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Eukaryotic translation initiation machinery can operate in a bacterial-like mode without eIF2

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Unlike bacteria, a specialized eukaryotic initiation factor (eIF)-2, in the form of the ternary complex eIF2–GTP–Met-tRNA$_i^{Met}$, is used to deliver the initiator tRNA to the ribosome in all eukaryotic cells. Here we show that the hepatitis C virus (HCV) internal ribosome entry site (IRES) can direct translation without eIF2 and its GTPase-activating protein eIF5. In addition to the general eIF2- and eIF5-dependent pathway of 80S complex assembly, the HCV IRES makes use of a bacterial-like pathway requiring as initiation factors only eIF5B (an analog of bacterial IF2) and eIF3. The switch from the conventional eukaryotic mode of translation initiation to the eIF2-independent mechanism occurs when eIF2 is inactivated by phosphorylation under stress conditions.

The bacterial translation initiation mechanism uses only three initiation factors: IF1, IF2 and IF3 (ref. 1). In recent years, their functional analogs have been identified in eukaryotic cells; namely, eIF1A, eIF5B and eIF1, respectively[2]. In spite of this similarity, the molecular mechanisms of translation initiation in bacteria and eukaryotes are believed to be significantly different. The principal distinctive feature of eukaryotic translation is the scanning of the mRNA 5′ untranslated regions (5′ UTRs)[3]. The initial recognition of the mRNA occurs through the interaction between the 5′ terminal m^{7}G cap and the cap binding complex eIF4F. The ribosome is loaded onto the 5′ end of the mRNA and is then thought to migrate downstream (to 'scan') to locate an AUG codon in a favorable context. The factors eIF1 and eIF1A are indispensable for scanning. The helicase activity of eIF4F, stimulated by eIF4B, unwinds secondary structures within the mRNA during scanning. Another distinctive feature is the presence of eIF2, which, in the form of the ternary complex eIF2–GTP–Met-tRNA$_i^{Met}$, brings the initiator tRNA to the ribosome[3]. There is no analog of eIF2 in bacteria. The role of eIF2 is broader than just delivering Met-tRNA$_i^{Met}$ to the ribosome, as phosphorylation of eIF2 is known to be central to the global regulation of protein synthesis under stress conditions and during virus infection[4].

This complicated machinery operates not only in the case of standard cap-dependent mRNAs but also with mRNAs whose translation initiation uses the binding of ribosomes to IRESs. These structural elements bind diverse components of the translation initiation apparatus and thereby direct ribosome binding to the vicinity of the initiation codon. Although most of the well-studied IRESs from picornaviruses do not require the cap binding factor eIF4E, their molecular mechanisms of initiation are no simpler than that for standard cap-dependent mRNAs, and they frequently require additional mRNA binding proteins[5]. The only exception, among IRES elements using methionine-based translation initiation, are the IRES elements of HCV RNA and the HCV-like IRESs from some other flavi- and picornaviruses. They bind directly to the 40S ribosomal subunit and position it close to the AUG codon so that no scanning is required, and this strongly resembles the prokaryotic mode of AUG selection. Consequently, the cap binding complex eIF4F and the scanning factors eIF1 and eIF1A are not required. For this type of IRES element, the 48S translation initiation complex can be formed with just eIF2–GTP–Met-tRNA$_i^{Met}$ (refs. 6,7).

It was intriguing to know whether the unique features of the HCV IRES are also involved in the formation of the final 80S initiation complex. Here we have explored the mechanism of 80S complex formation on the HCV IRES using totally purified components of the translational apparatus from mammalian cells. We have found that, in addition to the conventional eIF2-dependent mode of translation initiation, the HCV IRES can use a bacterial-like, eIF2-independent mechanism. In this case, it is apparently eIF5B, the homolog of prokaryotic IF2 (ref. 8), that promotes initiator tRNA binding to the ribosomal P site. Inactivation of eIF2 by phosphorylation, in response to various treatments, has a much smaller effect on HCV IRES-mediated translation than on cap-dependent translation. The switch from the eukaryotic to a bacterial-like mode of translation initiation occurs when eIF2 is inactivated.

