Hepatitis C virus intragenomic interactions are modulated by the SLVI RNA structure of the core coding sequence

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Abstract

Several RNA interactions are thought to play a role in the regulation of the hepatitis C virus (HCV) life cycle. Most of these interactions involve the 5BSL3.2 domain and therefore occur at the 3¢ end of the viral genomic RNA. A long-range interaction has also been described between 5BSL3.2 and the 5¢ untranslated region (UTR). Another interaction involves the SLVI stem loop of the core coding region and the 5¢UTR. We aimed to analyse the role of this SLVI domain, which likely interferes with others interactions. By evaluating RNA stability, translation and RNA synthesis, we showed that the SLVI stem loop extensively modulates the effect of the interactions mediated by the 5BSL3.2 domain and strongly affects the IIId/5BSL3.2 interaction. Numerous interactions in HCV genomic RNA have been described in the UTRs and the coding sequence but their roles are poorly understood. We showed that the SLVI domain located in the core coding sequence plays an important role in the translation of the polyprotein, but also in the modulation of long-range RNA interactions centred on the 5BSL3.2 domain. The SLVI domain has been absent from most studies, especially from the extensively used subgenomic replicon; our data highlight the importance of this domain in the studies of these long-range interactions in the HCV life cycle.

INTRODUCTION

Hepatitis C virus (HCV) is a causative agent of chronic liver disease. According to the World Health Organization (WHO), 3 % of the global population (approximately 180–185 million people) is persistently infected by this pathogen [1, 2], which provokes serious medical pathologies: hepatosteatosis, liver fibrosis, liver cirrhosis and hepatocellular carcinoma [3–5]. Although specific inhibitors against HCV have been used recently [6], the precise mechanisms governing HCV replication are not fully understood. A member of the *Hepacivirinae* genus within the *Flaviviridae* family (www.ictvonline.org/virusTaxonomy.asp), HCV is an enveloped RNA virus of positive polarity with a genome of approximately 9.5 kb flanked by two untranslated regions (UTRs; 5¢UTR and 3¢UTR). Located between the untranslated regions, a long ORF encompasses a polyprotein of approximately 3000 amino acids. The structural proteins are encoded in the 5¢-terminal region, while the nonstructural (NS) proteins are encoded in the 3¢-terminal region of the ORF [7].

The translation of this ORF is mediated by the 341 nt-long 5¢UTR, which functions as an internal ribosome entry site (IRES). The 3¢UTR is composed of three structural domains: an approximately 40 nt-long variable region downstream of the HCV coding sequence, a poly(U/UC) tract of heterogeneous length, and a highly conserved 98 nt-long sequence named 3¢X [8]. The UTRs are among the most conserved parts of the viral genome due to their multiple functions in viral translation and replication.

Until 2005, most of the studies on HCV replication were performed with the HCV replicon model. Flanked by the two HCV UTRs, this construction is a subgenomic RNA that is able to replicate autonomously in Huh7 cells [9]. The transcript is composed of two cistrons; the first cistron begins with the HCV IRES, followed by a sequence encoding a selection marker (neomycin phosphotransferase) or a reporter gene (luciferase), and the second cistron begins with the encephalomyocarditis virus (EMCV) IRES, followed by the 3¢ end of the HCV genome, which includes the viral components of the replication complex (RC), NS3 to NS5B (the polymerase) and the 3¢UTR. This construct has contributed greatly to improving knowledge of the functions of the viral proteins and RNA sequences involved in both translation and viral RNA replication, in particular, the requirement of the 5¢ and 3¢ UTRs in viral replication. However, in addition to the non-coding sequences, other *cis*-acting RNA elements have been broadly documented to

play an essential role in the replication of RNA viruses, including some flaviviruses [10], the Qbeta bacteriophage [11], aichi virus [12], human rhinovirus [13], poliovirus [14], Sindbis virus [15], tombusvirus [16] and giardia virus [17]. Such elements have also been described for HCV. In the coding sequences, these elements were identified because the codon bias at the third position is reduced despite the high variability of the virus. This sequence conservation, combined with computer predictions, suggested that in addition to acting as coding sequences, some regions of the genome adopt specific conserved stem-loop conformations necessary for productive infection. These secondary structures are actually engaged in specific tertiary conformations that are thought to lead to long-range intra- or intermolecular interactions important for replication. Yet, their role has not been clearly elucidated.

All these interactions are shown in Fig. 1. In the 5¢UTR, the sequence from nt 24 to 38 is complementary to that at nt 428 to 442 at the base of stem loop (SL) VI of the core gene sequence [18], called 5¢UTR/SLVI hereinafter. Additionally, the 3¢ part of the NS5B coding sequence contains a pseudo-loop called 5BSL3.2 that is at the centre of several long-range interactions. The apical loop (AP) of the 5BSL3.2 domain (nt 9284 to 9290, AP sequence) is thought to interact with the SL2 loop of the 3¢X region (SL sequence) to form a kissing-loop complex [19], hereinafter called the AP/SL interaction. Additionally, another interaction involving the same structure was suggested to occur between the internal loop (IL) of the 5BSL3.2 domain (nt 9292–9306, IL sequence) and a region located 200 nt upstream [nt 9106–9120, upstream sequence (US)] [20], hereinafter called the US/IL interaction. Finally, an interaction between the 5BSL3.2 internal loop and the 5¢UTR (loop IIId), hereinafter called the IIId/IL interaction, has also been described recently [21].

