The functional RNA domain 5BSL3.2 within the NS5B coding sequence influences hepatitis C virus IRES-mediated translation

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Abstract

Hepatitis C virus (HCV) translation is mediated by an internal ribosome entry site (IRES) located at the 5' end of the genomic RNA. The 3' untranslatable region (3'UTR) stimulates translation by the recruitment of protein factors that simultaneously bind to the 5' end of the viral genome. This leads to the formation of a macromolecular complex with a closed loop conformation, similar to that described for the cap-translated mRNAs. We previously demonstrated the existence of a long range RNA-RNA interaction involving subdomain IIId of the IRES region and the stem-loop 5BSL3.2 of the CRE element at the 3' end of the viral genome. The present study provides evidences that the enhancement of HCV IRES-dependent translation mediated by the 3'UTR is negatively controlled by the CRE region in the human hepatoma cell lines Huh-7 and Hep-G2 in a time-dependent manner. Domain 5BSL3.2 is the major partner in this process. Mutations in this motif lead to an increase in IRES activity by up to 8-fold. These data support the existence of a functional high order structure in the HCV genome that involves two evolutionarily conserved RNA elements, domain IIId in the IRES and stem-loop 5BSL3.2 in the CRE region. This interaction could have a role in the circularisation of the viral genome.

Keywords: RNA-RNA interaction; hepatitis C virus (HCV); internal ribosome entry site (IRES); cis-*acting replication element (CRE); domain 5BSL3.2; domain IIId; IRES-dependent translation.*

Abbreviations:

HCV, hepatitis C virus; IRES, internal ribosome entry site; UTR, untranslatable region; CRE, *cis*-acting replicating element.

INTRODUCTION

Hepatitis C virus (HCV) infection is the major worldwide cause of post-transfusion non-A, non-B hepatitis [1, 2]. Infection frequently leads to cirrhosis and hepatocellular carcinoma [3]. HCV is currently the sole member of the *Hepacivirus* genus, a member of the family Flaviviridae. Its genome is composed of a 9.6 kb-long, (+) polarity, single-stranded RNA molecule encoding a single open reading frame (ORF) [1, 4, 5]. This is flanked by highly conserved 5'- and 3'- untranslatable regions (5'UTR, 3'UTR), extensively folded elements with critical roles in the HCV cycle [6-12]. After virus entry, the genomic RNA acts as mRNA to generate a single polyprotein that is co- and post-translationally processed to yield structural and non-structural viral proteins. The translation process is initiated by an alternative mechanism to cap-dependent translation that requires an internal ribosome entry site (IRES). This element is mostly located at the 5'UTR but spans a short stretch of the ORF (Fig. 1a) [13, 14]. The IRES operates by the direct recruitment of the 40S ribosomal subunit, governed by specific structural elements of the IRES in the absence of initiation factors [15-17]. Both initiation and subsequent elongation steps are influenced by the presence of functional domains located at the 3'UTR of the viral RNA [12, 18-22]. Though the mechanism by which this stimulation occurs is not fully understood, it has been proposed that protein factors might bring together both ends of the viral RNA by simultaneous binding to both regions [18, 20, 23-31], thus ensuring the translation of intact molecules [32]. The resulting complex would resemble the circular forms of cellular mRNAs that are translated by a cap-dependent mechanism [33, 34]. As an alternative to this protein bridge formation, RNA elements at the two ends of some viral genomes can interact with one another in the absence of protein factors to promote efficient circularisation [35, 36]. The acquisition of a closed-loop conformation would be involved not only in the regulation of viral translation and replication, but also in the switch between these events.

We have previously described the existence in HCV genomic RNA of a higher order structure involving two evolutionarily well conserved domains [37]: the apical loop of domain IIId in the IRES, and the internal loop of 5BSL3.2 in the *cis*-acting replication element (CRE) region (Fig. 1). Domain IIId is an essential element for IRES-mediated translation owing to its direct binding to the 40S ribosomal subunit during the initiation step [38-41]. It is composed of two short helixes connected by an E-loop, capped by an apical loop (Fig. 1) [42, 43]. Domain 5BSL3.2 is embedded in a tertiary structure composed of three stem-loop elements that define the CRE region at the 3' end of the coding sequence [44, 45]. 5BSL3.2 is a structurally indispensable element for replication [44-46]. It is composed of two helical fragments connected by an essential internal loop and closed by an apical loop (Fig. 1a). This conformation provides an excellent anchoring site for distant RNA regions. In addition to its binding to the IRES region, an apical loop-apical loop interaction between 5BSL3.2 and 3'SLII in the 3'UTR occurs [46]. The crosstalk established between these functional domains, IIId, 5BSL3.2 and 3'SLII, suggests they have critical roles in the switch between essential steps during HCV infection, such as the initiation of translation and replication.

In an effort to elucidate the significance of the long range IIId-5BSL3.2 interaction in the viral cycle, the influence of domain 5BSL3.2 in IRES-dependent translation in cell culture was studied. The results clearly show that the CRE region downregulates the translation-enhancing effect mediated by the 3'UTR

in hepatic cells transfected with chimeric reporter RNAs with different 5' and 3' ends. Moreover, the HCV CRE by itself drastically blocks HCV IRES-dependent translation. The data suggest that the internal loop of domain 5BSL3.2 within the CRE region is an essential participant in this regulation. Indeed, our results demonstrate that 5BSL3.2 may be a key modulating agent during HCV initiation of translation, and also support the hypothesis of direct RNA-RNA contact between both ends of the viral genome.

MATERIALS AND METHODS

DNA templates and RNA synthesis

The DNA template for RLuc RNA was generated as previously described [47].

A plasmid containing the ICU reporter construct was obtained as follows. The 5' end of the HCV genome spanning nucleotides 1-698 was obtained by amplification of the plasmid pU5'HCV-691 [48] with primers asHCV-698+HindIII and 5'T7pHCV (see Table 1 for oligonucleotide sequences). The amplification product was subsequently cloned in the *Eco*R1 and *Hin*dIII restriction sites of the pGL3 Basic vector (Promega) to yield the construct pGL-I. The 3' end of the viral genome harbouring residues 9181-9606 was then added at the 3' end of the *fluc* gene. For this, the fragment HCV9181-9606 was amplified from plasmid pU3'HCV-9181 [37] with primers HCV-9181+Xba1 and 3'HCV+BamH1, and cloned in the restriction sites *Xba*1 and *Bam*H1 of the pGL-I vector to yield the construct pGL-ICU. The oligonucleotide sequences are shown in Table 1.