RESULTS

80S complex can be formed on the HCV IRES without eIF2

Previous work showed that the 48S initiation complex can be formed on the HCV IRES with 40S subunits and the eIF2–GTP–Met-tRNA$_i^{Met}$ complex only. The factors eIF1 and eIF4 were dispensable[6]. The role of eIF3 remained unclear; it was suggested to be involved in the next step of initiation. As eIF2 was the crucial component for 48S complex

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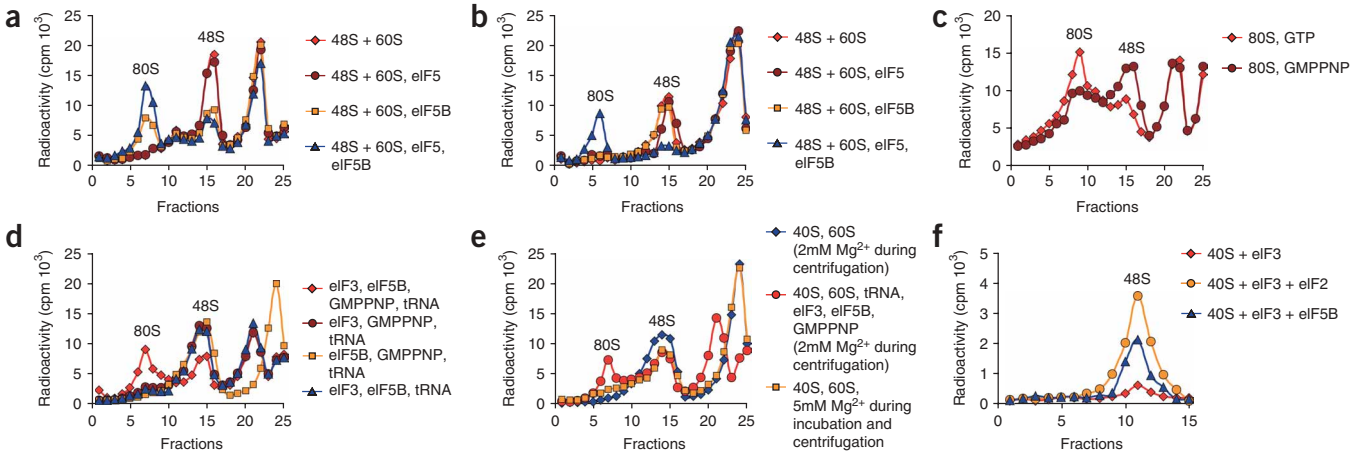

Figure 1 Assembly of translation initiation complexes on the HCV IRES and β-globin mRNAs and their analysis by sucrose density gradient centrifugation. (**a**) 80S complex reconstitution on the [^{32}P]-labeled HCV IRES in the presence of GTP, Met-tRNA$_i^{Met}$, 40S, 60S, eIF2 and eIF3 with omission of eIF5 or eIF5B as indicated. (**b**) 80S complex reconstitution on β-globin mRNA in the presence of GTP, Met-tRNA$_i^{Met}$, 40S, 60S, eIF1, eIF1A, eIF2, eIF3, eIF4A, eIF4B and eIF4F with or without eIF5 or eIF5B as indicated. (**c**) 80S complexes assembled on the HCV mRNA with the complete set of factors, with GTP being substituted for its nonhydrolyzable analog GMPPNP. (**d**) 80S complexes formed with GMPPNP, 40S, 60S, eIF3 and eIF5B or with omission of one of these factors. (**e**) 80S complexes reconstituted on the HCV IRES RNA in sucrose gradients containing 2 mM or 5 mM Mg^{2+} (see text). (**f**) 48S ribosomal complexes reconstituted on the HCV IRES with GMPPNP, [^{35}S]-labeled Met-tRNA$_i^{Met}$, 40S and eIF3 with either eIF2 or eIF5B as indicated.