Thus far, all of these interactions have been studied in the cellular context but without the SLVI structure, although it interacts with the 5¢UTR region under physiological conditions. In this study, we delineated the influence of the 5¢ interaction (5¢UTR/SLVI) on interactions involving the 5BSL3.2 domain using a model developed in the laboratory [22]. This model is designed to analyse translation and replication levels independently by using a mini-genome reporter gene and a constitutive expression of the replication complex. We found that the SLVI stem loop extensively modulates the effect of the interactions mediated by the 5BSL3.2 structure and strongly affects the IIId/5BSL3.2 internal loop interaction.

RESULTS

Approach

Two cellular models are available to study HCV replication: the virus system and the infectious replicon system. However, for both of these systems, translation and replication are coupled. When a mutation reduces translation activity

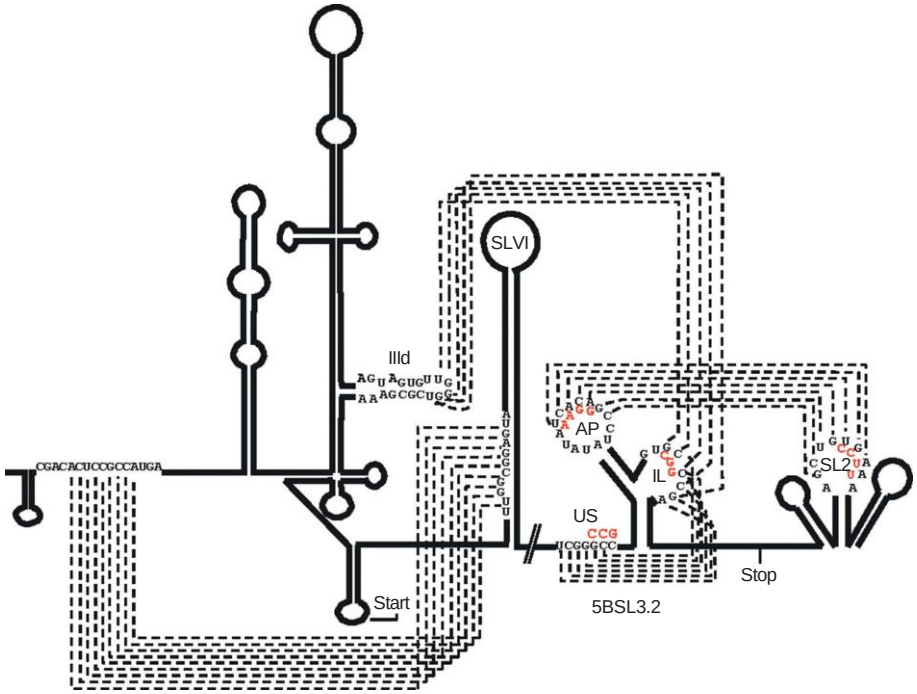

Fig. 1. Schematic of HCV intragenomic interactions. The untranslated regions are shaded in grey. The region encompassing the start codon to the SLVI sequence belongs to the core coding sequence. The sequence from the 5BSL3.2 region to the stop codon belongs to the NS5B-coding sequence. The other components of the ORF are not shown.

the expression of viral proteins will be hampered, and therefore the production of replication complexes will decrease. Consequently, viral RNA replication will also be directly impaired. The mini-genome model used in this work allows the separate study of replication and translation. A mini-genome is an RNA molecule carrying viral UTRs that flank a reporter gene. The replication of this RNA is ensured by the RC constitutively produced by replicon-harbouring Huh7 cells. As a consequence, in this system, variation in the translation efficiency of the mini-genome does not impact the quantity of RC produced, and therefore the level of replication is not affected. We are thus able to analyse the effect of mutations in untranslated regions on the stability, translation and replication of the viral RNA. In this study, the 3¢ region of the NS5B coding sequence (278 nt) was introduced into the mini-genome after the stop codon of the reporter gene to exclusively study the regulating role of this region as a non-coding sequence. Because in previous studies [22, 23] we have shown that the 5BSL region is not strictly required for the replication of the RNA template by the replication complex, we postulate that this sequence is a regulator of the critical steps involved in the viral life cycle. Because the NS5B sequence is located downstream of the stop codon of this RNA template, it is possible to replace any nucleotide of the sequence without affecting the reporter protein. Consequently, this construct allowed us to insert mutations that abolished or restored one or multiple interactions and to measure the implication on replication or translation efficiency. The terminology used for the 3¢ mutations is detailed in Table 1.

Mutations were introduced in two sets of mini-genomes: the first contained only the 5¢UTR and therefore is called 'miniG' in the following work, while the second contained the 5¢UTR followed by the SLVI structure (a part of the core coding sequence) and is called 'SLVI miniG' (Fig. 2).

Mutants of the apical loop of the 5BSL3.2 region ('mutAP') or of the SL2 stem loop ('mutSL') both abrogate the 5BSL3.2/SL2 interaction. The corresponding mutant restoring the interaction is called 'mutAP-SL' in the following report. To abolish the interaction between the 5BSL3.2 domain and the US (nt 9107–9112), mutants of the IL of 5BSL3.2 ('mutIL') or the US ('mutUS') were generated. The corresponding mutant restoring this interaction is therefore called 'mutUS-IL'. Finally, the mutant abrogating the two interactions involving the 5BSL3.2 domain (the AP and the IL) is called 'mutAP-IL'. All of these RNA mini-genomes were transfected into either naive Huh7 (Huh7 QR) cells or Huh7 cells bearing replicons, to analyse the inherent RNA stability, translation and replication activity of each mutant.

