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The regulation of hepatitis C virus (HCV) internal ribosome-entry site-mediated translation by HCV replicons and nonstructural proteins

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Hepatitis C virus (HCV), the global leading cause of chronic liver disease, has a positive-sense, ssRNA genome that encodes a large polyprotein. HCV polyprotein translation is initiated by an internal ribosome-entry site (IRES) located at the 5′ end of the viral genome, in a cap-independent manner, but the regulatory mechanism of this process remains poorly understood. In this study, we characterized the effect of HCV nonstructural proteins on HCV IRES-directed translation in both HCV replicon cells and transiently transfected human liver cells expressing HCV nonstructural proteins. Using bicistronic reporter gene constructs carrying either HCV or other viral IRES sequences, we found that the HCV IRES-mediated translation was specifically upregulated in HCV replicon cells. This enhancement of HCV IRES-mediated translation by the replicon cells was inhibited by treatment with either type I interferon or ribavirin, drugs that perturb HCV genome replication, suggesting that the enhancement is probably due to HCV-encoded protein function(s). Reduced phosphorylation levels of both eIF2α and eIF4E were observed in the replicon cells, which is consistent with our previous findings and indicates that the NS5A nonstructural protein may be involved in the regulatory mechanism(s). Indeed, transient expression of NS5A or NS4B in human liver cells stimulated HCV IRES activity. Interestingly, mutation in the ISDR of NS5A perturbed this stimulation of HCV IRES activity. All these results suggest, for the first time, that HCV nonstructural proteins preferentially stimulate the viral cap-independent, IRES-mediated translation.

INTRODUCTION

Hepatitis C virus (HCV) is the leading cause of chronic liver disease globally and is estimated to infect about 170 million people around the world (Alter, 1997). Chronic HCV infection frequently leads to liver fibrosis and cirrhosis, and is associated with the occurrence of hepatocellular carcinoma (Hoofnagle, 1997). There is no vaccine available for HCV, and the current therapies, either interferon (IFN)-α monotherapy, or a combination of IFN-α and ribavirin, have been unable to eliminate HCV replication in the majority of patients (Moradpour & Blum, 1999). However, the molecular mechanisms of HCV replication and persistence remain poorly characterized, since neither an adequate animal model, nor an efficient cell culture system for HCV infection and replication, are currently available (Gale & Beard, 2001). Further studies aiming at dissecting the molecular aspects of HCV life-cycle are thus of particular importance.

A *Flaviviridae* family member, HCV possesses a positive-sense, ssRNA genome of about 9600 nucleotides (Reed & Rice, 2000). The HCV genome consists of highly conserved 5′- and 3′-noncoding regions (NCR), and a large open reading frame (ORF) that encodes a polyprotein of approximately 3010 amino acids. The polyprotein is processed co- and post-translationally by both host and viral proteases into at least 10 structural (core, E1, E2 and p7) and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Recent studies also show the existence of an alternative ORF within the core-coding region that encodes a novel HCV protein of unknown function (Varaklioti *et al.*, 2002; Walewski *et al.*, 2001; Xu *et al.*, 2001). The 5′ terminus of the HCV genome possesses a complex secondary structure that functions as an internal ribosome-entry site (IRES) to mediate viral protein translation in a cap-independent manner (Hellen & Pestova, 1999; Rijnbrand & Lemon, 2000). The HCV IRES was mapped between about 40 and 370 nucleotides from the 5′-end of the genome, including most of the 5′-NCR and a small beginning portion of the core-coding

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region (Honda *et al.*, 1996; Reynolds *et al.*, 1995; Rijnbrand *et al.*, 1995). Three distinct elements have been shown to be involved in HCV IRES-mediated translation: (1) integrity of the global structure of HCV IRES (Hellen & Pestova, 1999); (2) the 3′-terminal region of the HCV genome; (3) *trans*-acting cellular factors that interact with the HCV IRES element and assist in translation initiation. Furthermore, the HCV core protein-coding sequence, but not the core protein, has been suggested to modulate HCV IRES-directed translation efficiency, possibly through long-range RNA–RNA interaction (Honda *et al.*, 1999; Wang *et al.*, 2000). However, another recent study suggested that core protein caused autogenous inhibition of HCV IRES translation, which may imply a mechanism for switching from translation to RNA replication and/or encapsidation during the virus life-cycle (Zhang *et al.*, 2002). Interestingly, HCV IRES-mediated translation seems to be regulated by cell cycle-associated mechanisms, and it is known that the HCV IRES activity varies with the cell cycle (Honda *et al.*, 2000; Pietschmann *et al.*, 2001).

Being a highly conserved, virus-specific mechanism, the IRES-mediated translation of HCV constitutes an excellent target for development of the next generation of antiviral drugs for HCV (Jubin, 2001). However, because of the lack of an efficient *in vitro* infection system, the regulatory mechanisms of HCV IRES-mediated translation still remain poorly characterized. Previous studies focused on the roles of HCV genomic RNA sequences and structures, and *in trans* cellular proteins, in regulation of HCV IRES-directed translation. Few studies have characterized the effects of HCV-encoded proteins on HCV translation, and it remains largely unknown whether any HCV-encoded nonstructural protein(s) can regulate IRES-directed translation, as part of the virus's strategies to control its life-cycle. In this study, using both the HCV replicon cells and human liver cells transiently expressing HCV nonstructural proteins, we investigated the function of HCV nonstructural proteins in regulating HCV IRES-mediated translation. We also characterized the possible molecular mechanisms underlying the phenotype and suggest a novel mechanism by which HCV modulates its cap-independent translation and facilitates virus persistence.

