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Analysis of hepatitis C virus/classical swine fever virus chimeric 5′NTRs: sequences within the hepatitis C virus IRES are required for viral RNA replication

Chantal B. E. M. Reusken, Tim J. Dalebout, Peter Eerligh, Peter J. Bredenbeek and Willy J. M. Spaan

Molecular Virology Laboratory, Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, LUMC E4-P, PO Box 9600, 2300 RC Leiden, The Netherlands

Correspondence
Willy Spaan
W.J.M.Spaan@LUMC.NL

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Hepatitis C virus (HCV) is classified in the genus *Hepacivirus* of the family *Flaviviridae*, whose members have a single-stranded RNA genome of positive polarity, which encodes a single polyprotein. Within this family, HCV is closely related to viruses of the genus *Pestivirus*, which includes classical swine fever virus (CSFV). Translation of the hepaci- and pestiviral polyprotein is initiated by internal entry of ribosomes, promoted by the 5′NTR. The secondary and tertiary RNA structures of the HCV and pestivirus 5′NTRs are well conserved, despite the fact that their sequences differ significantly from one another. By analogy with other positive-stranded RNA viruses, the 5′NTR of HCV is likely to contain *cis*-acting determinants for replication as well as the determinants for translation. Studies on both signals could be complicated, as these signals might overlap. In this study, this problem was addressed by constructing chimeric HCV/CSFV 5′NTRs. A two-step analysis of these 5′NTRs was performed: (a) in a translation assay, which provided the possibility to study translation independently of the possible effects on replication; and (b) in a replication assay, in which were studied only the chimeric 5′NTRs for which IRES-dependent translation was demonstrated. An overlap was observed between HCV RNA elements involved in these processes. Exchange of domain II had a minor effect on the translation efficiency of the chimeric 5′NTRs, while replication of subgenomic replicons with these chimeric 5′NTRs was abolished. Exchange of domain III subdomains severely decreased translation activity, while replication was maintained.

INTRODUCTION

Hepatitis C virus (HCV) is classified in the genus *Hepacivirus* of the family *Flaviviridae*, which also includes the genera *Flavivirus* and *Pestivirus* and the unclassified GB viruses. Viruses of this family are enveloped and have a single-stranded RNA genome of positive polarity, which encodes a polyprotein that is cleaved by host peptidases and viral proteinases into mature viral proteins. The structural proteins, including the core and glycoproteins, are encoded in the amino-terminal one-third of the polyprotein, while the nonstructural proteins, including helicase, proteinase and polymerase activities, are encoded in the carboxy-terminal two-thirds of the polyprotein. Within the *Flaviviridae*, HCV is most closely related to the pestiviruses, which include classical swine fever virus (CSFV), and GB virus B (GBV-B) (reviewed by Wengler *et al.*, 1995; Rosenberg, 2001).

Two different mechanisms for translation initiation are found within the *Flaviviridae*. While the polyprotein of flaviviruses is translated in a cap-dependent fashion, translation of the HCV, pestivirus and GBV-B polyprotein is initiated by internal entry of ribosomes, which is promoted by the 5′NTR (Poole *et al.*, 1995; Rijnbrand *et al.*, 1995, 2000; Tsukiyama-Kohara *et al.*, 1992; Wang *et al.*, 1993). These 5′NTRs contain IRES elements that have similar structures. Four major structural domains can be distinguished in the HCV 5′NTR (Fig.1a) in which the 5′ border of the IRES element was mapped between nt 38 and 46 (Honda *et al.*, 1999; Reynolds *et al.*, 1995; Rijnbrand *et al.*, 1995) and the 3′ border at the AUG start codon was mapped at nt 342 (Rijnbrand *et al.*, 2001). Domain III comprises the core of the IRES and participates in the formation of a pseudoknot structure essential for IRES activity (Wang *et al.*, 1995). Although there is some controversy about the importance of domain II in IRES-dependent translation, it is generally considered to be essential for its activity (Honda *et al.*, 1996b; Reynolds *et al.*, 1996; Rijnbrand *et al.*, 1995).

