HCV IRES manipulates the ribosome to promote the switch from translation initiation to elongation

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The internal ribosome entry site (IRES) of the hepatitis C virus (HCV) drives noncanonical initiation of protein synthesis necessary for viral replication. Functional studies of the HCV IRES have focused on 80S ribosome formation but have not explored its role after the 80S ribosome is poised at the start codon. Here, we report that mutations of an IRES domain that docks in the 40S subunit's decoding groove cause only a local perturbation in IRES structure and result in conformational changes in the IRES–ribosome 40S subunit complex. Functionally, the mutations decrease IRES activity by inhibiting the first ribosomal translocation event, and modeling results suggest that this effect occurs through an interaction with a single ribosomal protein. The ability of the HCV IRES to manipulate the ribosome provides insight into how the ribosome's structure and function can be altered by bound RNAs, including those derived from cellular invaders.

HCV is the most common blood-borne virus in the United States and is estimated to infect ~3% of the world’s population. HCV’s genome is a single-stranded, positive-sense RNA molecule; thus, after release of the genome into the cytoplasm, the first step of viral replication is translation of a single open reading frame to yield the viral proteins. The HCV genomic RNA is therefore similar to cellular mRNA, serving as the template for protein synthesis by translation machinery. However, unlike cellular mRNAs, which originate in the nucleus and are capped and polyadenylated on their 3′ ends, HCV uses an IRES RNA at the 5′ end of the viral genome to hijack the cellular translation machinery. The IRES sequence is highly conserved among HCV isolates and genotypes. This conservation underscores the importance of the HCV IRES RNA to the viral replication cycle and reflects the specificity of the interactions between the IRES RNA and the translation machinery.

Mechanistic studies of the HCV IRES have focused on how it assembles an 80S ribosome at the AUG start codon, and they have revealed a mechanism different from canonical translation initiation. In the canonical pathway, 43S particle binding causes a local perturbation in IRES and ribosome structure. The AUG in the 40S subunit decoding groove is recognized by the eukaryotic initiation factor 4F (eIF4F) complex, followed by binding of the 43S particle to the 40S subunit and eIF3. Subsequent GTP hydrolysis, eIF release and binding of a 60S subunit yield an 80S ribosome placed directly at the start codon. In addition, the HCV IRES can, under conditions of cellular stress, use eIF2-independent pathways to generate 80S ribosomes.

The function of HCV IRES is conferred by its structure. The IRES adopts an extended global architecture, within which specific RNA structural domains drive different stages of 80S ribosome formation. The IRES RNA is thus an active manipulator of the translation machinery, not just a binding site for the ribosome and factors.

Among the aforementioned IRES domains, dII is particularly notable: it induces changes in the conformation of the 40S subunit, and it docks within the ribosome's decoding groove to interact with ribosomal protein rpS5. This is known to contact E-site tRNA, and we therefore set out to study the role of dII subdomain dIIb, the part of the IRES that penetrates deep into the ribosome's decoding groove. We have discovered that dIIb stabilizes the IRES–40S subunit complex and inhibits the first round of ribosomal translocation. The modeled position of this domain (adjacent to rpS5) suggests this effect may be due to altered contact with rpS5. This is, to our knowledge, the first evidence that a step after 80S ribosome assembly is directly
influenced by the HCV IRES, and it may have implications for the understanding of translation initiation in general.

**RESULTS**

**IRES dIIb affects the rate of protein synthesis**

We have previously reported that mutation of the dIIb apical loop (Fig. 1d) changes the configuration of the HCV RNA in the decoding groove of 40S subunit–HCV IRES RNA complexes and reduces IRES-driven protein synthesis. This is consistent with other studies showing that dIIb is important for HCV IRES–mediated translation initiation. To monitor protein production as a function of time, we measured the ability of three IRES RNAs in which dIIb was mutated to translate a downstream luciferase (LUC) reporter sequence (uncapped and not polyadenylated) in rabbit reticulocyte lysate (RL) reporter over a 90-min time course (Fig. 1d,e). All three dIIb mutants produced LUC at a rate that was slower than that of wild-type (WT) IRES RNA but faster than that of an IRES mutant lacking dII (ΔIII). We observed differences in total LUC production after only 15 min (Fig. 1f), and the mutants continued to make protein at a roughly constant rate but more slowly than WT. These results indicate that the activity of the IRES with dIIb mutations is inhibited but not abolished. Assuming identical rates of elongation and ribosome termination on each RNA sample, we conclude that the dIIb mutations slow, but do not halt, translation initiation.

