Δ L1 suggests that the 5-nucleotide bulge may play a role in positioning the PKR dimer on domain II. The smaller decrease in activation for the L2 and L3 mutants suggests that these regions may also help position PKR for activation, although the size, rather than the sequence, of the loop may be the dominating factor. In summary, activation assays on the six RNA mutants support interaction of much of domain II with PKR, including non-canonical regions, consistent with the above footprinting experiments.

Effect of NS5A on PKR activation by HCV IRES domains

Activation of PKR in HCV-infected cells is antagonized by interaction with NS5A, an HCV non-structural protein.⁴⁷⁻⁴⁹ NS5A is an RNA-binding protein that prefers single-stranded GU-rich segments of the 3'-UTR of HCV.⁵⁰ We sought to elucidate a possible connection between NS5A binding and regulation of PKR by HCV IRES RNA.

Inspection of HCV IRES sequence reveals several GU-rich segments, which are present within domains III and IV (Fig. 1).⁵⁰ Notably, there are no stretches of GU-elements in domain II. We performed native gel-mobility shift assays of NS5A with domain II, domains III-IV, and full-length IRES, as well as 79 bp control (Fig. 8). We find that domains III-IV and full-length IRES bind NS5A tightly, forming multiple complexes with an apparent K_d of $\sim 0.13 \mu\text{M}$, based on loss of approximately half of the unbound RNA to shifted species (Fig. 8a). This number is slightly weaker than published values for NS5A binding to model rU₁₅ and rG₁₅ oligonucleotides and agrees with values for NS5A binding to the 3'-NTR of HCV.⁵⁰ In contrast, domain II binds NS5A much less tightly, forming just a single microshift, which might be indicative of kinetically labile binding (Fig. 8b). Additionally, 79 bp control appears to have essentially no interaction with NS5A, as expected (Fig. 8c). These observations support the aforementioned GU-content of the given domains. Given these results, it seemed possible that NS5A could attenuate PKR activation by domains III-IV specifically.

Studies have shown that responsiveness in HCV-1b-infected individuals to interferon (IFN) depends on a particular sequence within NS5A (codons 2209-2248), termed the interferon sensitivity determining region (ISDR).⁵¹⁻⁵³ Cell culture studies have revealed that PKR activation is inhibited through its interaction with the NS5A ISDR.⁵⁴⁻⁵⁶ This property gives NS5A the potential to inhibit PKR activation independent of RNA binding.

To test for NS5A-mediated inhibition of PKR, we conducted activation assays in the presence of increasing concentrations of NS5A (0-4 μM) and either domain II, domains III-IV, or 79 bp control. As shown in Fig. 9 a, c, profiles for NS5A inhibition of PKR activation were nearly identical for domains III-IV and domain II, with NS5A providing up to 5-fold inhibition for each domain. This suggests that NS5A inhibits PKR activation independent of its ability to bind RNA. Consistent with this notion, NS5A was nearly as effective in inhibiting activation of PKR by 79 bp control (Fig. 9 b, c). The mechanism for the RNA-independent mode of NS5A inhibition of PKR activation appears to center on direct interaction of NS5A with the inactive dephosphorylated form of PKR (M.R.S Hargittai and CEC, manuscript in preparation). We note that there is a 20-30% increase in PKR activation at the lowest NS5A concentrations for each of the tested RNAs that precedes the major, inhibitory arm. The origin of this stimulation is unclear.

Lastly, we examined whether NS5A interferes with activated PKR's ability to phosphorylate its natural substrate, eIF2 α . This was tested by varying the order of addition of PKR, dsRNA, NS5A, and eIF2 α (Fig. S5). When NS5A is added after autophosphorylation of PKR, but prior to addition of eIF2 α (Fig. S5, compare lanes 3 and 4 to lanes 5 and 6), no attenuation of eIF2 α phosphorylation is observed, indicating that NS5A is unable to prevent downstream events. This supports the ability of NS5A to interact with PKR but not eIF2 α .

Discussion

Hepatitis C virus IRES RNA has a complex and highly conserved structure that contains long base paired stretches that could potentially activate PKR, although many of these regions are interrupted by numerous imperfections. There has been some question about the role of HCV IRES RNA as either an activator or an inhibitor of PKR. In this report, we

presented evidence that full-length HCV IRES RNA both inhibits PKR activation by a dsRNA mimic, poly I:C, and activates PKR, with activity depending on IRES concentration. We examined individual segments of the HCV IRES for activation of PKR and found that domains III-IV alone activate PKR, consistent with another report, albeit less potently than full-length IRES. Surprisingly, we also found that domain II is a more robust activator than domains III-IV despite the fact that it has fewer base pairs and contains several internal loops and bulges. Mutational analysis and protein mapping techniques were employed to investigate the structural basis of activation by this atypical PKR activator. In an effort to elucidate the importance of PKR activation by domain II, the interaction of NS5A with individual domains of HCV IRES RNA was also studied. We found that, while NS5A binds domains III-IV and full-length IRES much more tightly than domain II, NS5A inhibits PKR equally in the presence of each of these various IRES elements. In the Discussion, we present evidence for structural mimicry of A-form dsRNA by domain II of the IRES, and a model for how domain II and NS5A may act to regulate host and viral translation during HCV infection.

Domain II has A-form helical properties

It is well-established that in addition to perfect dsRNA, PKR can bind and be activated by RNAs with complex secondary structural features, including internal loops, bulges, and pseudoknots, as well as single-stranded and non-Watson-Crick motifs.^{7,12-14,57} It may be the dynamic nature of many RNA structures that enables PKR to accommodate these structural imperfections; for example, A-bulge-induced bends in the middle of RNA helices are straightened upon PKR binding.⁵⁷ Additionally, noncontiguous helices within RNA can combine to activate PKR.^{12,57} The unifying principle in activation of PKR by these various RNAs may be maintenance of an overall A-form, or dsRNA-like, topology. However, little direct testing of this idea has been conducted.

Domain II of HCV RNA contains four short helical regions, as well as internal loops, bulges, and mismatches, and yet has the ability to activate PKR (Figs. 1, 3b). We propose that PKR still binds and is activated by domain II, despite absence of long base paired stretches, because the overall conformation of several of these loop regions are largely A-form. We first make a comparison between L4 of domain II and a loop from another RNA known to facilitate binding of a dsRBD, followed by a comparison between the P2-P4 section of domain II and a model A-form helix.

We found p20-dependent protection from nuclease digestion at several nucleotides in the apical loop (L4) of domain II (Fig. 6a, c). Mutation of this loop to an unstructured U7-loop resulted in decreased activation of PKR (Fig. 7). These data prompted a detailed inspection of the NMR structure of domain II³⁹, which revealed that the apical loop, L4, is fairly structured, with U80, A81, C84, and A85 turned in towards the helix. In addition, U80 and A85 interact via the 2'-hydroxyl of U80 hydrogen bonding with the N6 of A85, and U86 is extruded (Fig. 10a). Thus, L4 resembles a tetraloop having sequence AGCC.

There is precedent for other dsRBDs to interact with RNA terminal loops, including those from Staufen, RNase III, Drosha, and ADAR 2.⁵⁸⁻⁶¹ In particular, Feigon and co-workers found that the dsRBD of Rnt1p RNaseIII binds AGNN tetraloops, wherein the conserved A and G do not form sequence-specific contacts with the protein but rather help orient the remainder of the tetraloop to form non-sequence specific minor-groove contacts primarily via the NN nucleotides.^{59,62} We thus considered the possibility that L4 of domain II, which conforms to the AGNN motif, binds the dsRBD of PKR in a similar fashion.

