Unlike for cellular mRNAs and other viral internal ribosome entry sites (IRESs), the eIF3 subunit e is not required for the translational activity of the HCV IRES

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Viruses depend on the host cell translation machinery for their replication, and one common strategy is the presence of internal ribosome entry sites (IRESs) in the viral RNAs, using different sets of host translation initiation factors. The hepatitis C virus (HCV) IRES binds eukaryotic translation initiation factor 3 (eIF3), but the exact functional role of the eIF3 complex and of its subunits remains to be precisely defined. Toward this goal, here we focused on eIF3 subunit e. We used an in vitro assay combining a ribosome-depleted rabbit reticulocyte lysate and ribosomes prepared from HeLa or Huh-7.5 cells transfected with either control or eIF3e siRNAs. eIF3e silencing reduced translation mediated by the 5′UTR of various cellular genes and HCV-like IRESs. However, this effect was not observed with the bona fide HCV IRES. Silencing of eIF3e reduced the intracellular levels of the c, d, and l subunits of eIF3 and their association with the eIF3 core subunit a. A pulldown analysis of eIF3 subunits associated with the HCV IRES disclosed similar effects and that the a subunit is critical for binding to the HCV IRES. Carrying out HCV infections of control and eIF3e-silenced Huh-7.5 cells, we found that in agreement with the in vitro findings, eIF3e silencing does not reduce HCV replication and viral protein expression. We conclude that unlike for host cellular mRNAs, the entire eIF3 is not required for HCV RNA translation, favoring viral expression under conditions of low eIF3e levels.

In addition to biochemical studies, the mechanistic role of each eIF was also clarified by the discovery of viral strategies to bypass the requirement of some eIFs through the presence in the viral RNAs of IRESs that were classified in four major classes based on their structure and their dependence on different sets of eIFs (2). Type I, represented by the poliovirus IRES, requires all eIFs, except eIF4E, and the ribosome to classically scan a part of the 5′UTR. For type II, which includes the encephalomyocarditis virus (EMCV), the translation initiation factor eIF4F complex, which is the biggest translation initiation factor, is composed of 13 subunits (named eIF3a to eIF3m) in mammals (11). However, some authors now do not consider the loosely-associated eIF3f as a true subunit (12, 13). eIF3 is tightly bound to the 40S and allows ribosome recruitment to the mRNA by interacting with the eIF4F complex. In this regard, it has been shown that several eIF3 subunits, including eIF3e, also known as INT6, interact with eIF4G (14, 15).

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This article contains Figs. S1–S5 and Table S1.

5 The abbreviations used are: IRES, internal ribosome entry site; PABP, poly(A)-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; m.o.i., multiplicity of infection; HCV, hepatitis C virus; eIF, eukaryotic initiation factor; EF-P, cricket paralysis virus; EMCV, encephalomyocarditis virus; CSFV, classical swine fever virus; AEV, avian encephalomyelitis virus; SVV, Seneca Valley virus; HAV, hepatitis A virus; PVDF, polyvinylidene difluoride; DCV, Drosophila C virus; FFV, focus-forming unit; PL, poly-L-lysine; RRL, rabbit reticulocyte lysate; uRRL, untreated rabbit reticulocyte lysate; AFM, atomic force microscopy; miRNA, micro-RNA.

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without intervention of the scanning process (2). Type III IRESs, exemplified by the hepatitis C virus (HCV) IRES, correspond to highly-ordered structures that bind directly the 40S ribosome subunit (16–19), eIF3 (20–23), the ternary complex (eIF2–Met-tRNA\textsubscript{Met}–GTP), and other factors. These HCV-like IRESs regroup equivalent RNA structures from flaviviridae, such as the classical swine fever virus (CSFV), and also the picornaviridae such as Seneca Valley virus (SVV) and avian encephalomyelitis virus (AEV). Finally, the dicistroviridae family represents type IV IRESs with the cricket paralysis virus (CrPV) intergenic IRES prototype. This sequence corresponds to complex tertiary structures that are able to recruit the ribosome without any eIF (24–26). The hepatitis A virus (HAV) IRES eIFs’ exact requirement is still not very clearly established, and it represents an additional class on its own.

So far, the functional role of eIF3 regarding translation initiation driven by viral IRESs is not clearly established. This is essentially due to the lack of specific inhibitors and to the difficulty to carry out knockdown experiments in living cells, as silencing of even one subunit is often toxic. Concerning \textit{in vitro} analyses, depletion of eIF3 remains challenging as the complex is tightly bound to the ribosome (27).\textsuperscript{6} Concerning the HCV IRES, it has been known for nearly 20 years that this IRES strongly interacts with eIF3 (28–30). Although contacts between this RNA sequence and six of the 13 eIF3 subunits have been reported (22, 29), this binding seems to depend mainly on the eIF3a and -c subunits (23). Recent structural analyses by cryo-EM revealed that the CSFV IRES, an HCV-like IRES, by binding eIF3 displaces it from the 40S ribosome that interacts directly with the IRES to start translation at a specific position (30). These authors proposed that this mechanism also causes an advantage for HCV and HCV-like viruses, as eIF3 binding to the viral RNA could hinder competitive translation of cellular mRNAs (30).