Table 1. Terminology used for 3¢ mutations

Mutant	Interaction(s) affected (location within the viral genome)
AP	Abrogation 5BSL3.2/3¢UTR (3¢/3¢)
SL	Abrogation 5BSL3.2/3¢UTR (3¢/3¢)
AP-SL	Restoration 5BSL3.2/3¢UTR (3¢/3¢)
US	Abrogation 5BSL3.2/upstream sequence (3¢/3¢)
IL	Abrogation of both 5BSL3.2/upstream sequence (3¢/3¢) and 5¢UTR/5BSL3.2 (5¢/3¢)
US-IL	Restoration 5BSL3.2/upstream sequence (3¢/3¢) and abrogation 5¢UTR/5BSL3.2 (5¢/3¢)
AP-IL	Abrogation of all interactions involving 5BSL3.2

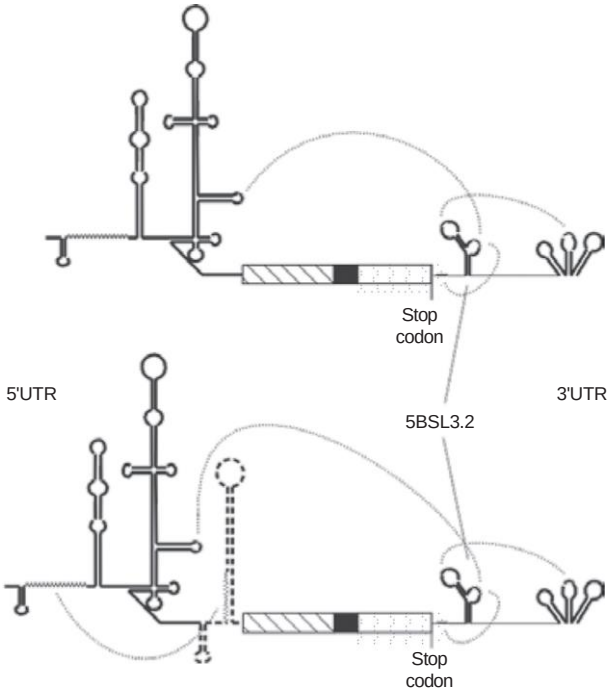

Fig. 2. Schematic of the mini-genomes used in this study. The mini-genomes contain, from 5¢ to 3¢, the hygromycin phosphotransferase (hatched) in fusion with the 2A autoprotease (black) and EGFP (dotted) coding sequences. After the stop codon, the last 532 nucleotides of the HCV genome are present, including the last 94 amino acids of the NS5B region and then the 3¢UTR with its variable sequence, the polyU/C and the 3¢X domain. For the 5¢UTR mini-genome (top), only the first 16 amino acids of the capsid are present. For the 5¢UTR-SLVI mini-genome, the 61 first amino acids of the core protein are present, allowing the 5¢UTR/SLVI interaction.

Effect of SLVI on RNA stability, translation and replication

First, the stability of RNA containing or not containing the SLVI structure was evaluated in Huh7-QR cells using RT-qPCR for RNA mini-genome quantification. The miniG or SLVI miniG mini-genomes were transfected into Huh7-QR cells, and the copy number of each RNA mini-genome was quantified 96 h post-transfection (Fig. 3a). The results did not show any significant difference in stability between the two mini-genomes, indicating that the presence of SLVI does not impact RNA stability.

Second, we compared the EGFP expression mediated by HCV IRES activity using flow cytometry 72 h post-transfection. Our data show that the level of translation was

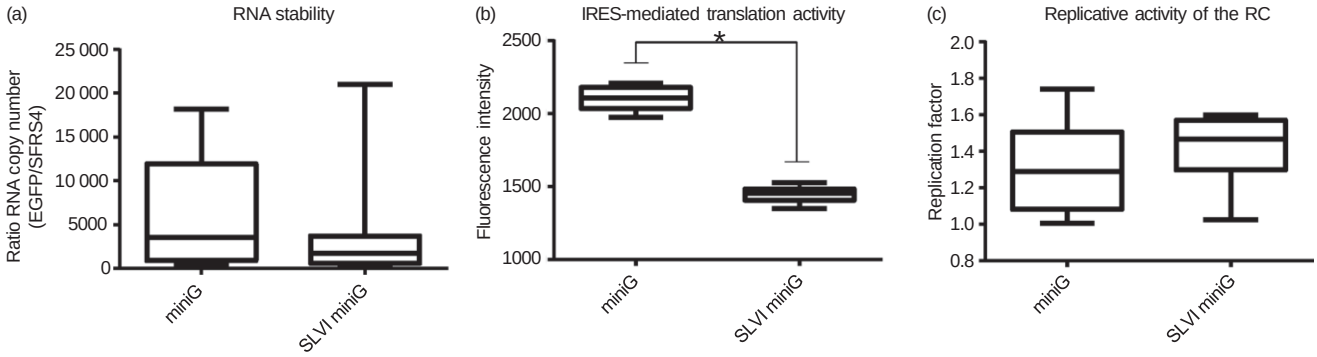

Fig. 3. Stability, translational and replicative activity of miniG and SLVI miniG. Both mini-genomes were transfected into naive (a, b) or replicon-bearing Huh7 cells (c). RNA stability was evaluated using triplicate RT-qPCR reactions (copy number of EGFP transcript/copy number of SFRS4 transcript in Huh7 cells) 96 h post-transfection (a). The translation efficiency was evaluated by flow cytometry 72 h post-transfection and normalized with the RNA stability calculated in (a). The percentage of fluorescent cells is shown (b). The replication factor (EGFP transcript copy number/SFRS4 copy number) was calculated after RT-qPCR 96 h post-transfection (c), (box and whiskers from 25th to 75th percentiles with n=5 independent experiments, *P<0.05 using Mann–Whitney–Wilcoxon test).

significantly decreased for the SLVI miniG. This result suggests that the 5¢UTR/SLVI interaction facilitated by the presence of the SLVI structure negatively regulates HCV-IRES activity (Fig. 3b).