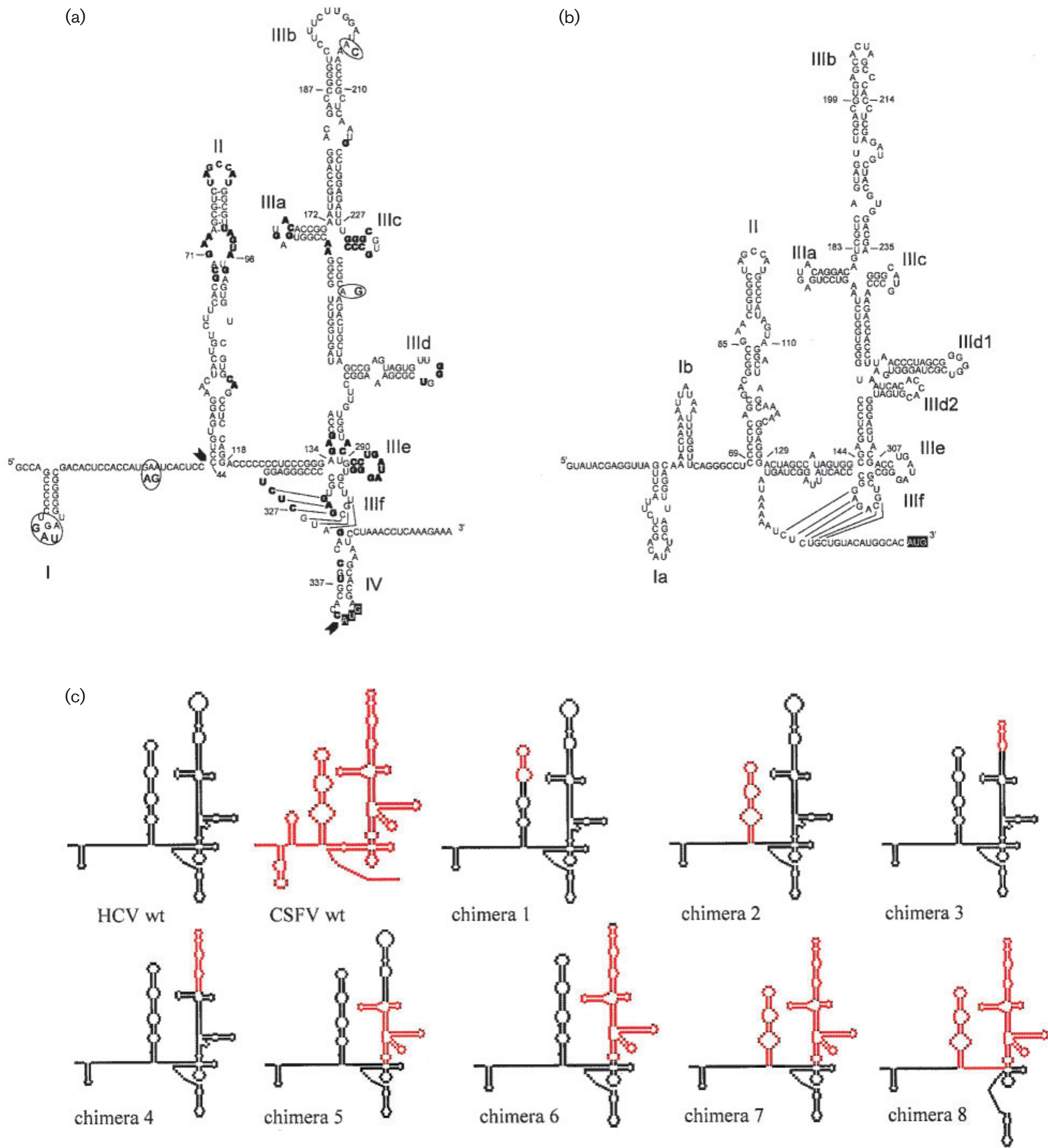

Fig. 1. (a) Primary and predicted secondary structure of the HCV genotype 1A 5′NTR (EMBL accession number AF009606). Structural domains are labelled I, II, III and IV. Hairpin loops are indicated by letters. Short nucleotide sequences that are conserved between HCV and pestiviruses are indicated in bold. Arrowheads refer to the 5′ and 3′ boundaries of the IRES element. Numbers refer to positions fused to CSFV sequences in the chimeric 5′NTRs (see Table 1). Circled nucleotides indicate differences between the genotype 1a sequence and the genotype 1b sequence (EMBL accession number AJ238799). The AUG start codon of the HCV polyprotein is indicated with a black box. (b) Primary and predicted secondary structure of the CSFV 5′NTR (c-strain, EMBL accession number Z46258). Structural domains are labelled I, II and III. Hairpins are indicated by letters. Numbers refer to positions fused to HCV sequences in the chimeric 5′NTRs. The AUG start codon is indicated with a black box. (c) Schematic presentation of the predicted secondary structure of the HCV, CSFV and chimeric HCV/CSFV 5′NTRs. HCV-derived sequences are shown in black, whereas CSFV-derived sequences are shown in red. Names of different chimeras are indicated below each representation.

Stem–loop IV is not essential for IRES activity but the stability of this structure inversely correlates with efficiency of translation (Honda *et al.*, 1996a). The secondary and tertiary RNA structures of the HCV and pestivirus 5′NTRs are well conserved (Fig. 1a, b), the main differences between their structures being the absence of stem–loop IV, an additional stem–loop IIId and an additional stem–loop I in pestiviruses (Fletcher & Jackson, 2002; Honda *et al.*, 1999; Rijnbrand & Lemon, 2000). Although the structures are similar, their sequences differ significantly from one another. A few short stretches of high sequence identity can be found, mainly in unpaired regions (Fig. 1a). HCV and pestivirus IRES elements can be distinguished from other viral IRES elements by their mechanism