**IRES–40S subunit complex conformation is influenced by dIIb**

Cryo-EM reconstructions of HCV IRES–40S subunit complexes show that binding of full-length WT HCV IRES induces structural changes in the subunit, but the ΔIII mutant does not cause this conformational change. This raised the question of what the conformation of the 40S subunit would be when bound to the dIIb mutant RNAs generated for this study. To answer this, we visualized IRES RNAs with the apical GCC (nucleotides 82–84) in dIIb deleted (ΔGCC) in complex with the 40S subunit by EM, using both negative staining (to assess sample purity, homogeneity and concentration) and cryo-EM (to generate a three-dimensional (3D) structure) (Fig. 2a). We obtained the reconstruction of the complex at a resolution comparable to previous HCV IRES–40S subunit reconstructions (17–20 Å). Comparison of the ΔGCC IRES–40S subunit and WT IRES–40S subunit structures revealed notable differences in the position and orientation of IRES domains and in the conformation of the 40S subunit (Fig. 2b). Specifically, in the mutant, dII did not loop away from the subunit’s surface to contact the side of the head and enter the decoding groove; rather, it lay across the platform. This change in the position of dII was accompanied by a rotation of the overall IRES orientation relative to the body of the 40S subunit. This cryo-EM structure does not eliminate the possibility that the IRES location and conformation is an average of several similar structures. In other words, the GCC deletion might cause the conformation of the IRES and its position on the 40S subunit to be more dynamic, which could explain why structural features visible in the WT IRES are not seen in the ΔGCC IRES and why the IRES appears to be rotated relative to the subunit. However, this possibility does not affect the conclusion that this relatively small deletion mutation at the tip of dII alters the IRES–40S subunit interaction, which is consistent with our observation that the ΔGCC mutation lowers, but does not eliminate, IRES activity. When the 40S subunit was bound to the ΔGCC mutant IRES, some features of its conformation were similar to the WT IRES–bound state, but some were markedly different. In both states, the latch formed between ribosomal RNA (rRNA) helices 18 and 34 was closed (this latch is open in apo-40S)23,31. However, the entry site (where mRNA enters the decoding groove) was much more open in the ΔGCC mutant IRES complex; this feature was clearly visible in class averages assembled from individual particle images (Fig. 2a). This result is consistent with the idea that the ΔGCC mutation changes the conformation of the IRES–40S complex and suggests that the ΔGCC and other IRES mutants can bind and functionally interfere with the WT IRES–40S complex in different ways.
with a previous study showing that mutation of dIIb alters the configuration of the mRNA in the decoding groove. In summary, the dIIb mutant IRES binds the 40S subunit using the same side of the subunit as the WT IRES. But the structures are considerably different; thus, deletion of the apical nucleotides of dIIb substantially affects the global conformation of the complex. These changes are different from those caused by ΔdII, suggesting that mutation of dIIb perturbs translation initiation differently from the ΔdII mutation.

dIIb influences a step after 80S ribosome formation

The structure of the mutant IRES–40S complex and the translation initiation efficiencies of all three dIIb mutants raises the question of whether dIIb influences a step after 80S ribosome formation.

Figure 2 EM of HCV IRES mutant ΔGCC in complex with the 40S subunit. (a) Representative electron micrographs of ΔGCC IRES–40S complexes, negatively stained (top) and embedded in vitrified ice (bottom). Inset, six classes of projection averages of particles in vitrified ice showing different views of the complex calculated using MSA and MRA programs in IMAGIC54. The readily observed open entry tunnel is indicated (arrow). Scale bar for inset, 10 nm. (b) Structures of WT (top, purple)23 and ΔGCC (bottom, red) HCV IRES bound to the 40S ribosomal subunit (yellow). Solvent-accessible (left), exit-channel (middle) and solvent-inaccessible (right) sides are shown. Key structural features are labeled (H, head; PT, platform; BK, beak; B, body). The entry and exit sites for mRNA as well as the location of dII are indicated.

Figure 3 WT and mutant IRES ribosome assembly assays and position of domain IIb. (a) Graph of radiolabeled IRES RNA migration through a sucrose gradient after 15 min incubation in RRL followed by ultracentrifugation. Free, IRES RNA not bound by any translation components. (b) Amount of 80S complex formed at time points from 0.5 min to 10 min. Error bars represent s.e.m. from three independent experiments. (c) Top, cryo-EM reconstruction of the full-length HCV IRES RNA (purple) bound to mammalian 40S subunit (yellow)23. Bottom, crystal structure of 40S subunit from Tetrahymena thermophila32 (yellow) and the NMR structure of HCV IRES dII 33 (purple) placed into the cryo-EM reconstruction (not shown). RpS5 is shown in green. (d) Comparison of the orientation of E-site bound tRNA (blue) and HCV IRES dII (purple) within the decoding groove. The position of dII was determined on the basis of the model shown in d22,24, and the E-site tRNA position, from a crystal structure of the T. thermophila 70S ribosome55, is shown in green, and its β-hairpin and the tRNA anticodon (AC) loop are indicated. (e) Close-up view of the position of dIIb (purple) near the β-hairpin of RpS5 (green). The nucleotides that were mutated in this study are shown in blue.
which step in initiation is affected by these mutations. Removal of domain II or replacement of the entire dIIb apical loop with an ultra-stable UUCG tetraloop has been reported to inhibit 80S ribosome formation, which suggested that our targeted dIIb mutations would do the same. To test this, we assayed ribosome assembly in RRL, assessing this, we characterized the structures of the dIIb mutant RNAs using NMR. We generated WT and mutant samples comprising nucleotides 76–100 of the HCV IRES, using NMR. We generated WT and mutant samples comprising nucleotides 76–100 of the HCV IRES (Fig. 3a), which contain dIIb and are identical to RNAs used to solve the structure of dII 33. Comparison of the one-dimensional 1H spectra obtained in water showed very little difference in the chemical shifts or relative intensity of the peaks obtained for WT and mutant RNAs (Fig. 4b). We observed the largest chemical shift in the imino protons of G87 and G88, which are adjacent to the apical loop of dIIb. The visibility of these imino proton peaks in all spectra indicates that base-pair formation is unaltered by dIIb mutation.

