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Dynamic refolding of IFN-γ mRNA enables it to function as PKR activator and translation template

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Interferon-γ mRNA activates the RNA-dependent protein kinase PKR, which in turn strongly attenuates translation of interferon-γ mRNA. Unlike riboswitches restricted to noncoding regions, the interferon-γ RNA domain that activates PKR comprises the 5′ UTR and 26 translated codons. Extensive interferon-γ coding sequence is thus dedicated to activating PKR and blocking interferon-γ synthesis. This implies that the PKR activator is disrupted by ribosomes during translation initiation and must refold promptly to restore PKR activation. The activator structure harbors an essential kink-turn, probably to allow formation of a pseudoknot that is critical for PKR activation. Three indispensable short helices, bordered by orientation-sensitive base pairs, align with the pseudoknot stem, generating RNA helix of sufficient length to activate PKR. Through gain-of-function mutations, we show that the RNA activator can adopt alternative conformations that activate PKR. This flexibility promotes efficient refolding of interferon-γ mRNA, which is necessary for its dual function as translation template and activator of PKR, and which thus prevents overexpression of this inflammatory cytokine.

The RNA-activated protein kinase PKR is an intracellular sensor of stress. Double-stranded RNA produced during viral replication activates PKR, which phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF2α) to inhibit translation, resulting in apoptosis of infected cells and preventing spread of the virus. Intracellular levels of PKR rise upon exposure to interferons, rendering PKR an essential mediator of interferon-induced antiviral responses[1–4].

Short sequences within mRNA can contribute to inflammatory cytokine gene regulation by activating PKR, as shown for tumor necrosis factor (TNF)-α and interferon-γ (IFN-γ)[5–7]. IFN-γ is a cytokine that is critical for protective immunity yet causes diseases when produced in excess[8–10]. Human IFN-γ mRNA strongly activates PKR, resulting in phosphorylation and inactivation of eIF2α and attenuation of IFN-γ mRNA translation by up to 30-fold[6]. Two distinct properties thus coevolved within this mRNA: encoding IFN-γ and limiting IFN-γ protein production through PKR activation.

Here, we first investigated how a cellular mRNA is able to activate the stress kinase PKR. Except for the essential role of a pseudoknot located within the 5′ untranslated region (5′ UTR)[6], the nature of the IFN-γ RNA activator of PKR has remained unknown. The pseudoknot stem, which is 5–7 base pairs in length[6], cannot bind or activate PKR, as the requirement for binding is minimally 16–18 base pairs of double-helical RNA[11] and the requirement for activation is minimally 33 base pairs of double-helical RNA[12,13]. We show that three additional short helices are essential for PKR activation. Alignment of these helices with the pseudoknot stem creates double-helical RNA long enough to engage the tandem RNA-binding domains of PKR needed for *trans*-autophosphorylation of the PKR dimer and activation[2]. A noncanonical kink-turn within the PKR activator is necessary, apparently to bring remote RNA sequences that form the pseudoknot stem into proximity. These features are phylogenetically conserved.

Riboswitches are noncoding RNA domains, predominantly within the 5′ UTR, that assume alternate structures upon binding specific metabolites[14–19]. Our finding is that the activator of PKR in IFN-γ mRNA comprises not only the entire 5′ UTR but also the first 26 translated codons. This implies that during IFN-γ synthesis, when ribosomes disrupt its structure, the mRNA must undergo dynamic refolding to enable its dual function as translation template and activator of PKR. Through gain-of-function mutations, we demonstrate that the PKR activator structure can adopt alternative RNA conformations in two domains essential for PKR activation. In the predominant structure of the activator, the AUG initiation codon is paired with the 5′ end and juxtaposed directly to the cap, rendering it accessible for translation initiation and concomitant denaturation of the PKR activator. This flexibility promotes facile alternation of the mRNA between two functional states—template for translation and activator of the PKR kinase—thus permitting tight regulation of IFN-γ synthesis.

RESULTS

Structural features of the RNA activator

The ability to activate PKR lies within the 5′-terminal 203 nucleotides harboring the 124-nucleotide 5′ UTR and the start of the IFN-γ open reading frame (ORF)[6]. Structure probing of this RNA fragment by spontaneous cleavage analysis[20] showed that recombinant human PKR (rPKR) induced increased cleavage or protection from cleavage at many

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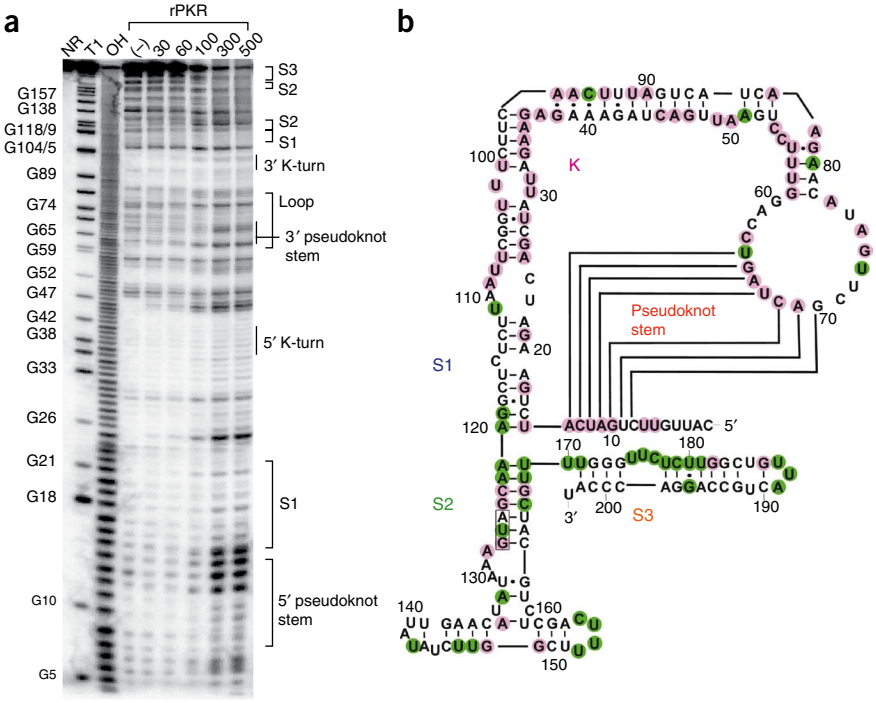