formation, it was logical to assume that the assembly of the final 80S initiation complex on the HCV IRES occurred according to the standard mechanism: (i) hydrolysis of GTP in the eIF2–GTP–Met-tRNA$_i^{Met}$ ternary complex triggered by eIF5 (the GTPase-activating protein for eIF2); (ii) dissociation of the eIF2–GDP complex from the 48S complex; (iii) association of the 48S complex lacking eIF2 with the 60S subunit mediated by factor eIF5B with concomitant release from the ribosome of all factors except eIF5B; and finally (iv) hydrolysis of the second molecule of GTP bound to eIF5B, releasing eIF5B and resulting in formation of the final 80S initiation complex competent for synthesis of a polypeptide (for review see ref. 9). Indeed, addition of conventional ribosome joining factors eIF5 and eIF5B along with 60S subunits to preformed 48S complexes on HCV RNA led predictably to 80S complex formation[10] (**Fig. 1a**). Unexpectedly, however, omitting eIF5, a trigger of GTP hydrolysis on eIF2, did not abolish 80S complex formation (**Fig. 1a**). The possibility of contamination with eIF5 within the system was ruled out by western blotting analysis of eIF2 and eIF5B preparations, by using a recombinant C-terminal fragment of eIF5B$_{587–1220}$ instead of native factor (data not shown), and finally by the inability of β-globin mRNA to form 80S complexes when eIF5 was omitted (**Fig. 1b**).

This finding could be explained by eIF5B functioning instead of eIF5, as was suggested for 80S complex formation on AUG triplets[11–13]. However, control experiments in which the conversion of the 48S complex to the 80S complex should have been blocked by the presence of GMPPNP (a nonhydrolyzable analog of GTP) also resulted in some 80S complex formation, although at a reduced level (**Fig. 1c**), indicating at least a partial independence of 80S assembly on GTP hydrolysis. As follows from the standard mechanism for 80S complex formation described above, the conversion of the 48S complex into the 80S requires GTP hydrolysis by eIF2. Therefore, we asked whether there is an obligatory requirement for any eIF2, if there was no strict requirement for GTP hydrolysis? Indeed, we found in the presence of only ribosomal subunits, eIF3, eIF5B and Met-tRNA$_i^{Met}$ that 80S translation initiation complexes were formed even in the presence of GMPPNP, thereby eliminating the possibility that 80S complexes are formed owing to residual eIF2 activity. However, eIF3 and eIF5B were indispensable (**Fig. 1d**). The functional role of eIF3 in this very simplified process of 80S assembly is not yet clear. Perhaps, eIF3 is required for correct ribosome–mRNA complex architecture. The need for GTP or its nonhydrolyzable analog (**Fig. 1d**) is not surprising as they are required for eIF5B to adopt an active conformation[11,14].

Recently, a 'factorless' translation initiation process has been reported for the HCV IRES at elevated Mg^{2+} concentrations[15]. Although we performed the assays for 80S complex formation at physiological ion concentrations, sucrose gradients used to analyze translation initiation complexes routinely contained increased concentrations of Mg^{2+} (6 mM). However, the control experiment showed that decreasing the Mg^{2+} concentration to 2 mM during centrifugation did not abolish eIF2-independent 80S assembly (**Fig. 1e**). In the absence of eIF3 or eIF5B, we failed to observe any 80S complex formation under our experimental conditions.

eIF5B promotes tRNA binding to 40S–eIF3–HCV IRES complexes

Although the experiments presented above clearly showed that eIF5B and eIF3 function together to produce an 80S complex, they do not indicate which factor, if either, is required to stabilize Met-tRNA$_i^{Met}$ binding to the 40S–HCV IRES complex. Indeed, one cannot exclude the possibility that, unlike its bacterial homolog IF2, eIF5B may carry only the subunit joining function and that Met-tRNA$_i^{Met}$ may bind only to preformed 80S–HCV IRES–eIF5B–GTP complexes. To clarify, we investigated whether [^{35}S]-Met-tRNA$_i^{Met}$ can bind to the complex of the 40S ribosomal subunit with the HCV IRES + eIF3 in the absence of eIF5B. We found strong evidence that eIF5B is absolutely required to place Met-tRNA$_i^{Met}$ on the 40S subunit (**Fig. 1f**).