We then compared the replicative activity of the HCV replication complex in the presence of these two mini-genomes as a template. RT-qPCR was performed 96 h post-transfection for both of the mini-genomes in replicon-bearing Huh7 cells. The replication factor was calculated for both of these mini-genomes as previously described [22]. As shown in Fig. 3(c), both RNA templates are replicated with the same efficiency by the RC. These results indicate that the 5¢UTR/SLVI interaction does not affect RNA stability and RNA replication but substantially decreases the translation level.

RNA stability of the 5BSL3.2 mutants

The 5BSL3.2 loop (Fig. 1, AP) is known to interact with the SL2 stem loop of the 3¢UTR. The 5BSL3.2 IL (Fig. 1) also interacts with the 5¢UTR IIId loop. Additionally, another interaction between the IL and an US has been described (Fig. 1). Thus, we sought to characterize the potential effect of the 5BSL3.2 interactions on both mini-genomes, miniG and SLVI miniG. For that purpose, several mutations that either disrupt or restore the interactions between 5BSL3.2 and the other regions were introduced into the mini-genome in the presence or absence of the SLVI structure. To evaluate the RNA stability of each construct, the wild-type (WT) and mutant RNA mini-genomes were transfected into Huh7-QR cells, and the residual RNA molecules were quantified 96 h post-transfection using RT-qPCR and then compared with the corresponding WT construct. For both types of RNA mini-genome, the ratio (copy number of EGFP transcripts per copy of SFRS4 transcript) was normalized to the corresponding WT mini-genome. For both mini-genomes, the results showed a slightly but significantly higher stability for the mutAP (~25 %) compared with the WT mini-genome (Fig. 4a). The mutAP mutant affected the RNA stability of both types of mini-genome regardless of the presence of the SLVI domain, clearly indicating that mutation of the 5BSL3.2 AP sequence increases RNA stability. While the stability of the mutAP-SL mutant was increased in the absence of SLVI compared with that of the WT genome (25 %), it was unchanged in the presence of SLVI. Despite the weak differences observed, the stability was indeed significantly modified for some mutants depending on the presence of the 5¢UTR/SLVI interaction. Similarly, the stability of mutIL (20 %) and mutUS-IL (20 %) was increased without SLVI, whereas it was decreased for mutIL in the presence of SLVI (Fig. 4). Altogether, these results suggest that the AP sequence tends to decrease RNA stability independently of the presence of the SLVI domain and that the presence of the SLVI domain predominantly influences the stability of the US-IL interaction.

Translational activity dependent on SLVI

We then analysed the potential effect of those interactions on HCV IRES-mediated translation. The mini-genomes were transfected into naive Huh7 cells, and the translational activity of the HCV IRES was evaluated by measuring EGFP expression using flow cytometry with naive Huh7 cells 72 h post-transfection. The translation activity was expressed by normalizing the EGFP signal with the respective stability previously quantified for the WT mini-genome. Unexpectedly, with the miniG RNA (Fig. 5a), our data showed that the translation activity was inversely correlated with RNA stability: the IRES-mediated translation was significantly weaker for the mutants exhibiting an increase in RNA stability (mutAP, mutAP-SL, mutUS and mutUS-IL). When SLVI was present, this inverse correlation was only observed for the AP mutant (Fig. 5b). Notably, the translation activity was significantly decreased for most of the AP mutants,

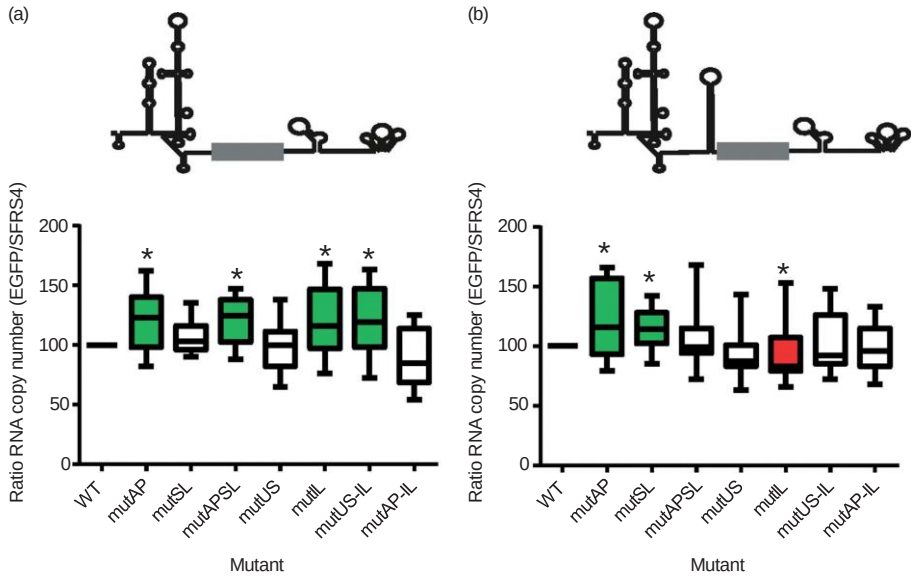

Fig. 4. Modulation of RNA mini-genome stability by mutant interactions. miniG (a) or SLVI miniG (b) were transfected into naive Huh7 cells. The translation efficiency was evaluated by flow cytometry 72 h post-transfection and normalized to WT levels. The ratio (copy number of EGFP transcripts/copy number of SFRS4 transcripts) was measured 96 h post-transfection by RT-qPCR and normalized to that of the corresponding WT mini-genome (box and whiskers from 25th to 75th percentiles with n=5 independent experiments, *P<0.05 using a Mann–Whitney–Wilcoxon test). Significant increase is denoted by a green box and significant decrease by a red box.

with mutAP ~10 % and mutAP-SL ~10 % for the miniG or mutAP ~25 %, mutAP-SL ~25 % and mutAP-IL ~20 % for the SLVI miniG, while the SL mutation never affected the translational activity of either type of mini-genome. This suggests that translation is controlled by the AP sequence independent of the AP/SL interaction; the recently described interaction between AP and the IIId loop is possibly involved [24].