Figure 1 Structure of the PKR activator element in human IFN-γ mRNA. (**a**) In-line structure probing of the 5′-terminal 203 nucleotides of IFN-γ mRNA was done as described in Methods. Gel shows representative pattern of spontaneous cleavages in the 5′ end–labeled RNA incubated alone (–) or with rPKR (nM) as shown. G (T1) and alkali (OH^-) ladders serve as size markers; NR, input RNA. (**b**) Structure supported by repeated in-line probing. Pseudoknot stem and loop, K-turn motif (K), helices S1, S2, S3 and bifurcation loop are shown; AUG codon is boxed. Purple and green colors denote nucleotides showing enhanced and reduced cleavage, respectively, induced by rPKR.

positions in the RNA, which is evidence for direct interaction with the kinase or conformational changes occurring upon binding (**Fig. 1a**,**b**; **Supplementary Fig. 1**). Stacking of the pseudoknot stem with contiguous helix S1 extends the double-stranded structure to 12–15 base pairs (**Fig. 1b**). Helix S2 is formed by pairing of the last four 5′ UTR nucleotides and the AUG initiation codon with seven downstream nucleotides located inside the ORF, whereas helix S3 is generated entirely from ORF sequences. We considered that these four helices could stack to form double-stranded RNA of sufficient length to permit *trans*-autophosphorylation of the PKR dimer needed for its activation.

A-U pair at end of helix S1 is critical for PKR activation

Helix S1 contains an A-U pair at the helical junction. Replacement of this pair by G-C resulted in near-total loss of the ability to activate PKR (phosphorylated 68 kDa band) and phosphorylate eIF2α (38 kDa band) (GC-J, **Fig. 2a**). The PKR band migrated as a doublet, reflecting the fact that phosphorylation of PKR occurs at multiple sites[2]. Disruption of helix S1 in mutant s1b RNA impaired the ability to activate PKR; this was most evident at low RNA concentration. Restoration of an inverted, perfect helix of nine base pairs in s1ab failed to restore full PKR activation (**Supplementary Fig. 2**). These mutations correspondingly enhanced translation efficiency of the intact IFN-γ mRNA in cells (**Fig. 2b**). Mere inversion of the A-U pair sufficed to abolish PKR activation (UA-J, **Fig. 2c**). By contrast, replacement of all of S1 other than this A-U pair left the ability to activate PKR intact (s1T, **Fig. 2d**). Thus, orientation of the A-U pair at the helical junction in S1 is critical for PKR activation. Folding of the RNA requires flexibility in this base pair, probably to permit stacking of helices.

U-A pair at end of helix S3 is critical for PKR activation

Disruption of helix S3 eliminated the ability of the RNA to activate PKR (s3a, **Fig. 2e**). A single nucleotide change, A202U, sufficed to abolish PKR activation. The phenotype of A202U was observed also for full-length IFN-γ mRNA transcript (data not shown). Although U171A retained activity, this mutation did not rescue the phenotype of A202U in U171A A202U, which shows that the U171-A202 pair at the base of helix S3 is orientation sensitive. Hence, helix S3 is essential for PKR activation, and the nucleotides at its base are critical.

The properties of helix S3 show that the PKR activator structure in IFN-γ mRNA extends well beyond the 5′ UTR, reaching 78 nucleotides into the ORF. Thus, IFN-γ mRNA evolved such that its ORF has dual use, allowing the RNA to fold into a conformation that enables PKR activation and translation inhibition in addition to its role as template for IFN-γ synthesis.

A K-turn motif is necessary for PKR activation

A kink-turn (K-turn) is characterized by an asymmetric bulge bordered by two helices, with the potential for non-Watson-Crick base pairing, typically between sheared G•A pairs[21]. Purine-purine pairing between positions 38–39 and 95–96 in the IFN-γ 5′ UTR, though in several aspects distinct from the canonical motif, could kink the RNA by about 120° to bring the remote sequences that create the pseudoknot stem into proximity (**Fig. 1**). Because formation of a K-turn should be sensitive to mutations that affect purine-purine pairing[21], we replaced G38 by C. This single-nucleotide substitution substantially reduced PKR activation at low RNA concentration, leaving residual activity that became apparent only at a higher concentration (**Fig. 3a**). The mutant phenotype was more pronounced when rPKR was used for activation (G38C, **Fig. 3b**). Like dsRNA[2], IFN-γ mRNA activates PKR at low concentrations but inhibits activation at high concentrations[6], apparently because the PKR monomers then bind to different RNA molecules, precluding their dimerization, which is necessary for kinase activation[2]. Translation efficiency of intact G38C mRNA in transfected BHK-21 cells exceeded that of wild-type mRNA by more than sixfold and even surpassed that of d1 mRNA, a mutant almost totally defective in PKR activation[6] (**Fig. 3c**).

Thus, a K-turn motif has a critical role in PKR activation and downregulation of IFN-γ mRNA translation. A G•A/A•G quartet characterizes most K-turns in prokaryotic RNA[21], as distinct from the G•A/A•A motif in human IFN-γ mRNA. Generation of a G•A/A•G quartet in A95G mutant IFN-γ mRNA impaired the ability of the RNA to activate PKR to the same extent as G38C (**Fig. 3a**), showing that the G•A/A•A motif is essential. K-turns typically have G-C pairs bordering the bulge[21], yet PKR activation was not affected by U92C, which converted the G42•U92 pair into G-C, or by G36C, which abolished the G36-C97 pair (**Fig. 3d**).