A structural comparison between the NMR structures of AGCC from L4 and AGAA from Rnt1p-snr47h RNA complex^{59,62} was carried out (Fig. 10a, b). We consider first

positioning of the bases, followed by positioning of the sugar-phosphate backbone. Overall, the relative positions of the bases in the two tetraloops are quite similar: the A's at the 5'-end of the loop (A81 from domain II and A15 from snR47h) are oriented inwards and stack over the helix below; the G's at position 2 of the loop (G81 from domain II and G16 from snR47h) are flipped out of the loop; and the remaining two bases roughly stack over the 3'-end of the helix. In the AGAA tetraloop, the highly conserved G16 is in the *syn* conformation about the glycosidic bond with the rare C2'-endo sugar pucker. These conformational characteristics do not lead the nucleobase of G16 to make direct contact with Rnt1p, but they do allow for exposure of the non-bridging phosphate oxygens between G16 and A17. While the corresponding G in L4 (G82) is *anti* in most of the NMR structures entered into the pdb, the sugar pucker of G82 in the majority of the 12 NMR structures is also C2'-endo resulting in similar exposure of the non-bridging phosphate oxygens between G82 and C83 (Fig. 10b).

Next, the sugar phosphate backbones of AGCC and AGAA were overlaid. We found an rmsd between the two backbones of just 1.57 Å (Fig. 10b). Moreover, visual inspection of the overlay reveals good superposition of the 2'-hydroxyls and phosphates. Given that PKR interacts with 2'-hydroxyls and phosphates rather than base moieties,⁴⁵ this suggests that the structured nature of the L4 loop may contribute to activation of PKR by domain II through a mechanism similar to that by which AGNN tetraloops are recognized by Staufen and RNase III.

It should be noted that the effects of the L4-L5 mutation to domain II on PKR activation are subtle, suggesting that PKR binding to the L4 tetraloop may not be critically dependent upon specific recognition of the structural details described above. Indeed, for Rnt1p RNase III, binding affinity for an AGNN versus a nonspecific tetraloop was not drastically different.^{59,62} The stem of the snR47 construct used in the Rnt1p-snR47h NMR structure contains only 15 bp, while in the NMR structure of Staufen dsRBD bound to a RNA tetraloop, the stem is only 14 bp. Given that the dsRBD motif typically requires 16 bp of dsRNA for binding, the minimal nature of the constructs used in these two RNA constructs may in fact “force” the dsRBD of Rnt1p and Staufen to interact with structured loop sequences. This may also be the case for interaction of L4 from HCV IRES domain II with PKR, in which the absence of 33 bp within the stem of domain II required for functional dimerization of PKR necessitates binding to the structured tetraloop. Thus, HCV IRES domain II may be an example of a biological RNA that presents a minimal construct for PKR activation.

Next, we turn our attention to the portion of domain II between P2 and P4. As mentioned, it is possible that activation of PKR by domain II is facilitated by A-form structural mimicry. In fact, the loops in this region of domain II are largely symmetrical—L2 is a 2×2 loop and L3 is a 3×4 loop—opening the possibility for nearly bulge-free non-Watson-Crick base pairing. Indeed, the NMR structure of domain II reveals a stretch of noncanonical base pairs within L2 and L3.³⁹ In particular, L2 consists of two pyrimidine-pyrimidine base pairs, U63•C104 and U64•U103, while L3 contains three non-Watson-Crick base pairs, A73•A93 (AA N7 symmetric), A72•U95 (AU Hoogsteen), and G71•A96 (sheared GA). In fact, the only base in L2 or L3 that does not directly participate in base pairing is G94, which was the only base within L3 that was not protected from p20-dependent in-line cleavage (Fig 6c). These observations suggest that, despite their non-canonical nature, PKR may recognize the base pairs in L2 and L3 as dsRNA. We do note, however, that the identity of the bases in these loops seems to be relatively unimportant, as substitutions in L2 and L3, which maintained their symmetrical geometries, led to only minor effects on activation.

To further explore A-form mimicry within L2 and L3, we overlaid the P2-P4 portion of domain II with a crystal structure of a model 19 bp A-form dsRNA⁶³ (Fig. 10c, d). The

backbones of these two structures gave an overall rmsd of 3.03 Å. In particular, the lower portion of these structures, which contains L2, overlays very well. Visual inspection of this region (Fig. 10d) reveals very similar positioning of the 2'-hydroxyls within L2 and model dsRNA, the only significant difference being the width of the two helices, which is ~ 11 Å between 2'-hydroxyls for the A-form helix and ~9 Å for L2, induced by the smaller pyrimidine-pyrimidine base pairs.⁶⁴

The deviation between domain II and model dsRNA is greater near L3, likely due to the curvature of domain II induced by L3. Visual inspection of this overlay region, however, still suggests that the 2'-hydroxyl group positions within L3 are highly similar to those of an overall A-form geometry, with only a few exceptions (Fig. 10d, lavender residues). One exception in particular, G94 (Fig. 10d, arrow), is not protected by p20, as described above. In summary, domain II from P2 through P4, appears to mimic A-form dsRNA, and contribute a potential 22 A-form-like base pairs.

Below P2 are L1 and P1. In contrast to L2 and L3, L1 is a 5-nucleotide bulge, which leads to a kink in the backbone, although this region is likely flexible as is characteristic of large bulges. The role of L1 in activation is unclear. Protections mapped onto the NMR structure in Fig. 6 suggest that the dsRBMs of a PKR dimer interact extensively with domain II, with the exception of L1; also, L1 can be deleted with just a slight loss in activation. Thus L1 likely plays little direct role in activation, although it remains somewhat surprising that it can be tolerated in this activating element.

At the base of domain II is the P1 pairing, which contributes 8 Watson-Crick base pairs. Together with the 22 A-form-like base pairs from P2 through P4, this gives 30 base pairs total. Assuming that L4 interacts productively with p20, as proposed above, an effective total number of base pairs is ~33, which is the minimum number needed for activation of PKR.⁴ Similarly, addition of base-paired segments leading to this value were found for the IFN- γ mRNA pseudoknot.¹² Thus the ability of shorter base paired segments and symmetrical loops to sum to a PKR activating total appears to be a common theme.

Lastly, we briefly consider the weaker activation of PKR by domain III-IV of HCV IRES. This domain contains more extensive helical regions than domain II (Fig. 1), yet it is a less potent activator of PKR (Fig. 3). We suggest that this may be due to the branched nature of this domain, which contrasts with the unbranched domain II. In particular, structural motifs within domain III of the IRES appear to resemble motifs in well-studied PKR-inhibitory RNA structures. Specifically, the geometries of the four-way junction from IIIabc and the three-way junction comprised of the branched IIIcd stem loop from HCV IRES (Fig. 1) parallel portions of the inhibitory Epstein-Barr EBER1 RNA and adenovirus VAI RNA.^{65,66} EBER1 contains a 4-way junction comprised of two stem regions and two branched stem-loops, with one shorter than the other, while the central domain of VAI RNA contains a three-way junction that is critical for PKR inhibition.^{16,67} Thus, the two multi-junction regions in domain III may cause PKR to adopt a combination of activating and inhibiting binding geometries, leading to its poorer activation.