\begin{figure}
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\caption{Effect of eIF3e silencing on translation initiation factors in HeLa cells. A, schematic representation of the 13 subunits constituting the eIF3 translation initiation factor. The a and c subunits, which are determining for binding to the HCV IRES, are circled. Additionally, subunits observed to contact RNA by proteolysis experiments are underlined. B, effect of eIF3e silencing (siRNA I6.3 at 10 nM) on protein level of eIF3a and other eIFs or cellular proteins as indicated on the left side. Whole-cell extracts were prepared 72 h after transfection of control (ctrl) or eIF3e (3e) siRNAs as described under “Experimental procedures” with equal numbers of cells for both conditions. Signals corresponding to the indicated proteins and validated by the position of a molecular weight band shown on the right were quantified. Signal intensities were normalized with respect to control and indicated on top of the blots.}
\end{figure}

\textbf{Results}

eIF3e down-regulation did not affect the HCV IRES-driven translation

Several cryo-EM studies have led to an anthropomorph model of the eIF3 general organization (Fig. 1A). To study the

\textsuperscript{6} B. Panthu, personal observation.
functional consequences of elf3e on translation initiation efficiency, this subunit of the elf3, which corresponds to the right arm, was silenced by transfecting HeLa cells with elf3e siRNAs. As evaluated by Western blotting with a specific antibody, this led to an efficient 5–10-fold reduction in the amount of elf3e (Fig. 1B). The amount of the core elf3a subunit and of other translation initiation factors, such as elf4A1 and elf2α, was weakly affected (Fig. 1B). To understand better how elf3 interferes in the activity of the HCV IRES, we tested the functional effect of the elf3e knockdown (elf3eKD). This was first done using the in vitro assay that we recently developed (31) by combining riboproteome-free rabbit reticulocyte lysate and a ribosomal fraction prepared from HeLa cells transfected with control or elf3e siRNAs (Fig. 2A). To measure the translation capability of such an altered riboproteome, we used in vitro-transcribed mRNA from which translation is driven by several 5’UTRs upstream of the luciferase reporter gene; the CrPV IRES, which does not require elf3 for translation, was translated in parallel as a positive control. The in vitro assay was carried out using mRNAs harboring a 5’ cap and a 50-nucleotide 3’ poly(A) tail (+/+), only a 5’ cap (+/−), only a 3’ poly(A) tail (−/+), or lacking both of these elements (−/−).

These cellular RNA constructs produced variable amounts of luciferase (Table S1), and all those known to be dependent on elf3 for translation initiation were less efficiently translated, usually with a 60–70% decrease (Fig. 2B). For the PABP mRNA, this reduction even reached 90%. This confirms in our experimental setting that translation initiation is clearly altered by elf3eKD, as reported previously by Wagner et al. (12), by looking at the monosome/polysome ratio. In this assay, we tested the activity of the HCV IRES. Differently from what was observed with the constructs derived from cellular RNAs, the activity of the HCV IRES-driven translation was not decreased when the elf3eKD riboproteome was used, despite the fact that this sequence has been shown to bind the elf3 complex (Fig. 2C). This was true whether or not a 5’ cap and/or a poly(A) tail was added to the reporter mRNA. As observed in the previous experiment, elf3eKD enhanced the activity of the CrPV IRES. Such an effect was also observed for the HCV construct lacking a 5’ cap and a poly(A) tail but to a much lesser extent. The observed effects were not related to alteration of mRNA stability as controlled by quantitative RT-PCR. Indeed, the amount of sensitive or insensitive mRNAs did not vary during more than 1 h, and for analyses, translation was blocked after 30 min of incubation (Fig. S1). We also tested several viral IRESs that are known to trigger translation initiation with only a subset of translation initiation factors. This experiment was carried out with a higher amount of RNA due to weaker activities except for the Drosophila C virus (DCV) IRES. We observed that the activity of picornavirus-derived IRESs (HAV and EMCV), as well as that of several HCV-like IRES, was reduced similarly to what was observed with the cellular 5’UTR constructs (Fig. 2D, constructs AEV, SVV, and CSFV). By contrast, translational activity driven by the DCV and HCV IRESs was not affected.

These results led us to conclude that the full elf3 function did not appear to be required for HCV IRES-mediated translation initiation and that the HCV IRES is able to recruit the ribosome for effective translation with no need for the elf3e subunit. By contrast, the complete elf3 complex was necessary for full functionality of the other HCV-like and HAV IRESs that were tested.