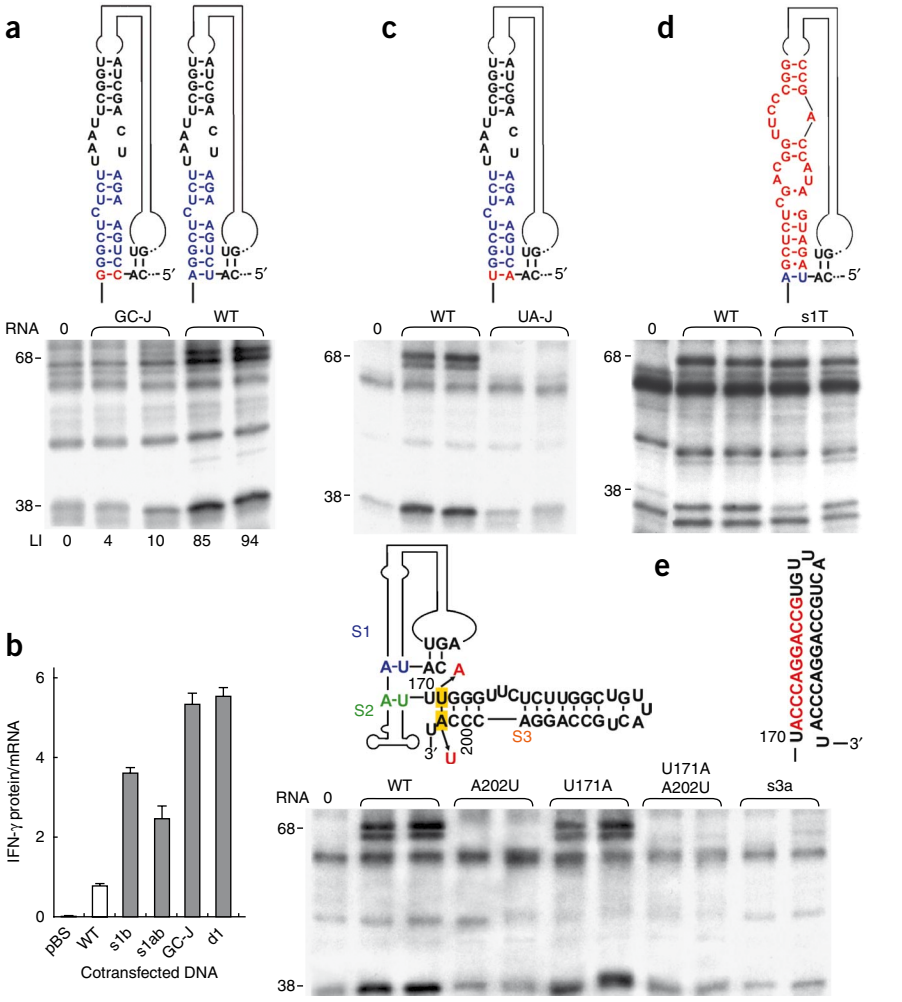


Figure 2 A-U pairs at the base of helices S1 and S3 are critical for PKR activation. (**a**) 203-nucleotide 5′ transcripts were purified, and activation of PKR by 0.1 and 0.25 ng μl^{-1} wild-type (WT) or mutant RNA was assayed, using rabbit reticulocyte ribosomal fraction. Mutated bases in S1 are shown in red. Positions of PKR (68 kDa) and eIF2α (38 kDa) bands are indicated. LI, labeling intensity of PKR band in autoradiogram. (**b**) BHK-21 cells were transfected as described in Methods with 1 μg each of pSV2CAT and pBS, wild-type or mutant phIFN-γ-2 as indicated. d1 lacks ten 5′-terminal nucleotides, carries the A14C mutation and is substantially impaired in PKR activation[6]. IFN-γ and CAT mRNA and IFN-γ protein were quantitated. Relative translation efficiency is expressed in arbitrary units as IFN-γ protein/mRNA ratio ± s.d. A representative of four independent experiments is shown. (**c**) Activation of PKR as in **a**, using WT and UA-J transcript. (**d**) Activation of PKR as in **a**. In s1T, all but the A-U pair at the base of S1 were replaced by a theophyllin aptamer and slippery helix[33]. (**e**) Activation of PKR as in **a**, using WT and S3 mutant transcripts as indicated. Representative experiments are shown.

Alternative RNA conformations in the loop activate PKR

We next mutated stem M3, which borders the loop (**Fig. 4a**, left). The m3b mutant was impaired in PKR activation, whereas m3a and compensatory double mutant m3ab retained activity (**Fig. 4b**,**c**). This asymmetric phenotype suggested that the RNA may fold into alternative conformation aM3 in which loop size would increase by only one nucleotide, through creation of two short stem loops (**Fig. 4a**, right). In that case, m3a should not affect RNA conformation, whereas m3b should extend the loop size to 30 nucleotides. To examine this point, we created m3c, which compensates m3b in the aM3 conformation by restoring base pairing yet resides within the loop in the M3 conformation. m3c restored the ability of m3b to activate PKR (m3bc, **Fig. 4c**,**d**). m3c activated PKR as expected, as it should not affect loop size in the M3 conformation.

These results support the notion that alternative RNA conformations M3 and aM3 exist, through the activation of PKR by m3c and m3ab, accounted for only in the M3 conformation, and by m3a and m3bc, accounted for only in the aM3 conformation. Because U56 through U58 appear unpaired in structure probing, whereas G59 through C62 and G74 are paired (**Fig. 1**), the aM3 conformation is predominant.

We created deletions vg1 and vg2, which eliminate either of the short stem loops in aM3 without affecting loop size. In the M3 conformation, vg1 and vg2 increase loop size to 30 and 31 nucleotides, respectively, and accordingly should impair activity, as for m3b. However, vg1, vg2 and double mutant vg12 (**Fig. 4a**, right) retained most of the ability of the RNA to activate native rabbit PKR (**Fig. 4e**) and activated rPKR at least as effectively as the wild type (**Supplementary Fig. 3**), further supporting alternative conformation aM3. The stem loops flanking the pseudoknot loop are dispensable for PKR activation.

The notion that excessive length of the pseudoknot loop impairs PKR activation is supported by several results. Removal of the two short stem loops left pseudoknot loop size and activation of PKR intact (**Fig. 4e**; **Supplementary Fig. 3**). Mutation m3b opens one short stem and adds 11 nucleotides to the pseudoknot loop, causing loss of PKR activation, which could be rescued by restoring the stem loop in m3bc (**Fig. 4c**,**d**). By contrast, m3a leaves the stem loops largely intact and thus retains pseudoknot loop size and remained able to activate PKR (**Fig. 4a**–**c**).

Alternative RNA conformations in helix S2 activate PKR

Disruption of base pairing in helix S2 abolished the ability of the RNA to activate PKR (s2a, **Fig. 5a**). Restoration of base pairing by an inverted helix failed to restore activity (s2ab). However, s2abAU-J regained the ability to activate PKR, showing that orientation of the apical A121-U169 pair is critical (**Fig. 5b**). S2 contains ORF sequences, including the AUG initiation codon, which is dispensable for PKR activation (s2i and s2abAU-J). The bifurcation loop adjoining the AUG codon (**Fig. 1**) is also expendable: PL RNA, in which this loop was replaced by a pentaloop, activated PKR nearly as well as wild-type RNA (**Fig. 5a**).