Possible roles for PKR in HCV replication

It is known that NS5A regulates PKR activation throughout the lifecycle of HCV. Observation that NS5A can equally inhibit activation of PKR by domains II and III-IV suggests possible models for involvement of these IRES domains in PKR activation. In Table 1, we present possible modes for regulation of host and virus translation through PKR and NS5A interaction. Early in the viral lifecycle, when NS5A levels are low, PKR could be activated by the IRES leading to phosphorylation of eIF2 α and inhibition of cap-dependent host translation. However, viral translation, which has been shown to function independent

of PKR phosphorylation,^{32,68,69} would continue unabated, leading to high levels of viral proteins (Table 1, column 2). Later in the viral lifecycle, when NS5A levels have increased, PKR activity would be inhibited, leading to upregulation of host translation but still allowing high levels of viral translation. In this way, early in infection translation would be predominantly of viral proteins, but host proteins would be made available to the virus for later stages of replication.

We demonstrated that NS5A binds tightly to domains III-IV but only weakly to domain II. One possible consequence of this is that low levels of NS5A could be sequestered by binding to domains III-IV, allowing PKR activity to remain high via activation by domain II. In fact, the spacing of the bands in Figure 8, suggest that domains III-IV can titrate 3 or more NS5A proteins, consistent with the presence of 5 GU-rich elements in this region.⁵⁰ Only later in infection, when NS5A levels become high enough to exceed the concentration of binding sites, would PKR be bound by NS5A and be inhibited. Weaker binding of NS5A to PKR than RNA is suggested by the 50% values for RNA binding and PKR inhibition (Figs. 8a and 9c). This model for the interaction of PKR, NS5A, and IRES elements is consistent with observation that domain II is required for both replication and translation, whereas domains III-IV are only required for translation. Indeed, it has previously been suggested that domain II could function as a switch between translation and replication.²⁶ Given the evidence here presented, it is possible that this domain II regulation of HCV translation and replication is mediated by differential interaction with PKR and NS5A.

Materials and Methods

RNA Sequences

The 79 bp (dsRNA-79) control was prepared by transcribing opposing strands of pUC19 and annealing as previously described.⁷ The remaining RNAs were prepared by in vitro transcription from a linearized plasmid, a double-stranded PCR product, or a hemi-duplex DNA template (see below). Following are the sequences of RNA used in this study.

1-388 (full-length IRES):

```
5'GCCAGCCCCCGAUUGGGGGCGACACUCCACCAUAGAUCACUCCCCUGUGAGG
AACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUC
GUGCAGCCUCCAGGACCCCCCUCCCGGGAGAGCCAUAGUGGUCUGCGGAACC
GGUGAGUACACCGGAAUUGCCAGGACGACCGGGUCCUUUCUUGGAUCAACCC
GCUCAUAGCCUGGAGAUUUGGGCGUGCCCCCGCGAGACUGCUAGCCGAGUAG
UGUUGGGUCGCGAAAGGCCUUGUGGUACUGCCUGAUAGGGUGCUUGCGAGUG
CCCCGGGAGGUCUCGUAGACCGUGCACCAUGAGCACGAAUCCUAAACCUCUAA
AGAAAAACCAAAGGGCGCGCCGC
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1-130 (domains I-II):

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5'GCCAGCCCCCGAUUGGGGGCGACACUCCACCAUAGAUCACUCCCCUGUGAGG
AACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUC
GUGCAGCCUCCAGGACCCCCCUCCC
```

131-388 (domains III-IV):

```
5'GGGAGAGCCAUAGUGGUCUGCGGAACCGGUGAGUACACCGGAAUUGCCAGG
ACGACCGGGUCCUUUCUUGGAUCAACCCGCUCAUAGCCUGGAGAUUUGGGCG
UGCCCCCGCGAGACUGCUAGCCGAGUAGUGUUGGGUCGCGAAAGGCCUUGUG
GUACUGCCUGAUAGGGUGCUUGCGAGUGCCCCGGGAGGUCUCGUAGACCGUG
CACCAUGAGCACGAAUCCUAAACCUCUAAAGAAAAACCAAAGGGCGCGCCGC
```

39-119 (domain II): (two additional G's at the 5'-end included to prime transcription are in bold)

5'**GG**ACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUG
GCGUUAGUAUGAGUGUCGUGCAGCCUCCAGGA

Domain II mutants: (additions or substitutions are indicated by underlines, and deletions are indicated by dashes)

L4-U5:

5'**GG**ACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUUUUUUUG
GCGUUAGUAUGAGUGUCGUGCAGCCUCCAGGA

Δ2bp: 5'**GG**ACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAA-CG-
CUAGCCAUG-CG-UAGUAUGAGUGUCGUGCAGCCUCCAGGA

+2bp: 5'

GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCCGGUCUAGCCAUG
GGCCGGUUAGUAUGAGUGUCGUGCAGCCUCCAGGA

G71A/G94A: 5'

GGACUCCCCUGUGAGGAACUACUGUCUUCACGCAGAAAGCGUCUAGCCAUGG
CGUUAAAUAUGAGUGUCGUGCAGCCUCCAGGA

C104U: 5'**GG**ACUCCCCUGUGAGGAACUACUGUCUUC

ACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUUGUGCAGCCUCCAG
GA

ΔL1: 5' **GG**ACUCCCCUGUGAGG-----

CUGUCUUCACGCAGAAAGCGUCUAGCCAUGGCGUUAGUAUGAGUGUCGUGCA
GCCUCCAGGA

PCR primers (T7 promoter is underlined, additional 5'-end G's are in bold)

1-130 TS: 5'GAAATTAATACGACTCACTATAGCC

1-130 BS: 5'GGGAGGGGGGGTCCTGGA

131-388 TS: 5'GAAATTAATACGACTCACTATAGGGAGAGCCATAGTGGTCTG

131-388 BS: 5'GCGGCGCGCCCTTTGG

39-119 TS: 5' GAAATTAATACGACTCACTATAGGACTCCCCTGTGAGGAAC

39-119 BS: 5'TCCTGGAGGCTGCACGAC

Protein Preparation

Full-length PKR and the dsRBD (p20) containing N-terminal His₆ tags were purified from *E. coli* BL21(DE3) Rosetta cells (Novagen) as described elsewhere.^{14,45} Because full-length PKR was isolated in phosphorylated form, the protein was dephosphorylated prior to activation assays by treatment with λ-phosphatase (NEB).^{14,70} His-Δ-NS5A, which contains amino acids 2005-2419 of the HCV polyprotein) was prepared as previously described.⁷¹ This construct has the 32 amino acid, N-terminal membrane-anchor domain deleted.

RNA Preparation

Full-length HCV IRES (1-388) was transcribed by combining 1.5 μ g of linearized pUC18 plasmid containing a T7 promoter⁷² with 1.8 μ g of T7 RNA polymerase, along with 40 mM Tris (pH 8), 33 mM Mg(OAc)₂, 40 mM DTT, 2 mM spermidine, and 7 mM of each NTP and incubating at 37 °C. After 3.5-4 h, the reaction was quenched by adding 95% (v/v) formamide loading buffer. RNA was then purified by fractionating on a polyacrylamide denaturing gel (7 M urea, 1X TBE). The transcript was identified by UV shadowing, excised from the gel, and eluted overnight at 4 °C in 1X TEN₂₅₀. The RNA was then ethanol precipitated and resuspended in 1X TE buffer (pH 7.5) and stored at -20 °C. RNA concentration was determined spectrophotometrically.