METHODS

Cell culture and plasmid construction. The HCV subgenomic replicon construct (Fig. 1A) has been described previously, and the Huh7-derived HCV replicon cells were generated and maintained as previously described (Blight *et al.*, 2000; Lohmann *et al.*, 1999). The Huh7 human haematoma cells were cultured in DMEM supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 10 % foetal calf serum, at 37 °C in the presence of 5 % CO_2. The Tet-On Huh7 cell line was constructed using the Tet-On Gene Expression System (Clontech) according to the manufacturer's instructions, and was maintained in DMEM supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1}, 10 % foetal calf serum and 200 μg G418 ml^{-1}, at 37 °C in the presence of 5 % CO_2. In order to construct the expression plasmids carrying the individual replicon nonstructural protein genes, the individual replicon nonstructural protein coding sequences were amplified by PCR from pHCVneo17 (Blight *et al.*, 2000), and inserted into the *Sac*II and *Xba*I sites of pTRE vector (Clontech), resulting in pTRE-NS3, pTRE-NS4A, pTRE-NS4B, pTRE-NS5A and pTRE-NS5B. The following PCR primers were used (sequences in 5′ to 3′): GTACCGCGGATGGCGCCTATTACG and TCGTCTAGACGTGACGACCTCCAGGTCAG (for NS3 coding sequence); CGACCGCGGATGAGCACCTGGGTG and ACGTCTAGAGCACTCTTCCATCTCATCG (for NS4A coding sequence); TTACCGCGGATCGCCTCACACCTCCCTTAC and ACGTCTAGAGCATCCCGTGGAGCAGTC (for NS4B coding sequence); GTACCGCGGATGTCCGGCTCGTGGCTAAG and ACGTCTAGAGCAGCAGACGACGTCCTCAC (for NS5A coding sequence); and GTACCGCGGATGTCGATGTCCTACACATGG and GCTTCTAGATCGGTTGGGGAGTAGATAG (for NS5B coding sequence). pCDNA3-NS5A-1a, pCDNA3-NS5A-1b1 and pCDNA3-NS5A-1b5 have been described previously (Gale *et al.*, 1997, 1998, 1999). The pTRE-*β*-galactosidase construct will be described in a future publication (G. K. Geiss and others).

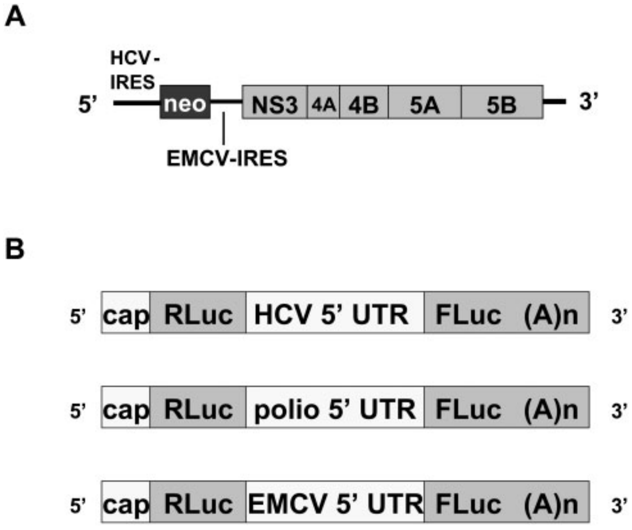

Fig. 1. (A) The HCV RNA replicon construct. See text for detailed description of the construct. Neo, neomycin (G418) resistance gene. (B) The dual-luciferase reporter constructs containing the HCV 5′ UTR (top), the poliovirus 5′ UTR (middle) and the EMCV 5′ UTR (bottom). Translation of the upstream Renilla luciferase gene (Rluc) is mediated by the 5′ cap structure. Translation of the downstream firefly luciferase gene (Fluc) is mediated by different viral IRES elements located between the RLuc and FLuc sequences. (A)n, poly(A) tail.

Luciferase reporter constructs and luciferase assays. The dual-luciferase reporter constructs containing the HCV IRES, the poliovirus IRES and the encephalomyocarditis virus (EMCV) IRES have been described previously (Collier *et al.*, 1998; Creancier *et al.*, 2001; Poulin *et al.*, 1998) (Fig. 1B). 2×10^5 cells in 35 mm tissue culture plates were transiently transfected with 1 μg of each plasmid DNA using SuperFect (Qiagen) according to the manufacturer's instructions. The dual-luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions, on a Beckman Coulter LS6500 scintillation counter. All the luciferase assays were performed in triplicate and mean values are shown.

Interferon and ribavirin treatment. Human type I IFN was purchased from Access. Ribavirin was purchased from Sigma. For IFN treatment, Huh7 and HCV replicon cells were incubated with media containing type I IFN (5 and 50 IU ml^{-1}) for 24 h at 37 °C in the presence of 5 % CO_2. For ribavirin treatment, Huh7 and HCV replicon cells were incubated with media containing ribavirin (400 and 1000 μM) for 24 h at 37 °C in the presence of 5 % CO_2.