Plasmids containing the reporter constructs pGL-IU, pGL-ICUd3.1, pGL-ICUd3.2, pGL-ICUd3.3, pGL-ICU3.2ALinv, pGL-ICUd3.2dIL, pGL-ICU3.2R and pGL-ICU3.2m were generated by site-directed mutagenesis using the Phusion site-directed mutagenesis kit (Finnzymes) from the template vector pGL-ICU with the following primers: for the pGL-IU, asFLuc and HCV-9383; for the pGL-ICUd3.1, HCV-9260 and asHCV-9215; for the pGL-ICUd3.2, HCV-9311 and asHCV-9262; for the pGL-ICUd3.3, HCV-9352 and asHCV-9320; for the pGL-ICU3.2ALinv, HCV-9277ALinv and asHCV-9276; for the pGL-ICU3.2dIL, HCV-9303 and asHCV-9294; for the pGL-ICU3.2R, HCV-9313 and asHCV-3.2R; and for the pGL-ICU3.2m, HCV-9315 and asHCV-3.2mut. A DNA vector encoding the sequence for the reporter ICU-d3.1+3.3, pGL-ICU-d3.1+3.3 was obtained by site directed mutagenesis as noted above by amplification from the plasmid pGL-ICUd3.1 with the primers HCV-9352 and asHCV-9320.

Molecules T7pICU, T7pIU, T7pICU-d3.1, T7pICU-d3.2, T7pICU-d3.3, T7pICU-3.2ALinv, T7pICU-d3.2, T7pICU-d3.2dIL, T7pICU-3.2R and T7pICU-3.2m were amplified from their respective plasmid constructs using the primers 5'T7pHCV and 3'HCV to achieve the precise 3' termini.

DNA templates encompassing the sequences for the IP and the IC constructs, T7pIP and T7pIC respectively, were obtained by PCR from the plasmid pGL-ICU. For the T7pIP molecule, primers 5'T7pHCV and asFLuc-PolyA were used in the amplification reaction; the T7pIC variant was generated with the oligonucleotides 5'T7pHCV and asHCV-9321.

DNAs encoding the truncated forms of the reporter RNA molecules for the IRES - F, FP, FC, FU, FCU – were constructed as follows. The variants F, FC and FCU were obtained by PCR from the vector pGL-ICU. For the molecule T7pF, primers T7pFLuc and asFLuc were used; the sequence T7pFC was

amplified using the oligonucleotides T7pFLuc and asHCV-9321; the construct T7pFCU was generated with the primers T7pFLuc and 3'HCV.

All the RNA molecules used in the transfection assays were obtained as described by Romero-López *et al*. [47].

Cell lines and culture conditions

The human hepatoma Huh-7 cells used were a kind gift of Dr. Rafael Aldabe. Cell lines HepG2, HeLa and HEK-293 were provided by the *Centro de Instrumentación Científica* (University of Granada, Spain). Cell culture conditions were essentially the same for all cell types. Briefly, cell monolayers were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen) and 1 mM sodium pyruvate (Sigma), at 37ºC in a 5% CO_2 atmosphere.

Transfection assays

Cell transfections were essentially performed as previously described [47]. Briefly, 24 h before transfection, 90000 cells were seeded onto a 24-well plate to reach 90% confluency. A mixture containing 1 µg of the reporter constructs and 0.25 µg of the cap-RLuc was then prepared with 50 µl of Opti-MEM® (Invitrogen) and 2 µl of transfection reagent (TransFectin™, Bio-Rad) for well-to-well normalisation. This lipid-RNA complex was added to the cell culture. RNA molecules were denatured prior to transfection by heating for 2 min at 95ºC and cooling quickly at 4ºC for 10 min. Translational efficiency was determined at the indicated time points by measuring *Firefly* and *Renilla* luciferase activities using the Dual-Luciferase™ reporter assay system (Promega).

RNA stability assays

For RNA stability studies, Huh-7 cells were transfected as indicated above. At 6 h and 18 h post-transfection, the cells were washed with PBS and total cellular RNA was extracted using Trizol reagent as previously described [49], following the manufacturer instructions. Relative levels of the reporter constructs were detected by quantitative RT-PCR and normalized to that obtained for the reference mRNA GAPDH, as reported by Romero-López *et al*. [49]. PCR was performed using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The results were analysed using ABI PRISM 7000 SDS software v.1.1 from Applied Biosystems.

RESULTS

The HCV CRE domain inhibits IRES-dependent but not cap-dependent translation

Distant RNA-RNA interactions promote changes in the global RNA conformation that may mediate important events for the biology of the host molecule. In an attempt to elucidate the contribution that the IRES-CRE connection plays during viral translation, chimeric constructs were designed containing the HCV IRES domain fused to the reporter gene *fluc* encoding the *Firefly* luciferase protein (named molecule I; Fig. 2a). Different 3' tails were attached to this sequence to evaluate their effect over IRES-dependent translation: the CRE region to generate the so-called IC construct, and the whole 3' end containing the CRE plus the 3'UTR to yield the ICU variant. To further validate the experimental model, two chimeras were generated that increase HCV IRES function: IP, with a polyA tail fused to the 3' end of construct I [19], and IU, composed of molecule I fused to the 3'UTR [12, 18]. The precise 3' ends of these constructs without additional extra sequences were obtained by PCR-generated templates [12], which were subsequently transcribed and used for the transfection of the hepatoma cell line Huh-7 along with the RNA encoding for the RLuc protein (translated in a cap-dependent manner). Luciferase units were measured at 6 h post-transfection at the time of maximum IRES activity [22] and at 18 h.

Soon after transfection an enhancement in IRES-mediated translation occurred for both the IP and IU control constructs. This data strongly agrees with those obtained by other authors, confirming the reproducibility of the experimental system [12, 19]. More importantly, a drastic reduction in HCV IRES function was observed for the IC construct compared to the I molecule. This effect was suppressed by the presence of the 3'UTR in the ICU molecule (Fig. 2b), in which translation was even clearly increased with respect to the control RNA I. At 18 h post-transfection, the molecules IC, IU and IP showed similar behaviour to that observed soon after transfection. Surprisingly, the ICU construct showed a 33% reduction in its activity compared to that detected at 6 h post-transfection. This points to a time-dependent inhibitory activity for the CRE element, even in the presence of the translational enhancer 3'UTR. No significant changes were detected for the cap-RLuc RNA, showing that the different 3' tails do not affect cap-dependent translation in *trans*.