To rule out that the absence of effect observed with the HCV IRES was related to a dosage effect, we have varied the added amount of control and elf3eKD riboproteomes to a fixed concentration of mRNA translated (Fig. 3, A, C, and E). We have also performed the complementary experiment in which the mRNA concentration was increased with a fixed amount of riboproteome (Fig. 3, B, D, and F). These experiments were carried out with three test constructs whose expression was driven by the β-globin (HBB) 5’UTR, HCV IRES, and CrPV IRES. As shown before for the β-globin mRNA, the translation efficiency was reduced by ~60% when elf3e was silenced, and this effect was not affected either by varying the amount of the riboproteomes (Fig. 3A) or by the increase in the mRNA amount, except at the highest concentrations used in the case of the elf3eKD riboproteome (Fig. 3B). Hence, for this construct elf3e is clearly necessary for efficient translation. However, for both HCV and CrPV IRESs, we noticed a clear dose-response effect with the elf3eKD riboproteome (Fig. 3, C and E). Indeed, increasing the elf3eKD riboproteome raised translation efficiency, in a linear manner for the CrPV IRES and up to a certain point for the HCV IRES (Fig. 3, E and C). Similarly, translation efficiency dropped for both constructs when the mRNA concentration was increased (Fig. 3, D and F). As it is known that IRES-driven translation is sensitive to salt concentration in RRL and even in a hybrid-reconstituted system (31), it is noteworthy that increasing amounts of riboproteome were diluted in an equal volume of riboproteome resuspension buffer (typically 1 μl at a desired riboproteome concentration in 10 μl of final mix, see buffer R2 under “Experimental procedures”) to maintain a constant magnesium and potassium concentration in the mix when riboproteome or mRNA quantities were varied. Nevertheless, if elf3e, and by extension intact elf3, has a positive role in the translation of test cellular mRNAs, these results show that it is dispensable for the recruitment of the ribosome at the initiation codon when this event is driven by the HCV or CrPV IRES. For both, increasing the ribosomes/RNA ratio has a positive effect on translation efficiency that is not observed for the β-globin reporter constructs, possibly due in this case to a limiting factor in the complete initiation process.

**elf3e silencing strongly alters the elf3 complex**

To assess precisely the consequences of elf3e silencing on the elf3 complex, we performed immunoprecipitation experiments with an antibody directed against elf3a and analyzed by Western blotting the presence of several other elf3 subunits in the immunoprecipitates. To check on elf3e silencing, we performed analysis by Western blotting of control extract serial dilutions using an IR imaging system that allows precise quantification of protein loads, which confirmed the high efficiency of the elf3e depletion in the transfected cells (Fig. S2). As observed previously, silencing of elf3e did not reduce the abundance of the a subunit and also of the b and i subunits (Fig. 4A, lanes 1 and 2). As expected, the e signal was reduced, and the chemiluminescent revelation slightly underestimated this effect (Fig. 4A and Fig. S2). This was also the case, although...
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more weakly, for the c, d, and l subunits. In previous studies, Walsh and Mohr (32) observed in normal human diploid fibroblasts a similar effect for eIF3c, -d, and -h, and more recently, Wagner et al. (12) reported that eIF3e knockdown reduced eIF3c, -d, -h, -k, and -l. After immunoprecipitation, the association of the b and i subunits with eIF3a was clearly not affected and, if anything, was slightly enhanced by eIF3e knockdown, whereas the c, d, e, and l signals were reduced (Fig. 4A, lanes 4 and 5). Previous studies have shown that the right arm of eIF3 that is composed of the e, d, k, and l subunits forms a subcomplex and that its presence in the eIF3 complex relies on a c–e interaction. Our observations show that eIF3e is necessary for

Figure 2. In vitro translation assays with eIF3eKD ribosomal fraction. A, a schematic representation of the method used to work in vitro that associates a ribosomes-depleted rabbit reticulocyte lysate (ribFreeRRL) and a ribosomal fraction prepared from HeLa cells transfected either with control or anti-eIF3e siRNAs (riboproteome). B–D, various RNAs with the luciferase-coding sequence downstream of cellular 5′UTRs (H2B, H2A, p53, cyclin D2, PAPB, c-myc (MYC), GAPDH, β-globin (HBB)) or of viral IREs (CrPV, DCV, AEV, HAV, SVV, CSFV, HCV, and EMCV) were translated using the in vitro hybrid system with either control (siRNA ctrl, white bar) or eIF3eKD (siRNA 3e, gray bar) HeLa riboproteomes. For each construct, the presence of a cap at the 5′ end and a poly(A) tail at the 3′ end is annotated by the symbol (+/−) and corresponds to the native context of the mRNA from which it is derived, except for D, where HCV that is normally uncapped and unpolyadenylated (HCV −/−) was artificially modified with a cap (HCV +/+ ) or with a poly(A) tail (HCV +/− ) or both (HCV +/+ ). For histone RNAs, there was no poly(A) tail, but the 3′ stem-loop (SL) of these genes was present. Translation was carried out for 30 min at 30 °C with 10 ng (B and C) or 100 ng (D, except DCV 10 ng) of RNA and 1 μg of riboproteome. Results are expressed as ratio of inhibition relative to the mean of values obtained with siRNA control that was set arbitrarily to 1. Graphs represent the values obtained in three independent experiments (circle, square, and triangle), and the bars correspond to the mean ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns > 0.05 (t test).
the presence of c in the eIF3 complex likely due to the direct interaction between both subunits. Therefore, eIF3e appears to play a key role in anchoring the right arm and head of eIF3. Given this strong alteration, it is not surprising that translation of many cellular and viral mRNAs is sensitive to eIF3e depletion. As eIF3 is known to bind to the HCV IRES, we also performed RNA pulldown with the HCV IRES. This RNA sequence was hybridized with a small biotinylated oligonucleotide that specifically targets the IRES sequence. The biotinylated RNA was then used to affinity purify the eIF3 complex. The eIF3 complex was subsequently analyzed by Western blotting to determine its composition and abundance. The results showed that the eIF3 complex from eIF3-depleted cells contained reduced levels of eIF3e compared to control cells, confirming the importance of eIF3e in the anchoring of the eIF3 complex. Overall, these findings provide new insights into the role of eIF3e in translation initiation and highlight the potential therapeutic targets for modulating translation efficiency.
**elf3e is not necessary to the HCV IRES activity**

Similar to what was observed by immunoprecipitating elf3a, elf3e depletion did not affect association of the a and b subunits of elf3 to the HCV IRES, and the signals corresponding to the c, d, and e subunits were markedly reduced (Fig. 4B, lanes 3 and 4). In agreement with previous observations, this indicates that the a subunit plays an important role in the binding to the HCV IRES. This association does not necessitate the c or e subunits. These observations support the notion that the HCV IRES does not require the entire elf3 complex and can support translation with a strongly-altered complex lacking the head and right arm subunits.