Transcription templates for HCV 1-130, 39-119 and 131-388 were prepared by PCR amplification of the target sequence from the pUC18 plasmid using appropriate TS and BS DNA primers. Templates for each RNA included a T7 promoter sequence at the 5'-end. Additionally, one to two G's were included at the start of the RNA sequence in order to promote transcription. RNA was transcribed directly from the PCR products using Ambion Inc. T7 transcription kits and purified as described above. HCV 39-119 mutants were prepared by transcription from a hemi-duplex DNA template (IDT) with a T7 promoter⁷³ using Ambion T7 kits and purified. Prior to each experiment, RNAs were thawed and then renatured by heating to 90 °C for 3 min, followed by slow cooling to room temperature for 10 min.

5'-end-labeling of RNA

For use in gel-shift and structuring mapping/footprinting assays, RNAs were 5'-end labeled with ³²P. In order to remove the 5'-triphosphate, RNA was first treated with calf intestinal alkaline phosphatase (CIP), as previously described.⁷ The RNA was 5'-end-labeled with PNK (New England Biolabs) and again purified by gel electrophoresis, excised, eluted overnight at 4 °C, and ethanol precipitated. The RNAs were resuspended in 1X TE, and the concentration was determined using a liquid scintillation counter (Beckman).

PKR Activation Assays

RNAs were tested for their ability to activate PKR autophosphorylation and eIF2 α phosphorylation.⁷ PKR was first treated with λ phosphatase (New England Biolabs) at 30 °C for 1 hr followed by addition of the phosphatase inhibitor, sodium orthovanadate. Next, 0.6 μ M dephosphorylated PKR was incubated with RNA at various concentrations in 20 mM HEPES (pH 7.5), 4 mM MgCl₂, 100 mM KCl, 100 μ M ATP (Ambion), 1.5 mM DTT, and 1.5 μ Ci [γ -³²P]-ATP for 10 min at 30 °C. In certain cases, 3 μ M eIF2 α (10-fold excess over PKR) was added and incubated for an additional 10 min. In PKR inhibition studies by HCV IRES RNA, poly I:C (Sigma) of approximate size 30-200 bp was used as an activator of PKR. HCV IRES RNA and poly I:C were added prior to dephosphorylated PKR and reaction components. All reactions were quenched by addition of SDS loading buffer, then loaded on 10% SDS-PAGE gels. Gels were dried and exposed to a storage PhosphorImager screen, then scanned on a Typhoon PhosphorImager and quantified using ImageQuant (Molecular Dynamics). In all experiments, a negative control containing TEK₁₀₀ instead of RNA and a positive control containing 0.01 or 0.1 μ M 79 bp dsRNA were included. Data were normalized after subtracting a background value averaged from different portions of the gel.

Native-Gel Mobility-Shift Assays

To determine the binding affinity of the dsRBD of PKR (p20) or NS5A for certain RNAs, excess p20 or NS5A (0.05-5 μ M) and trace amounts of 5'-³²P-end labeled RNA (~ 2 nM)

were incubated with 1 mg/mL herring sperm DNA, 10 mM NaCl, 25 mM HEPES (pH 7.5), 5 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.1 mg/mL bovine serum albumin, and 0.01 % NP-40 for 30 min at 22 °C.⁴⁵ Samples were loaded onto 0.5X TBE native gels (29:1 crosslink) at 16 °C while the gel was running, and fractionated for 2-2.5 hr. Gels were dried and exposed to storage PhosphorImager screens overnight.

Enzymatic Structure Mapping

Trace amounts of 5'-³²P-end labeled RNAs were digested in the presence of single-strand-specific (RNase T1, RNase A) or double-strand-specific (RNase V1) nucleases under native conditions (20 mM HEPES, pH 7, 100 mM NaCl, 4 mM MgCl₂) for 15 min at 37 °C. To generate the T1 ladder, labeled RNA was incubated under denaturing conditions in 0.01 U/μL T1, 18 mM Na-citrate (pH 3.5), 0.9 mM EDTA, and 6 M urea for 30 min at 50 °C.¹³ For the hydrolysis ladder, labeled RNA was incubated in 100 mM Na₂CO₃/NaHCO₃ and 2 mM EDTA for 4 min at 90 °C. All reactions were immediately quenched by addition of an equal volume of 0.2 M EDTA/formamide/0.2% SDS loading buffer and boiled before fractionating on a 12% polyacrylamide, 8.3 M urea sequencing gel. Nuclease concentrations that gave limited digestion were as follows: 0.01 U/μL RNase T1 (Ambion), 1 ng/mL RNase A (Ambion), 1.6 U/μL RNase V1 (Pierce).

Nuclease Footprinting

5'-³²P-end labeled RNAs were treated under the same native conditions as in structure mapping experiments, with the following additions: 130 ng tRNA^{Phe}, 1.5 μM BSA, and 10 μM p20. Prior to treatment with nucleases, the samples were incubated at 22 °C for 30 min to allow the RNA and p20 to bind. Appropriate nucleases were then added at the same concentrations as in the structure mapping experiments. Digestions for RNase A and RNase V1 proceeded at 37 °C for 15 min and 22 °C for 30 min, respectively. Samples were quenched with an equal volume of 0.2 M EDTA/formamide/0.2% SDS loading buffer and loaded on a 12% polyacrylamide, 8.3 M urea sequencing gel.

In-line Probing

In-line probing experiments rely on self-cleavage of the RNA at slightly elevated pH by in-line attack of the 2'-hydroxyl. Patterns of cleavage often change in the presence of proteins as well. 5'-³²P-end labeled RNAs were mixed with the following components: 50 mM Tris (pH 8.3), 20 mM MgCl₂, 100 mM KCl, and p20 (0.625, 1.25, 2.5, 5, or 10 μM).^{46,74} The samples were then incubated at 27 °C for 40 h, followed by addition of an equal volume of formamide/1.5 mM EDTA/10 M urea loading buffer. Loading buffer was also added to an additional sample of labeled RNA that was not subjected to reaction. Samples were then fractionated on a denaturing (8.3 M urea) 12% gel, along with T1 and hydrolysis ladders, as per structure mapping and footprinting experiments. The gel was analyzed on ImageQuant by drawing a line through each of the lanes and plotting the raw counts.

Inhibition of PKR Activation by NS5A

Following is the general approach taken for studies on inhibition of PKR by NS5A, although changes to the order of addition were also explored, as described in the legend to Figure S5. A final concentration of 2.5 μM domain II or domains III-IV, or a final concentration of 0.1 μM 79 bp RNA, was pre-incubated with various concentrations of NS5A (stored in protein storage buffer (PSB): 50 mM HEPES pH 7.5, 50 mM NaCl, 0.1% NP-40, and 1 mM DTT) for 10 minutes at 30 °C. In the 0 μM NS5A lanes, an equivalent volume of PSB was added to keep buffer concentrations consistent across lanes. Dephosphorylated PKR and activation assay reaction components (see above) were then added and incubated at 30 °C for an

additional 10 min. Reactions were quenched and analyzed as per PKR activation assays (see above).