Elongation with 80S complexes assembled without eIF2

The functionality of the 80S initiation complexes assembled from 40S and 60S ribosomal subunits, eIF3, eIF5B and Met-tRNA$_i^{Met}$, that is, their ability to be engaged into the subsequent step of translation, the elongation of the polypeptide chain, had to be proven. To this end, we changed the seventh triplet of the HCV ORF to a UAA stop codon and

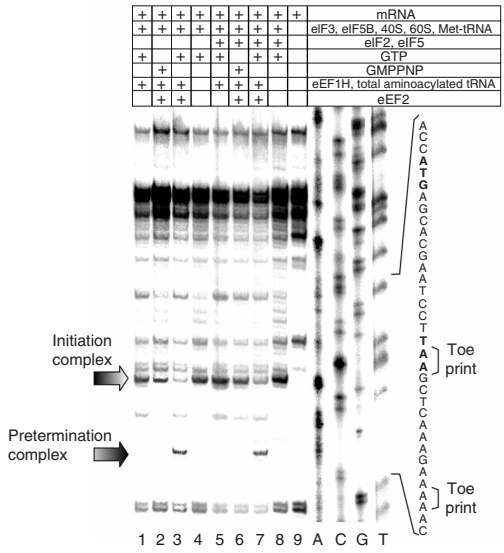

Figure 2 80S complexes formed without eIF2 are competent for translational elongation. 80S complexes were assembled from 40S, 60S, eIF3, eIF5B, eIF2 and eIF5 as indicated on the HCV mRNA bearing a termination codon at the seventh amino acid position, and then elongation factors were added. The migration of ribosomes to the termination codon was assayed by toeprinting. Initiation and termination codons indicated by bold letters and relevant reverse-transcription stops are indicated by arrows.

then reconstituted the translation elongation process from totally purified components[16]. We identified the presence of pretermination ribosomal complexes at the nucleotide triplet preceding the termination codon by toeprint assay. This technique is based on the primer extension inhibition of reverse transcription from an oligodeoxynucleotide, which is hybridized 3′ to the codon of an mRNA positioned in the P site of the ribosome. The arrest of reverse transcription always occurs at the same position, +16 nt to +18 nt 3′ of the first nucleotide residue of the codon occupying the P site of the ribosome. Identical elongation factor–dependent stops of the ribosomes can be clearly seen in **Figure 2**, regardless of whether the 80S initiation complexes were formed with the complete set of eIFs (including eIF2) or with just eIF3 and eIF5B (compare lanes 3 and 7). Thus, the 80S initiation complexes formed in the absence of eIF2 were totally functional and not an artifact.

eIF2-independent mechanism operates in cell-free system

Notably, in our *in vitro* experiments with the reconstitution system, eIF2 seemed to inhibit GTP hydrolysis–independent formation of the 80S complex (data not shown). In cell extracts, with their balanced concentrations of translation initiation components, the HCV IRES does not form 80S initiation complexes in the presence of non-hydrolyzable analogs of GTP[6,17,18]. This could be due to a higher affinity of the ternary complex for the 40S subunit in this extract, implying that eIF2 outcompetes eIF5B for the 40S.

To address the physiological relevance of these data, we inactivated eIF2 via phosphorylation and studied the effect on HCV IRES–driven translation. Rabbit reticulocyte lysate (RRL) was treated with double-stranded RNA (dsRNA) to induce PKR[19] (also known as eIF2AK2) and supplemented with GMPPNP to block the eIF2-dependent initiation pathway at the 48S complex stage. Control assays were not treated with dsRNA but also contained GMPPNP. No 80S complexes were assembled on the HCV IRES-containing mRNA in control lysates (**Fig. 3a**). In contrast, in dsRNA-treated RRL, 80S complexes were formed on the HCV IRES. This was not the case for the β-globin mRNA, a typical cellular mRNA that can use only the canonical eIF2-dependent pathway of translation initiation (**Fig. 3b**). It is clear that eIF2 had been inactivated, at least partially, because the amount of 48S complexes assembled on the β-globin mRNA was reduced. The phosphorylation of eIF2 in the presence of dsRNA was confirmed by western blotting with anti–phosphor-eIF2α(Ser51) antibodies (**Fig. 3c**). This indicates that, under normal conditions (that is, when eIF2 is fully active), the translation initiation on the HCV IRES proceeds primarily through the eIF2-dependent pathway. It also means that eIF2 inactivation should result in a switch between the translation initiation modes used by HCV from eIF2-dependent to eIF2-independent, rather than in a severe inhibition of its translation.