Antibodies and immunoblot assays. Antibodies specific to HCV NS3, NS4A and NS5B were described previously (Tomei *et al.*, 1993). The antibody specific to HCV NS5A was purchased from ID Labs. Antibodies specific to phosphorylated eIF2α (Ser51), total eIF2α, phosphorylated eIF4E (Ser209), total eIF4E, phosphorylated Erk1/2 (Thr180/Tyr182) and total Erk1/2 were previously described (He *et al.*, 2001). For immunoblot analysis, cell lysates were collected and protein concentrations were determined as described previously (He *et al.*, 2001). Equal amounts of cell lysates were resolved on SDS-PAGE (12 %), followed by electroblotting to nitrocellulose membrane (Schleicher and Schuell). The immunoblot analysis was performed as previously described (He *et al.*, 2001). The relative levels of protein phosphorylation were determined by quantifying the immunoblots with ImageQuant (version 5.1). The signals from the phospho-specific immunoblots were normalized against their individual control signals and the ratio of phospho-specific signal to control signal was calculated as previously described (He *et al.*, 2001).

RESULTS

HCV replicon specifically up-regulates HCV IRES-mediated translation

In this study we utilized the recently developed HCV replicon system to study the effect of HCV nonstructural proteins on HCV IRES-mediated translation. The HCV replicon cells are Huh7 human hepatoma cells stably transfected with self-replicating, subgenomic HCV RNA constructs carrying the HCV nonstructural coding region (Fig. 1A), and express the nonstructural proteins (NS3, NS4A, NS4B, NS5A and NS5B) (Blight *et al.*, 2000; Lohmann *et al.*, 1999). Expression of the HCV proteins (NS3, NS4A, NS5A and NS5B) and replication of the subgenomic construct in the replicon cells were confirmed by immunoblot and RT-PCR analysis, respectively (data not shown).

We examined the effect of HCV replicon on HCV IRES-mediated translation using an established dual-luciferase reporter gene construct containing a genotype 1b HCV IRES element (Fig. 1B). In this bicistronic reporter construct, translation of the upstream Renilla luciferase gene is initiated in a cap-dependent mechanism, while translation of the downstream firefly luciferase gene is mediated by an HCV IRES element contained within the HCV 5′NTR in a cap-independent manner. This construct allows internally controlled quantification of the IRES-directed translational level since the two luciferase reporters are apparently translated from the same transcripts. The HCV-IRES dual-luciferase reporter construct was transiently transfected into either the HCV replicon cells (clone 10A) (Blight *et al.*, 2000) or Huh7 cells, the parental control cell line. Cell lysates were collected at different time-points post-transfection and dual-luciferase assays were performed on the lysates (Fig. 2A). The relative IRES-activity levels were expressed as the ratio of the activity of firefly luciferase (IRES-dependent) over that of Renilla luciferase (cap-dependent). The activity of the HCV IRES in Huh7 cells was arbitrarily taken as 100 % with the activity of the IRES in replicon cells expressed relative to this. As shown in Fig. 2(A), at 12 h post-transfection, there was no significant difference in HCV IRES-directed translational levels between the replicon and Huh7 cells. However, at 24 h post-transfection, an almost 2-fold increase in relative HCV IRES activity was observed in the replicon cells over that of Huh7 cells. In our experiments the effects of NS proteins on IRES were not observed at early time-points after transfection, which is probably due to the recovery phase of cells following the rather cytotoxic transfection procedure. Examination of the absolute Renilla and firefly luciferase activity levels showed that HCV replicon caused an increase in the IRES-dependent firefly luciferase activity, but not in cap-directed Renilla luciferase reporter activity (data not shown). In addition, no significant difference in the global cellular protein synthesis rates was observed between Huh7 and replicon cells by pulse-labelling analysis (data not shown). This suggests that the HCV replicon cells specifically stimulate HCV IRES-directed translation, but not cap-dependent global mRNA translation. Similar, but more dramatic, results (a 5·5-fold increase) were acquired with another HCV replicon cell line clone (clone Huh-9-13) (Lohmann *et al.*, 1999), although slightly different kinetics was observed in this cell line (Fig. 2B). The difference in relative HCV IRES activity levels and kinetics may be caused by variations in either experimental conditions and/or replicon clones. Collectively, these results suggest that the stimulatory effect of HCV replicon on HCV IRES activity is not clone-specific, and is thus likely due to HCV-encoded function(s).

Since numerous viruses employ an IRES-mediated translational initiation mechanism (Gale *et al.*, 2000), this raised the question of whether the HCV replicon only stimulates the HCV IRES-mediated translation specifically, or if it may also enhance the activity of other viral IRES elements. We thus compared the effects of HCV replicon on the activity of HCV, poliovirus and EMCV IRES, using dual-luciferase reporter constructs that contain these different viral IRES elements (Fig. 1B). The different reporter constructs were transfected into both HCV replicon cells (clone 10A) and Huh7 cells, and the relative IRES activity levels were determined at 24 h post-transfection as described above (Fig. 2C, D). The activities of the viral IRES in Huh7 cells were arbitrarily taken as 100 % with the IRES activities in replicon cells expressed relative to this. The HCV replicon caused a 3- to 5-fold increase in HCV IRES activity levels, while either only a slight increase in poliovirus IRES activity (Fig. 2C) or no significant difference in EMCV IRES activity (Fig. 2D) was observed. These results indicate that HCV replicon specifically stimulates HCV, but not other viral