**Mutations in the HCV IRES mir122-binding site affect the translation efficiency but not elf3e-independent translation**

To further evaluate the independence of the HCV IRES on elf3e, as observed using the in vitro assay, we performed experi-
**CrPV IRES upon addition of the elf3eKD riboproteome (Fig. S3). The knockdown of elf3e could also be evidenced in Huh-7.5 although less pronounced than in HeLa cells, likely due to poorer transfection efficiency (Fig. S4).** Then, we compared translation of the HCV IRES construct with mutated miR-122–binding sites to WT. As expected, the loss of the miR-122–binding sites resulted in a marked decrease in the HCV IRES translational efficiency (Fig. 5B), but the RNA remained unaffected by the depletion of the elf3e subunit. To assess sequence folding, we also used an atomic force microscopy (AFM) approach. The HCV IRES was produced by in vitro transcription and analyzed by AFM after deposition on mica surfaces to get nanometer resolution (Fig. S5). These data showed a structure that is similar to that observed by structure probing, molecular modeling, and cryoEM by others (35). This structure is in agreement with a folding in three main stem-loops as represented in Fig. 5A.

Taken together, these observations confirmed the lack of necessity of elf3e for the HCV IRES activity, and they showed that it was not due to an incorrect folding of the sequence used in the in vitro assays.

**elf3e is dispensable for HCV life cycle from replication to infectious particle production**

Considering data obtained so far using the in vitro assay, we further addressed the question of the requirement of elf3e and by extension of an intact elf3 for expression of HCV in the context of viral infection. Huh-7.5 cells were transfected with control or elf3e siRNAs, and 3 days later, the HCV JC1 strain was used to infect the cells (see Fig. 6A for details of the experimental procedure). As shown, elf3e was efficiently silenced 3 days post-transfection, and this status was maintained for up to 6 days (Fig. 6, B and C). Consistent with the elf3e silencing, a clear decrease in overall translation was observed by monitoring total protein synthesis labeled with a pulse of [35S]methionine (Fig. 6D). As observed in HeLa cells, elf3eKD affected several other elf3 subunits, including elf3d and -h (Fig. 7A). In contrast to the situation occurring in HeLa cells (Fig. 4), elf3c was modestly affected, whereas elf3i was reduced by elf3eKD. These effects on the elf3 subunits were observed in both mock- and HCV-infected cells (Fig. 7A). However, to our surprise, HCV infection also led to a reduction in the amount of several elf3 subunits, including elf3d, -e, -h, and -i (Fig. 7A).

Various structural (E2 and core) or nonstructural proteins (NS2 and NS5A), which result from processing of a single polyprotein, were analyzed by immunoblot after normalization with respect to cell number (Fig. 7B). Translation of all these proteins is under the dependence of the 5’ IRES. Whereas under HCV infection conditions, elf3e silencing led to a decrease in several cellular translation initiation factors such as elf4E, PABP, elfA1, and elf2e, possibly due to the marked decrease in elf3e as a consequence of the combined effects of infection and siRNA transfection, none of the viral protein levels were reduced (Fig. 7B). This absence of effect of elf3eKD on viral expression was also observed by flow cytometry monitoring the expression level of the NS5A protein (Fig. 7, C and D).