Structural Comparisons

RMSD calculations between domain II and 19 bp A-form helix were conducted as follows. Initially, visual inspection was carried out in PyMOL⁷⁵ where the structural similarities between an A-form helix crystal structure (PDB ID: **1QC0**) and the domain II lowest energy NMR structure (PDB ID: **1P5O**) became apparent. RMSD's were calculated in VMD version 1.8.7⁷⁶ using a selection script and the measuring utility. The sugar phosphate backbone of domain II nucleotides 59-73, 95-105, and 107-109 were fit to A-form nucleotides 1-15, 24-34, and 36-38, respectively. Additionally, the S-turn of domain II was accommodated by fitting the hydroxyl oxygens of domain II nucleotides 90-93 to nucleotides 19-17 and 23 of the A-form helix. RMSD calculations between the apical loops of domain II average energy NMR structure (PDB ID: **1P5P**) and snR47h (PDB ID: **1T4L**) were conducted in a similar fashion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Balachandran S, Roberts PC, Brown LE, Truong H, Pattnaik AK, Archer DR, Barber GN. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity*. 2000; 13:129–141. [PubMed: 10933401]
2. Williams BR. PKR; a sentinel kinase for cellular stress. *Oncogene*. 1999; 18:6112–6120. [PubMed: 10557102]
3. Samuel CE. Antiviral actions of interferons. *Clin Microbiol Rev*. 2001; 14:778–809. table of contents. [PubMed: 11585785]
4. Manche L, Green SR, Schmedt C, Mathews MB. Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol*. 1992; 12:5238–5248. [PubMed: 1357546]
5. Cole JL. Activation of PKR: an open and shut case? *Trends Biochem Sci*. 2007; 32:57–62. [PubMed: 17196820]
6. Garcia MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, Esteban M. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. *Microbiol Mol Biol Rev*. 2006; 70:1032–1060. [PubMed: 17158706]
7. Nallagatla SR, Hwang J, Toroney R, Zheng X, Cameron CE, Bevilacqua PC. 5'-triphosphate-dependent activation of PKR by RNAs with short stem-loops. *Science*. 2007; 318:1455–1458. [PubMed: 18048689]
8. McCormack SJ, Thomis DC, Samuel CE. Mechanism of interferon action: identification of a RNA binding domain within the N-terminal region of the human RNA-dependent P1/eIF-2 alpha protein kinase. *Virology*. 1992; 188:47–56. [PubMed: 1373554]
9. Green SR, Mathews MB. Two RNA-binding motifs in the double-stranded RNA-activated protein kinase, DAI. *Genes Dev*. 1992; 6:2478–2490. [PubMed: 1364113]
10. Ryter JM, Schultz SC. Molecular basis of double-stranded RNA-protein interactions: structure of a dsRNA-binding domain complexed with dsRNA. *EMBO J*. 1998; 17:7505–7513. [PubMed: 9857205]

11. Heinicke LA, Wong CJ, Lary J, Nallagatla SR, Diegelman-Parente A, Zheng X, Cole JL, Bevilacqua PC. RNA dimerization promotes PKR dimerization and activation. *J Mol Biol.* 2009; 390:319–338. [PubMed: 19445956]
12. Cohen-Chalamish S, Hasson A, Weinberg D, Namer LS, Banai Y, Osman F, Kaempfer R. Dynamic refolding of IFN-gamma mRNA enables it to function as PKR activator and translation template. *Nat Chem Biol.* 2009; 5:896–903. [PubMed: 19801993]
13. Bevilacqua PC, George CX, Samuel CE, Cech TR. Binding of the protein kinase PKR to RNAs with secondary structure defects: role of the tandem A-G mismatch and noncontiguous helices. *Biochemistry.* 1998; 37:6303–6316. [PubMed: 9572845]
14. Zheng X, Bevilacqua PC. Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. *RNA.* 2004; 10:1934–1945. [PubMed: 15547138]
15. Sharp TV, Schwemmle M, Jeffrey I, Laing K, Mellor H, Proud CG, Hilse K, Clemens MJ. Comparative analysis of the regulation of the interferon-inducible protein kinase PKR by Epstein-Barr virus RNAs EBER-1 and EBER-2 and adenovirus VAI RNA. *Nucleic Acids Res.* 1993; 21:4483–4490. [PubMed: 7901835]
16. Clarke PA, Schwemmle M, Schickinger J, Hilse K, Clemens MJ. Binding of Epstein-Barr virus small RNA EBER-1 to the double-stranded RNA-activated protein kinase DAI. *Nucleic Acids Res.* 1991; 19:243–248. [PubMed: 1673026]
17. McCormack SJ, Samuel CE. Mechanism of interferon action: RNA-binding activity of full-length and R-domain forms of the RNA-dependent protein kinase PKR--determination of KD values for VAI and TAR RNAs. *Virology.* 1995; 206:511–519. [PubMed: 7530396]
18. Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A. Internal ribosome entry site within hepatitis C virus RNA. *J Virol.* 1992; 66:1476–1483. [PubMed: 1310759]
19. Wang C, Sarnow P, Siddiqui A. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. *J Virol.* 1993; 67:3338–3344. [PubMed: 8388503]
20. Pestova TV, Hellen CU. Functions of eukaryotic factors in initiation of translation. *Cold Spring Harb Symp Quant Biol.* 2001; 66:389–396. [PubMed: 12762041]
21. Reynolds JE, Kaminski A, Carroll AR, Clarke BE, Rowlands DJ, Jackson RJ. Internal initiation of translation of hepatitis C virus RNA: the ribosome entry site is at the authentic initiation codon. *RNA.* 1996; 2:867–878. [PubMed: 8809014]
22. Honda M, Beard MR, Ping LH, Lemon SM. A phylogenetically conserved stem-loop structure at the 5' border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. *J Virol.* 1999; 73:1165–1174. [PubMed: 9882318]
23. Kieft JS, Zhou K, Jubin R, Murray MG, Lau JY, Doudna JA. The hepatitis C virus internal ribosome entry site adopts an ion-dependent tertiary fold. *J Mol Biol.* 1999; 292:513–529. [PubMed: 10497018]
24. Zhao WD, Wimmer E. Genetic analysis of a poliovirus/hepatitis C virus chimera: new structure for domain II of the internal ribosomal entry site of hepatitis C virus. *J Virol.* 2001; 75:3719–3730. [PubMed: 11264361]
25. Kieft JS. Viral IRES RNA structures and ribosome interactions. *Trends Biochem Sci.* 2008; 33:274–283. [PubMed: 18468443]
26. Kim YK, Kim CS, Lee SH, Jang SK. Domains I and II in the 5' nontranslated region of the HCV genome are required for RNA replication. *Biochem Biophys Res Commun.* 2002; 290:105–112. [PubMed: 11779140]
27. Otto GA, Puglisi JD. The pathway of HCV IRES-mediated translation initiation. *Cell.* 2004; 119:369–380. [PubMed: 15507208]
28. Kolupaeva VG, Pestova TV, Hellen CU. An enzymatic footprinting analysis of the interaction of 40S ribosomal subunits with the internal ribosomal entry site of hepatitis C virus. *J Virol.* 2000; 74:6242–6250. [PubMed: 10864633]
29. Spahn CM, Kieft JS, Grassucci RA, Penczek PA, Zhou K, Doudna JA, Frank J. Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit. *Science.* 2001; 291:1959–1962. [PubMed: 11239155]