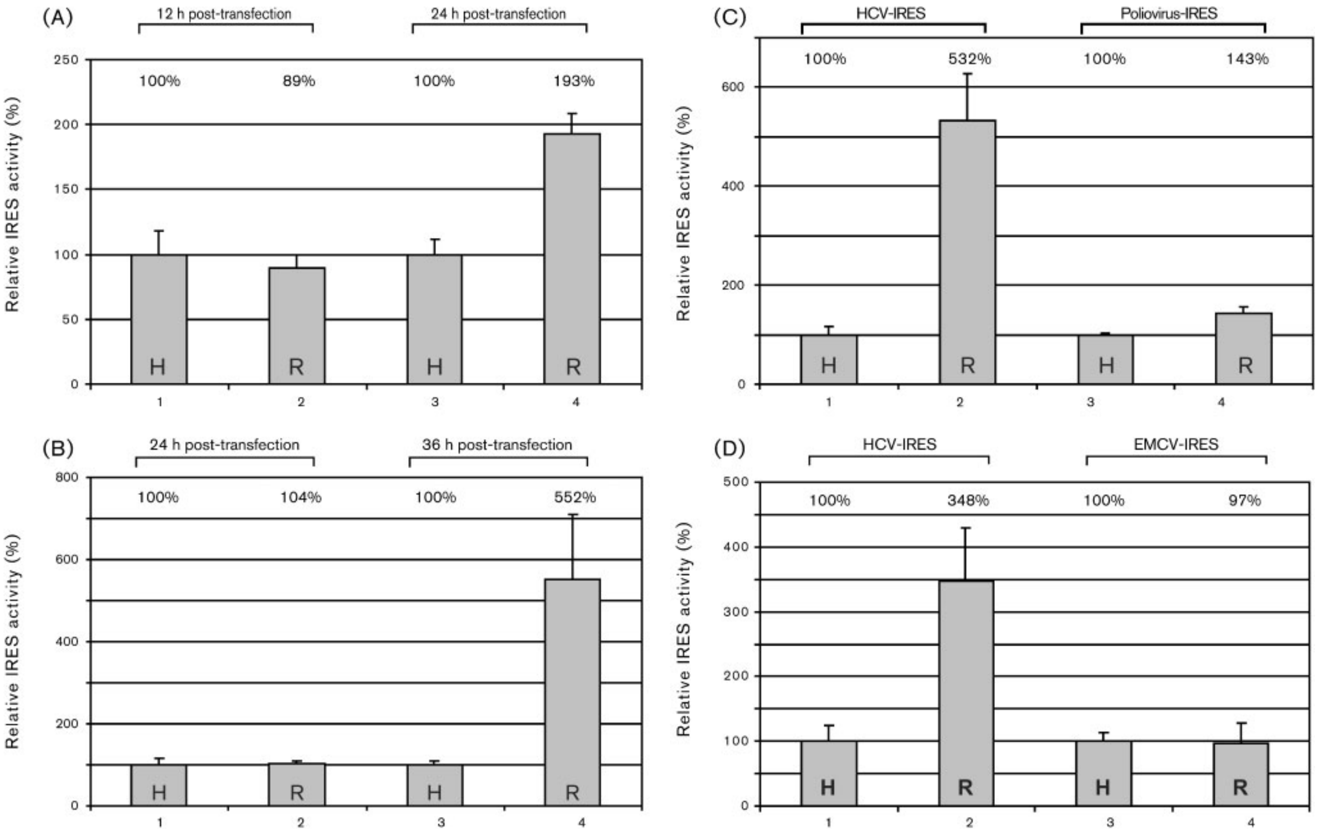

Fig. 2. HCV replicon cells specifically enhanced HCV IRES-mediated translation. (A) Huh7 (H) and HCV replicon cells (R) (clone 10A) were transfected with the dual-luciferase reporter construct containing the HCV IRES sequence (Fig. 1B). Cell lysates were collected at 12 and 24 h post-transfection and subjected to dual-luciferase assays. The relative IRES activities were expressed as the ratio of firefly luciferase activity over that of the Renilla luciferase. The activity of the HCV IRES in Huh7 cells was arbitrarily taken as 100 % with the activity of the IRES in replicon cells expressed relative to this. The experiment was performed in triplicate and mean values are presented. The error bars in all the figures represent standard deviations. (B) Huh7 (H) and HCV replicon cells (R) (clone Huh-9-13) were transfected with the dual-luciferase reporter construct containing the HCV IRES sequence. Then cell lysates were collected at different time-points post-transfection and subjected to dual-luciferase assays as described above. The activity of the HCV IRES in Huh7 cells was arbitrarily taken as 100 % with the activity of the IRES in replicon cells expressed relative to this. (C) Huh7 (H) and HCV replicon cells (R) (clone 10A) were transfected with the dual-luciferase reporter construct containing either the HCV IRES or the poliovirus IRES sequences (Fig. 1B). Cell lysates were collected at 24 h post-transfection and subjected to dual-luciferase assays as described above. The experiment was performed in triplicate and mean values are presented. The activities of the viral IRES in Huh7 cells were arbitrarily taken as 100 % with the IRES activities in replicon cells expressed relative to this. (D) Huh7 (H) and HCV replicon cells (R) (clone 10A) were transfected with the dual-luciferase reporter construct containing either the HCV IRES or the EMCV IRES sequences (Fig. 1B). Cell lysates were collected at 24 h post-transfection and subjected to dual-luciferase assays as described above. The experiment was performed in triplicate and mean values are presented. The activities of the viral IRES in Huh7 cells were arbitrarily taken as 100 % with the IRES activities in replicon cells expressed relative to this.

IRES-mediated translation, and the underlying mechanism is thus likely to be HCV-specific.