Modeled local interaction between dIIb and rpS5
To determine which interactions of dIIb with the ribosome are disrupted by mutation, we modeled the placement of dII on the ribosome by docking a 40S subunit structure22 into the cryo-EM density of the complete WT HCV IRES–40S subunit complex22,23 (Fig. 3c). Our placement of dII is consistent with a previously published model based on an IRES–80S complex24, with additional detail provided by the crystal structure of a 40S subunit. We compared our model with the crystal structure of a bacterial ribosome bound to tRNAs to determine how similar the contacts with the 40S subunit are between our modeled dII and a bound E-site tRNA (Fig. 3d).

The putative location of our dIIb mutations adjacent to the β-hairpin of rpS5 suggests that they disrupt a specific contact between dIIb and rpS5. Previous chemical probing of these mutant RNAs showed no global change in IRES secondary structure, but it remains possible that the mutations change the overall structure of dII. To assess this, we characterized the structures of the dIIb mutant RNAs using NMR. We generated WT and mutant samples comprising nucleotides 76–100 of the HCV IRES (Fig. 4a), which contain dIIb and are identical to RNAs used to solve the structure of dII 33. Comparison of the one-dimensional 1H spectra obtained in water showed very little difference in the chemical shifts or relative intensity of the peaks obtained for WT and mutant RNAs (Fig. 4b). We observed the largest chemical shift in the imino protons of G87 and G88, which are adjacent to the apical loop of dIIb. The visibility of these imino proton peaks in all spectra indicates that base-pair formation is unaltered by dIIb mutation.
The spectrum of mutant ΔapexC (dIIb mutant with only apical C83 deleted) was most similar to that of the WT, suggesting that the ΔapexC mutation elicits the post–80S ribosome functional effect with the smallest change in loop structure. On the basis of our prediction that the single deleted nucleotide (C83) would contact the β-hairpin of rpS5 (Fig. 3e), we selected this mutant for additional NMR experiments. When we overlaid the WT and ΔapexC spectra obtained by 2D 1H-NOESY in water, the portions that contained the imino-imino cross-peaks overlapped almost perfectly, with the largest shift occurring at the G87 imino (Fig. 4c). Likewise, we observed only small chemical-shift changes in spectra showing the cross-peaks between the imino and other protons (Fig. 4d). For example, cross-peaks between the G87 imino and C79 amino protons were shifted but intense (Fig. 4d), again confirming that this base pair at the base of dIIb still forms in the mutant RNA (Fig. 3e). The ΔapexC mutation elicits the post–80S ribosome functional effect with the smallest change in loop structure. On the basis of our prediction that the single deleted nucleotide (C83) would contact the β-hairpin of rpS5 (Fig. 3e), we selected this mutant for additional NMR experiments. When we overlaid the WT and ΔapexC spectra obtained by 2D 1H-NOESY in water, the portions that contained the imino-imino cross-peaks overlapped almost perfectly, with the largest shift occurring at the G87 imino (Fig. 4c). Likewise, we observed only small chemical-shift changes in spectra showing the cross-peaks between the imino and other protons (Fig. 4d). For example, cross-peaks between the G87 imino and C79 amino protons were shifted but intense (Fig. 4d), again confirming that this base pair at the base of dIIb still forms in the mutant RNA (Fig. 3e). Changes to the spectra were limited to nucleotides adjacent to the deletion, showing the localization of structural changes to the dIIb apical loop. This result is consistent with published chemical-probing data, based on selective 2′-OH acylation analyzed by primer extension, for the ΔapexC and other mutant RNAs, in that changes in the chemical probing pattern were limited to the apical loop in the IRES–40S subunit complex22 (Fig. 4f). Taken together, our data show that C83 deletion—and, we would predict, the other dIIb mutations shown in Fig. 1—induces a local structural perturbation that, we propose, disrupts dIIb interaction with the β-hairpin of rpS5 and is accompanied by a global change in the structure of the IRES–40S complex and the inhibition of a step after 80S ribosome formation.