The relative intracellular amounts of the constructs were determined by real time RT-PCR at 6 h and 18 h post-transfection to preclude that variations in translation efficiency might be due to differences in RNA stability (Fig. 2c). No significant changes in the amount of RNA were detected for different reporter molecules, indicating that the 3' tail modulates IRES translational efficiency without affecting RNA stability.

A new set of variants was generated to further investigate the specificity of the inhibitory effect exerted by the CRE element on IRES-dependent protein synthesis. With that aim, the IRES region was exchanged for the cap-analogue structure to generate a collection of molecules harbouring the reporter gene *fluc* fused to different 3' ends (Fig. 3a). Translation efficiency was checked 18 h post-transfection to achieve the CRE inhibitory effect. As expected, the presence of the polyA tail and the 3'UTR induced cap-dependent FLuc protein synthesis [22], confirming the suitability of the experimental model (Fig. 3b).

The CRE region alone was unable to affect cap-dependent translation (molecule FC), but it attenuated the translational enhancement exerted by the 3'UTR (construct FCU). Taken together, these data support the idea that the CRE element alone influences IRES activity without perturbing cap-dependent translation.

IRES-dependent translation is inhibited by the CRE element both in hepatic and non-hepatic cells

We performed the same set of experiments in three additional cell lines, including hepatic (Hep-G2) and non-hepatic (HeLa and HEK-293) models, to further study the effects of the cellular environment on the activity of the CRE region (Fig. 4).

Molecules containing either the polyA tail or the 3'UTR stimulated IRES-mediated translation (Fig. 4), in good agreement with previous data obtained for the Huh-7 cell line (Fig. 2b). Similarly, the IC construct was clearly defective for IRES-mediated translation compared to the molecule RNA I. Again, this inhibitory effect was even detected in the presence of the translational enhancer 3'UTR. Interestingly, the ICU variant showed a significant reduction in its activity at 18 h post-transfection only in hepatic cells Huh-7 and Hep-G2. These results indicate that the CRE region blocks IRES activity on its own, independent of the cell type and that the addition of the 3'UTR attenuates this drastic effect in a cell line and time-dependent way. This prompted our use of Huh-7 cells as the exclusive model for subsequent analysis.

The domain 5BSL3.2 within the CRE region interferes with IRES activity

The structural elements 5BSL3.1, 5BSL3.2 and 5BSL3.3 were independently deleted from the ICU variant with the aim of identifying the essential domains for the inhibitory effect (Fig. 5a). All experiments were performed at 18 h post-transfection, the maximum inhibitory effect of the CRE region.

The deletion of domain 5BSL3.1 or 5BSL3.3 did not report significant differences in IRES translational efficiency with respect to construct ICU (Fig. 5b). Interestingly, variants lacking element 5BSL3.2 were associated with a five-fold increase in IRES activity compared to that observed for the ICU molecule. A double deletion mutant was generated lacking domains 5BSL3.1 and 5BSL3.3 to corroborate the influence of the 5BSL3.2 domain on IRES function (Fig. 5a). This RNA molecule showed similar translational efficiency to that shown by the single mutants (Fig. 5b). Taken together, these data suggest that domain 5BSL3.2 is the main agent responsible for the IRES inhibitory effect induced by the CRE region.

The internal loop of domain 5BSL3.2 is required for the regulation of HCV IRES function

Domain 5BSL3.2 consists of different structural elements that interact with distant regions in the HCV genome – possibly investing this domain with a regulatory role in essential steps in the viral cycle [37,

45, 46, 50]. A set of reporter constructs was generated to further analyse the involvement of these structural constituents in IRES-dependent translation. Mutations in the apical or internal loop were included in the ICU variant (Fig. 6a) and the constructs tested for their translational efficiency at 18 h post-transfection.

The molecule harbouring the sequence of the apical loop in reverse orientation had no significant effect on IRES-dependent protein synthesis in Huh-7 cells (Fig. 6b). In contrast, the deletion of the internal loop promoted a drastic (up to 8-fold) stimulation of IRES activity. These results strongly suggest that the internal loop of 5BSL3.2 is the main agent responsible for the regulation of IRES function. Two additional variants with different modifications of the internal loop were constructed to investigate this possibility (Fig. 6a): ICU-3.2R, with a non-related sequence in the internal loop described to abolish viral replication [46], and ICU-3.2m, with three modified residues (CCC to GGG) that impede the interaction of 5BSL3.2 with domain IIId of the IRES [37]. Both of these RNA molecules enhanced IRES function by up to 7-fold with respect to the ICU molecule (Fig. 6b).

Taken together, these data suggest a functional role for the internal loop of 5BSL3.2 in IRES-dependent translation and support the notion of tertiary contacts between this element and the apical loop of domain IIId of the IRES.

DISCUSSION

It has been shown that mRNA must have a circular conformation if translation is to be efficient. The acquisition of this geometry is promoted by the interaction between the termini in capped and polyA mRNAs. This structure is conserved by cellular protein factors that act as molecular bridges between the ends of mRNA, helping to preserve its physical integrity (for a review see [51]). However, many viral RNAs lack cap and/or polyA elements. For the members of the *Flavivirus* genus, the acquisition of the closed loop conformation is promoted by direct RNA-RNA interactions that involve complex high order structural regions [52, 53]. In the case of HCV, a closely related virus, we previously demonstrated the existence of an interaction between the specific functional domains IIId and 5BSL3.2 of the two highly conserved IRES and CRE regions at opposite ends of the genomic RNA [37]. Both sites are associated with essential functions in viral translation and replication. The present work analyses the impact of the interaction IRES-CRE in protein synthesis and demonstrates the implication of domain 5BSL3.2 in IRES-dependent translation.

A collection of reporter RNA molecules was generated to investigate the role of the different structural elements at the 3' end of the HCV genome in IRES-dependent translation. The folding of these domains in the chimeric constructs was evaluated by secondary structure prediction with the MFold software [54]. These in *silico* analysis showed that the expected conformation of the HCV genomic domains was preserved in all the variants tested (data not shown), though we cannot rule out the existence of alternative conformations promoted by the cellular environment. The use of monocistronic constructs avoids collateral *cis*-effects derived from the presence of additional regulatory domains [55]. In addition, the integrity of the 3' end, which is critical for the reproducibility of results, was assessed using DNA templates obtained by PCR [12, 56]. The time points for assaying the activity of the reporter gene were

taken into account [12]; the IRES reaches its maximum activity soon after transfection, which might be important when testing the regulatory effects of the elements under study.