Expression of the HCV viral RNA was also tested at 3 days post-infection by RT-qPCR analyses that showed no significant
**Figure 6. Cell response to eIF3e silencing under HCV infection conditions.** A, schema of the experimental design. Huh-7.5 cells were transfected with siRNA, and 72 h later cells were infected with the HCV JFH1 strain at a m.o.i. of 0.5. Biochemical analyses were performed 3 days after transfection and 3 days after infection. B, expression of eIF3e and calnexin (CNX) was analyzed by immunoblot at 3 days (time of infection) and 6 days (3 days after infection). C, level of the eIF3e mRNA at the time of infection was determined by RT-qPCR and is represented as percentage to cells transfected with control siRNA (points of three independent experiments with mean ± S.D.). D, [35S]methionine labeling of Huh-7.5 cells 6 days after siRNA transfection. Cells were either mocked or HCV-infected at day 3. Total proteins were separated by SDS-PAGE, and the gel image was acquired by phosphorimaging (upper panel). Quantification of the total radioactivity present in protein lane was performed with the ImageQuant software (middle upper panel). The amount of loaded proteins was monitored by TCE labeling of the gel (middle lower panel). The amount of eIF3e and RPL11 proteins was analyzed by immunoblot (lower panels).
Figure 7. Effect of elf3e silencing on HCV infection in a hepatocyte cell line. A and B, effect of siRNAs targeting elf3e on protein levels of various elf3 subunits (A) and other viral and cellular proteins (B), in mock or JFH1-infected cells, was determined by immunoblot at day 3 post-infection with various antibodies as indicated on the right. Whole-cell extracts were prepared as described under “Experimental procedures” with equal numbers of cells for both conditions. C, expression of NS5A in HCV-infected cells transfected with control (sictrl) or elf3e (si3e) siRNAs was analyzed by flow cytometry at 3 days post-infection. D, levels of NS5A in both types of cells were quantified by measuring the mean fluorescence intensity of NS5A-positive cells. Results are expressed as percentage of the value in control cells (points of three independent experiments with mean ± S.D.). E and F, levels of elf3e mRNA (E) and HCV RNA (F) in mock- or HCV-infected cells were determined 48 h siRNA post-transfection on an aliquot of cells that were further split and 72 h post-infection (120 h post siRNAs transfection) by RT-qPCR and represented as percentage of the value in cells transfected by control siRNA and mock-infected. The points of six independent experiments are represented with mean ± S.D. except for one point at 48 h, which was not measured. G, extracellular infectivity was determined from supernatants of infected cells at 3 days post-infection and represented as percentage of the value in cells transfected by control siRNA.
eIF3e is not necessary to the HCV IRES activity

decrease in the amount of the viral RNA (Fig. 7F) when eIF3e was silenced (Fig. 7E). Finally, we examined whether eIF3e\textsuperscript{KD} could affect the production of infectious particles by acting on steps downstream of the viral replication, such as assembly and secretion of viral particles. We did not detect any difference in the production of infectious particles (Fig. 7G), confirming that eIF3e is not required for the HCV life cycle.

Therefore, and in agreement with the in vitro data, eIF3e silencing did not impact HCV replication and protein expression.

**Discussion**

Viral IRESs have the ability to initiate translation by using only a subset of the many factors that are used for classical cap-dependent translation. This is the case for the HCV IRES that drives the expression of the viral proteins resulting from processing of a single polyprotein. Until recently, it was considered from several studies that the eIF3 translation initiation factor was required for the functionality of this IRES. Indeed, it has been clearly shown that eIF3 binds the IIIabc stem–loop domain of this RNA motif, and mutations in this sequence lead to complete inactivation of the IRES. Hence, the HCV IRES was considered as a cap-independent but eIF3-dependent translation initiator. Consequently to detailed cryo-EM structural studies, this notion has been recently challenged by Hashem et al. (30), who proposed a different view. Indeed, by looking at the contacts between eIF3 and the CSFV IRES, they observed that the RNA stem-loops of the IIIabc domain interact mainly with the a and c subunits of eIF3, which are known to also associate with the 40S small ribosomal subunit. In the anthropomorphistic representation of eIF3, eIFa and eIF3c represent the left arm and head, respectively (Fig. 1A). These observations led the authors to propose a model in which the interaction of the IRES with eIF3 is not important for recruiting this initiation factor but for displacing it from the ribosomal small subunit that is directly loaded on the start codon by interacting with other RNA sequences of the IRES, the direct binding of eIF3 to the viral RNA also having the advantage to hinder cellular translation. Whereas most of the data were obtained with the CSFV IRES that interacts more strongly with eIF3, these authors considered that this model is also likely true for the HCV IRES. However, it is important that in vitro analyses have shown that eIF3 is needed for assembly of the 80S complex on the CSFV IRES, suggesting that this initiation factor is likely to play some role in translation initiation at a later step after recruitment of the 40S (28).

To have a more functional view of these questions, we took advantage of the hybrid RRL system that we have designed in previous studies. With this experimental procedure it is possible to deplete the fraction containing the ribosomes and associated factors by preparing a so-called riboproteome from cells transfected with specific siRNAs. Another noticeable advantage of this system is also to look at the effect of increasing amounts of riboproteome, as this type of analysis is unreachable in cellulo. In this study, this was achieved by silencing eIF3e. Transfection of cells with a modified siRNA targeting eIF3e led to an efficient reduction in the intracellular amount of this eIF3 subunit, but its depletion also affected the assembly and composition of eIF3 as a whole as observed previously by others (12, 32). All the subunits on the right side of the eIF3 were coarsely affected. This includes the right arm consisting of eIF3d that recruits eIF3d to the complex and the right leg, including eIF3l and eIF3k. Smith et al. (36) have proposed that the assembly of eIF3 results from a sequence of events starting from the interaction of eIF3a with the eIF3b, -g, and -i subcomplex, followed by an association with eIF3c and -h. At this step, the eIF3e and -d complex can enter the complex followed by association of eIF3k and -l (36). The observed reduction in eIF3e, -d, and -l is in very good agreement with this sequence as eIF3e plays a pivotal role for entry in the complex of the other affected subunits. Other eIF3 subunits were affected, yet to a lesser extent. The results of our in vitro experiments show that with eIF3e the right side of the complex is important for cap-dependent translation driven by the 5′ UTR of many cellular genes. The effect observed ranged between a 60 and 90% decrease, which was quite strong and more pronounced than what was observed in vivo in transfected cells. The reason for this difference is not clear and could relate to a stabilizing role of the intracellular milieu and/or other factors. This observation is in agreement with the previously reported interactions of eIF3e with eIF4G1 (14) and in the case of the histone genes with MIF4GD that binds stem-loop binding protein associated with the 3′ stem-loop motif specific to the histone mRNAs (37). When viral IRESs were tested, as expected the CrPV IRES was completely insensitive to eIF3e knockdown, as was the DCV IRES. In contrast, several other HCV-related IRESs, such as those of AEV, SVV, and CSFV, were sensitive to the depletion of eIF3e. For CSFV, our observations are in good agreement with the results of Liu et al. (38), who have recently reported that eIF3e is necessary for the full in vivo activity of the CSFV IRES. By strictly considering the model of Hashem et al. (30), this is surprising. Indeed, if the only function of the binding of eIF3 to the IIIabc domain of the IRES is to displace it from the 40S ribosomal subunit, a negative effect of eIF3 alteration would not be expected. Therefore, our observations indicate that even bound to the IRES, some of the eIF3 subunits, especially those of the right side, still play a positive role in the initiation of translation, possibly through contacts with other translation factors and the ribosome. Alternatively, the eIF3 subcomplex persisting after eIF3e silencing could be less efficient in stabilizing the viral IRES in a configuration favoring recruitment and the start of the ribosome. Future in vitro studies with reconstituted eIF3 subcomplexes should help to discriminate between these two possibilities.