HCV IRES–driven translation in stressed transfected cells

The *in vitro* experiments were complemented with those performed using transfected cells. First, we treated HEK293T cells with various reagents that elicited eIF2 phosphorylation either through activation of various eIF2 kinases (PKR by dsRNA transfection, PERK (also known as eIF2AK3) by DTT[20] or HRI (also known as eIF2AK1) by sodium arsenite[21]) or by inhibition of PP1 phosphatase (by okadaic acid[22]). Then, we transfected the cells with a mixture of a standard cap-dependent mRNA and the HCV IRES–containing monocistronic mRNA. All these treatments affected the HCV IRES–driven translation much less than that directed by a standard 5′ UTR (**Fig. 4**). The translation directed by certain picornavirus IRESs, such as that of human rhinovirus (HRV) or encephalomyocarditis virus (EMCV), were inhibited by these treatments to the same extent as cap-dependent translation (**Fig. 4**; for EMCV, data not shown). Notably, the HCV RNA translation was found to be resistant to treatment with interferon-α (IFNα), widely used in HCV therapy. Thus, consistent with other previously reported data[23–25], eIF2 inactivation does not lead to severe inhibition of HCV IRES–driven translation.

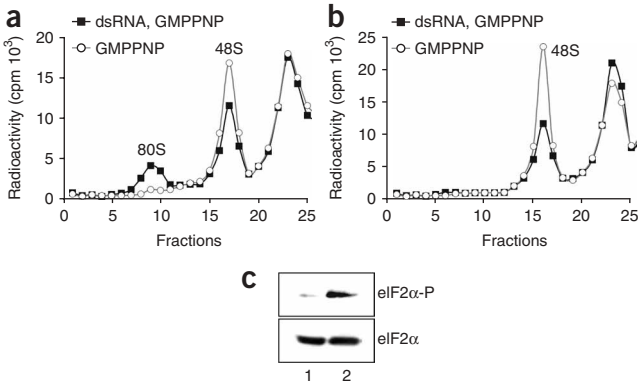

Figure 3 eIF2 phosphorylation activates the alternative pathway for 80S formation on the HCV IRES in RRL. (**a**,**b**) Sucrose gradient centrifugation of ribosome complexes formed on HCV mRNA (**a**) and β-globin mRNA (**b**) incubated in RRL first treated with dsRNA and GMPPNP. (**c**) Western blot showing eIF2 phosphorylation in equal amounts of untreated (lane 1) or dsRNA-treated (lane 2) RRL, using antibodies specific for eIF2 phosphorylated at Ser51.

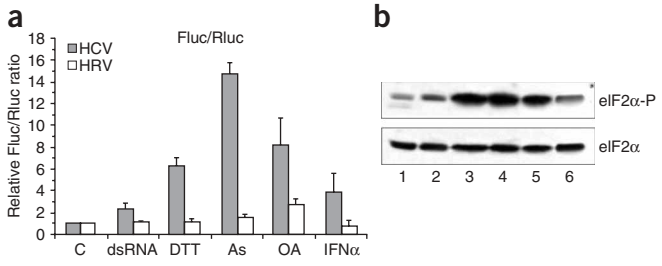

Figure 4 HCV IRES–directed translation is relatively resistant to eIF2 inactivation *in vivo*. HEK293T cells were treated with 2 μg ml^{-1} dsRNA, 2.5 mM DTT, 2 mM sodium arsenite (As), 1 μM okadaic acid (OA) or 1,000 IU ml^{-1} IFNα as indicated (C, control), and then (**a**) transfected with the mixture of capped Rluc and HCV-Fluc or HRV-Fluc mRNAs, harvested 2 h later and assayed for reporter protein expression (results are means ± s.d. of three representative experiments performed in triplicate) or (**b**) lysed and probed for eIF2 phosphorylation or total eIF2 amount (lanes: 1, untreated; 2, plus dsRNA; 3, plus DTT; 4, plus arsenite; 5, plus OA; 6, plus IFNα.

DISCUSSION

Initiation factor eIF2 is a pivotal component of the translation initiation apparatus in all eukaryotic cells. It is absolutely required for Met-tRNA$_i^{Met}$ delivery to ribosomal complexes formed with all eukaryotic mRNAs except for in a few exotic cases[16,26,27]. Its activity is suppressed by phosphorylation of its α subunit as a result of the host-cell response to viral infection or stresses[4,24]. However, some viral mRNAs are efficiently translated under these conditions. This is exactly the case for HCV RNA, whose translation is reported to be refractory to reduced eIF2–GTP–Met-tRNA$_i^{Met}$ ternary complex availability[25].