In contrast to s2a, mutation of the complementary strand in S2 left the ability of the RNA to activate PKR intact (s2b, **Fig. 5a**). This asymmetric phenotype indicated a potential for alternative base pairing. Because the leading strand of S2 is also complementary to the 5′-terminal seven nucleotides of IFN-γ mRNA (**Fig. 5c**), formation of alternative helix aS2 might explain the ability of s2b to activate PKR. To test this, we created as2a, in which the leading strand of S2

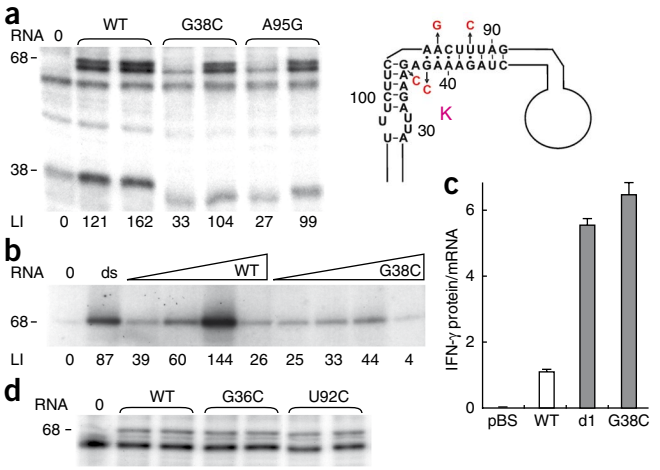


Figure 3 A K-turn motif is essential for PKR activation. (**a**) 203-nucleotide 5′ transcripts of wild-type and mutant IFN-γ mRNA were purified, and activation of PKR by 0.1 and 0.25 ng μl^{-1} RNA was assayed, using rabbit reticulocyte ribosomal fraction. Mutated bases are shown in red. (**b**) rPKR (100 ng per lane) was used for activation by wild-type or G38C RNA transcript at 0.05, 0.1, 0.25 and 0.5 ng μl^{-1}; ds, poly (rI:rC) (0.25 ng μl^{-1}). LI, labeling intensity of PKR band in autoradiograms. (**c**) Translation efficiency of wild-type and mutant IFN-γ mRNA in transfected BHK-21 cells was determined as for **Figure 2b**. Relative translation efficiency is expressed in arbitrary units as IFN-γ protein/mRNA ratio ± s.d. (**d**) Activation of PKR by 0.025 and 0.05 ng μl^{-1} wild-type or mutant RNA transcripts was assayed as in **a**.

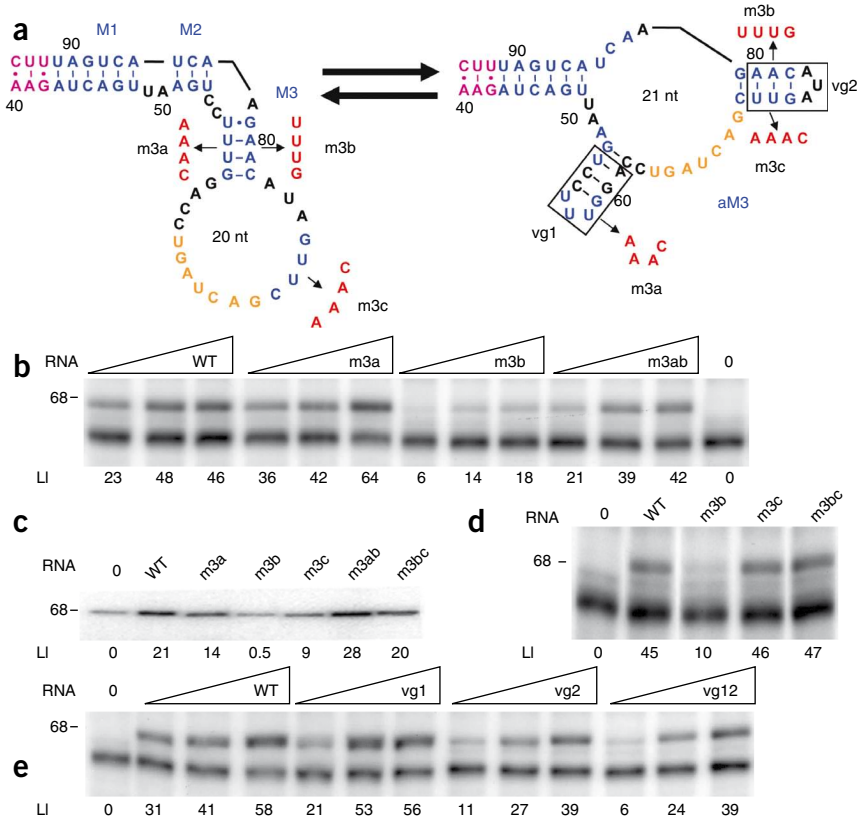
cannot pair with the opposite strand or with the 5′ end. This mutation substantially reduced PKR activation, whereas compensatory mutation of the 5′-terminal six nucleotides restored it (as2ab, **Fig. 5c**,**d**). Mutation of the 5′ end alone leaves S2 intact. Indeed, as2b retained partial activity, showing that helix S2 suffices for PKR activation. We conclude that to activate PKR, human IFN-γ mRNA must form either of two alternative helices: S2 or aS2.

We next analyzed the distribution of the mRNA between S2 and aS2 conformations by structural analysis. The downstream strand of S2 should be paired in aS2b RNA but not in aS2ab RNA, and in wild-type RNA it should be paired only in the S2 conformation. Indeed, the core of this sequence, nucleotides 164–168, was protected from spontaneous cleavage in aS2b RNA but accessible in aS2ab RNA (**Fig. 5e**). In wild-type RNA, these nucleotides were relatively accessible, supporting a predominance of the aS2 conformation.

In S2 and aS2, the apical A-U pairs are essential for PKR activation. The A121U mutation, which destroys both base pairs, did not activate PKR (**Supplementary Fig. 4a**,**b**). U169A abrogated PKR activation (**Supplementary Fig. 4b**), as did U7A (**Fig. 5f**), which led to a sixfold enhancement of mRNA translation (**Fig. 5g**). A121U failed to rescue U169A or U7A (**Supplementary Fig. 4b**), showing that both apical A-U pairs are orientation sensitive.

Even when PKR activation is through helix S2, the 5′ end remains essential. Deletion of the first six nucleotides (d6) resulted in attenuation, with truncation through U7 (d7) inducing complete loss of PKR activation (**Fig. 5h**). Thus, the free 5′ end has a role in PKR activation independent of aS2 formation; it probably stabilizes the adjacent pseudoknot stem when the RNA is in the S2 conformation.

Although PKR can be activated by short stem loop RNA in a 5′-triphosphate–dependent manner[22], our results do not support this mechanism for IFN-γ mRNA. Activation of PKR by *in vitro* transcribed IFN-γ RNA was resistant to alkaline phosphatase treatment (**Supplementary Fig. 5**), and PKR-dependent translational repression of capped mRNA was observed in transfected cells (**Figs. 2b**, **3c** and **5g**).