In contrast to the other HCV-related IRESs tested, that of HCV itself is insensitive to eIF3e silencing. In this way, it behaves similarly to the CrPV IRES, which is independent of eIF3 and has not been reported to bind this complex. Johnson et al. (39) have recently shown that the knockdown of eIF3h strongly affects eIF3k and -l but that, like eIF3e silencing, it does not alter the HCV and CrPV IRES activity. Hence, for HCV, subunits of the right side of eIF3 are not necessary for direct 40S recruitment and 80S assembly to the initiation codon. In this line, it is interesting to mention that several differences exist between the CSFV and HCV IRESs. For instance, stem 1 of the pseudoknot is interrupted in CSFV, whereas in HCV it forms a
single fully base-paired stem. Furthermore, in the 40S ribosome-interacting part consisting of domains IIIId, IIIe, and pseudoknot, the CSFV IRES has an extra subdomain (IIIId2) that is absent from the HCV IRES (30). The exact RNA motifs at the basis of the difference in elf3 dependence that we observed could be identified in future studies by testing chimeric constructs with various combinations of CSFV and HCV IRESs domains. In agreement with the in vitro results obtained with riboproteomes prepared from both the HeLa and Huh-7.5 cell lines, elf3e silencing in living cells did not affect the amount of the HCV RNA nor the synthesis of viral proteins. In agreement with these observations, no difference was observed in the infectivity of the viral particles produced from elf3e-silenced cells, which confirms nicely as this subunit is not critical for HCV replication.

Intriguingly, the immunoblot experiments carried out to monitor the various eIF3e subunits revealed that HCV infection led to a decrease in the abundance of several of them, including elf3e and -d. This effect of infection is therefore close to that observed when elf3e was silenced. The reason for this remains unclear, but elf3e down-regulation might also have consequences on the maintenance of several elf3 subunits. By considering the results of the in vitro assays, this likely does not affect HCV translation, although it could be a means to hinder translation of cellular mRNAs giving a competitive advantage to the viral RNA. Along this line, two other important proteins, PABP and elf2e, were also observed to be down-regulated by HCV infection in Huh-7.5 cells, in agreement with the reported relative insensitivity of the HCV IRES sensitivity to these factors in hepatoma cell lines (40, 41). How infection affects all these proteins important for translation will represent an interesting line of investigation. Moreover, the effect of HCV infection on elf3e could be important to consider with respect to the oncogenic effect of this virus. Indeed, INT6/elf3e has first been identified as the product of a gene described as a site of insertion of the mouse mammary tumor virus in preneoplastic and neoplastic lesions of the mammary gland in CZECH II mice (42). Reduced expression of INT6/elf3e has also been reported in nonsmall cell lung carcinoma and in breast tumors (43, 44). Among the various effects resulting from INT6/elf3e silencing, an alteration of the DNA damage response has been shown (45, 46). Hence, in addition to the effect of some viral proteins (47, 48), a possible role of elf3e down-regulation in genomic instability would be interesting to explore in infected hepatocytes.

In conclusion, our observations bring new clues about the functional relationship between the HCV-like IRESs and translation initiation factors and show that the relationship between these RNA sequences and elf3 is far more complex than initially thought. These results also open new and interesting perspectives about the virus–host interactions leading to carcinogenesis associated with HCV.

**Experimental procedures**

**Cell culture and reagents**

HeLa and Huh-7.5 cells were grown in Dulbecco’s modified minimal essential medium (Invitrogen, France) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum.