of ribosome entry (reviewed by Hellen & Pestova, 1999; Hellen & Sarnow, 2001). The 40S ribosomal subunit is able to bind specifically to the IRES in the absence of any additional translation initiation factors, in such way that the initiation codon is placed directly in the ribosomal P site. Toeprinting of 40S subunits to HCV and CSFV IRES elements showed that ribosomal binding consists of at least two distinct steps: first, the initial attachment, which involves stem 1 of the pseudoknot structure and the loop of subdomain IIId (IIId1 for CSFV); and second, the placement of the AUG start codon in the ribosomal P site. Determinants for this step include stem 2 of the pseudoknot and domains II and IIIa. Besides the small ribosomal subunit, additional translation factors, like eIF3, which interacts specifically with domain IIIb, and noncanonical factors, like PTB, hnRNPL and La, are recruited. These latter proteins are not essential for IRES activity (reviewed by Hellen & Pestova, 1999; Hellen & Sarnow, 2001).

By analogy with other positive-stranded RNA viruses with IRES-driven genome expression, the 5′NTR of HCV is expected to contain both translation and replication elements. Studies on these *cis*-acting signals are complicated, as these signals potentially overlap, as has been suggested in two recent studies using replicons with HCV/poliovirus chimeric 5′NTRs (Friebe *et al.*, 2001; Kim *et al.*, 2002). In this study, we used a different approach to study 5′NTR determinants for HCV replication. Although the studies cited analysed truncated HCV NTRs, we constructed HCV/CSFV chimeric 5′NTRs by swopping HCV domains for their exact CSFV counterparts. Using this strategy, we were able to study the various HCV domains as an integrated part of a functional IRES consisting of RNA elements of two closely related viruses. Our studies showed an overlap between HCV RNA elements involved in translation and replication. Domain II was found to be crucial for replication, while domain III was found to modulate replication. Both domains are part of the HCV IRES element.