The results showed that the CRE region promoted a drastic reduction in the efficiency of IRES function (Fig. 2). This effect was even detected in the presence of the 3'UTR at 18 h post-transfection, suggesting a dual regulatory activity for the CRE region by the attenuation of the 3'UTR translational enhancement in a time-dependent way and by directly affecting to IRES activity. This hypothesis is reinforced by the observation that the CRE region alone had no effect on cap-dependent protein synthesis, but its inhibitory effect over the 3'UTR translational enhancement was still present (Fig. 3). Our results point to a complex interplay between the three regions. This is also supported by previous data reporting the existence of a kissing apical loop-apical loop interaction between the 5BSL3.2 domain of the CRE and the 3'SLII domain of the 3'UTR [46]. It should be noted that the IRES activity results for the construct carrying the whole 3' end of the HCV genome at 18 h post-transfection correlated perfectly with those reported by Lourenço *et al*. [21], who detected no effect of the whole 3' end over IRES-dependent translation at a single time point of 24 h after transfection. The results obtained from the time-course assays at early post-transfection allowed the detection of the CRE regulatory effect on IRES function. To our knowledge, this is the first report describing this phenomenon. Data obtained for the control constructs harbouring the 3'UTR or a polyA tail were consistent with those reported by other groups [12, 21, 22].

The inhibitory effect exerted by the CRE did not seem to depend on the cell line. Nevertheless, the regulatory activity of the whole 3' end in the ICU variant is restricted to hepatic cells (Fig. 4). This suggests that the interplay between the three regions IRES, CRE and 3'UTR occurs under specific cell conditions and likely requires additional factors as well as RNA-RNA interactions. HCV preferentially replicates and propagates in hepatocytes [21, 57-60], which suggests that liver cells may supply these factors (protein and RNA) essential to the viral cycle. This is in good agreement with previous observations that point to rabbit reticulocyte lysate being an inappropriate system for evaluating IRES function [12, 21, 22, 26] (data not shown). It is remarkable that Lee *et al*. [44] found no effect of the CRE region on protein synthesis, probably because they used an *in vitro* system that might not have been able to reproduce the results obtained in hepatic cells.

Mutational analysis of 5BSL3.2 in the CRE region showed that changes in the internal loop resulted in the stimulation of IRES activity by up to 8-fold, depending on the construct used (Figs. 5, 6). This region is involved in the establishment of a long range RNA-RNA interaction with domain IIId of the IRES [37], the key element for the binding of the 40S ribosomal subunit. It seems likely that mutations interrupting this contact would favour an increase in ribosome recruitment, thus leading to more efficient IRES function. It is also noteworthy that similar modifications lead to deleterious replicating genomes [46, 50]. This suggests a dual role for domain 5BSL3.2 in two different events of the viral cycle.

The effects detected for the CRE and the 3'UTR regions over the IRES activity are assumed to be preferentially exerted in *cis*, though we cannot exclude intermolecular contacts involving both termini of the RNA molecules. This hypothesis is supported by previous data demonstrating that the IRES translational enhancement mediated by the 3'UTR only operates in *cis* [18]. Moreover, we have shown that the 3'UTR is unable to suppress in *trans* the translation inhibition effect exerted by the CRE domain (unpublished data). In addition, the location of 5BSL3.2 along the transcript could be relevant to play its

regulatory action over IRES-dependent translation (Romero-López *et al*., unpublished results). These observations support the existence of a close loop conformation mediated by a long distant RNA-RNA contact between the IRES and the CRE region, which could be preserved by cellular factors bound at the 5' and the 3' ends of the viral genome. The circular form would determine the translation and replication of intact HCV RNA molecules and could direct the switch between viral translation and replication.

In summary, the present data reinforce the idea of a direct RNA-RNA interaction between domain IIId of the IRES and 5BSL3.2 in the CRE region previously described by our group [37]. They also support the role of the CRE domain as a modulating agent in IRES-dependent translation and point to domain 5BSL3.2 as a key element in the regulation of viral protein synthesis. It is tempting to propose roles for this element in essential steps of the infective cycle and in the switch between viral translation and replication.

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FIGURE LEGENDS

Fig. 1 a) Sequence and secondary structure of the 5' and the 3' ends of the HCV genome. The 5'UTR plus domains V and VI located in the core coding sequence are included. The 3' end of the viral genomic RNA is organized into two structural elements: the CRE region containing the three stem-loops 5BSL3.1, 5BSL3.2 and 5BSL3.3; and the 3'X-tail, linked by a hypervariable sequence and the polyU/UC stretch. Numbers refer to the nucleotide positions of the HCV Con1 isolate. Start and stop translation codons at positions 342 and 9371 respectively are shown in bold. b) Theoretical kissing interaction involving the apical loop of domain IIId and the internal loop of 5BSL3.2

Fig. 2 a) Diagram of the IRES reporter constructs. The 3' end of the HCV genome harbouring the CRE region plus the 3'UTR was attached to the IRES-FLuc fusion, as shown above. Harmful mutants for the 3' tail are shown below. The IP variant includes an artificial polyA tail at the 3' end of the sequence. b) The 3' tail influences IRES-dependent translation. Huh-7 cells were co-transfected with the RNA molecules indicated in a) and the RNA RLuc. IRES function was measured as the activity of FLuc protein and normalized against that obtained for RLuc. Luciferase activity in the variant I is established as 100%. Data points are the mean of at least three independent experiments. c) RNA stability control. RNA reporter constructs were transfected into Huh-7 cells and total cellular RNA was isolated at the indicated times using Trizol reagent. Quantitative RT-PCR was performed with specific primers plus the Taqman probe for the HCV IRES or the GAPDH mRNA. The results were analysed using ABI PRISM SDS Software 1.1 (Applied Biosystems). * indicates significant differences ($p<0.05$) between the translational efficiency of different constructs. Data are the mean of three independent experiments

Fig. 3 a) Diagram of the cap reporter constructs. Different fragments of the 3' end of the HCV genome were fused to the FLuc coding sequence, which is translated in a cap-dependent way. The FP variant harbours at its 3' end a synthetic polyA tail. b) Molecules shown in a) were co-transfected with the RLuc RNA and relative luciferase units were measured at 18 h post-transfection as indicated in Fig. 2b. Molecule F was established as providing 100% translation (control). Data are the mean of at least three independent experiments