The effect of HCV replicon on HCV IRES-mediated translation is sensitive to both IFN and ribavirin treatment

However, it was unclear whether the enhancement of HCV IRES activity by replicon cells was caused by HCV-encoded function(s) or by adaptive cellular mutations that occurred during the replicon cell line construction process. We next attempted to address this question by inhibiting replication of the HCV replicon in clone 10A replicon cells with IFN and ribavirin treatment. Both IFN and ribavirin are used in the current therapies for HCV infection (Hoofnagle, 1999), and have been shown to perturb HCV genome replication in different cell culture systems, including the replicon system (Blight *et al.*, 2000; Chung *et al.*, 2001; Frese *et al.*, 2001; Maag *et al.*, 2001). If the HCV IRES-stimulatory effect of the replicon cells were due to HCV-encoded protein function(s), we would speculate that anti-HCV drug treatment, which inhibits HCV genome replication and protein expression, would therefore inhibit the IRES-stimulatory effect of HCV replicon cells.

In the experiment shown in Fig. 3(A), both Huh7 and clone 10A replicon cells were transiently transfected with the HCV IRES-containing dual-luciferase reporter construct, immediately followed by treatment either with different concentrations of type I IFN (lanes 3–6) or with ribavirin (lanes 7–10). Untreated cells were used as controls (lanes 1 and 2). At 24 h post-transfection and drug treatment, cell lysates were collected and dual-luciferase assays were performed to determine relative IRES activity levels as described above. The activity of the HCV IRES in untreated Huh7 cells was arbitrarily taken as 100 % with the activities of the IRES under other conditions expressed relative to this. As shown in Fig. 3(A), the drug treatment did not have a significant effect on relative HCV IRES activity in the parental Huh7 cells (lanes 3, 5, 7 and 9), while in replicon cells 2- to 3-fold inhibitions of HCV IRES activity were observed (lanes 4, 6, 8 and 10). The inhibition of relative HCV IRES activity in replicon cells was primarily caused by a decrease in IRES-directed firefly luciferase activity level, while the cap-dependent Renilla luciferase activity level remained almost unchanged (Fig. 3B). These results suggest that the mechanism(s) responsible for the enhancement of HCV IRES activity by replicon cells is sensitive to anti-HCV drug treatment, and is thus likely to be an HCV-encoded function(s), rather than adaptive cellular mutations in the replicon cells. Inhibition of HCV replicon RNA levels by IFN treatment in our experiments was observed by RT-PCR analysis (data not shown), which is consistent with the results of previous studies (Blight *et al.*, 2000; Chung *et al.*, 2001). IFN treatment was also found to reduce HCV protein levels in the replicon cells (data not shown). All these results argue forcefully in supporting the notion that HCV upregulates its own cap-independent translation process.

The NS5A protein stimulates HCV IRES activity

Knowing that the HCV replicon upregulates HCV IRES activity, we next examined which HCV nonstructural protein(s) was responsible for the function. The individual HCV nonstructural protein coding regions (NS3, NS4A, NS4B, NS5A and NS5B) were subcloned from the parental HCV replicon plasmid construct and expressed separately in Huh7 cells by transient transfection, in combination with the HCV IRES-containing dual-luciferase reporter gene. As