By contrast, translation was hampered for most of the IL mutants without the SLVI domain and was unchanged in the presence of SLVI. This suggests that the SLVI region potentially affects the IIId/IL interaction because none of the IL mutants have any effect on translation with the SLVI miniG.

Replicative activity of the RNA constructs

It is generally hypothesized that these previously described interactions regulate viral RNA synthesis activity. Therefore, their function in replication was analysed in the presence of the viral RC. Each of the miniG and SLVI miniG RNAs, both containing the specific 5BSL3.2 mutations indicated, were transfected into replicon-bearing Huh7 cells to ensure the presence of the RC [22, 25]. The mini-genome quantity was evaluated by RT-qPCR 96 h post-transfection. For both types of RNA mini-genome, the ratio (copy number of EGFP transcripts per copy of SFRS4 transcript) was normalized to the stability of each mini-genome and then to the WT mini-genome.

For the miniG RNAs, the results showed that the mutAP, mutIL and mutUS-IL mutants were significantly less efficiently replicated by the RC compared with the WT mini-genome, with a decrease of between ~15 and 20 % (Fig. 6a). In the presence of the SLVI, our results clearly showed that all the AP mutants, but also the SL mutant, were significantly less efficiently replicated than the WT, with a decrease of ~40 to 20 %. (Fig. 6b). This demonstrates the importance of this AP sequence in the mechanism of RNA replication.

Additionally, similar to the translation analysis, the mutIL and mutUS-IL mutants exhibited a defect in RNA replication compared with the WT in the absence of the SLVI domain. Yet, no significant difference in the replication of these mutants was observed in the presence of the SLVI region. Again, this supports the hypothesis that the presence of the SLVI stem loop interferes with the IIId/IL interaction.

DISCUSSION

In this study, we performed a functional analysis of the different long-range RNA–RNA interactions described for the HCV genome. Two templates were compared: an RNA template similar to the replicon (miniG) and a template similar to the full-length virus (SLVI miniG) (Fig. 2). The goal of this study was to characterize the effect of the SLVI interaction with the genomic 3¢ end on the RNA synthesis and IRES-mediated translation of HCV. All of the interaction(s) other than that involving the SLVI structure caused relatively slight modifications of viral activity, as expected from

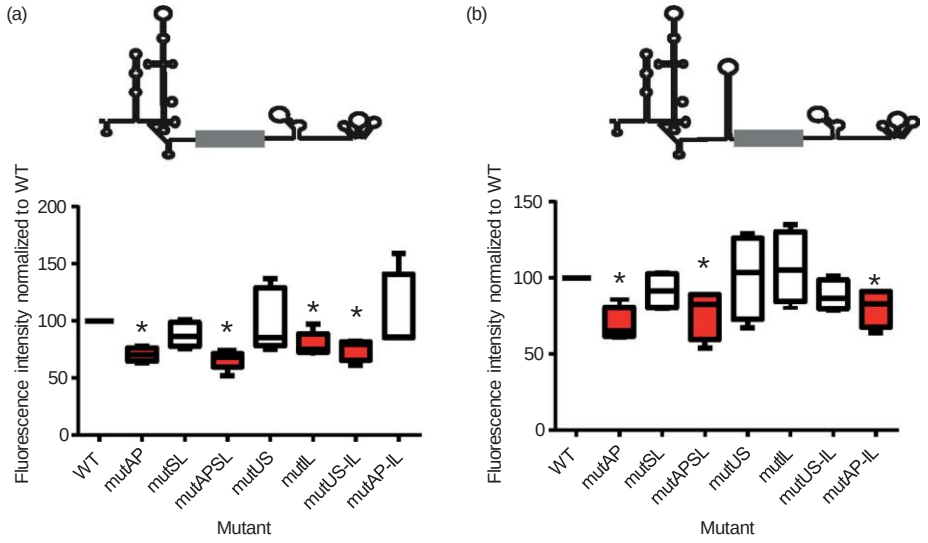

Fig. 5. Modulation of HCV IRES-mediated translation by mutant interactions. miniG (a) or SLVI miniG (b) were transfected into naive Huh7 cells. The translation efficiency was evaluated by flow cytometry 24 h post-transfection and normalized to that of the corresponding WT mini-genome. The translation value was normalized to the stability of each mini-genome in naive Huh7 cells, which was calculated in Fig. 4 (box and whiskers from 25th to 75th percentiles with n=5 independent experiments, *P<0.05 compared with the corresponding WT mini-genome using a Mann–Whitney–Wilcoxon test). Significant decrease is denoted by a red box.

our previous results, where we showed that the mini-genome without 5BSL3.2 is also replicated by the HCV RC [22, 23]. Indeed, despite the absence of the SLVI in the replicon model, this RNA molecule is capable of autonomous replication, clearly demonstrating that the interaction is not essential for establishing an RNA replication system.