Fig. 4 Effect of the 3' tail on IRES-dependent translation in different cell lines. Hep-G2, HeLa and HEK-293 cells were co-transfected with the molecules shown in Fig. 2a and RLuc RNA. They were harvested at 6 h and 18 h post-transfection and luciferase activity measured as indicated in Fig. 2b. Data normalized against to those obtained for the variant I and represent three independent assays

Fig. 5 a) Deletion mutants of the three stem-loops of the CRE region constructed from the ICU molecule (Fig. 2a). b) Variants shown in a) were co-transfected with the RLuc RNA in Huh-7 cells. The

translational efficiency of the IRES region was evaluated at 18 h post-transfection as indicated in Fig. 2b. The control reaction was set for the ICU construct. Means correspond to four independent experiments

Fig. 6 a) Mutations targeting the apical loop or the internal loop of domain 5BSL3.2. The ICU construct (Fig. 2a) was modified at the positions of the 5BSL3.2 domain shown in bold to generate a set of variants. The sequence and secondary structure of the wild-type stem-loop is shown above. b) The effects of the mutations in 5BSL3.2 were analysed by co-transfection of the molecules shown in a) (above) with the cap-RLuc RNA in Huh-7 cells. Relative luciferase activity was quantified at 18 h post-transfection as described in Fig. 2b. Values were normalized against those obtained for the ICU construct and represent the mean of four independent experiments

Table

Table 1 Oligonucleotides used in this study

Oligonucleotide	**5'-3' sequence**
5'T7pHCV	TAATACGACTCACTATAGCCAGCCCCCTGATGG
asHCV-698+HindIII	TAT*AAGCTT*CCAAATTGCGCGACCTAC
HCV-9181+Xba1	AAT*TCTAGA*GGGCAGTAAGGACCAAGCTCA
3'HCV+BamH1	TATGGATCCACTTGATCTGCAGAGAGGCCA
asFLuc	TCTAGAATTACACGGCGATCTTTCCGC
HCV-9383	CTAAACACTCCAGGCCAATAGGCCATC
HCV-9260	TTACAGCGGGGGAGACATATATCACAG
asHCV-9215	GATTGGAGTGAGTTTGAGCTTGGTCCT
HCV-9311	GTTCATGTGGTGCCTACTCCTACTTTCTGTA
asHCV-9262	TAACCAGCAACGAACCAGCTGGATAAATCCAA
HCV-9352	TCTATCTACTCCCCAACCGATGAACGG
asHCV-9320	CCACATGAACCAGCGGGGTCGGGCA
HCV-9277ALinv	CCGACACTATATTGTCTCGTGCCCGACCCCGCTGGT
asHCV-9276	TGTCTCCCCCGCTGTAACCAGCAACGA
HCV-9303	CCCCGCTGGTTCATGTGGTGCCTACTCCTACTT
asHCV-9294	GAGACAGGCTGTGATATATGTCTCCCCCGCTGT
HCV-9313	CATGTGGTGCCTACTCCTACTTTCT
HCV-9315	ATGTGGTGCCTACTCCTACTTTCTGTAGGGGTA
asHCV-3.2mut	GAACCAGCGGGGTCCCCGACGAGACAGGCT
HCV-9352	TCTATCTACTCCCCAACCGATGAACGG
asHCV-9320	CCACATGAACCAGCGGGGTCGGGCA
3'HCV	ACTTGATCTGCAGAGAGGCCA
asFLuc-PolyA	(A_{24})TCTAGAATTACACGGCGATCTTTCCGC
asHCV-9321	ACCACATGAACCAGCGGG
T7pFLuc	TAATACGACTCACTATAGGGATGGAAGCCAAAAACA

* T7 promoter is underlined. Restriction sites are noted in italics.

Figure 1
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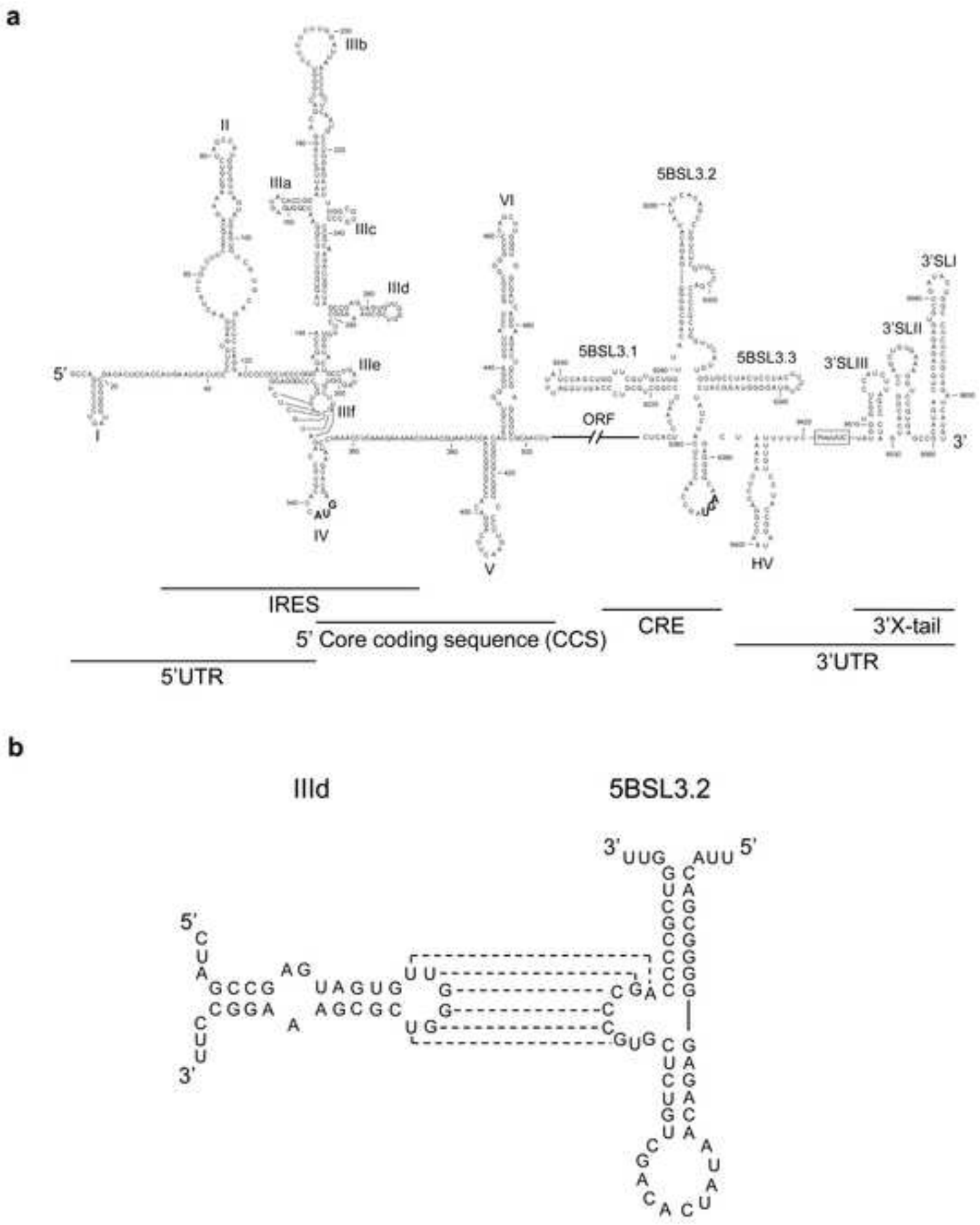