METHODS

Cells. Huh-7 hepatocellular carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % heat-inactivated FCS, 2 mM L-glutamine and 1 % nonessential amino acids. For cell lines carrying HCV replicons, 500 μg active compound G418 ml^{-1} (geneticin, Life Technologies) was added to the medium.

Construction of dual-luciferase reporter plasmids. All dual-luciferase reporter constructs were generated by a (fusion) PCR-based strategy from either pDualLuc-HCVwt or pDualLuc-HCVwt/ΔC, which contain consecutively the T7 promoter, the firefly luciferase ORF, HCV genotype 1a (nt 1–342 or 1–389), the *Renilla* luciferase ORF, the HCV genotype 1a 3′NTR, the hepatitis δ ribozyme and the T7 terminator in a pBluescript backbone (Fig. 2a). Table 1 gives an overview of the exact composition of the different dual-luciferase reporter constructs with respect to the 5′NTR preceding the *Renilla* luciferase ORF. All fragments generated by PCR were sequenced completely upon cloning.

Construction of HCV replicon cDNAs with HCV genotype 1a or chimeric HCV genotype 1a/CSFV 5′NTRs. All replicon cDNAs were generated from pFK-I_{389}neo/NS3-5′/5.1 (Krieger *et al.*, 2001). Using a PCR-based approach, an unique *Xba*I site was introduced directly upstream (mutation of nt 11 028–11 036 into TCTAGAAC) of the T7 promoter to yield pFKNeo5.1/*Xba*I. The replicon with the genotype 1a 5′NTR was generated by replacement of the *Xba*I–*Nru*I (nt 11 028–269) fragment of pFKNeo5.1/*Xba*I by a *Xba*I–*Nru*I PCR fragment corresponding to the equivalent part in the HCV genotype 1a 5′NTR, as present in pDualLuc-HCVwt (see above). Replicons with the HCV/CSFV chimeric 5′NTRs were all constructed by replacement of the *Xba*I–*Asc*I (nt 11 028–391) fragment of pFKNeo5.1/*Xba*I by a *Xba*I–*Asc*I fragment obtained by fusion PCR using the corresponding chimeric HCV/CSFV dual-luciferase reporter plasmids (see above and Table 1) as template. A PCR fragment corresponding to nt 291–699 of pFKNeo5.1 was used as downstream primer. All fragments generated by PCR were sequenced completely upon cloning.

***In vivo* expression studies.** The vaccinia virus recombinant vTF7-3 (Fuerst *et al.*, 1986), which expresses the T7 RNA polymerase, was used to infect Huh-7 cells. Cells were grown in 12-well plates until ~75 % confluency was reached. Cells were then infected at an m.o.i. of 10 in DMEM containing 1 % FCS. After incubating at 37 °C for 1 h, the cells were washed twice with PBS. For each set of two (duplo) wells, 100 μl LipofectACE reagent–OptiMEM (1 : 19) mixture was added to 100 ng of plasmid DNA in 100 μl OptiMEM and incubated for 15 min at room temperature. OptiMEM (800 μl) was then added to the mixture and 450 μl was added to each of the two wells. At 7 h post-transfection, cells were rinsed with PBS and lysed using 250 μl passive lysis buffer (Dual-Luciferase Reporter assay, Promega). Cells were stored at −80 °C and thawed just before reporter activities were measured with the dual-luciferase reporter assay using a luminometer. In all experiments, $n \geqslant 4$.

***In vitro* transcription.** Transcripts of HCV replicon cDNA were generated and treated essentially as described by Lohmann *et al.* (1999).

Electroporation and selection of G418-resistant cell lines. Electroporation of Huh-7 cells and colony counting was done as described by Lohmann *et al.* (1999). To correct for differences in transfection efficiency, 500 ng pGL3 control-plasmid (Promega), which carries the firefly luciferase gene under the control of the SV40 promoter/enhancer sequences, was included in every electroporation. For each replicon tested, $n \geqslant 3$.