**Figure 5** Biochemical analysis of AUG docking and potential frameshift. (a) Denaturing sequencing gel of the reverse transcription and toeprinting of WT and mutant IRES RNAs; the relevant part of the gel is magnified at right. Lanes 1–4, dideoxy sequencing reaction; lanes 5, 7, 9 and 11, free IRES; lanes 6, 8, 10 and 12, IRES–80S complexes (formed by incubation in RRL with cycloheximide (CHX)). Nucleotide numbers (bullets; left), the A of the AUG (black arrow; +1) and the toeprint (blue arrow; +15 and +16) are indicated. (b) Graph of quantified, normalized and background-corrected IRES–80S toeprints as in a. Positions +1 (black) and +15 and +16 (blue) are indicated. pknot, pseudoknot; dIV, domain IV. (c) Cartoon representation of the uncapped, unpolyadenylated monocistronic LUC reporter; the RNA region between the viral AUG and the LUC AUG (both shown in red) is positioned in all of these complexes and that the mutations probably cause incorrect placement of the AUG start codon. To test this, we used primer extension inhibition (toeprinting) analysis10,34–36 on WT and dIIb mutant RNA in the unbound and 80S ribosome–bound states (the latter is formed in RRL with cycloheximide) (Fig. 5a, compare lanes 5, 7, 9 and 11 with 6, 8, 10 and 12). As expected, we observed no toeprint for unbound RNAs; however, when bound to the 80S ribosome, all mutants produced similar toeprints at nucleotide positions +15 and +16 downstream of the AUG initiation codon (where A is in position +1) (Fig. 5a,b). This indicates that the AUG is properly positioned in all of these complexes and that the mutations probably affect a step after 80S ribosome assembly on the IRES.

**AUG start codon placement is unaffected by dIIb mutations**

To determine which step after 80S ribosome formation is affected by dIIb mutation, we first explored the possibility that the mutations cause incorrect placement of the AUG start codon. To test this, we used primer extension inhibition (toeprinting) analysis10,34–36 on WT and dIIb mutant RNA in the unbound and 80S ribosome–bound states (the latter is formed in RRL with cycloheximide) (Fig. 5a, compare lanes 5, 7, 9 and 11 with 6, 8, 10 and 12). As expected, we observed no toeprint for unbound RNAs; however, when bound to the 80S ribosome, all mutants produced similar toeprints at nucleotide positions +15 and +16 downstream of the AUG initiation codon (where A is in position +1) (Fig. 5a,b). This indicates that the AUG is properly positioned in all of these complexes and that the mutations probably affect a step after 80S ribosome assembly on the IRES.

**dIIb mutants initiate in the correct reading frame**

Mutations to the β-hairpin of rpS7 have been shown to increase frameshift rate27. We therefore considered the possibility that the dIIb mutants, by disrupting contact with rpS5, could cause frameshift during or after the first translocation event and, thus, an apparent decrease in translation. To test this, we conducted translation assays using reporters to which one or two nucleotides were added after the IRES start codon but before the start codon of the LUC open reading frame. If the ribosome sometimes slips out of frame on the dIIb mutant RNAs, the addition of these nucleotides would rescue a 1- or 2-nucleotide frameshift (Fig. 5c). We did not observe a partial rescue (Fig. 5d); in fact, introduction of these nucleotides decreased...
translation efficiency even more than the dIIb mutations. Hence, the decreased translation initiation efficiency observed for the dIIb mutants is not caused by frameshifting.

dIIb mutation does not affect peptide bond formation

Because slowed initial peptide bond formation after 80S ribosome assembly would decrease the overall rate of protein production and explain a decrease in protein synthesis (Fig. 1e), we hypothesized that dIIb mutations affect peptide bond formation. We tested the ability of 80S ribosomes formed on dIIb mutants to catalyze peptide bond formation using the aminoglycoside puromycin to accept an amide linkage from the amino acid on the P-site tRNA when the A site of the ribosome is vacant10,38 (Fig. 6a,b). We generated 80S ribosome complexes in RRL supplemented with [35S]Met using WT and dIIb mutant IRES RNAs. Error bars represent s.e.m. for three independent duplicate experiments.

The first translational event is promoted by dIIb

We hypothesized that mutation of dIIb allows 80S ribosome formation and peptide bond formation but inhibits the first round of translocation, in which the P site–bound initiator tRNA and start codon AUG move to the E site. To test this hypothesis, we used toeprinting analysis to directly detect ribosome movement on the WT and mutant IRES RNAs. We conducted these assays in RRL and in the absence of the antibiotic hygromycin B, which inhibits translocation13. In the absence of hygromycin B, all IRES RNAs produced a toeprint that prevents binding of primers or reverse transcriptase. We observed slightly higher toeprint intensity for the dIIb mutants than for WT IRES RNAs, indicating that more 80S ribosomes were paused at this initial stop. However, we did not observe strong stops caused by elongating ribosomes, probably because these ribosomes move to a position that prevents binding of primers or reverse transcriptase.