Here we report that, in addition to the conventional eIF2-dependent mechanism of the initiation complexes formation, the HCV IRES can be assembled into 80S initiation complexes without eIF2 participation. This alternative pathway requires only eIF3 and eIF5B as initiation factors and does not require GTP hydrolysis (**Fig. 1c**,**d**). The 80S complexes thus formed are fully competent for translation elongation (**Fig. 2**). Notably, we observed 80S complex formation in the cell-free system independently of GTP hydrolysis under conditions where eIF2 had been inactivated (**Fig. 3**). Similarly, in living cells the HCV IRES was resistant to eIF2 phosphorylation induced under various stress conditions and to treatment with IFNα (**Fig. 4**).

Presumably, under normal (nonstressful) conditions, HCV uses predominantly the canonical eIF2-dependent pathway, as the HCV IRES was unable to form eIF2-independent 80S complexes in RRL, unless eIF2 phosphorylation was induced (**Fig. 3a**). This implies that the switch from a conventional mode of translation initiation to an alternative one occurs when eIF2 is inactivated by phosphorylation, an important mechanism by which host cells counteract viral infection.

Our data provide, for the first time, a mechanistic explanation for the phenomenon of reduced sensitivity of the HCV RNA translation to the eIF2 inactivation observed previously[23–25] and may be relevant to the well-known resistance of HCV to interferon-based therapy[28]. The eIF2-independent pathway of the HCV IRES–directed translation may represent one more line of defense used by this virus against host antiviral responses.

The possibility of using such an alternative pathway might be explained by the unique features of the HCV IRES. Specifically, a stable mRNA–40S complex is formed in which the initiation codon is placed near the P site of the ribosome, and thus no scanning of the 5′ UTR occurs. This is also true for pestiviruses (for example, classical swine fever virus[6]) and for certain picornaviruses (for example, porcine teschovirus[7] and avian encephalomyelitis virus[29]), whose 5′ UTRs contain IRESs with structural elements that resemble domains of the HCV IRES. *In vitro* reconstitution of initiation complexes on these IRESs revealed functional similarities to the HCV example: apart from the 40S subunit, they also bind directly to eIF3 and have similar simplified mechanisms for the formation of the 48S translation initiation complexes[6,7]. However, whether these features are necessary and sufficient for the ability to use an eIF2-independent pathway of translation initiation remains to be established.

Other picornaviruses (such as HRV or EMCV) contain two other distinct types of IRES elements, and both apparently fail to use the eIF2-independent pathway (**Fig. 4**). At least in the case of the EMCV IRES, the scanning of the whole initiation region is also not required to place the 40S ribosomal subunit in the vicinity of the start codon, although within the starting window some limited scanning may be needed, because deletion of the authentic initiator codon leads to initiation from downstream AUG codons[30]. Unlike in the HCV example, the formation of a stable complex of the EMCV IRES with the 40S ribosomal subunit absolutely requires the eIF4A-directed helicase reaction and, more importantly, the codon-anticodon interaction[31]. Otherwise, the accommodation of EMCV mRNA in the 40S ribosomal channel does not occur. One cannot exclude that this accommodation reaction requires a concerted action of eIF4 factors and the ternary complex. Moreover, in the case of the HCV IRES, the region of the mRNA that will be loaded into the P site is strictly defined sterically by the 40S–IRES complex solely and not by initiation factors. This explains the narrowness of the starting window on the HCV mRNA[32] and its ability to use non-AUG codons as initiation