Phylogenetic conservation of the RNA activator

Structure probing supports a predominance of alternative conformations aM3 and aS2 in the RNA activator of PKR in human IFN-γ mRNA (**Figs. 1** and **5e**). In this secondary structure, four short helices can be aligned: S1, the pseudoknot stem, aS2 and S3 (**Fig. 6a**). This alignment creates helical

Figure 4 Alternative RNA conformations in the loop mediate PKR activation. (**a**) Alternative folding of stems M1, M2, M3 and aM3, colored blue. Pseudoknot loop size is indicated; nucleotides that pair in the pseudoknot stem are shown in orange. Mutated bases are shown in red. Boxes mark deletions vg1, vg2 and double deletion vg12 in the aM3 conformation. (**b**) 203-nucleotide wild-type 5′ transcript of IFN-γ mRNA and corresponding mutant transcripts were purified, and activation of PKR by 0.05, 0.1 and 0.25 ng μl^{-1} RNA was assayed, using rabbit reticulocyte ribosomal fraction. (**c**) rPKR (100 ng per lane) was used for activation by 0.1 ng μl^{-1} wild-type or mutant RNA transcripts. (**d**) Activation of PKR by 0.25 ng μl^{-1} wild-type or mutant RNA transcript was assayed as in **b**. (**e**) Activation of PKR by 0.05, 0.1 and 0.25 ng μl^{-1} wild-type or mutant RNA transcript was assayed as in **b**. LI, labeling intensity of PKR band in autoradiograms.

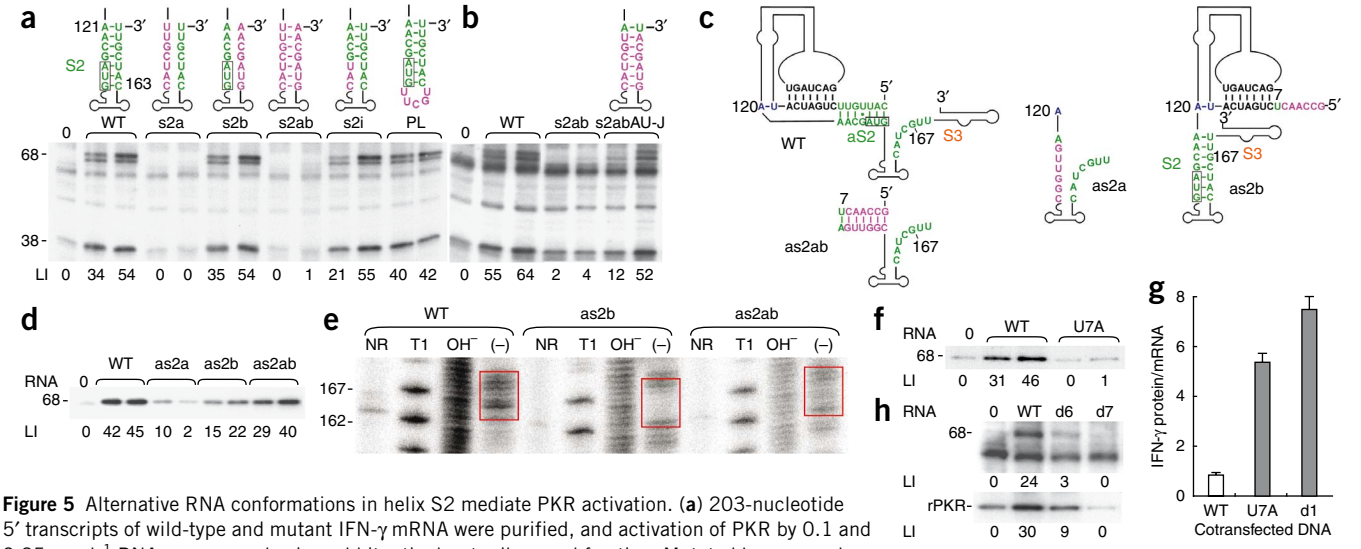


Figure 5 Alternative RNA conformations in helix S2 mediate PKR activation. (**a**) 203-nucleotide 5′ transcripts of wild-type and mutant IFN-γ mRNA were purified, and activation of PKR by 0.1 and 0.25 ng μl^{-1} RNA was assayed using rabbit reticulocyte ribosomal fraction. Mutated bases are shown in magenta. The AUG initiation codon is boxed. (**b**) Activation of PKR by 0.1 and 0.25 ng μl^{-1} wild-type or mutant RNA transcript was assayed as in **a**. Mutated bases are shown in magenta. (**c**) Alternative helices aS2 and S2, colored green. Mutated bases are shown in magenta. (**d**) rPKR (100 ng per lane) was used for activation by 0.1 and 0.25 ng μl^{-1} RNA transcripts shown in **c**. (**e**) In-line structure probing of the downstream strand of S2 in 5′ end–labeled wild-type and mutant RNA transcripts was done as in **Figure 1**. (**f**) rPKR (100 ng per lane) was used for activation by 0.1 and 0.25 ng μl^{-1} RNA transcript. (**g**) Translation efficiency of wild-type and mutant IFN-γ mRNA in transfected BHK-21 cells was determined as for **Figure 2b**. Relative translation efficiency is expressed in arbitrary units as IFN-γ protein/mRNA ratio ± s.d. (**h**) Activation of PKR by 0.1 ng μl^{-1} wild-type, d6 or d7 RNA transcript was assayed using rabbit reticulocyte ribosomal fraction (upper panel) or rPKR (100 ng per lane) (lower panel). LI, labeling intensity of PKR band in autoradiograms.

RNA of about 33 base pairs, achieving the minimum length shown to activate PKR[12,13]. The AUG initiation codon pairs with 5′-terminal nucleotide adjoining the cap, leaving no free 5′ end. This AUG is embedded within an extended double-stranded domain. Orientation-sensitive A-U pairs are located at each joint between the four helices: A120-U15 and A121-U7 may allow alignment of S1 and aS2 with the pseudoknot stem, and A202-U171 may allow alignment of S3 with aS2. The bifurcation loop and its apical A-U helix stabilize alignment of S3 with aS2. The apical A-U helix A is supported by the loss of PKR activation exhibited by U169A RNA and U170A RNA (**Supplementary Fig. 4b**,**c**). Moreover, A128–A130 were relatively resistant to spontaneous cleavage (**Supplementary Fig. 1**).