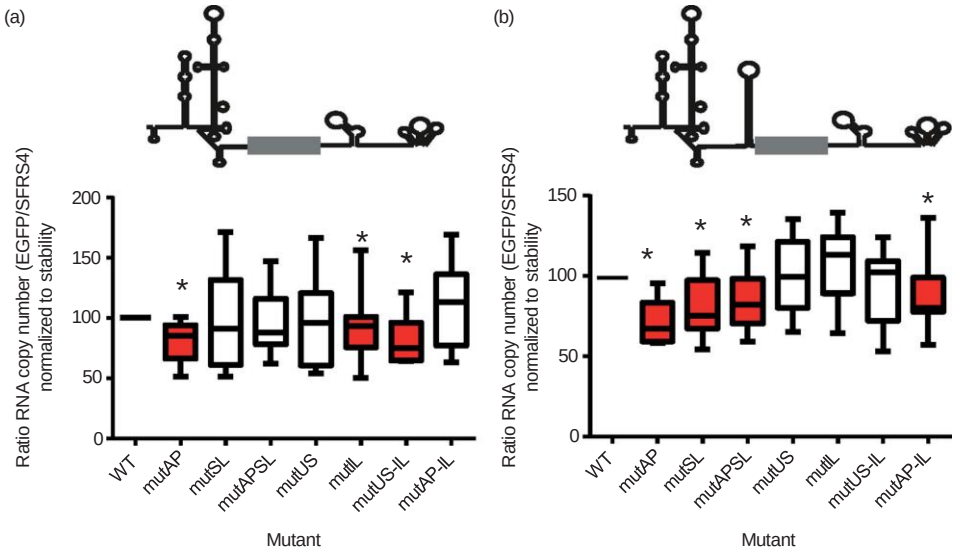

Fig. 6. Modulation of RNA mini-genome replication by 5BSL3.2 mutations. miniG (a) or SLVI miniG (b) were transfected into replicon-bearing Huh7 cells. The ratio (copy number of EGFP transcripts/copy number of SFRS4 transcripts) was measured 96 h post-transfection by RT-qPCR and normalized to that of the corresponding WT mini-genome. The ratio of replicon-bearing cells was normalized to the stability of each mini-genome in naive Huh7 cells, which was calculated in Fig. 4 (box and whiskers from 25th to 75th percentiles with n=5 independent experiments for each mini-genome, *P<0.05 compared with the corresponding WT mini-genome using a Mann–Whitney–Wilcoxon test). Significant decrease is denoted by a red box.

Analysis of IRES-mediated translation

Our data show that the SLVI stem loop (probably through the 5¢UTR/SLVI interaction) primarily decreases HCV IRES activity in the WT context (Fig. 3). When the AP sequence is mutated (mutAP and mutAP-SL), the translation is hampered but mutation of SL has no effect. This result supports a previous study showing that the AP/SL interaction promotes translation [26]. Nevertheless, our data show that the mutation of only one partner of the interaction (the 5BSL3.2 AP but not the SL2 SL) leads to a decrease in IRES-mediated translation, indicating that the sequence, more than the interaction, is important. A new interaction, described by Fricke *et al*., between AP and the IIId loop could indeed explain this result [24]. However, the mutIL and mutUS-IL mutants show opposite behaviour depending on the presence of the SLVI region. For both of these mutants, the translation is decreased in the absence of SLVI, likely preventing genome circularization by the IIId/IL interaction [21, 27]. Because the SLVI stem loop has already decreased the translation (Fig. 3), the mutation of the IL sequence does not have any obvious effect (Fig. 5b). This implies that genome cyclization (interaction IIId/IL) promotes viral protein expression but that the 5¢UTR/SLVI interaction likely prevents this cyclization. We hypothesize that the 5¢UTR/SLVI interaction is itself regulated by other interactions, such as those described by Fricke and colleagues [24], or by the varying mir122 quantities in the different compartments of the cell mediated by this interaction [28].

Analysis of RNA replication

All the AP mutants are replication-deficient in the presence of the SLVI, whereas only the mutAP mutant leads to a replication decrease for the miniG. Unlike stability, the mutAP-SL mutant, which supposedly restores the interaction, does not lead to a replication level similar to the WT with the SLVI miniG. This confirms again that RNA synthesis is dependent on the 5BSL3.2 AP sequence and does not rely on the AP/SL interaction. This result supports previous studies suggesting that the AP sequence is involved in replication because viral polymerase is thought to bind the 5BSL3.2 domain [29–32]. A previous study also showed that the binding of the EWSR1 factor to 5BSL3.2 is essential for replication [33]. Our work suggests that a specific protein/RNA interaction could be mediated by the AP sequence. As described above with translation activity, the replication analysis shows that the presence of the SLVI region affects the IIId/IL interaction. Similarly, the IIId/IL interaction also increases RNA synthesis when paired with a template that is unable to perform the 5¢UTR/SLVI interaction (without SLVI). Therefore, both translation and replication studies clearly show that the 5¢UTR/SLVI interaction interferes with HCV RNA circularization that is mediated by the IIId/IL interaction. This conclusion also confirms the importance of the SLVI domain when performing analysis of the replication mechanism.

In the context of the SLVI miniG, the mutSL mutation also decreases the replication activity. Because we previously showed that the AP/SL interaction is likely not involved, this replication defect could be related to genome dimerization [34]. Indeed, the nucleotide sequence of SL2 that is involved in the APSL interaction also belongs to the sequence mediating the dimerization of the viral RNA. We recently showed that genome dimerization is needed for RNA synthesis [34]. *In vitro* studies indicate that the SL2 mutant (used in this work) is not able to dimerize on SDS-PAGE, unlike the WT sequence (data not shown), supporting the hypothesis that the effect on replication could be due to the abolition of dimerization.