RNA labelling in cell culture. Of the various replicon cell lines, $0{\cdot}5 \times 10^6$ cells were incubated for 14 h in the presence of 50 μCi [^{3}H]uridine and 2 μg actinomycin D in 750 μl medium. Total RNA was isolated using Trizol reagent (Invitrogen), according to the

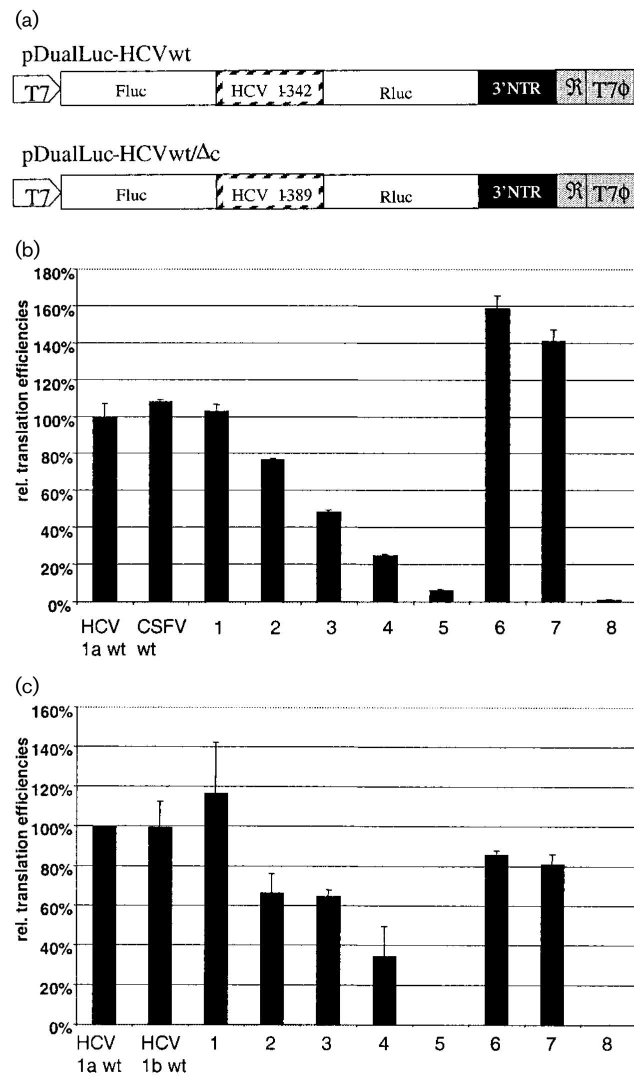

Fig. 2. Analysis of HCV/CSFV chimeric IRES element-mediated translation in human liver cells. (a) Schematic presentation of the basic bicistronic reporter plasmids used in these experiments. The dual-luciferase plasmids contain the T7 promoter (arrowhead), the *Photinus pyralis* luciferase ORF (Fluc, open box), the HCV, CSFV or chimeric HCV/CSFV 5′NTR with IRES element under study (hatched box), either without (pDualLuc-HCVwt) or with (pDualLuc-HCVwt/Δc) the first 48 nt of the HCV ORF, the *Renilla reniformis* luciferase ORF (Rluc, open box), the HCV genotype 1a 3′NTR (black box), the hepatitis δ ribozyme and the T7 terminator sequence (grey box). (b) Relative translation initiation efficiencies (ratio Rluc : Fluc) of HCV, CSFV and chimeric HCV/CSFV IRES elements following DNA transfection into vTF7-3-infected Huh-7 cells when studied in the context of pDualLuc-HCVwt. Chimeric IRES elements are indicated by numbers corresponding to the numbers used in Fig. 1(c). All activities were normalized to the activity of the HCV genotype 1a wild-type 5′NTR, which was set at 100 %. In all experiments, $n \geqslant 4$. (c) Relative translation initiation efficiencies (ratio Rluc : Fluc) of wild-type HCV, wild-type CSFV and chimeric HCV/CSFV IRES elements following DNA transfection into vTF7-3-infected Huh-7 cells when studied in the context of pDualLuc-HCVwt/Δc. All activities were normalized to the activity of the HCV genotype 1a wild-type 5′NTR, which was set at 100 %. In all experiments, $n \geqslant 4$.

manufacturer's instructions, and analysed by denaturing agarose gel electrophoresis. Gels were prepared for autoradiography as described by Bredenbeek *et al.* (1993).