In contrast, we did not observe these strong downstream toeprint stops on the mutant RNAs (Fig. 6e, lanes 5, 7, 9 and 11). We did not see stops caused by elongating ribosomes, probably because these ribosomes move to a position that prevents binding of primers or reverse transcriptase. We observed slightly higher toeprint intensity for the dIIb mutants than for WT IRES RNA, indicating that more 80S ribosomes were paused at this initial assembly location (Fig. 6f). When present, hygromycin B captured ribosomes that had undergone one or two rounds of translocation, as indicated by the presence of toeprint stops at positions +20, +21 and +22 (Fig. 6e, lane 6). In contrast, we did not observe these strong downstream toeprint stops on the mutant RNAs (Fig. 6e, lanes 8, 10 and 12). Again, we quantified multiple replicates to correct for loading differences and found that 80S ribosomes formed on dIIb mutants were
slow to move from their initial positions (Fig. 6g). Specifically, 33.3% of the WT RNA was left in an untranslocated state, whereas 54.9%, 58.0% and 62.0% of AGCC, ΔpexC and GCC-AUU (dllb mutant with GCC at nucleotides 82–84 replaced with AUU) did not translocate, respectively. Thus, mutations in dllb decreased translocation by ~50%, which is consistent with the activity measurements presented in Figure 1. We conclude, therefore, that mutation of HCV IRES dllb results in 80S ribosomes that have a reduced ability to undergo the first round of translocation. This suggests that an intact dllb is needed for the first translocation step, at which initiation transitions to elongation.

DISCUSSION

We have found that mutations in the apical loop of dll of the HCV IRES inhibit the first translocation event after the formation of the 80S ribosome. HCV IRES-driven 80S ribosome formation and the first step of translocation can therefore be decoupled, indicating that dll has a function that is distinct from previously defined roles of HCV IRES domains. The involvement of dllb in the first round of translocation could be explained by two broad mechanisms. First, the position of dll in the E site requires it to move to make way for the P-site tRNA to translocate, as evidenced in cryo-EM reconstructions of the HCV IRES bound to a 40S subunit and 80S ribosome23,24. On the basis of cryo-EM reconstruction of the HCV IRES–80S ribosome, it has been suggested that contacts between a different portion of dll and the L1 stalk of the large subunit could facilitate dll displacement from the E site, but this IRES-ribosome contact and its potential function have not been investigated24. Also, a recent crystal structure of HCV IRES domain IIa bound to an inhibitor suggests that conformational changes in domain IIa or other parts of dll not studied here may affect movement of the domain from the E site40, but this has not been demonstrated functionally. In dllb, mutations could potentially inhibit dll displacement, and this would slow translocation by sterically hindering the movement of tRNA. This 'failure-to-move' phenotype, then, could be ascribed to the loss of a specific IRES-rpS5 interaction necessary for dll ejection from the E site, although an analogous role for rpS5 in tRNA ejection has not been reported. This idea does not eliminate the possibility that the L1 stalk and dll conformational changes also help move dll. The second potential explanation is that dllb actively promotes an event within the ribosome that is important for the first round of translocation—again, probably through a specific interaction of dllb with rpS5 and subsequent conformational changes in the IRES–ribosome complex. Mutation of dllb, then, might either interfere with this event or render the IRES unable to actively promote it. These broad mechanisms are consistent with our structural and functional data, and they are not mutually exclusive.

Given that the HCV IRES dllb is positioned to interact directly with the β-hairpin of rpS5, examining known functions of rpS5 (and bacterial ortholog rpS7) could give insight into how dllb influences translocation. During elongation, rpS5 has a role in maintaining the reading frame and in overall fidelity37,41,42. In bacteria, truncation of the β-hairpin of rpS7 results in destabilization of the E-site tRNA and an increased number of frameshift and reverse translocation events37. However, our data show no evidence of frameshift induced by the dllb mutants. Although not explicitly shown to be dependent on rpS5 or rpS7, the fidelity of aminoacyl-tRNA selection in the A site43 was recently shown to be influenced by the presence of tRNA in the E site during elongation, but other studies find little or no evidence for this44–46. It seems unlikely, then, that the presence of dllb directly influences entry of A-site tRNA. Overall, we find no known role for rpS5 that readily explains the observed effects of HCV IRES dllb mutation, consistent with the notion that the IRES is co-opting this ribosomal feature to manipulate the complex in a noncanonical way or that rpS5 has a yet-undiscovered role in translation initiation.