**DNA constructs**

The β-globin (HBB), GAPDH, MYC, PABP, cyclin D2, p53 5′ UTR, foot-and-mouth disease virus, HAV, AEV, DCV, HCV, and CrPV IRESs p0-renilla constructs were described previously (31), as well as the HCV IRES p0-renilla harboring a mir122 mutation (33). Plasmids PSL-H2A and PSL-H2B were used for PCR amplification of H2A and H2B 5′ UTR upstream of the firefly reporter gene (37). The 5′ oligonucleotides included the T7 promoter sequence (5′-GTAATACGACTCACTATAGGGAAAGCCCGATGTTTATTTA-3′ for H2AC and 5′-GTAATACGACTCATAAGGCAATGGAAGACGCCAAA-3′ for H2BG), and the 3′ oligonucleotides were located downstream of the 3′ stem–loop elements (5′-TAGGGGCTCTGAAAGAG-3′ for H2AC and 5′-TTTATGTGAGAC-TTGAATGG-3′ for H2BG). PCR products were precipitated, dried, and resuspended in water before direct in vitro transcription. pFK-JFH1/6/C-846_dg was a kind gift of Bartenschlager and co-workers (49).

**In vitro transcription**

RNAs were transcribed using the T7 RNA polymerase from templates linearized either at the AflII (for polyadenylated mRNAs) or at the EcoRV restriction sites (for nonpolyadenylated mRNAs). Uncapped mRNAs were obtained by using 1 μg of linear DNA template, 20 units of T7 RNA polymerase (Promega), 1.6 mM of each ribonucleoside triphosphate, 3 mM DTT in transcription buffer (40 mM Tris-HCl, pH 7.9, 6 mM MgCl₂, 2 mM spermidine, and 10 mM NaCl). For capped mRNAs, the rGTP concentration was reduced to 0.32 mM, and 1.28 mM m7GpppG cap analog (New England Biolabs) was added. The transcription reaction was carried out at 37 °C for 2 h, and the mRNAs were precipitated with ammonium acetate at 2.5 m final concentration. The RNA pellet was resuspended in 30 μl of RNase-free water, and RNA concentration was determined by reading the absorbance using Nanodrop technology. RNA integrity was checked by electrophoresis on nondenaturing agarose gel. HCV RNA was transcribed as described previously (50).

**HCVcc production and titration**

HCVcc production procedures were described previously (50). Supernatant infectivity titers were determined as focus-forming units (FFU/ml). Serial dilutions of supernatants were used to infect Huh-7.5 cells; FFUs were determined 3 days post-infection by counting NS5A-immunostained foci.

**Combining Huh-7.5 siRNA transfection and HCV infection**

Huh-7.5 cells were transfected with siRNA and 48 h post-transfection were plated in 6-well plates. 72 h post-transfection, cells were infected at a m.o.i. of 0.5. At 72 h post-infection, cells were washed with PBS, harvested, counted, and either lysed for Western blotting or qPCR analysis or fixed for flow cytometry analysis. Supernatant was also harvested and used for virus titration.
**Western blotting and autoradiography**

Cell extracts were migrated on a 12% SDS-PAGE, transferred to a PVDF membrane, and blotted using the following: anticalnexin (Sigma, catalog no. C7617); anti-NS5A 9E10 and anti-NS5B 6H6 (kind gifts from C. Rice, Rockefeller University, New York); anti-E2 3/11 (kind gift from J. McKeating, University of Oxford, UK); anti-core C7-50 (Thermo Fisher Scientific, catalog no. MA1-080); anti-β-actin (Sigma, catalog no. A2228); anti-eIF3a (Cell Signaling Technology, catalog no. 3411S); anti-eIF3b (Novus, catalog no. NB100-93302); anti-eIF3c (Cell Signaling Technology, catalog no. 20685S); anti-eIF3d (51); anti-eIF3e (C-20 (52)); anti-eIF3f (BioLegend, catalog no. 638202); anti-eIF3g (Biolegend, catalog no. 646102); anti-eIF3h (Cell Signaling Technology, catalog no. 3413S), anti-eIF3i (BioLegend, catalog no. 646702); anti-eIF3j (Cell Signaling Technology, catalog no. 3261S); anti-eIF3k (51); anti-eIF4A1 (Cell Signaling Technology, catalog no. 3490); anti-PABP (Sigma, catalog no. P6246); anti-RPL11 (Cell Signaling Technology, catalog no. 2217S). The proteins of interest were revealed by enhanced chemiluminescence detection (SuperSignal West Pico, Thermo Fisher Scientific or ECL Prime, GE Healthcare) or IR fluorescence (LI-COR fluorescent secondary antibodies). Signals were quantified using ImageJ (autoradiograms), Image Lab (ChemiDoc Imaging System, Bio-Rad), or Image Studio (Odyssey Clx, LI-COR). To detect bulk translation after [35S]methionine labeling, gels were dried and subjected to autoradiography for 12 h using Kodak Biomax films (Thermo Fisher Scientific), or Image Lab (ChemiDoc Imaging System, Bio-Rad) or Image Studio (Odyssey Clx, LI-COR). The signal was quantified by using a Molecular Dynamics Phospholimager FLA 5100 (Fuji).

**Flow cytometry analysis**

Cells were fixed and stained using Cytofix/Cytoperm (BD Biosciences) according to manufacturer’s instructions. NSSA staining was achieved with 9E10 antibody (kind gift from C. Rice, Rockefeller University, New York).

**Luciferase activity**

Luciferase activity was measured using the Renilla or firefly luciferase assay system (Promega) and a Mithras luminometer (Berthold Technologies) with 50–µl substrate injection and the 10-s signal integration program.