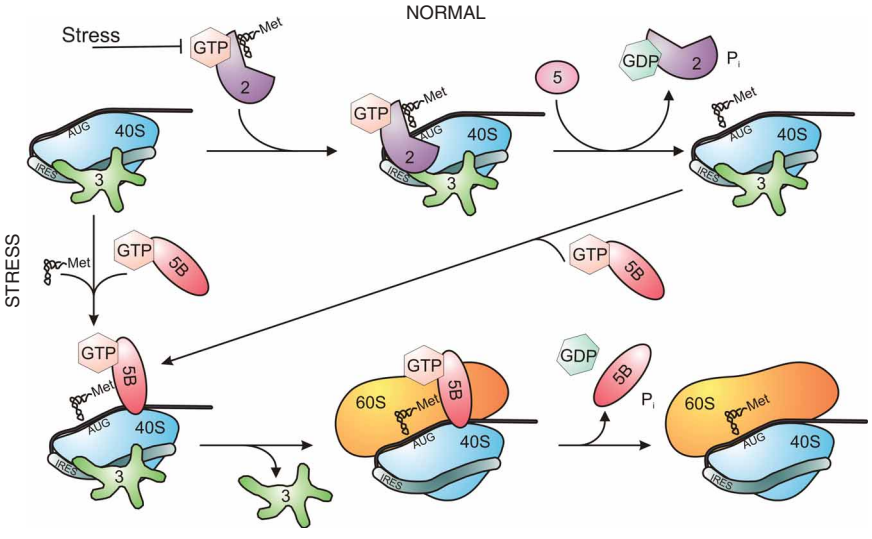

Figure 5 Alternative pathways of the 80S initiation complex formation on the HCV IRES. Initiation factors are denoted by numbers: 2, eIF2; 3, eIF3; 5, eIF5; 5B, eIF5B. Under stress eIF2 is inhibited, but eIF5B (left) can act instead of the normal initiation pathway (top).

sites[33]. This is also probably why an eIF2-independent pathway is possible for the HCV IRES.

From a mechanistic point of view, the complex between the 40S subunit, eIF3, the initiator tRNA and the mRNA is a direct intermediate within the conventional 80S formation pathway that is formed after eIF5-induced GTP hydrolysis and eIF2 dissociation. Thus, the eIF2-independent pathway described here may be regarded as a simplified mechanism, in which this intermediate is formed not with the participation of eIF2, but rather directly by cooperative tRNA and eIF5B binding. Notably, the omission of the preceding steps makes it possible for the HCV IRES to achieve the post-initiation 80S complex formation using the hydrolysis of only one GTP molecule. **Figure 5** schematically represents the proposed bacterial-like mechanism along with the canonical one.

In fact, we cannot exclude the existence of some other proteins able to stabilize the codon-anticodon interaction, which could enter the initiation complex at this step in a manner similar to eIF5B. One such candidate may be eIF2A, which has been shown to deliver Met-tRNA to the ribosome in an AUG-dependent fashion[34]. The role of this protein in eukaryotic translation is obscure.

The situation with the HCV IRES seems to be analogous to that observed for prokaryotes, in which mRNAs can form sufficiently stable complexes with small ribosomal subunits without Met-tRNA$_i^{Met}$. As for the bacterial IF2 (ref. 1), eIF5B here has two roles: first it promotes binding of the initiator tRNA to ribosomes (**Fig. 1f**), and second it couples the ribosomal subunits (**Fig. 1d**), in spite of the fact that, in contrast to IF2, no direct binding of eIF5B to initiator tRNA has been reported. Notably, our data provide biochemical evidence that eIF5B stabilizes tRNA binding to the 40S subunit, which was previously suggested on the basis of genetic studies[35]. The need for the only eukaryote-specific factor eIF3 in this process is not entirely clear. Perhaps, for HCV-like IRESs the eIF3 is required for the mRNA–ribosome complex to adopt the appropriate architecture. Thus, the data presented here throw one more bridge across the evolutionarily different modes of translation initiation. They also may suggest that the origin of eIF2 in evolution is relevant to the appearance of ribosomal scanning in eukaryotes.

METHODS

Ribosome subunits, initiation factors and Met-tRNA$_i^{Met}$. We isolated 40S and 60S subunits plus eIF4F, eIF5, eIF5B and eEF1H from RRL, purified eIF2 and eIF3 from Krebs-2 ascites cells, and expressed eIF1, eIF1A, eIF4A, eIF4B and MetRS in *Escherichia coli* as described[16,36,37]. We expressed eIF5B$_{587–1220}$ as suggested[10] and additionally purified it on MonoQ HR 5/5 and Superdex-70 columns, followed by His-tag removal with thrombin (Novagen). Rabbit eEF2 was a gift from L.P. Ovchinnikov. We prepared Met-tRNA$_i^{Met}$ and labeled it with [^{35}S]methionine (GE Healthcare) using MetRS expressed in *E. coli* as described[16]. We freed the S100 supernatant from ascites cells from nucleic acids by passing it through DEAE cellulose at 0.25 M KCl and ammonium sulfate precipitation and used it as a source of mammalian aminoacyl tRNA synthetases. To aminoacylate total calf liver tRNA (Novagen), we used a protocol similar to that used for the aminoacylation of individual tRNA$_i^{Met}$.