Mutation of dllb disrupts a putative interaction with rpS5, changes the structure of the IRES–40S subunit complex and inhibits the first translocation step. These observations suggest that a specific rpS5–dllb interaction induces allosteric changes that propagate throughout the ribosome. Indeed, there is evidence for a network of interactions within the ribosome that could propagate allostery, and also for similar conformational changes induced by bound initiation factors eIF1 and eIF1A. Specifically, rpS5 interacts with rRNA helices 29, 30 and 42 (refs. 47,48), and these helices interact with eIF1A49. Another network links rpS5 to the eIF1 binding site through rpS14 and rRNA helix 23 (refs. 41,50,51). This is notable because binding of eIF1 and eIF1A induces a conformational change in the 40S subunit that strongly resembles the change induced by the WT HCV IRES23,31, and eIF1A is known to act with eIF5B after 80S formation to commit the ribosome to elongation52,53. This last point raises the possibility that the HCV IRES dllb may induce the same effect as eIF1, eIF1A and eIF5B to promote a late step during initiation. This (speculative) notion is appealing because a minimal reconstitution of HCV IRES–driven translation initiation does not require eIF1 or eIF1A50, so dllb could substitute for these absent factors. Although not a part of this study, higher-resolution structures of HCV IRES mutants in complex with
80S ribosomes and chemical probing of the tRNA in these complexes before and after translocation could provide insight into the putative allosteric changes associated with this translocation-slowing phenotype.

We would like to propose the following model to explain the role of HCV IRES dIIb in events that occur within the IRES–ribosome complex before and during the first translocation step (Fig. 7): first, the IRES assembles an 80S ribosome such that the ribosome is poised at the start codon with an initiator tRNA in the P site. Within this ribosome, dIIb contacts the β-hairpin of rpS5, thereby stabilizing the ribosome in a conformation that is conducive to translocation. Delivery of aminoacyl-tRNA to the ribosome by eukaryotic elongation factor 1A (eEF1A) and subsequent peptide bond formation are then followed by rapid and efficient eEF2-catalyzed translocation. In the dIIb mutants studied here, the mutation induces a local change in the apical loop structure that perturbs the interaction with rpS5, affecting 40S subunit conformation. The dIIb mutant IRES–40S complex is still capable of forming an 80S ribosome, but one whose conformational equilibrium is shifted such that its ability to translocate is inhibited. Although aminoacyl-tRNA may still be delivered to the A site and a peptide bond formed, the mutant-bound ribosome stalls at the start site. However, the ribosome samples conformations, so these ribosomes are not permanently stalled; rather, they occasionally sample a productive state in which they are able to translocate.

In summary, our data support a model in which dIIb selects a productive state from the conformational ensemble, and ribosomes bound to IRESs with mutated dIIb spend more time in an unproductive state and transition to elongation less efficiently.

Our data open another door to understanding the intricacies of translation initiation and ribosome function. The ribosome is fundamentally a Brownian machine that samples many conformational equilibria, providing efficiency and directionality. Thus, the ribosome is programmed to be manipulated by its binding partners. This inherent characteristic of the ribosome is crucial for canonical translation processes and allows subtle and robust regulation of ribosome function. Our results reveal that these principles are exploited by a single loop of the HCV IRES, supporting the view of the HCV IRES as a dynamic manipulator of the translation machinery and lending insight into how the translation machinery works in cap-dependent and cap-independent pathways.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The cryo-EM map of the mutant HCV IRES–rabbit 40S subunit complex has been deposited in the Electron Microscopy Data Bank with accession number 5527.

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AUTHOR CONTRIBUTIONS

M.E.F. conducted all biochemical experiments. J.S.K. and M.E.F. conducted and analyzed the NMR experiments. B.S.V., D.S., T.G. and J.S.K. conducted the cryo-EM experiments, with structural calculations by B.S.V. Results were interpreted by M.E.F., B.S.V., J.S.K. and T.G. M.E.F. and J.S.K. designed the overall study and wrote the manuscript. All authors contributed to figure construction.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plasmid construction and cloning. We constructed pUC19-based plasmids containing the HCV genotype 1b wild-type (WT; nucleotides 40–372) and ΔIII mutant (nucleotides 119–372) sequences flanked by a 5′ hammerhead and 3′ hepatitis delta ribozyme as previously described. Plasmids with the WT or mutant HCV IRES between two luciferase-encoding genes were made by PCR amplification of the desired sequence and ligation into the EcoRI and NcoI sites of plasmid pRL (gift of A. Wills). The plasmid used to generate RNA for toeprinting analysis (containing 85 additional 3′ nucleotides on the wild-type HCV genotype 1a as well as the primer binding site) was a kind gift of P. Lukavsky. We generated the genotype 1b ΔIII mutant used in toeprinting by PCR amplification of the desired sequence and ligation into the HindIII and Xbal sites of this plasmid. All mutants were made using the QuikChange mutagenesis kit (Stratagene).