RESULTS

Experimental strategy

To study *cis*-acting replication signals in the HCV 5′NTR, we needed to dissect these signals from the translation signals as present in the IRES element. Therefore, we constructed a set of eight chimeric 5′NTRs by swopping HCV (sub)domains for their exact CSFV counterparts (Fig. 1c and Table 1). By exchanging HCV domains for the corresponding CSFV domains, we hoped to maintain the requirements for IRES activity, thereby creating the possibility to analyse the replicative role of the various HCV domains in the context of a functional IRES.

For domain II, two chimeras were constructed: chimera 1, in which the apical part was replaced; and chimera 2, in which the complete domain II was replaced. For domain III, a set of four chimeric 5′NTRs was generated: chimeras 3 and 4, in which, respectively, the apical part and the complete subdomain IIIb were swopped; chimera 5, in which subdomains IIIa, IIIc, IIId and the basal stem of domain III were swopped; and chimera 6, in which the complete domain III, with the exception of subdomains IIIe and IIIf, was swopped. Furthermore, two chimeras were constructed with a combined substitution of domains II and III: chimera 7, in which domains II and III were exchanged; and chimera 8, in which, in addition to domains II and III, the intermediate region was exchanged, thereby disrupting the pseudoknot structure.

We performed a two-step analysis of these 5′NTRs: (a) in a translation assay, which provided the possibility to study IRES efficiencies independent of possible effects on replication; and (b) in a replication assay, in which we studied only the chimeric 5′NTRs for which IRES-dependent translation was demonstrated.

Activity of the HCV/CSFV chimeric IRES elements

The translation activity of the HCV/CSFV chimeric 5′NTRs was analysed in a bicistronic context (pDualLuc-HCVwt, Fig. 2a) in which the firefly luciferase is translated by a 5′ end-dependent mechanism and the *Renilla* luciferase is expressed under the control of the IRES element under study. Luciferase expression of the various constructs was analysed in DNA transfected and vTF7-3-infected Huh-7 cells. The relative translation efficiency of the IRES elements was calculated as the ratio of the luciferase activities (Fig. 2b).

We observed that exchange of the apical part of domain II for its CSFV counterpart (chimera 1) had no effect on translation, while replacing the complete domain (chimera

Table 1. Composition CSFV and chimeric HCV/CSFV 5′NTRs

5′NTR	Deleted HCV sequence (position)*	Replacement CSFV sequence (position)†	5′ fusion‡	3′ fusion‡
CSFV wild-type	1–341	1–373	–	–
Chimera 1	71–96	85–110	ACGCAgaact	tagtaTGAGT
Chimera 2	44–118	69–129	ACTCCccctc	gagggACCCC
Chimera 3	187–210	199–214	ACGACgtgag	cccacCTCAA
Chimera 4	172–227	183–235	ACCGGtcgtc	gacgaTGGGC
Chimera 5	134–171/228–290	144–182/236–307	CCGGGcgagc/gacagAATTG	AGATTggcat/gtacgGCCTG
Chimera 6	134–290	144–307	CCGGGcgagc	gtacgGCCTG
Chimera 7	44–118/134–290	69–129/144–307	ACTCCccctc/CCGGGcgagc	gagggACCCC/gtacgGCCTG
Chimera 8	44–290	69–307	ACTCCccctc	gtacgGCCTG

*Based on HCV genotype 1a; EMBL accession number AF009606.
†Based on CSFV c-strain; EMBL accession number Z46258.
‡HCV sequences are indicated in upper case; CSFV sequences are indicated in lower case.