Assembly and analysis of translation initiation complexes. We assembled and analyzed ribosomal complexes by sucrose-gradient centrifugation or toeprinting as described[6,36]. Briefly, we assembled 48S complexes on the β-globin mRNA by incubating 0.5 pmol of mRNA for 10 min at 30 °C in a 20-μl reaction volume that contained the reconstitution buffer (20 mM Tris-HCl, pH 7.5, 120 mM potassium acetate, 2.5 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT), 0.4 mM GTP or GMPPNP, 1 mM ATP or AMPPNP, 0.25 mM spermidine-HCl, Met-tRNA$_i^{Met}$ (5 pmol), eIF1 (10 pmol), eIF1A (10 pmol), eIF2 (8 pmol), eIF3 (5 pmol), eIF4A (10 pmol), eIF4B (6 pmol), eIF4F (2.3 pmol), 40S ribosomal subunits (2.5 pmol). For 80S assembly, we supplemented preformed 48S complexes with 60S subunits (2.5 pmol), eIF5B (3 pmol) and eIF5 (3 pmol) and incubated them for a further 10 min. In the case of the HCV mRNA, 48S and 80S complexes were assembled similarly, except we omitted eIF1, eIF1A, eIF4A, eIF4B, eIF4F and ATP (also see text). For *in vitro* translation elongation experiments, we added factors eEF1H (15 pmol), eEF2 (10 pmol) and total aminoacylated tRNA (2 μg) to preformed 80S complexes as described[16]. For sucrose gradient centrifugation analysis we used [^{35}S]Met-tRNA$_i^{Met}$ or mRNAs labeled co-transcriptionally with α-[^{32}P]UTP and centrifuged, assembled complexes through 5–20% (w/v) linear sucrose gradient for 3.5 h for 80S analysis or 4.5 h for 48S analysis at 135,000*g* in SW41 rotor (Beckman). For toeprinting, we used the primer 5′-GGGATTTCT GATCTCGGCG-3′. We analyzed the presence of assembled complexes by primer extension using AMV RT (Promega) essentially as described[36]. cDNA products were analyzed by electrophoresis through a 6% polyacrylamide sequencing gel. For *in vitro* eIF2 inhibition, we added 20 ng of synthesized dsRNA[16] to 14 μl of RRL (Promega) and incubated them for 15 min at 30 °C, then supplemented the lysate with 2mM GMPPNP-Mg^{2+} and analyzed ribosomal complexes as described previously[36].

Plasmids and *in vitro* transcription. Plasmids encoding mRNAs with β-globin[36] or HCV[6] 5′ UTRs for ribosomal complex assembly have been described. For cell transfections, we synthesized polyadenylated mRNAs using PCR products as templates, with the T7 promoter and 50-nt poly(A) tail being introduced by corresponding primers[38].

Studies in transfected cells. Cell-culture manipulations and RNA transfection procedures were performed as described[38]. We replated exponentially growing HEK293T cells to 24-well plates at density 1:2 and added IFNα where indicated to a final concentration of 1,000 IU ml^{-1}. After 12 h of growth, we added dsRNA (2 μg ml^{-1}) with Lipofectamine2000 (Invitrogen) for another 1 h, or added 2.5 mM DTT, 2 mM sodium arsenite or 1 μM okadaic acid (Calbiochem) for 30 min, followed by transfection of reporter mRNAs. We analyzed the reporter protein expression 2 h after transfection.

Western blotting. We grew cells for western blotting in parallel with cells for transfection, similarly treated them with the effectors and harvested them 30 min afterwards (or 1 h later in the case of dsRNA treatment). To address eIF2 phosphorylation, we resolved equal amounts of cell lysates by SDS-PAGE and probed them with the anti–phosphor-eIF2α(Ser51) antibodies (Cell Signaling) or anti-eIF2α antibodies (a gift from W.C. Merrick).

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AUTHOR CONTRIBUTIONS

I.M.T. and S.E.D. contributed equally to this work. I.M.T. and D.E.A. performed *in vitro* experiments; S.E.D. carried out cell transfection studies; and I.N.S., S.E.D. and I.M.T. wrote the article. All authors discussed the results and commented on the manuscript.

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