RNA preparation. We made RNAs for assembly assays and puromycin experiments using DNA generated by PCR using M13 (−41) forward and reverse primers (5′-GGTTTCCCAGTCACGAC-3′ and 5′-GGGAAACGCTATGACGATTG-3′, respectively) and the relevant plasmid template. The PCR products were used in vitro transcription reactions as described. We purified and concentrated RNA as described. Monocistronic Photinus luciferase RNAs were made from PCR templates using forward T7-HCV and reverse photinus primers (5′-TA ATACGACTCACTATAGGGCTCCCTGATGAACTACGCTCT-3′ and 5′-TTACCGCCGATTCCTTCGCCCTTCTTCTT-3′, respectively) using the T7 MegaScript kit (Ambion). RNAs were DNase treated, then purified with TRIzol and ethanol precipitation, respectively. We made RNAs for toeprinting from EcoRI-linearized plasmids in the same manner as the luciferase RNAs.

Radiolabeling RNA and primers. For assembly assays, we 5′ radiolabeled RNA as described and diluted it to approximately 1,000 c.p.m. μl⁻¹. DNA primers were 5′ radiolabeled in a reaction containing 800 pmol primer in the same conditions as the RNA, mixed with 20 μl 9 M urea loading buffer, loaded directly onto a 10% urea denaturing gel, purified and diluted to approximately 25,000 c.p.m. μl⁻¹.

Ribosome assembly assays. We diluted 5′-radiolabeled HCV IRES RNAs to ~1,000 c.p.m. μl⁻¹, heated to 85 °C for 30 s, then cooled on the desktop. 1 μl of this RNA was then added to a mixture containing 30 μl rabbit reticulocyte lysate (RRL), 0.5 μl amino acid mixture minus leucine, 0.5 μl amino acid mixture minus methionine (all provided in RRL translation kit, micrococcal-nuclease treated; Promega) and 18 μl RNase-free water. Reactions were incubated at 30 °C for the desired time, halted by the addition of ribosome association dilution buffer (50 mM Tris (pH 7.5), 50 mM NaCl, 5 mM MgCl₂ and 1 mM DTT) and placed on ice. The reactions were analyzed with 10–35% sucrose gradients. The gradient was centrifuged, and the 10–35% gradient was recovered. For 48S-bound IRES, 10.75 μl RRL was mixed with 0.5 μl Rnasin Plus and 0.5 mM dithiothreitol (DTT) and placed on ice. The reactions were analyzed with 10–35% sucrose gradients. The gradient was centrifuged, and the 10–35% gradient was recovered. For 80S-bound IRES, 10.8 μl RRL was mixed with 0.5 μl Rnasin Plus and 0.5 mM dithiothreitol (DTT) and placed on ice. The reactions were analyzed with 10–35% sucrose gradients. The gradient was centrifuged, and the 10–35% gradient was recovered. The gradient was centrifuged, and the 10–35% gradient was recovered.

Luciferase assays. We conducted translation assays as described with the following exceptions: for the time-point experiment, reactions were brought up to 125 μl volume so that 25 μl could be removed at each time point, halted with 200 μl cold 1× Passive Lysis Buffer (Promega) and placed at −80 °C to ensure no further activity.

Toeprinting assays. We completed toeprinting assays essentially as described. For 48S-bound IRES, 10.75 μl RRL was mixed with 0.5 μl Rnasin Plus and 0.5 mM guaninyl imidodiphosphate and incubated at 30 °C for 5 min, after which 0.5 μg toeprint RNA was added to a final volume of 15 μl. For 80S-bound IRES, 10.8 μl RRL was mixed with 0.5 μl Rnasin Plus (Promega) alone or with 3 mg ml⁻¹ cycloheximide or 2 mg ml⁻¹ hygromycin b and incubated at 30 °C for 5 min. This incubation was followed by addition of 0.5 μg toeprint RNA to a final volume of 15 μl. We made the ladder used for analysis with wild-type toeprint RNA reverse transcribed with SuperScript III Reverse Transcriptase (Life Technologies) with annealing and extension temperatures at 45 °C.