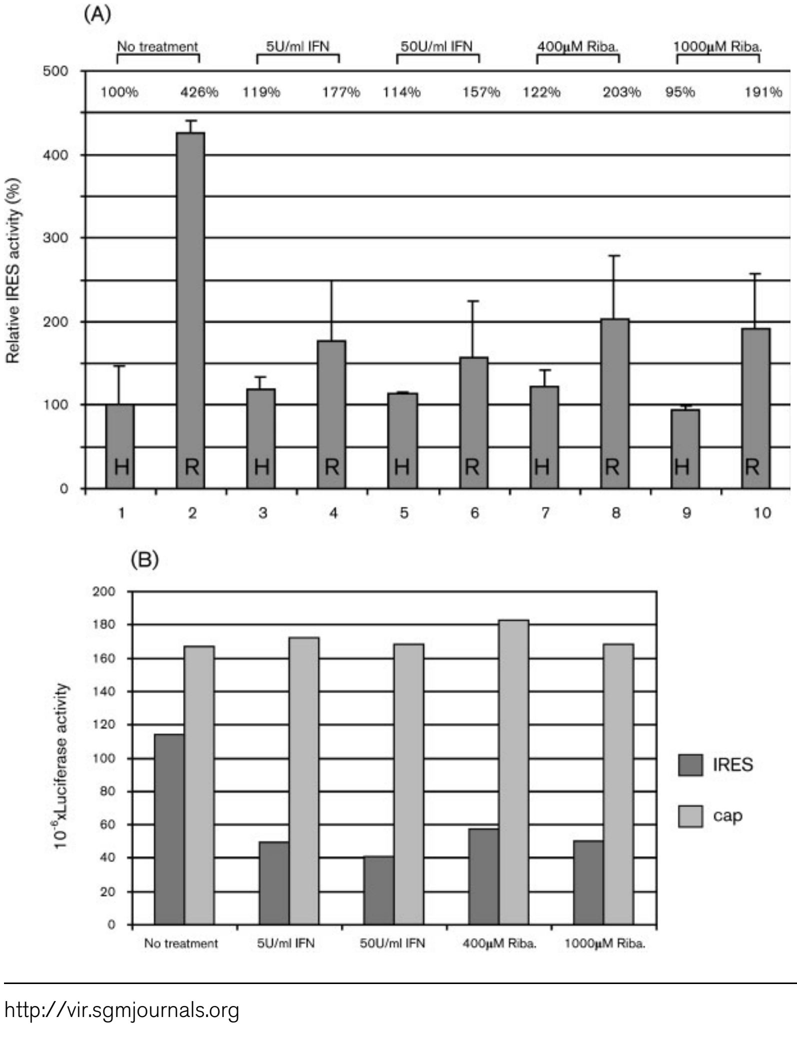

Fig. 3. The enhancement of HCV IRES-mediated translation by HCV replicon cells is inhibited by IFN and ribavirin treatment. (A) Huh7 (H) and HCV replicon cells (R) were transfected with the dual-luciferase reporter construct containing the HCV IRES sequence (Fig. 1B), and incubated with fresh medium containing different concentrations of human type I IFN or ribavirin. Cell lysates were collected at 24 h post-transfection and drug treatment and subjected to dual-luciferase assays as described in the legend to Fig. 2. The experiment was performed in triplicate and mean values are presented. The activity of the HCV IRES in untreated Huh7 cells was arbitrarily taken as 100 % with the activities of the IRES under other conditions expressed relative to this. (B) The average, absolute IRES- and cap-dependent luciferase activity levels of the untreated and drug-treated replicon cells described in (A) are shown.

shown in Fig. 4(A), expression of NS3, NS4A and NS5B did not seem to have any significant effect on the activity level of HCV IRES, as compared with the vector control. On the other hand, expression of NS5A increased HCV IRES-mediated translation level by about 2-fold. In addition, NS4B expression also seemed to increase HCV IRES activity levels to a lesser extent. These results indicate that NS5A and NS4B may be the HCV nonstructural proteins that upregulate HCV IRES activity, although it is possible different HCV proteins may function synergistically. The expression of the HCV nonstructural proteins was confirmed by immunoblot analysis (data not shown).

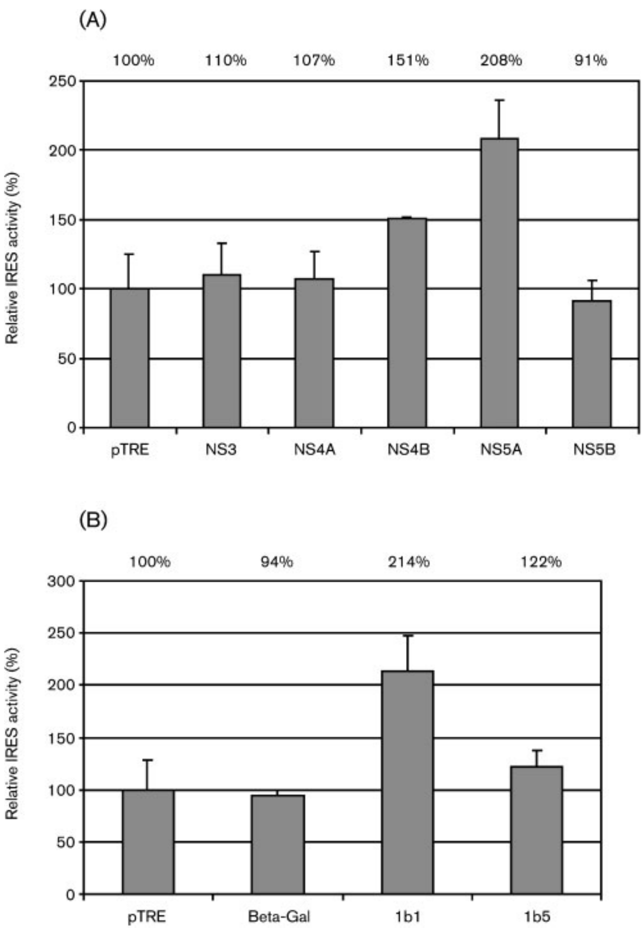

Fig. 4. HCV nonstructural proteins enhanced HCV IRES activity. (A) The pTRE vector, pTRE-NS3, pTRE-NS4A, pTRE-NS4B, pTRE-NS5A and pTRE-NS5B were separately transfected into Huh7 cells, in combination with the dual-luciferase reporter construct containing HCV IRES. Cell lysates were collected at 36 h post-transfection and subjected to dual-luciferase assays as described in the legend to Fig. 2. The experiment was performed in triplicate and mean values are presented. The activity of the HCV IRES in pTRE vector-transfected cells was arbitrarily taken as 100 % with the activities of the IRES under other conditions expressed relative to this. (B) The pTRE vector, pTRE-β-galactosidase, pTRE-NS5A-1b1 and pTRE-NS5A-1b5 were separately transfected into Huh7 cells, in combination with the dual-luciferase reporter construct containing HCV IRES, as described above. Cell lysates were collected at 36 h post-transfection and subjected to dual-luciferase assays as described in the legend to Fig. 2. The experiment was performed in triplicate and mean values are presented. The activity of the HCV IRES in pTRE vector-transfected cells was arbitrarily taken as 100 % with the activities of the IRES under other conditions expressed relative to this.

To gain insight into the molecular mechanism(s) by which NS5A protein enhances HCV IRES-directed translation, we tested the effect of different NS5A isolates on HCV IRES activity level. As shown in Fig. 4(B), an independent NS5A isolate, NS5A-1b1, which carries a 'wild-type', 'IFN-resistant' ISDR (IFN sensitivity-determining region) sequence (Gale *et al.*, 1997), increased IRES activity level to a similar extent (about 2-fold), as compared with both the vector control and the expression of an unrelated protein, β-galactosidase. Interestingly, in the same experiment, an NS5A isolate carrying a 'mutant', 'IFN-sensitive' ISDR sequence, NS5A-1b5 (Gale *et al.*, 1997), was defective in enhancing the activity of HCV IRES. The expression of both NS5A-1b1 and NS5A-1b5 was confirmed by immunoblot analysis and comparable expression levels were observed (data not shown). Therefore, the ability of NS5A to mediate IFN-resistance of HCV is correlated to its ability to regulate HCV protein translation and replication (He & Katze, 2002).