Interestingly, our study shows that the US/IL interaction is not directly involved in RNA stability, translation or replication. Although this interaction has been shown to be crucial for replicon replication [20], this result is in accordance with a recent study showing that the abolition of this interaction does not lead to any alterations in the translation or replication activity of the HCV replicon [26]. Our study shows that this lack of alteration is reproduced in the context of the SLVI domain.

In conclusion, this study highlights the major role of the SLVI structure: its presence modulates the effect of the interactions involving the 5BSL3.2 domain on RNA translation and RNA synthesis. In the literature, most of the studies have been performed without the SLVI SL and could lead to misinterpretation regarding both viral activities. Furthermore, additional interactions have been identified in a recent report [24]. Some of these have been suggested to strengthen RNA dimerization, meaning that they can potentially reinforce or even counterbalance the above-mentioned interactions. Others involve the IIId domain of the 5¢UTR region, which interacts with the SLVI domain, making the analysis of their individual roles more complex.

The potential role of all intragenomic interactions studied is summarized in Fig. 7, where we propose a model of their respective functions in the viral life cycle deduced from this analysis. Understanding the roles and functions of each previously described interaction would not only be beneficial regarding hepatitis treatment, but could also help to elucidate the mechanism of replication of members of the *Flaviviridae* family.

METHODS

Plasmid constructs

The pGEM-T/5UTR-H2AE-5BSL-3UTR vector was constructed from the previously described pGEM-T/5UTR-H2AE-3UTR vector [22] by inserting the last 278 nt of the HCV NS5B gene (nt 9100 to nt9378 with reference to the H77 complete genome sequence, GenBank accession no. AF011753 [20]) between the EGFP stop codon and the 3¢UTR to create the pGEM-T/5UTR-H2AE-5BSL-3UTR vector. The first 120 nt of the core (the SLV and SLVI structures) were then inserted in fusion with the H2AE polyprotein

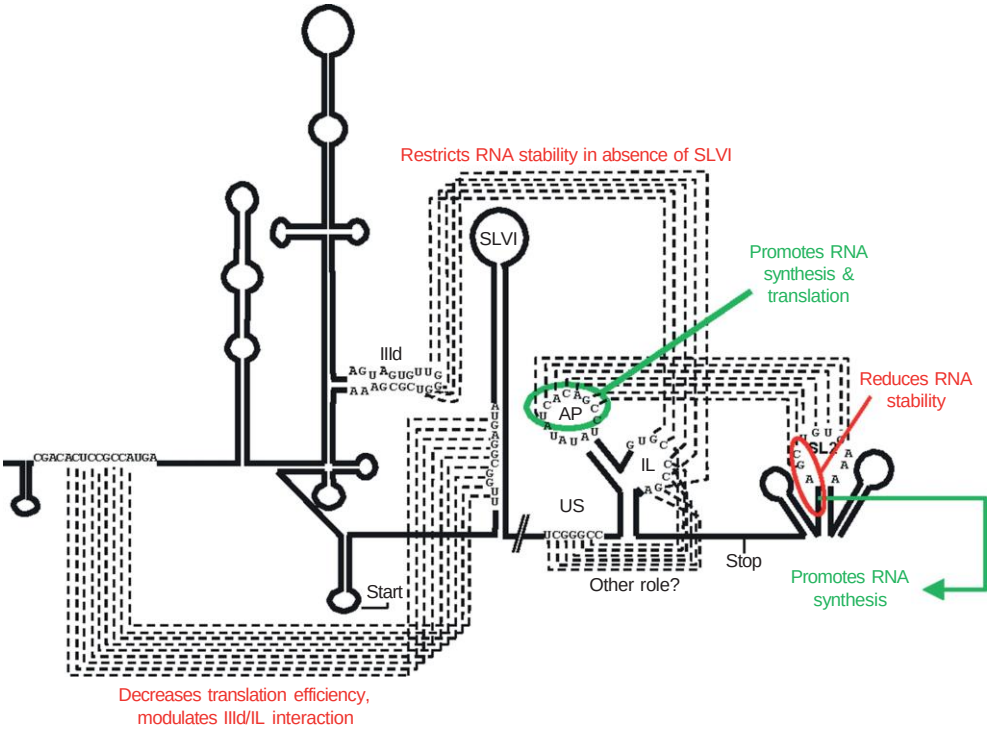

Fig. 7. Putative role of the interactions analysed in this study. The presence of the SLVI structure decreases translation activity (Fig. 3b) and modulates the IIId/IL interaction (Figs 4, 5 and 6). The IIId/IL interaction in the absence of the SLVI and AP/SL interactions restricts RNA stability (Fig. 4). The AP (Fig. 6) and SL2 sequences [34] (circled in green and red, respectively) promote RNA synthesis. The AP sequence also promotes translation and RNA synthesis (Fig. 4).

(Hygromycin phosphotransferase-2A-EGFP), resulting in the pGEM-T/5UTR(SLVI)-H2AE-5BSL-3UTR vector. The 5BSL3.2 and SL2 APs were mutated using site-directed mutagenesis of the pGEM-T/5UTR-H2AE-5BSL-3UTR vector by exchanging TCAC with AAAG in the 5BSL3.2 AP (mutAP mutant), GTGA with CCTT in the 3¢UTR SL2 (mutSL mutant), GGC with CCG in the US identified by Diviney *et al.* (mutUS mutant) [20] and GCC with CGG in the 5BSL3.2 IL (mutIL mutant) using the primers described in Table 2. Each double mutant (mutAP-SL and mutUS-IL) designed to restore the interactions, or mutants designed to abrogate both interactions (mutAP-IL), was also obtained using site-directed mutagenesis. The sequence of the mutated fragments was verified by DNA sequencing using the MWG Operon Sequencing facility (www.mwg-biotech.com/).