Puromycin assays. We first biotinylated 3′-truncated IRES RNAs (nucleotides 40–344, which stop after the AUG codon) using the 5′ EndTag Nucleic Acid Labeling System (Vector Laboratories). Briefly, 65 μg of RNA was phosphorylated with ATPγS using T4 polynucleotide kinase in a 20-μl reaction, at 37 °C for 1 h. Biotinylation then was carried out upon the addition of ~385 μg biotin (long arm) maleimide for 1 h at 65 °C. Reactions were then extracted with equal volume phenol-chloroform-isooamyl alcohol, pH 6.7 (25:24:1) (Fisher), precipitated with 1/10 volume 3 M acetic acid (pH 5.2) and three volumes 100% cold ethanol overnight. RNA was pelleted and washed with 70% ethanol, dried and resuspended in 10 μl RNase-free water. Concentration was determined by absorbance at 260 nm. To conduct the assay, we mixed 30 μl RRL with 16.5 μl RNase-free water and 1 μl [35S]methionine (>1,000 Ci mmol⁻¹) and incubated the mixture at 30 °C for 15 min to allow aminoacylation of initiator methionine tRNA. Biotinylated RNA (2.25 μg) was added to the reaction and incubated at 30 °C for 25 min for 80S ribosome formation. Reactions were mixed with one tube (0.6 ml) streptavidin paramagnetic beads (MagnetSphere, Promega), prewashed three times with 0.5× saline sodium citrate (SSC) buffer (7.5 mM trisodium citrate dehydrate (pH 7.2), 75 mM NaCl) and once with 300 μl ribosome association dilution buffer), resuspended in 50 μl ribosome association dilution buffer and incubated at 30 °C for 10 min. Complexes were then washed six times with 500 μl ribosome association dilution and resuspended such that reactions were split into duplicates with and without 1 μM puromycin. The assay was then carried out at 35 °C for 60 min. Puromycin was extracted with 50 μl 200 mM potassium phosphate buffer (pH 8) and 500 μl ethyl acetate with continuous shaking for 10 min at 35 °C. The upper ethyl acetate layer was removed and mixed with 7 ml ScintiSafe (Fisher) liquid scintillation fluid, and counts per minute were averaged between two 10-min count times. This method of immobilizing the IRES-80S ribosomes greatly reduced background levels of puromycin-[35S]methionine formation compared to results for 80S ribosomes purified by ultracentrifugation in sucrose gradients.

Nuclear magnetic resonance. We collected NMR spectra at 25 °C on a Varian 900 MHz spectrometer using Standard Varian Biopack pulse sequences for all experiments. This included both one-dimensional spectra as well as all homonuclear two-dimensional spectra employing a 3919 WATERGATE for water suppression. NOESY (Biopack pulse sequence, WBN0ESY) spectrum was collected with 256 indirect points. Two-dimensional data were processed using a Gaussian weighting function in the direct dimension and a sine-bell weighting function in the indirect dimension.

Negative-stain electron microscopy. We prepared the ΔGCC mutant IRES RNA as described above and the 405 ribosomal subunits from RLL as previously described. We assembled IRES–40S subunit complex as previously described. We applied the complex to freshly glow-discharged carbon coated 400 mesh copper grids and stained with 0.75% uranyl formate as described. Samples were viewed on a 120-kV transmission electron microscope (FEI, Hillsboro, OR). Images were recorded at a nominal magnification of ×40,000 using a bottom mount 4k × 4k Gatan slow-scan charge coupled device (CCD) camera.

Electron cryomicroscopy. Purified ΔGCC mutant IRES RNA in RNase-free water was heated to 70 °C for 2 min then cooled to room temperature. Buffer solution was added to a final concentration of 20 mM Tris-HCl (pH 7.4), 100 mM acetic acid, 2.5 mM MgCl₂, 1 mM DTT and 40 mM KCl. Purified 40S subunits were added at a 1:1 molar ratio with the IRES RNA to a final concentration of 500 nM complex. Complex was stored on ice until diluted (generally to 100 nM) and used in microscopy.

We prepared vitrified samples of the ΔGCC mutant IRES–40S subunit complex at 100 nM using an FEI Vitrobot. Briefly, 3.5 μl was applied to a Quantifoil Holey Carbon grid (Vitrobot chamber was at 4 °C and 100% humidity). After a 20-s pause, the grid was blotted with filter paper (force = 0, blot time = 2 s) and plunged into liquid ethane. Frozen samples were loaded onto a Gatan cryo-holder and inserted into an FEI Tecnai F20 operating at 200 kV equipped with a field emission gun. Images were collected at a nominal magnification of ×62,000 using
a 4k × 4k Tietz CMOS detector. Images were binned two times yielding a pixel size of 2.66 Å per pixel. Approximately 29,000 particles were selected from 1,790 images using Electron Micrograph Utility (http://cryoem.ucsf.edu/). Class averages were determined using five consecutive rounds of MSA (multivariate statistical analysis) and MRA (multireference alignment) in IMAGIC54. Contrast transfer function parameters for each image were determined using CTFFIND3 (ref. 60). An initial model for refinement and three-dimensional reconstruction was generated by filtering a previously published apo–40S reconstruction (EMD-1346) model to 40 Å31. Initial parameters were generated during cycles of randomized search and refinement using FREALIGN v. 8.08 (ref. 61). After initial parameters were determined, consecutive cycles of local refinement and reconstruction were carried out until there was no apparent improvement in the alignment. Resolution of the three-dimensional model was calculated with the program RMEASURE62 and determined to be ~17.5 Å. The density was normalized using MAPMAN63 and filtered to 20 Å using BFACTOR. Difference maps presented in the figures were calculated using MAPMAN63. We calculated the density corresponding to the WT IRES from a difference map between the WT IRES–40S and apo–40S23, and the mutant IRES was calculated from a difference map between the ΔGCC IRES–40S reconstruction and apo–40S23. Reconstructions and difference maps were assembled as displayed in Figure 2b using UCSF Chimera64.

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