HCV replicon cells show decreased levels of eIF2α and eIF4E phosphorylation

Our previous study (He *et al.*, 2001) showed that NS5A was able to decrease the phosphorylation levels of both eIF2α and eIF4E, suggesting a possible mechanism by which HCV may differentially regulate cap-dependent and -independent translation initiation. So we next examined the phosphorylation status of eIF2α and eIF4E in Huh7 and clone 10A replicon cells, in order to probe the possible molecular mechanisms by which HCV replicon stimulates HCV IRES activity. By performing immunoblot analysis with antibodies that specifically recognize the phosphorylated forms of eIF2α and eIF4E (Fig. 5A, B), lower phosphorylation levels of both eIF2α and eIF4E were detected in replicon cells than in Huh7 parental cells, which is consistent with our previous results. Importantly, HCV replicon did not seem to alter the total protein levels of eIF2α and eIF4E, as shown by immunoblot analysis with antibodies that recognize total eIF2α and eIF4E (Fig. 5A, B). However, it remains uncertain whether in replicon cells the modulation of eIF2α and eIF4E phosphorylation levels is due to NS5A action. Since it is known that the phosphorylation of eIF4E is regulated by the mitogen-activated protein kinase (MAPK) pathway, the activation level of the Erk1/2 MAPK was also compared in Huh7 and replicon cells, by performing immunoblot analysis with antibody specific for the phosphorylated, activated forms of Erk1/2 MAPKs. Consistent with the reduced level of eIF4E phosphorylation in replicon cells, the level of Erk1/2 MAPK phosphorylation observed in replicon cells was lower than in Huh7 cells (Fig. 5C) (He *et al.*, 2002).

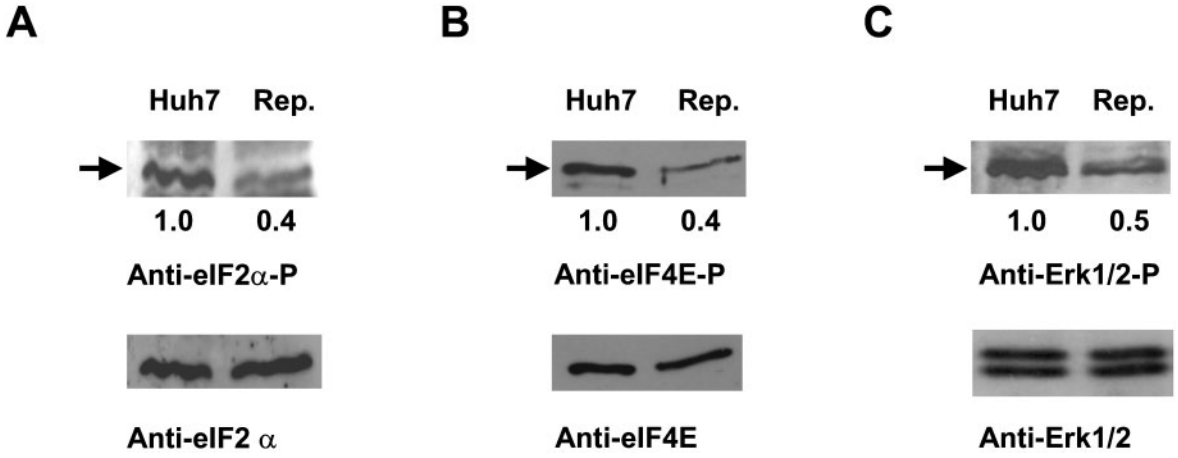

Fig. 5. The phosphorylation levels of eIF2α and eIF4E were decreased in the HCV replicon cells. Equal amounts (30 μg protein) of lysates from actively proliferating Huh7 and HCV replicon cells (Rep.) (~70 % confluent) were resolved on SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies specific to phosphorylated eIF2α (A, upper panel), phosphorylated eIF4E (B, upper panel) and phosphorylated Erk1/2 MAPKs (C, upper panel), respectively. The same membranes were stripped and reprobed with antibodies specific to eIF2α (A, lower panel), eIF4E (B, lower panel) and Erk1/2 MAPKs (C, lower panel), respectively. The numbers below the phospho-specific immunoblots denote the relative levels of eIF2α, eIF4E and Erk1/2 phosphorylation. The protein phosphorylation levels in Huh7 cells were arbitrarily taken as 1·0 with the protein phosphorylation levels in replicon cells expressed relative to this.

These results suggest that HCV replicon caused reduced phosphorylation levels of both eIF2α and eIF4E, which may play a role in the enhancement of HCV IRES activity in these cells. However, considering the fact that HCV replicon stimulated HCV IRES function specifically, and had no significant effect of other viral IRES elements, it is likely that an additional HCV-specific mechanism(s) is responsible for the HCV IRES-specific phenotype of the replicon cells.