30. Boehringer D, Thermann R, Ostareck-Lederer A, Lewis JD, Stark H. Structure of the hepatitis C virus IRES bound to the human 80S ribosome: remodeling of the HCV IRES. *Structure*. 2005; 13:1695–1706. [PubMed: 16271893]
31. Vyas J, Elia A, Clemens MJ. Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA. *RNA*. 2003; 9:858–870. [PubMed: 12810919]
32. Shimoike T, McKenna SA, Lindhout DA, Puglisi JD. Translational insensitivity to potent activation of PKR by HCV IRES RNA. *Antiviral Res*. 2009; 83:228–237. [PubMed: 19467267]
33. Clarke PA, Mathews MB. Interactions between the double-stranded RNA binding motif and RNA: definition of the binding site for the interferon-induced protein kinase DAI (PKR) on adenovirus VA RNA. *RNA*. 1995; 1:7–20. [PubMed: 7489491]
34. Minks MA, West DK, Benven S, Baglioni C. Structural requirements of double-stranded RNA for the activation of 2',5'-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. *J Biol Chem*. 1979; 254:10180–10183. [PubMed: 489592]
35. Kostura M, Mathews MB. Purification and activation of the double-stranded RNA-dependent eIF-2 kinase DAI. *Mol Cell Biol*. 1989; 9:1576–1586. [PubMed: 2725516]
36. Brown EA, Zhang H, Ping LH, Lemon SM. Secondary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. *Nucleic Acids Res*. 1992; 20:5041–5045. [PubMed: 1329037]
37. Wang C, Le SY, Ali N, Siddiqui A. An RNA pseudoknot is an essential structural element of the internal ribosome entry site located within the hepatitis C virus 5' noncoding region. *RNA*. 1995; 1:526–537. [PubMed: 7489514]
38. Lescoute A, Westhof E. Topology of three-way junctions in folded RNAs. *RNA*. 2006; 12:83–93. [PubMed: 16373494]
39. Lukavsky PJ, Kim I, Otto GA, Puglisi JD. Structure of HCV IRES domain II determined by NMR. *Nat Struct Biol*. 2003; 10:1033–1038. [PubMed: 14578934]
40. Dibrov SM, Johnston-Cox H, Weng YH, Hermann T. Functional architecture of HCV IRES domain II stabilized by divalent metal ions in the crystal and in solution. *Angew Chem Int Ed Engl*. 2007; 46:226–229. [PubMed: 17131443]
41. Zhao Q, Han Q, Kissinger CR, Hermann T, Thompson PA. Structure of hepatitis C virus IRES subdomain IIa. *Acta Crystallogr D Biol Crystallogr*. 2008; 64:436–443. [PubMed: 18391410]
42. Ehresmann C, Baudin F, Mougél M, Romby P, Ebel JP, Ehresmann B. Probing the structure of RNAs in solution. *Nucleic Acids Res*. 1987; 15:9109–9128. [PubMed: 2446263]
43. Lockard RE, Kumar A. Mapping tRNA structure in solution using double-strand-specific ribonuclease V1 from cobra venom. *Nucleic Acids Res*. 1981; 9:5125–5140. [PubMed: 7031604]
44. Brown TS, Bevilacqua PC. Method for assigning double-stranded RNA structures. *Biotechniques*. 2005; 38:368, 370, 372. [PubMed: 15786803]
45. Bevilacqua PC, Cech TR. Minor-groove recognition of double-stranded RNA by the double-stranded RNA-binding domain from the RNA-activated protein kinase PKR. *Biochemistry*. 1996; 35:9983–9994. [PubMed: 8756460]
46. Regulski EE, Breaker RR. In-line probing analysis of riboswitches. *Methods Mol Biol*. 2008; 419:53–67. [PubMed: 18369975]
47. Gale MJ Jr, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology*. 1997; 230:217–227. [PubMed: 9143277]
48. Gale MJ Jr, Kwieciszewski B, Dossett M, Nakao H, Katze MG. Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. *J Virol*. 1999; 73:6506–6516. [PubMed: 10400746]
49. He Y, Tan SL, Tareen SU, Vijaysri S, Langland JO, Jacobs BL, Katze MG. Regulation of mRNA translation and cellular signaling by hepatitis C virus nonstructural protein NS5A. *J Virol*. 2001; 75:5090–5098. [PubMed: 11333890]
50. Huang L, Hwang J, Sharma SD, Hargittai MR, Chen Y, Arnold JJ, Raney KD, Cameron CE. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J Biol Chem*. 2005; 280:36417–36428. [PubMed: 16126720]

51. Witherell GW, Beineke P. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. *J Med Virol.* 2001; 63:8–16. [PubMed: 11130893]
52. Pascu M, Martus P, Hohne M, Wiedenmann B, Hopf U, Schreier E, Berg T. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. *Gut.* 2004; 53:1345–1351. [PubMed: 15306598]
53. Schinkel J, Spaan WJ, Kroes AC. Meta-analysis of mutations in the NS5A gene and hepatitis C virus resistance to interferon therapy: uniting discordant conclusions. *Antivir Ther.* 2004; 9:275–286. [PubMed: 15134190]
54. Pflugheber J, Fredericksen B, Sumpter R Jr, Wang C, Ware F, Sodora DL, Gale M Jr. Regulation of PKR and IRF-1 during hepatitis C virus RNA replication. *Proc Natl Acad Sci USA.* 2002; 99:4650–4655. [PubMed: 11904369]
55. Xiang J, Martinez-Smith C, Gale M Jr, Chang Q, Labrecque DR, Schmidt WN, Stapleton JT. GB virus type C NS5A sequence polymorphisms: association with interferon susceptibility and inhibition of PKR-mediated eIF2 α phosphorylation. *J Interferon Cytokine Res.* 2005; 25:261–270. [PubMed: 15871663]
56. Gimenez-Barcons M, Wang C, Chen M, Sanchez-Tapias JM, Saiz JC, Gale M Jr. The oncogenic potential of hepatitis C virus NS5A sequence variants is associated with PKR regulation. *J Interferon Cytokine Res.* 2005; 25:152–164. [PubMed: 15767789]
57. Zheng X, Bevilacqua PC. Straightening of bulged RNA by the double-stranded RNA-binding domain from the protein kinase PKR. *Proc Natl Acad Sci USA.* 2000; 97:14162–14167. [PubMed: 11114159]
58. Ramos A, Grunert S, Adams J, Micklem DR, Proctor MR, Freund S, Bycroft M, St Johnston D, Varani G. RNA recognition by a Staufen double-stranded RNA-binding domain. *EMBO J.* 2000; 19:997–1009. [PubMed: 10698941]
59. Wu H, Yang PK, Butcher SE, Kang S, Chanfreau G, Feigon J. A novel family of RNA tetraloop structure forms the recognition site for *Saccharomyces cerevisiae* RNase III. *EMBO J.* 2001; 20:7240–7249. [PubMed: 11743000]
60. Stefl R, Allain FH. A novel RNA pentaloop fold involved in targeting ADAR2. *RNA.* 2005; 11:592–597. [PubMed: 15840813]
61. Zeng Y, Yi R, Cullen BR. Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. *EMBO J.* 2005; 24:138–148. [PubMed: 15565168]
62. Wu H, Henras A, Chanfreau G, Feigon J. Structural basis for recognition of the AGNN tetraloop RNA fold by the double-stranded RNA-binding domain of Rnt1p RNase III. *Proc Natl Acad Sci USA.* 2004; 101:8307–8312. [PubMed: 15150409]
63. Klosterman PS, Shah SA, Steitz TA. Crystal structures of two plasmid copy control related RNA duplexes: An 18 base pair duplex at 1.20 Å resolution and a 19 base pair duplex at 1.55 Å resolution. *Biochemistry.* 1999; 38:14784–14792. [PubMed: 10555960]
64. Zoll J, Tessari M, Van Kuppeveld FJ, Melchers WJ, Heus HA. Breaking pseudo-twofold symmetry in the poliovirus 3'-UTR Y-stem by restoring Watson-Crick base pairs. *RNA.* 2007; 13:781–792. [PubMed: 17449731]
65. Glickman JN, Howe JG, Steitz JA. Structural analyses of EBER1 and EBER2 ribonucleoprotein particles present in Epstein-Barr virus-infected cells. *J Virol.* 1988; 62:902–911. [PubMed: 2828685]
66. Ma Y, Mathews MB. Secondary and tertiary structure in the central domain of adenovirus type 2 VA RNA I. *RNA.* 1996; 2:937–951. [PubMed: 8809020]
67. Clarke PA, Pe'ery T, Ma Y, Mathews MB. Structural features of adenovirus 2 virus-associated RNA required for binding to the protein kinase DAI. *Nucleic Acids Res.* 1994; 22:4364–4374. [PubMed: 7971266]
68. Koev G, Duncan RF, Lai MM. Hepatitis C virus IRES-dependent translation is insensitive to an eIF2 α -independent mechanism of inhibition by interferon in hepatocyte cell lines. *Virology.* 2002; 297:195–202. [PubMed: 12083818]
69. Garaigorta U, Chisari FV. Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. *Cell Host Microbe.* 2009; 6:513–522. [PubMed: 20006840]