2) resulted in a 25 % reduction in IRES activity. The exchange of almost the entire domain III (chimera 6) resulted in an increase in translation activity (75 %), whereas the exchange of several stem–loop III subdomains in chimeras 3, 4 and 5 showed an inhibitory effect of 50, 75 and 95 %, respectively. When domains II and III were replaced simultaneously (chimera 7), a slight increase of activity was observed, while exchange of both stem–loops in combination with the intermediate sequence (chimera 8) rendered the IRES element inactive.

The chimeric 5′NTRs that were translationally active, i.e. chimeras 1–4, 6 and 7, were selected for further analysis in a replication assay based on the HCV subgenomic replicon system. However, since we observed that an HCV replicon lacking HCV nt 342–389, encoding the amino-terminal 16 aa of core protein, was not functional (see below), we had to include this sequence in our chimeric 5′NTR expression plasmids. To analyse whether these extended plasmids had similar translational characteristics as those of the initial plasmids, we analysed them in the translation assay (Fig. 2c). The translation characteristics of the 5′NTRs including the first 48 nt of the HCV ORF were shown to be essentially similar to those obtained with the corresponding plasmids lacking these nucleotides. Subsequently, we analysed the selected chimeras in the replication assay.

Activity of HCV/CSFV chimeric 5′NTRs in HCV subgenome replication

The effect of the chimeric 5′NTRs on HCV RNA replication was analysed using the G418-selectable replicon cell culture system for which it was shown that the number of G418-resistant colonies directly reflects the efficiency with which a replicon multiplies in cells (Bartenschlager & Lohmann, 2001; Lohmann *et al.*, 1999). In this replicon, the HCV 5′NTR precedes the neomycin ORF, which is expressed as an amino-terminal fusion protein with 16 aa of the core protein. The encephalomyocarditis virus (EMCV) IRES mediates the translation of the HCV NS3–NS5 ORF, which is followed by the 3′NTR. The cell culture-adapted HCV genotype 1b-based pFK-I_{389}neo/NS3-5′/5.1 cDNA (Fig. 3a) was used for our studies. This replicon has a high sensitivity of detection, thereby enabling the detection of mutants with a low efficiency of replication (Krieger *et al.*, 2001). Since the HCV/CSFV chimeric cassettes, as tested in the translation assay, lack HCV nt 342–389 encoding the amino-terminal 16 aa of the core protein, we first analysed whether the pFK-I_{389}neo/NS3-5′/5.1 replicon was still functional without this sequence. We observed that the presence of this region is a prerequisite for replicon activity (data not shown). Therefore, nt 342–389 were included in our chimeric cassettes.

Since the HCV/CSFV chimeric 5′NTRs were all based on the HCV genotype 1a 5′NTR and the HCV replicon is of genotype 1b origin, it was subsequently determined whether the replicon was still active when the authentic genotype 1b 5′NTR was replaced by the genotype 1a 5′NTR. The latter 5′NTR differs from the former at seven positions (Fig. 1a). We observed that the genotype 1b replicon containing the genotype 1a 5′NTR conferred G418 resistance to Huh-7 cells with a similar efficiency as that of the original genotype 1b replicon (Fig. 3b).

Subsequently, we analysed the relative replication efficiency of replicons containing the chimeric 5′NTRs that were translationally active (Fig. 3b). The number of colonies found for each replicon is normalized to the number of colonies found for the genotype 1b replicon with the 5′NTR from genotype 1a (Fig. 3b, 1a 5′NTR). Mutants containing the apical part of domain II (chimera 1) showed a severe reduction in colony formation to 1 % of the level seen with genotype 1a 5′NTR, while mutants containing the complete CSFV domain II (chimeras 2 and 7) failed to yield G418-resistant colonies at 3–4 weeks after electroporation. The mutant in which the apical part of subdomain IIIb was