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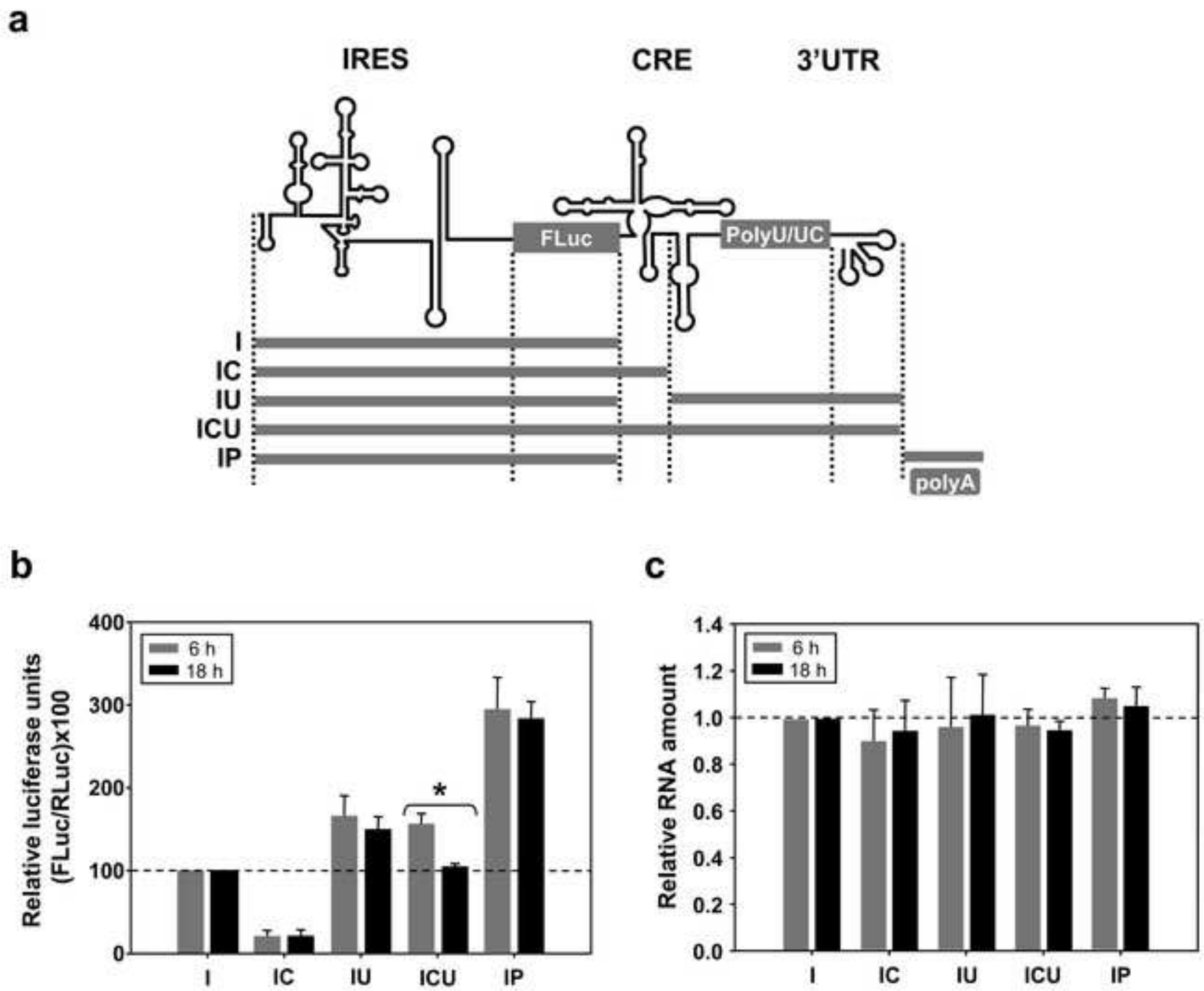