70. Matsui T, Tanihara K, Date T. Expression of unphosphorylated form of human double-stranded RNA-activated protein kinase in *Escherichia coli*. *Biochem Biophys Res Commun*. 2001; 284:798–807. [PubMed: 11396973]
71. Huang L, Sineva EV, Hargittai MR, Sharma SD, Suthar M, Raney KD, Cameron CE. Purification and characterization of hepatitis C virus non-structural protein 5A expressed in *Escherichia coli*. *Protein Expr Purif*. 2004; 37:144–153. [PubMed: 15294292]
72. Milligan JF, Uhlenbeck OC. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol*. 1989; 180:51–62. [PubMed: 2482430]
73. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res*. 1987; 15:8783–8798. [PubMed: 3684574]
74. Hunsicker A, Steber M, Mayer G, Meitert J, Klotzsche M, Blind M, Hillen W, Berens C, Suess B. An RNA aptamer that induces transcription. *Chem Biol*. 2009; 16:173–180. [PubMed: 19246008]
75. DeLano, WL. The PyMol molecular graphics system. DeLano Scientific; San Carlos, CA, USA: 2002.
76. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996; 14:33–38. 27–38. [PubMed: 8744570]

Abbreviations

BS-79	bottom strand of 79 bp RNA
C104U	domain II L2 single mutant
dsRBD	dsRNA binding domain of PKR
EBER1	noncoding Epstein-Barr virus encoded RNA 1
eIF2α	eukaryotic initiation factor 2 α
G71A/G94A	domain II L3 double mutant
HCV IRES	hepatitis C virus internal ribosome entry site
HIV TAR	human immunodeficiency virus trans activation-response region
IFN	interferon
ISDR	interferon sensitivity-determining region
L4-U5	domain II L4 mutant that replaces AGCCA with UUUUU
NS5A	HCV non-structural protein 5a
p20	20 kDa dsRNA binding domain of PKR
PKR	protein kinase R
poly I	C, polyinosinic:polycytidylic acid (synthetic dsRNA analog)
RNase A	T1, V1, ribonucleases
Rnt1p	RNase III from <i>S. cerevisiae</i>
snR47h	small nucleolar precursor substrate of Rnt1p
TS-79	top strand of 79 bp RNA
5'-UTR	5'-untranslated region
VAI	noncoding virus-associated RNA I from adenovirus
+2bp	domain II P4 mutant that adds two base pairs
Δ2bp	domain II P4 mutant that deletes two base pairs

19 bp	perfectly dsRNA with 19 bp with crystal structure
79 bp	perfectly dsRNA with 79 bp derived from pUC19
ΔL1	domain II mutant that deletes L1
λpp	lambda phosphatase

Fig. 1. Secondary structure of 5'-end of HCV RNA.^{22,23} Shown are nucleotides 1-388, which we refer to as 'full-length HCV IRES RNA'. Domains are numbered in Roman numerals, with domain II boxed. Translation start codon is boxed (domain IV). Inset: Secondary structure of domain II construct employed and determined in this study containing residues 39-119, with the two G nucleotides added at the 5'-end to aid in transcription priming in bold. Pairing (P1, P2...) and loop regions (L1, L2...) in domain II are labeled. Asterisk downstream of domain II indicates the 3'-end of domains I-II (1-130) construct and the 5'-end of domains III-IV (131-388) construct. Domain III is further labeled according to its subdomains IIIa-IIIf.

Fig. 3.

Activation of PKR by multiple domains of HCV IRES. (a) PKR activation assays of domains I-II (1-130) (left panel) and III-IV (131-388) (right panel). RNA concentrations for both panels were 0.3, 0.6, 1.3, 2.5, and 5.0 μM . (b) PKR activation assay of domain II (39-119) alone. RNA concentrations were 0.15, 0.3, 0.6, 1.3, 2.5, 5 and 10 μM . For both (a) and (b), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. A no-RNA lane and 79 bp dsRNA lane are included. Phosphorylation activities are normalized to the 79 bp lane and are noted under the gel. (c) Graphical representation of phosphorylation activities from panels (a) and (b) as a function of RNA concentration.

Fig. 4.

Activation of PKR and phosphorylation of eIF2 α by multiple domains of HCV IRES. (a) PKR/eIF2 α phosphorylation by full-length (1-388) HCV IRES. For both - and + eIF2 α lanes, IRES concentrations were 0.625, 1.25, and 2.5 μM . (b) PKR/eIF2 α phosphorylation by domains III-IV (131-388). For both - and + eIF2 α lanes, domains III-IV concentrations were 0.625, 1.25, 2.5, 5, and 10 μM . (c) PKR/eIF2 α phosphorylation by domain II (39-119). For both - and + eIF2 α lanes, domain II concentrations were 0.625, 1.25, 2.5, 5, and 10 μM . For all panels, 10% SDS-PAGE gels are presented, and positions of phosphorylated PKR (p-PKR) and phosphorylated eIF2 α (p-eIF2 α) are indicated. When present, eIF2 α was in 10-fold excess over PKR. PKR activation for all gels was normalized to the 79 bp (-eIF2 α) PKR band in panel (c), and eIF2 α phosphorylation for all gels was normalized to the 79 bp (+eIF2 α) eIF2 α band in panel (c). These values are provided under the gel.