The overall features of the 5′-terminal structure, including the K-turn, apical loop and helical domain formed by the pseudoknot stem and helices S1, aS2 and S3 bordered by A-U pairs, are conserved in IFN-γ mRNA of higher mammals (**Fig. 6a**,**b**; **Supplementary Fig. 6**). The potential to form stem loops in aM3 and the bifurcation loop with its apical helix A is also conserved. The K-turn, with its G•A/A•A quartet and adjacent non-Watson-Crick A40•C94 pair, is highly conserved, as is the potential for forming a new, long-range G42•A95 pair, which is facilitated upon kinking[23] and will stabilize the K-turn. G36C remained able to activate PKR (**Fig. 3d**); indeed, G36 is not conserved. Porcine IFN-γ mRNA also shows structural conservation; it activates PKR through the pseudoknot and induces eIF2α phosphorylation (**Fig. 6b**). Activation was attenuated by introducing a G•U pair in the pseudoknot stem (C68U), abolished when either central nucleotide was mutated and restored by compensatory mutation. The potential for alternative conformations shows high phylogenetic conservation (**Fig. 6**; **Supplementary Figs. 6** and **7**). A124-U4 pairing renders marmoset aS2 more stable than human aS2 (**Supplementary Fig. 6a**,**b**). Bovine and canine aS2 (**Supplementary Fig. 6c**,**d**) should be more stable than S2 (**Supplementary Fig. 7c**,**d**).

Mouse IFN-γ mRNA lacks sequence homology with other mammalian mRNAs, yet its 5′-terminal 208 nucleotides can be folded similarly, albeit without a K-turn and without potential for aS2 formation (**Fig. 6c**). Bioinformatic analysis indicated that GenBank cDNA entries for mouse mRNA (M28621 and K00083) did not reach the true 5′ end. Indeed, the equivalent 5′ transcript failed to activate PKR significantly (d17). We extended the 5′ end by 17 nucleotides based on the M28381 genomic sequence to yield an mRNA that can form a pseudoknot. The extended RNA vigorously activated PKR (wild type) (**Fig. 6c**). As for human IFN-γ mRNA[6], deletion of 5′-terminal nucleotides destroyed the pseudoknot necessary for PKR activation.

The structures in **Figs. 1** and **6a** represent alternative, functional conformations of the PKR activator (**Fig. 6d**).

DISCUSSION

Human IFN-γ mRNA attenuates its own translation through an unusual mechanism: the activation of PKR[6]. The mRNA contains a more potent activator of PKR than double-stranded RNA (**Fig. 3b**). Although activation of PKR by IFN-γ mRNA depends strictly on a pseudoknot[6], its short helix, which is five to seven base pairs long, can fulfill only part of the need for double-helical RNA in kinase activation, which raises the question of how this cellular mRNA activates PKR. We show that the RNA activator contains three short helices that align with the pseudoknot stem, each of which is essential for PKR activation (**Fig. 6a**). This alignment generates double-stranded RNA of sufficient length to bind the tandem RNA-binding domains needed for *trans*-autophosphorylation of the PKR dimer and PKR activation. Throughout its 203-nucleotide sequence, the PKR activator is sensitive to single nucleotide changes. These structural features show phylogenetic conservation among higher mammals, but they are most perfectly paired in the human mRNA. Apparently, IFN-γ mRNA evolved to acquire optimal ability to activate PKR, as we did not find any mutation that enhanced PKR activation.

Unlike riboswitches triggered upon binding of specific metabolites, which are confined to noncoding regions in mRNA or pre-mRNA[13,16],