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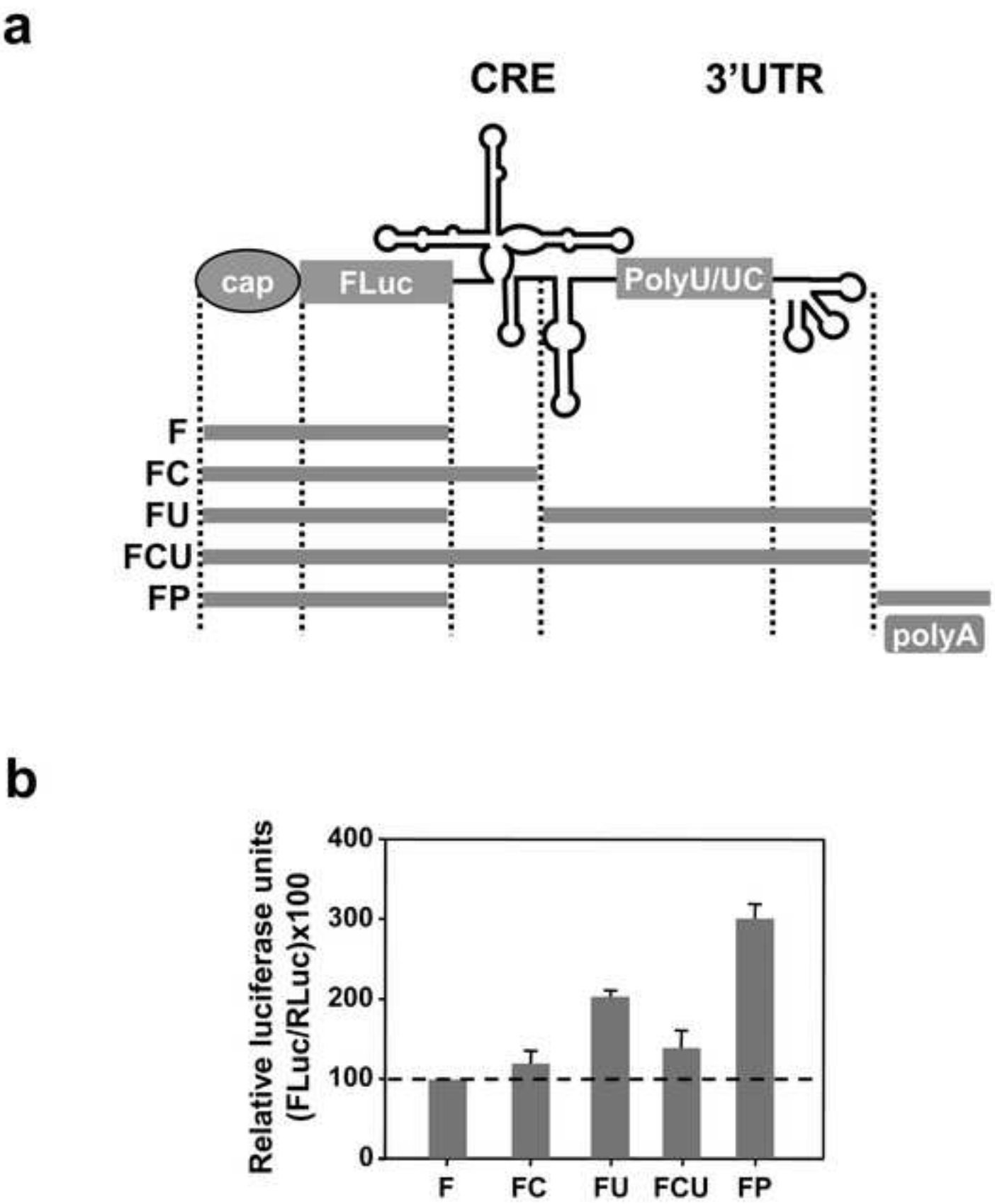

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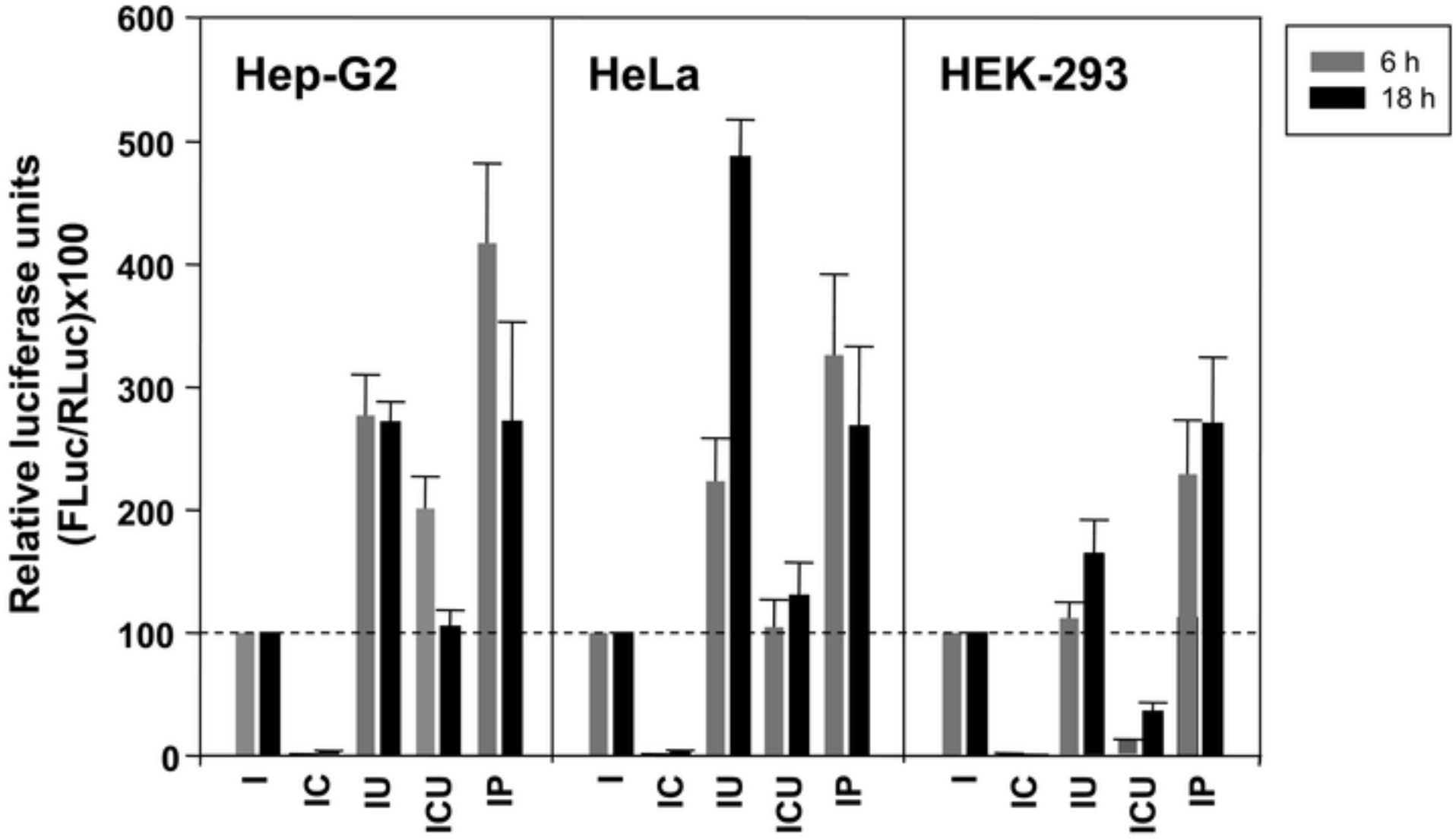