Mini-genome transcription

Two sets of RNA mini-genomes were obtained from pGEM-T/5UTR-H2AE-5BSL-3UTR (miniG) or pGEM-T/5UTR(SLVI)-H2AE-5BSL-3UTR (SLVI miniG) using PCR with the primers T7_5UTR_Start and 3UTR_Stop, which were designed to introduce a T7 RNA polymerase promoter in the correct orientation (Table 2). Then, *in vitro* transcription was performed as previously described [25], and the purity and integrity of the RNAs were determined using capillary electrophoresis on a Bioanalyzer 2100 (Agilent).

Cell culture and transient RNA transfection

All cell lines were cultured in Dulbecco's modified Eagle's medium (ThermoFisher) supplemented with 10 % heat-inactivated FCS and gentamycin (50 µg ml^{-1}) at 37° C in a 5 % CO_2 atmosphere. The Huh7/Rep 5.1 cell line was established after electroporation of the Rep5.1 RNA replicon (kindly provided by R. Bartenschlager – University of Heidelberg) and selected with 500 µg G418 ml^{-1}. The Huh7-QR cells originated from a Huh7/Rep5.1 cell line that was cured of the Rep5.1 replicon by a three-week treatment with 150 U interferon-a ml^{-1}.

Reverse transient transfections were performed by seeding 24-well plates with 18×10^4 of the appropriate cells (Huh7-QR or Huh7-Rep) per well 24 h before transfection. RNA transfection was performed using 1 µg of RNA combined with 3 µl of the transfection agent DMRIE-C according to the manufacturer's instructions (Invitrogen). For translation analysis, the mean fluorescence intensity was measured using flow cytometry (Becton-Dickinson LSR Fortessa, University of Bordeaux cytometry facility).

RNA isolation

Total RNA was extracted from two wells of a 24-well plate using TRI Reagent (Molecular Research Center) 96 h post-transfection according to the manufacturer's instructions,

Table 2. Oligonucleotides used in cloning and PCR experiments

The sequence corresponding to the T7 RNA polymerase promoter is underlined.

Oligonucleotide	Sequence (5¢→3¢)
T7_5UTR_Start	TAATACGACTCACTATAGGGCCAGCCCCCGATTGGGGGCGACAC
3UTR_stop	ACTTGATCTGCAGAGAGGCCAG
miniG_AP_S	GACATATAAAGGAGCCTGTCTCGTGCCCGACCCC
miniG_AP_As	GGTTACAGCGGGGGAGACATATAAAGGAGCCTGTCT
miniG_IL_S	GCCTGTCTCGTCGGCGACCCCGCTGGTTCATGTGG
miniG_IL_As	GAGACATATATCACAGCCTGTCTCGTCGGCGACCCC
miniG_US_S	AGACATCGCCGCAGAAGTGTCCGCGCTAGGCTAC
miniG_US_As	GACGAGTCTGGAGACATCGCCGCAGAAGTGTC
miniG_SL_S	GCTAGCTCCTTAAGGTCCGTGAGCCGCTTGACTG
miniG_SL_As	GCCCTAGTCACGGCTAGCTCCTTAAGGTCCGTGA
SFRS4_S	AAAAGTCGGAGCAGGAGTCA
SFRS4_As	CTCTTCCTGCTCTTCCTCTT
EGFP_S	GACCACATGAAGCAGCACGA
EGFP_As	GACGTTGTGGCTGTTGTAGT

and then resuspended in 50 µl DEPC-treated H_2O. RNA integrity was then determined using capillary electrophoresis on a Bioanalyzer 2100 (Agilent).

Quantitative RT-PCR

The EGFP_S and EGFP_As primers (Table 1) were designed to amplify a portion of the EGFP gene (225 nt). The HCV RNA copy number was normalized to the level of SFRS4 cellular RNA, which remains stable in HCV-infected cells [35]. The SFRS4_S and SFRS4_AS primers (Table 2) were used to amplify a portion of the housekeeping gene SFRS4 (182 nt). Reactions were performed with the MyIq apparatus (Biorad) in 20 µl using SYBR Green (Promega GoTaq 1-Step RT-qPCR System) with primers at 300 nM under the following conditions: step 1, reverse-transcription for 20 min at 37° C; step 2, 5 min at 95° C; step 3, 40 cycles of 5 s at 95° C, 20 s at 57° C and 10 s at 72° C. SYBR Green fluorescence was measured for 2 s at 80° C for SFRS4 and EGFP. The copy numbers of EGFP and SFRS4 were determined by comparison with specific, serially diluted standard transcripts that were included in the RT-PCR analysis. To avoid the effect of potential inhibitors in the extract, RT-qPCR reactions were performed on 5 µl of two dilutions (50–500) of cellular RNA to determine the copy number of the transcript being considered.

Data and statistical analysis

The data were obtained from five independent transfections for each type of mini-genome (miniG and SLVI miniG) with the seven 5BSL3.2 mutants. For each transfection of the WT and seven mutant mini-genomes, at least three RT-qPCR reactions (with two dilutions for each sample) were performed to accurately determine the copy number of EGFP and SFRS4 in naive and replicon-bearing Huh7 cells. Statistical analysis was performed with GraphPad software using a two-sided Mann–Whitney–Wilcoxon test.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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