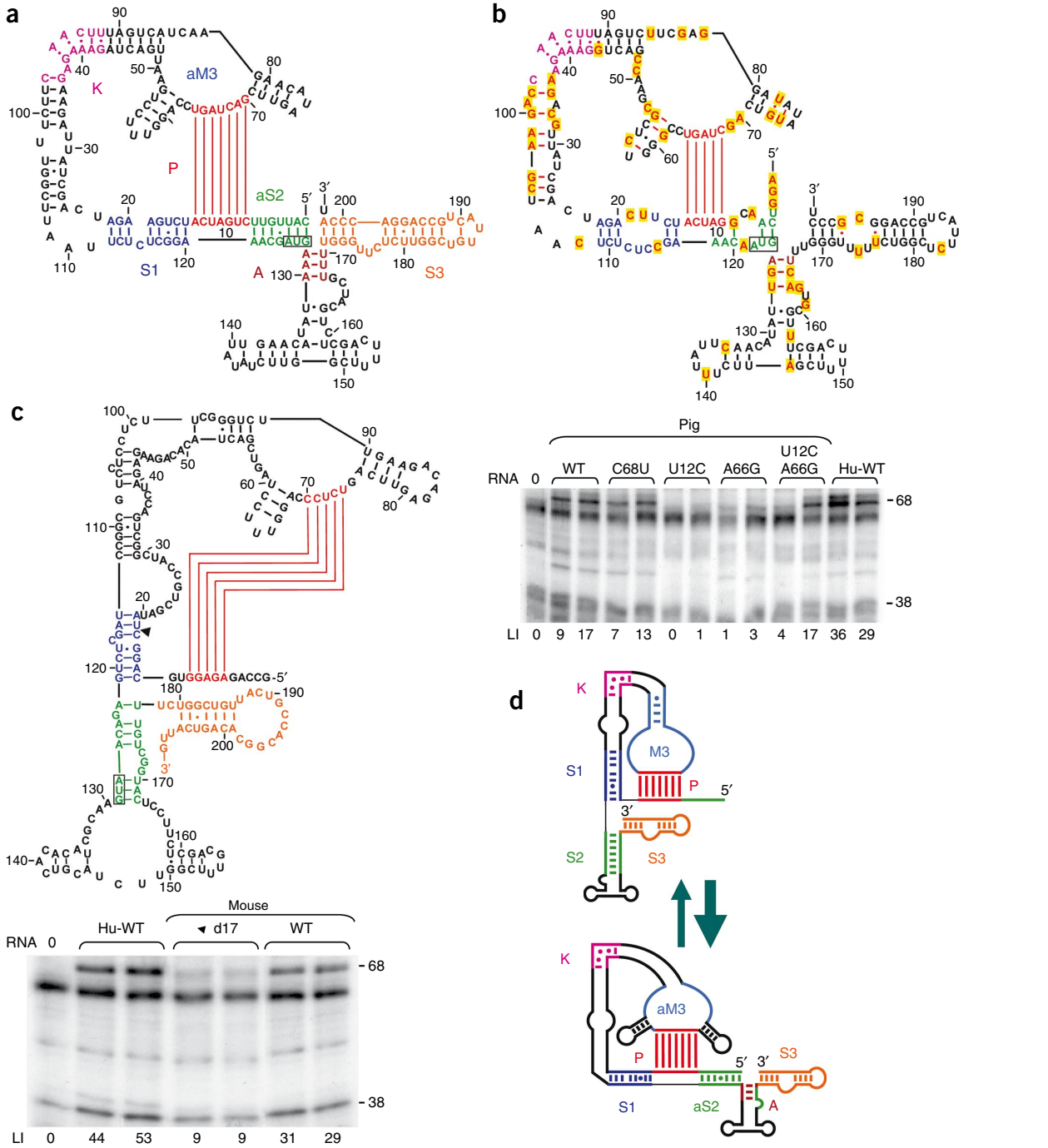


Figure 6 Phylogenetic conservation of the RNA activator of PKR in IFN-γ mRNA. (**a**,**b**) 5′-terminal sequences of *Homo sapiens* IFN-γ mRNA (**a**) and of *Sus scrofa* IFN-γ mRNA folded as for the human sequence (**b**). Pseudoknot stem (P), K-turn motif (K), helices S1, aS2, S3 and A are shown, with the pseudoknot loop in the aM3 conformation. Red color on yellow background denotes nucleotides that differ from the human sequence. (**b**) Wild-type and mutant 200-nucleotide *Sus scrofa* IFN-γ mRNA 5′ transcripts were purified, and activation of PKR by 0.1 and 0.25 ng μl^{-1} RNA was assayed using rabbit reticulocyte ribosomal fraction. Hu-WT, human wild-type control 203-nucleotide 5′ transcript. A representative experiment is shown. (**c**) *Mus musculus* IFN-γ mRNA folded as for the human sequence in **Figure 1**. Mouse 208-nucleotide (wild-type) and 191-nucleotide (d17) IFN-γ mRNA 5′ transcripts were purified, and activation of PKR was assayed as in **b**. A representative experiment is shown. (**d**) Alternative conformations of the activator of PKR in IFN-γ mRNA. Schematic representation of the alternative helical stacks that lead to PKR activation. Needed for PKR activation and indicated in color are pseudoknot stem P, kink-turn K, helices S1, S2, aS2 and S3, apical helix A and pseudoknot loop domains M3 and aM3.

the human IFN-γ mRNA structure that binds and activates PKR not only comprises the full 5′ UTR but also reaches deeply into the protein-coding region. A significant portion of the IFN-γ ORF is thus dedicated to PKR activation, resulting in inhibition of IFN-γ synthesis. This result implies that the PKR activator domain is disrupted by ribosomes during initiation of translation and must refold promptly to restore PKR activation. Our data support the concept that once the ribosome has moved on, the PKR activator is regenerated rapidly. Thus, even before translation by a given ribosome has been completed, activation of PKR may resume. This can account for the

strong translational inhibition observed for wild-type IFN-γ mRNA (**Figs. 2b**, **3c** and **5g**)[6]. Through gain-of-function mutations, we provide direct evidence that the PKR activator can adopt alternative, active conformations in the pseudoknot loop and in the helix harboring the AUG initiation codon (**Fig. 6d**). This flexibility promotes the efficient refolding of the IFN-γ mRNA domain that is necessary for facile alternation between its dual functional states—template for protein synthesis and activator of PKR.

Alternative pairing of the leading S2 strand with the first seven nucleotides of IFN-γ mRNA not only generates a helix that can stack onto the pseudoknot stem (aS2, **Figs. 5** and **6a**) but also places the AUG codon in the center of an extended double-stranded region and in direct juxtaposition to the cap. This placement renders the start codon readily accessible to the initiating 40S ribosomal subunit, ensuring that the PKR activator structure is denatured during the initiation step and facilitating its transition to translation template.

Each of the helices S1, S2, aS2 and S3 (**Figs. 1b** and **6a**) carries at one end an orientation-sensitive A-U pair that is critical for PKR activation. These A-U pairs may be recognized by PKR through RNA-protein contacts; indeed, PKR protected at least one of the nucleotides in each pair from cleavage (**Fig. 1b**). The A-U pairs may impart the flexibility that is necessary for stacking of the helices and the pseudoknot stem. If they undergo a complex three-way interaction, it is unlikely to be Watson-Crick-Hoogsteen pairing in *trans*[24,25], because noncanonical reverse Watson-Crick pairs do not occur adjacent to a G-C Watson-Crick pair as present in S1, S3 and at the junction of aS2 with the pseudoknot stem; but require additional bulged nucleotides between the Watson-Crick pair and the U or A position[26].

We describe a K-turn motif whose proposed role is to kink the RNA, thereby permitting strand annealing between remote sequences that form the pseudoknot stem. Nucleotides in this stem are relatively prone to spontaneous cleavage (**Fig. 1**), which is indicative of weak base pairing. Kinking of the RNA may overcome a critical energy threshold to permit pseudoknot stem formation. The potential for purine-purine pairing in a bulge, which is characteristic of K-turns[21], is present in the IFN-γ 5′ UTR, but the K-turn motif lacks the typical adjacent inverted G•A pairs. Instead, a G•A/A•A quartet is phylogenetically conserved. Single nucleotide changes in this quartet substantially reduced the ability of the RNA to activate PKR, as did the creation of a canonical G•A/A•G quartet. Unlike a standard K-turn that bends toward the minor groove, a noncanonical G•A/A•A quartet in the J9/9.0 junction of the *Azoarcus* group I intron bends toward the major groove in a reverse K-turn[27]. The G•A/A•A motif may impart sufficient instability to allow for facile alternation of IFN-γ mRNA between its dual states—translation template and activator of PKR. Although K-turns often are recognized by RNA-binding proteins[21], most of the nucleotides in the K-turn region became more prone to cleavage when PKR was present (**Fig. 1**). This may be explained by PKR binding elsewhere and inducing a conformational change in the K-turn region that enhances cleavage. Binding of PKR may stabilize the K-turn to transform the mRNA into a PKR activator.

Despite extensive lack of sequence homology, the ability to activate PKR through a pseudoknot is conserved in mouse IFN-γ mRNA (**Fig. 6c**). The mouse PKR activator resembles the human activator in the S2 conformation including the stem loops typical of aM3, yet cannot form the K-turn and alternative helix aS2. Rodents diverged early during the mammalian radiation[28,29], suggesting that the potential for alternative folding into the aS2 conformation developed later and that the S2 conformation is the primordial structure. The K-turn also may have evolved late—probably to facilitate efficient pseudoknot formation during dynamic refolding of the IFN-γ mRNA translation template. These additional structural elements in the PKR activator promote effective attenuation of IFN-γ production during immune responses.