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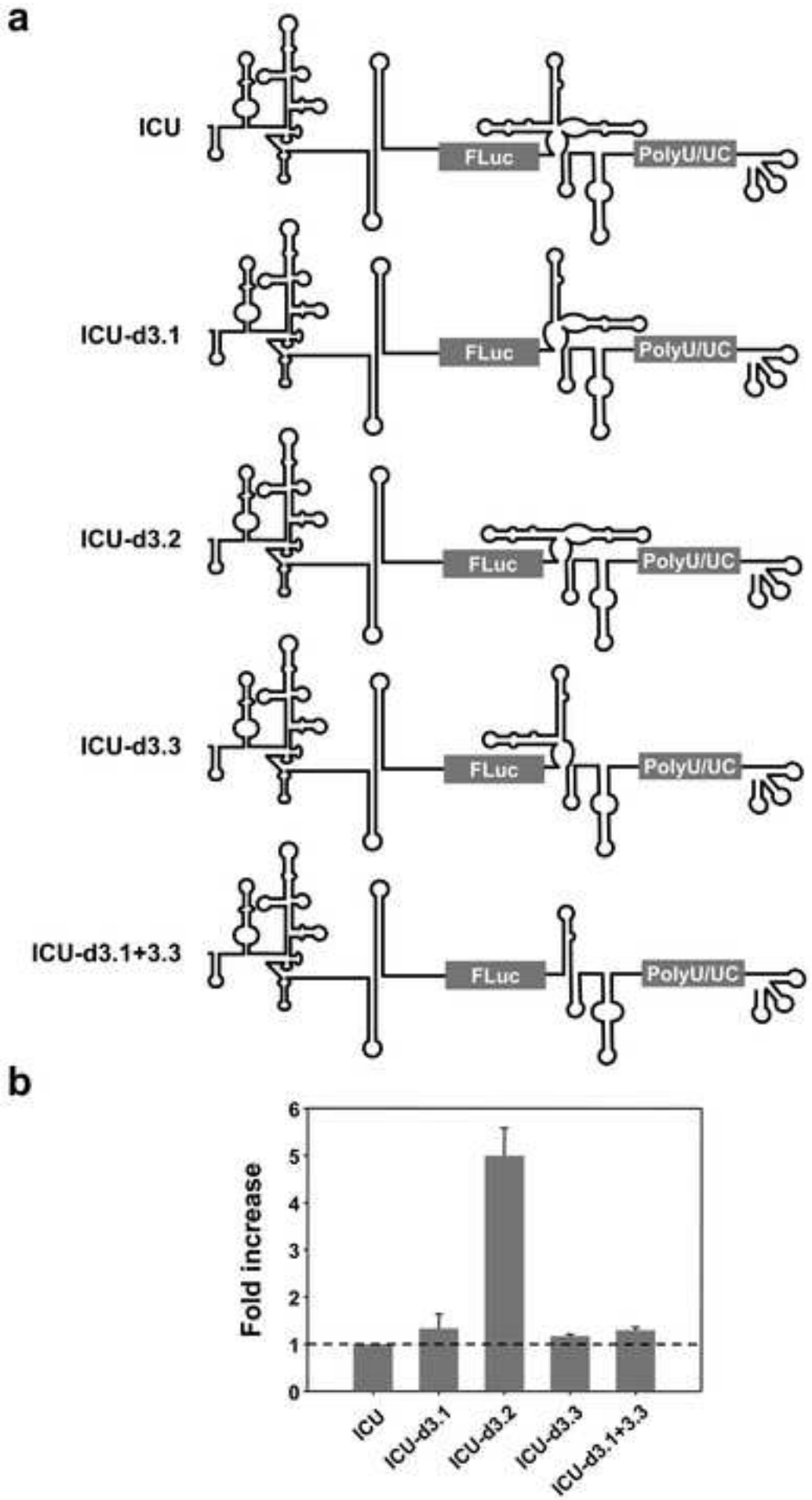

Figure 6

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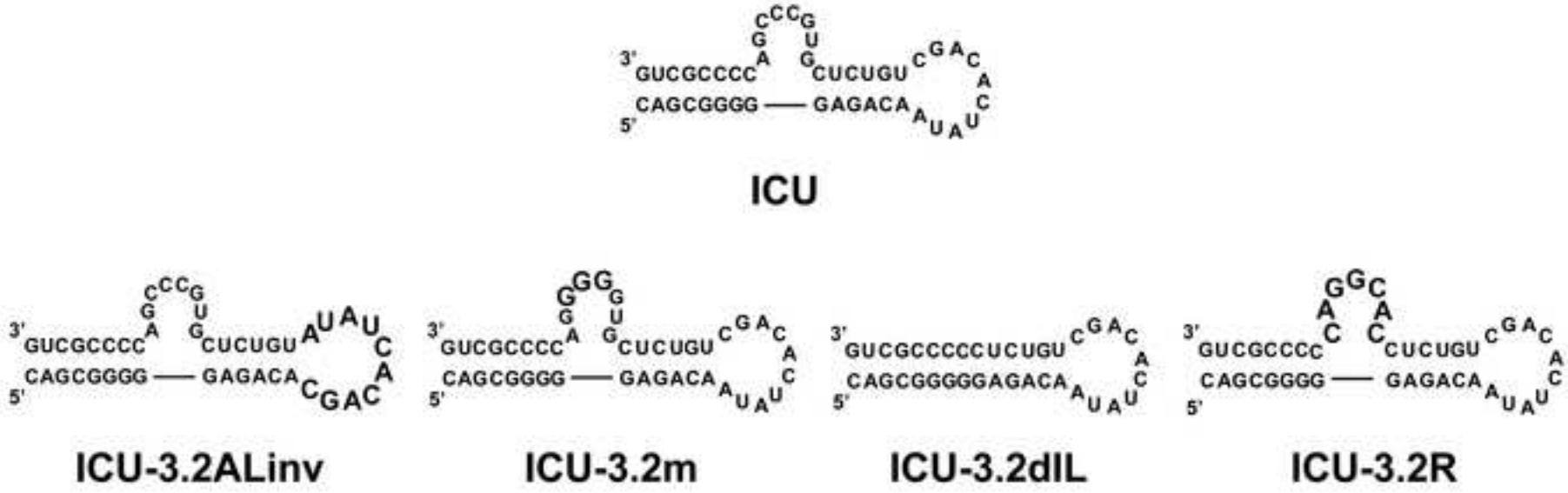

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