Fig. 5. Ribonuclease structure mapping of domain II. (a) RNA was 5'-end-labeled and subjected to limited nuclease digestion or hydrolysis. Denaturing 12% polyacrylamide gel is shown. Lanes are as follows: C is a control sample (no nuclease), OH⁻ is a limited alkaline digest, and T1, A, and V1 are limited digests with ribonucleases specific for single-stranded G, single-stranded C and U, and double stranded regions, respectively. Lanes 2 and 3 were performed under RNA-denaturing conditions (denoted "Den."), while lanes 4 and 5 were performed under RNA-native conditions (denoted "Nat."). T1 digestion was also performed under native conditions (not shown). Nucleotides are denoted to the left of the T1 denaturing lane, and pairing and loop regions of domain II are indicated to the right of the gel. (b)

Secondary structure of domain II with positions of cleavage by ribonucleases in panel (a) indicated by symbol. Legend is provided and symbol size is proportional to cleavage intensity.

Fig. 6. Footprinting of p20 onto domain II. (a) Ribonuclease footprinting. RNA was 5'-end-labeled and saturating p20 (10 μM) was added in indicated lanes. This was incubated at room temperature for 30 min followed by digestion with indicated ribonucleases under native conditions. Control lanes were not subjected to nuclease digestion. Denaturing 12% polyacrylamide gel is shown. Lanes are labeled as per Figure 5. Nucleotides are denoted in the T1 denaturing lanes, and regions of domain II are indicated next to sites of cleavage in both the RNase V1 and A digestion lanes. (b) In-line footprinting. Details are as in panel (a) with the following exceptions. RNA was subjected to partial digestion for 40 h at pH 8.3 in the presence of increasing concentrations of p20 (0, 0.625, 1.25, 2.5, 5, 10 μM). NR denotes no reaction. (c) Secondary structure of domain II, with positions of p20-dependent protection from RNase A, RNase V1, and in-line cleavage indicated. Legend is provided in the figure. (d) Stereoview of averaged domain II NMR structure (PDB ID: **1P5P**)⁶², with positions of p20-dependent protection of 2'-hydroxyls from RNase A, RNase V1, and in-line cleavage indicated. 2'-hydroxyls are depicted as spheres and colored as per the legend provided in the figure. In cases where protection from nuclease and in-line cleavage

occurred on the same 2'-hydroxyl (see panel (c)), bases were colored according to nuclease protections for simplicity.

Fig. 7.

Activation of PKR by domain II mutants. (a) Domain II mutants (L4-U5, Δ2bp, +2bp, G71A/G94A, C104U, and ΔL1) superimposed on secondary structure of domain II wild-type (WT). (b) PKR activation assays of P4 and L4 mutants (Δ2bp, +2bp, L4-U5). RNA concentrations for WT and mutants were 0.16, 0.3, 0.6, 1.5, 2.5, and 3.6 μM. Phosphorylation activities for both gels are normalized to 0.01 μM 79 bp in upper gel and are noted under the gel. (c) Graphical representation of phosphorylation activities from panel (b) as a function of RNA concentration. (d) Time course of PKR activation assays for L1, L2, and L3 mutants (ΔL1, C104U, G71A/G94A). Mutants and WT RNA were tested at 1.25 and 5 μM as indicated, and assays were conducted for 3, 5, and 10 min at each RNA concentration. Phosphorylation activities for both gels are normalized to 0.01 μM 79 bp in upper gel and are noted under the gel. (e) Graphical representation of phosphorylation activities from panel (d) as a function of time, at RNA concentration of 5 μM. For both (b) and (d), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated.

Fig. 8.

Native mobility gel-shift assays of NS5A binding to HCV domains. (a) NS5A binding to full-length IRES and domains III-IV. Trace amounts of radiolabeled RNA were incubated with the indicated concentrations of NS5A and analyzed by 6% native PAGE. (b) NS5A binding to domain II. Assay was conducted as in panel (a). (c) NS5A binding to 79 bp dsRNA. Bottom strand 79 bp was radiolabeled (p*BS-79 bp) and pre-annealed to excess unlabeled top strand RNA in all lanes except the first lane. Assay conducted as in panel (a) and analyzed by 8% native PAGE. Anomalous migration of single-stranded p*BS-79 as two bands is not unexpected, as BS-79 bp has the potential for strong self-structure, supported by migration as a single band on a denaturing gel and indirect evidence from *in vivo* experiments.⁷

Fig. 9. Inhibition of PKR activation by NS5A in the presence of domain II, domains III-IV, or 79 bp. (a) Inhibition of domain II- and domains III-IV-mediated activation of PKR by NS5A. Domain II and domains III-IV concentrations were 2.5 μM. NS5A concentrations were 0, 0.125, 0.25, 0.5, 1, 2 and 4 μM. (b) Inhibition of 79 bp dsRNA-mediated activation of PKR by NS5A. The concentration of 79 bp in all lanes was 0.1 μM, and concentrations of NS5A were 0.06, 0.125, 0.5, 1, 2, and 4 μM. For both panels (a) and (b), 10% SDS-PAGE gels are shown, with position of phosphorylated PKR (p-PKR) indicated. Phosphorylation activities were normalized to the lane in which NS5A was absent and are provided under the gels. (c) Graphical representation of the phosphorylation activities in panel (a) as a function of NS5A concentration.

Fig. 10.

Structural comparisons of domain II with AGNN loop and 19 bp dsRNA. (a) Secondary structures of domain II L4 and snR47h RNA apical loop employed for rmsd analysis. Numbering for snR47h is from the reference⁶². At left, domain II structure contains a portion of P4 and L4, depicted in its tetraloop-like form, as described in the text, in which U80 hydrogen bonds with A85 (dotted line), and U86 is extruded from the loop. The boxed region contains all the nucleotides assigned to L4 according to the phylogenetically determined secondary structure (see Fig. 1). Loop nucleotides included in the overlay indicated in red. At right, snR47h RNA AGNN tetraloop, with nucleotides included in the overlay indicated in purple. (b) Overlay of sugar-phosphate backbones from panel (a). Domain II L4 nucleotides AGCC are in red and snR47h AGAA tetraloop are in purple. Numbering for domain II (PDB ID: **1P5P**) is shown adjacent to ribose sugars, with snR47h (PDB ID: **1T4L**) numbering in parenthesis. All atoms shown were included in rmsd calculations. (c) Secondary structures of domain II P2-P4 region and 19 bp dsRNA employed for rmsd analysis. At left are the regions of domain II included in the comparison. The boxed regions contain all the nucleotides assigned to L2 and L3. Pairing within these loops, as described in the text, is denoted by dashed lines. Nucleotides compared in the overlay are described in the Methods section. (d) Stereoview of overlay of sugar-phosphate backbones from panel (c). This structure is sized such that loops and pairing elements approximately align with the secondary structures in panel (c). Lowest energy NMR structure of domain II is used (PDB ID: **1P5O**).³⁹ Domain II truncations are still in red and

19 bp RNA (PDB ID: **1QC0**) is in tan. 2'-hydroxyls are represented by spheres. Brackets indicate positions of L2 and L3, and the arrow indicates the 2'-hydroxyl of domain II G94. The nucleotides in domain II that were excluded from the alignment (A74, G75, C76, G94, and U106) are shown in lavender (see Methods). For clarity, nucleotides 20 and 21 of 19 bp RNA are not shown.

Table 1

Mediation of host and viral translation through NS5A-PKR interactions

Viral life cycle:	Early	Late
NS5A levels:	low	high
PKR activity:	high	low
Host translation:	low	high
Virus translation:	high	high