DISCUSSION

Viral protein synthesis is completely dependent on the host cell translational machinery since viruses do not encode or carry their own. Viruses have evolved not only strategies to reduce the impact of translational dependence on virus replication (Gale *et al.*, 2000), but also a remarkable variety of strategies to modulate the host cell translation apparatus, in order to optimize viral mRNA translation and replication (Thompson & Sarnow, 2000). Initiation of translation of most eukaryotic mRNAs is dependent on a 5′-terminal ‘cap’ structure. However, in a few families of viruses, initiation of viral protein translation is mediated through the internal binding of ribosomes onto the substrate mRNA in an internal ribosome entry site (IRES)-dependent manner (Gale *et al.*, 2000). IRES-mediated translation avoids the potential limitations posed by cap dependency and partially alleviates the competition for certain host factors from cap-dependent cellular mRNA translation, favouring the translation of viral mRNA and providing important advantages for virus replication. Different viral IRESs display a diversity in sequence and structure that can lead to specific requirement for a variety of IRES *trans*-acting factors, and these specific requirements could lead to different IRES regulatory mechanisms (Hellen & Sarnow, 2001). Specifically, the prototypic HCV IRES diverges in both length and structure from the three picornavirus groups represented by EMCV, poliovirus and hepatitis A virus (Gale *et al.*, 2000). This may partially contribute to the result that HCV replicon specifically stimulates HCV IRES activity, but not those of the two picornaviruses. It is also possible that certain HCV NS proteins specifically interact with HCV IRES, but not other viral IRES, either alone or in collaboration with host cell factors, to regulate HCV IRES-dependent translation.

In persistent viral infections, such as those by HCV or the DNA tumour viruses, constitutive modulation of host translational control pathways and release of translational suppression may make important contributions to viral pathogenesis/oncogenesis. However, little is known of the nature of viral translational programming as it pertains to persistent infection, although it clearly requires that the host mRNA translation remain sufficient to sustain the host cell and support virus persistence. Analyses of the mechanisms by which viruses may mediate persistence and latency suggest that host cell integrity and translational competence are maintained through (i) viral modulation of specific cellular mRNA translation and (ii) viral modification of host signalling and translational regulatory pathways (Gale *et al.*, 2000). In the case of HCV, our understanding of viral translational control mechanisms is further limited by the lack of efficient virus infection systems, and the current working models are based on results from *in vitro* and surrogate systems. Both NS5A and E2 proteins of HCV have been shown to interact with and inhibit PKR (Gale *et al.*, 1997, 1998; Taylor *et al.*, 1999). Inhibition of PKR-dependent eIF2α phosphorylation can be seen as a mechanism to ensure overall translational competence during virus infection. Our previous study also showed that NS5A protein inhibits eIF4E (the mRNA cap-binding protein) phosphorylation, through both Grb2- and PKR-dependent pathways (He *et al.*, 2001). The downregulation of eIF4E phosphorylation and activation may negatively affect the translation of at least a subset of cellular mRNAs, especially those that are more sensitive to the phosphorylation status of eIF4E, such as some genes regulating cell growth and stress response (Gingras *et al.*, 1999; Sonenberg & Gingras, 1998). So in addition to favouring viral protein synthesis, the modulation of eIF4E by NS5A may contribute to regulation of host cell growth and stress response, suggesting a new mechanism of viral pathogenesis.

The most important implication of this study is that HCV encodes nonstructural proteins, possibly NS4B and NS5A, that specifically enhance HCV IRES-directed translation. This is the first indication that an HCV nonstructural protein upregulates the activity of its own IRES, probably in order to facilitate viral protein synthesis and virus replication. Although the underlying molecular mechanism remains basically unclear, it is possible that HCV nonstructural proteins modulate viral IRES activity through interaction with host cell proteins involved in the translation machinery or translational control pathways. It is also possible that different HCV NS proteins may collaborate in this process. It would be interesting to test the effect of different combinations of HCV NS proteins on HCV IRES activity in future studies. (This would be technically challenging if we consider the number of NS proteins involved and the different possible combinations.) Interestingly, it is known that picornavirus leader and 2A proteinases enhance picornavirus IRES activity either indirectly, by cleaving eIF4G, as well as possibly directly, by an unknown mechanism that does not involve eIF4G cleavage (Hambidge & Sarnow, 1992; Macadam *et al.*, 1994; Ventoso & Carrasco, 1995). It seems that these different groups of viruses employ diversified mechanisms to achieve a common goal: to boost viral translation and replication. It is noteworthy that NS5A-1b1 and -1b5, two isolates related to different responses to IFN treatment in patients (Gale *et al.*, 1997), show different abilities to enhance HCV IRES activity. Thus the biological differences between HCV genotypes/isolates, such as IFN-resistance, may be due, in part, to the variations in IRES-dependent translation efficiency, which in turn influence virus replication. Eventually, the biological relevance of our results awaits careful examination in a biologically relevant virus infection system, which is still not available due to technical obstacles.

The IRES is the most conserved part of the HCV genome, and may play multifunctional roles in translation, replication or packaging of the viral genome (Hellen & Sarnow, 2001). Interestingly, the translation initiation process on the HCV IRES has simpler factor requirements than many other translation initiation mechanisms (only the cricket paralysis virus IRES has even simpler factor requirements that the HCV IRES) (Hellen & Pestova, 1999; Wilson *et al.*, 2000), indicating that HCV employs a very unique translation control mechanism, even among the various IRES-utilizing viruses. IRES-mediated translation is not a common feature among cellular mRNAs, and thus may represent a valid and new target for therapeutic intervention in viral mRNA translation (Jubin, 2001), and remains a promising new area for the development of effective antiviral compounds, which mostly has been limited to inhibitors of viral enzymes.

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