Through the RNA element characterized here, IFN-γ mRNA activates PKR, thus inducing eIF2α phosphorylation and inhibiting its own translation (**Figs. 2b**, **3c** and **5g**). During the immune response, basal PKR levels permit translation of newly induced IFN-γ mRNA. As IFN-γ and other inflammatory cytokines build up in the cell's microenvironment, they induce PKR to higher levels[1]. More extensive PKR activation by IFN-γ mRNA intensifies attenuation of IFN-γ production. Translational regulation does not result from repression upon binding of PKR to IFN-γ mRNA but from eIF2α phosphorylation by the activated kinase, because translation is restored in full by co-expression of the nonphosphorylatable mutant eIF2α S51A[6]. IFN-γ mRNA induces a local activation of PKR rather than a global inhibition[6]. Therefore, during an immune response the translation of other mRNA species should not be affected by IFN-γ mRNA.

The unusual structure of an RNA activator of PKR in a cellular mRNA apparently evolved to permit IFN-γ expression to levels that allow for protective immunity yet avoid excess associated with pathology. Activation of PKR by mRNA may be more ubiquitous. A *cis*-acting element in the TNF-α 3′ UTR is a PKR activator that renders mRNA splicing dependent on PKR activation[5]. Use of PKR activators within mRNA as controlling elements extends the range of mechanisms regulating inflammatory cytokine gene expression[7].

METHODS

Bifunctional expression vector for human IFN-γ mRNA. phIFN-γ-2 carries human IFN-γ cDNA under the T7 promoter within the myeloproliferative sarcoma virus (MPSV) LTR such that transcripts initiated in cells differ at the 5′ end by one additional nucleotide from *in vitro* transcripts; 3′ end generation is directed by the SV40 late polyadenylation sequence[6]. phIFN-γ-2 DNA was used directly to express IFN-γ mRNA with authentic 3′ sequence and poly(A) in transfected cells from the MPSV promoter. 5′-terminal 203-nucleotide T7 transcripts for activation of PKR were generated with a T7 transcription kit (Promega) using a PCR primer pair in which the 5′ primer contained the T7 promoter.

Mutant IFN-γ mRNA. To generate mutations in human IFN-γ mRNA, phIFN-γ-2 was used as template for PCR using phosphorylated mutant DNA primers and KOD polymerase (Novagen) to generate linear full-length plasmid DNA carrying blunt ends; the product was purified on a 1% agarose gel and self-ligated. Primer sequences will be supplied on request. DNA sequencing was used to verify the constructs.

Porcine and **murine IFN-γ mRNA.** Porcine IFN-γ cDNA[30] was provided by A. Billiau (Catholic University of Leuven). Mutations in porcine IFN-γ mRNA were generated as described for the human mRNA. Murine genomic DNA was purified from blood using MasterPure (Epicentre), and 5′-terminal IFN-γ sequence was cloned into pGEM-T (Promega) using PCR primers and a ReddyMix polymerase kit (ABgene) as specified by the manufacturers. T7 transcripts were generated as for human IFN-γ mRNA.

Activation of PKR. Uncapped RNA transcripts were purified twice by Sephadex G-50 gel chromatography followed by chromatography on CF-11 cellulose, washing with ethanol and eluting with water as described[31]. A rabbit reticulocyte ribosomal pellet was prepared, and activation of PKR was assayed using 2 μl of ribosome fraction as described[6], except that mixtures (20 μl) were resolved by SDS-10% PAGE. To express rPKR in a form that can be activated by RNA, human PKR cDNA was inserted into pHTT7K[32] between the NdeI and PstI sites and λ protein phosphatase cDNA (New England Biolabs) at the PstI site; PKR was expressed in *Escherichia coli* Rosetta(DE3)pLysS (Invitrogen) as full-length N-terminally hexahistidine-tagged protein. PKR was recovered from inclusion bodies by solubilization in 6 M urea buffer and loaded onto a His·Bind column (Novagen) that was eluted stepwise with imidazole. PKR recovered after dialysis was >98% pure on SDS-12% PAGE.

Activation of rPKR by RNA was determined in the presence of [γ-^{32}P]ATP in 50 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM $MgCl_2$ and 0.02 mM unlabeled ATP; mixtures (10 μl) were incubated for 20 min at 25 °C and resolved by SDS-10% PAGE. A master mix of rPKR or reticulocyte ribosome fraction and labeled ATP was distributed to all reaction vessels, and only the RNA was added individually. Data shown are representative and have been reproduced at least three times using independent RNA preparations. Labeling intensity of the PKR band in autoradiograms, in arbitrary units, was quantitated using TotalLab software (Nonlinear Dynamics), subtracting the signal generated upon incubation without RNA.

Translation efficiency in transfected cells. BHK-21 cells were transfected with >75% efficiency as described[6] except that a mixture of 1.4 μg of phIFN-γ-2 or pBS DNA and 0.6 μg pEGFP-N3 DNA (Clontech) was used for transfection. IFN-γ mRNA[10] and EGFP mRNA as internal standard were measured by RNase protection analysis. Secreted IFN-γ was quantitated in 45-h culture medium by ELISA (DuoSet, R&D Systems). Translation efficiency was expressed as ratio of IFN-γ protein over IFN-γ mRNA.

In-line structure probing of RNA. For in-line probing of the 5′-terminal 203 nucleotides of IFN-γ mRNA, we adapted previously described protocols[12,20]. About 1 nM 5′ ^{32}P-labeled RNA was incubated for 40 h at 25°C in 20 mM $MgCl_2$/50 mM Tris-HCl pH 8.3, 100 mM KCl, 1.5 mM dithiothreitol and 6% (v/v) glycerol in the absence or presence of rPKR. RNA cleavage products were resolved by 10% PAGE for 2–18 h.

Accession codes. Entrez Nucleotide: IFN-γ DNA sequences were deposited as part of previous studies for *Homo sapiens* (J00219), *Callithrix jacchus* (X64659), *Bos taurus* (Z54144), *Sus scrofa* (S63967), *Canis familiaris* genomic contig (NW_876250) and *Mus musculus* (M28621, K00083, M28381).

Note: Supplementary information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

S.C.-C. performed in-line structure probing and cloned mouse IFN-γ DNA; S.C.-C., A.H., D.W. and F.O. generated mutant RNA transcripts; L.S.N. and F.O. expressed recombinant PKR; R.K. and Y.B. prepared rabbit reticulocyte ribosomes; A.H., Y.B., D.W. and S.C.-C. assayed PKR activation; A.H. and Y.B. measured translation efficiency in cells; R.K. and S.C.-C. designed the study.

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