**Analysis of intracellular RNA levels**

Cells were harvested, and RNAs were extracted with Tri Reagent (Sigma) according to manufacturer’s instructions. The RNAs were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) and quantified with the FastStart Universal SYBR Green Master kit (Roche Applied Science) on an Applied StepOne Real-Time PCR apparatus. The sequences of the primers used for the RT-qPCR were as follows: HCV, 5'-TCTGGGAACCGGTAGTGA-3' and 5'-TCAGGCAAGT-CCACAAGGC-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AGGTGAGGCTCGAGTTCAAG-3' and 5'-TGGAAGATGTTGATGGGATTC-3'; and for eIF3e, 5'-TTCTTCAATCACCCAAAAGG-3' and 5'-TAGAACCTGGCGAGTCTTC-3'. RNA levels were normalized with respect to GAPDH RNA levels.

**Atomic force microscopy, surface preparation, and image analysis**

For the AFM imaging, the in vitro transcribed HCV IRESs were immobilized onto poly-L-lysine (PL)-mica surfaces. The PL functionalization of freshly cleaved mica disks (muscovite mica, grade V-1, SPI) was obtained by depositing 10 µl of 0.01% poly-L-lysine solution (Sigma) for 30 s before rinsing twice with 1 ml of milliQ Ultrapure® water and dried by nitrogen flow. A 5-µl droplet of the IRES solution (in 10 mM Tris, 1 mM MgCl2 buffer) was then deposited onto the PL-mica surface for 5 min, rinsed with 1 ml of milliQ-Ultrapure® water, and gently dried by nitrogen flow. The samples were visualized using a NanoScope V Multimode 8 AFM (Bruker AXS). The images have been obtained in Tapping Mode in air, using Diamond Like Carbon Spikes tips (resonant frequency ~150 kHz) or MPP1100 silicon probes (resonant frequency ~350 kHz) at scanning rates of 1–2 Hz over scan areas of 0.5–2 µm wide.

**Cells extract and fractionation**

All following steps were performed at 4 °C. 1.10⁶ HeLa or Huh-7.5 cells were collected by centrifugation at 1,000 × g for 5 min, rinsed three times with PBS, resuspended in an isovolume of hypotonic buffer (10 mM Hepes, pH 7.0, 10 mM CH3CO2K, 1 mM (CH3CO2)2 Mg, and 1 mM DTTP), and lysed using a Dounce (B-pestle). The lysate was then centrifuged at 16,000 × g for 10 min, and the supernatant was the cytoplasmic extract.

The ribosomal pellet was obtained by centrifugation of 300 µl of cytoplasmic extract through a 1M sucrose cushion for 2 h 15 min at 75,000 rpm using a TLA 100.3 rotor (Beckman), rinsed three times in buffer R2 (20 mM Hepes, pH 7.0, 10 mM NaCl, 25 mM KCl, 1.1 mM MgCl2, 7 mM β-mercaptoethanol), and resuspended in 30 µl of R2 buffer to get a 10 mg/ml final concentration.

**In vitro translation**

Unlabeled rabbit reticulocyte lysate (uRRL) (Promega) preparation was performed as described previously (31). All steps were performed at 4 °C. After centrifugation of 1 ml of uRRL for 2 h 15 min at 75,000 rpm using a TLA 100.3 rotor (Beckman), 900 µl of post-ribosomal supernatant (ribosome free RRL) was collected, frozen, and stored at −80 °C. The extent of ribosome depletion from reticulocyte lysate was checked by translating 27 nm of in vitro-transcribed capped and polyadenylated globin-Renilla mRNA in the S100 and validated when no luciferase activity could be detected. The reconstituted lysate was then assembled by mixing 5 µl of ribosome-free RRL supernatant (S100) with a range from 0.5 to 8 µg of ribosomal fraction derived from mock-transfected or eIF3e siRNA-transfected HeLa or Huh-7.5 cells. Typically, the standard reaction contains 5 µl of S100 with 1 µg of ribosomal fraction from given cells in a final volume of 10 µl. Upon reconstitution, the translation mixture was supplemented with 75 mM KCl, 0.75 mM MgCl2, 20 mM amino acids mix in a final volume of 10 µl. Each reaction was performed in triplicate, and a Student’s t test (unpaired, two tails) was performed (*, p < 0.05; **, p < 0.01; ***, p < 0.001, ns > 0.05).
Immunoprecipitation and RNA pulldown

Cells were lysed in Nonidet P-40 buffer with 300 mM NaCl (NP-40 300). For immunoprecipitation, extracts were incubated for 1.5 h with an antibody to elf3a diluted 1:250 at 4 °C. Sepharose protein A beads were added, and the mix was further incubated for 1 h. Beads were collected by centrifugation (2 min at 1000 × g) and washed three times with NP-40 300 buffer. Proteins were eluted in 1× SDS protein gel loading buffer for 10 min at 80 °C and denatured for 5 min before loading onto the gel. For RNA pulldown experiments, magnetic beads were washed once in blocking buffer (20 mM Hepes, pH 7.0, 300 mM NaCl, 0.1% Nonidet P-40) and were incubated for 2 h at 4 °C in blocking buffer complemented with 0.1 µg/µl tRNA and 1 µg/ml BSA. Beads were collected and washed twice in blocking buffer, and the proteins were prepared for the SDS protein gel as described above for immunoprecipitation.

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