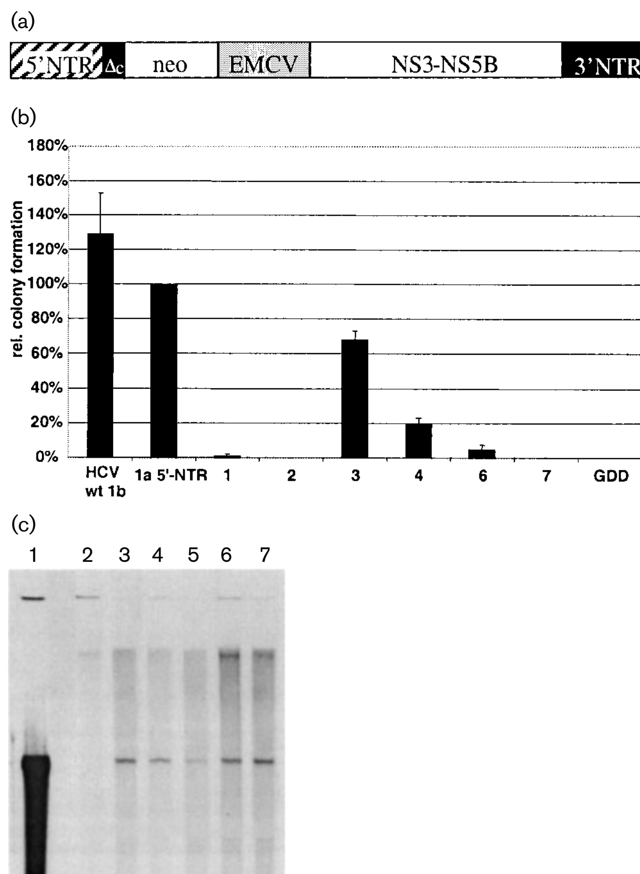

Fig. 3. Analysis of the ability of mutant HCV replicons to replicate in human liver cells. (a) Schematic presentation of the basic HCV replicon pFK-I_{389}neo/NS3-5′/5.1 (HCV wt), as used in these experiments. RNA contains the HCV or mutant 5′NTR (hatched box), the first 16 aa residues of HCV core (Δc, black box), the neomycin phosphotransferase ORF (neo, open box), the EMCV IRES (grey box), the HCV NS3–NS5B ORF (NS3–NS5B, open box) and the HCV 3′NTR (3′NTR, black box). (b) Colony formation by mutant HCV replicons. Huh-7 cells were electroporated with selectable HCV subgenomic RNAs in which the neomycin selection marker was expressed under the control of the different 5′NTRs. As a negative control, cells were electroporated with R5.1-NS5BGDD-SR, a pFK-I_{389}neo/NS3-5′/5.1 mutant with an inactivated replicase (GDD). At 3–4 weeks post-transfection and G418 selection, colonies were fixed, stained and counted. The number of colonies found for each replicon is normalized to the number of colonies counted for the genotype 1b replicon with the genotype 1a 5′NTR (1a 5′NTR), which was set at 100 %. In all experiments, $n \geqslant 3$. (c) G418-resistant colonies contain replicating HCV RNA. Cell lines generated from isolated colonies from chimeras 1, 3 and 4, and from the wild-type and genotype 1a 5′NTR HCV replicons were labelled with [^{3}H]uridine in the presence of actinomycin D for 14 h. Total RNA was isolated and analysed under denaturing conditions. As a size marker, the [^{3}H]uridine-labelled HCV wild-type replicon transcript was loaded. Lanes: 1, HCV pFK-I_{389}neo/NS3-5′/5.1 transcript; 2, mock; 3, genotype 1a 5′NTR; 4, HCV wild-type; 5, chimera 1; 6, chimera 4; 7, chimera 3.

replaced by the corresponding part of CSFV (chimera 3) gave a reduction in colony formation to 70 % of the level seen with genotype 1a 5′NTR, whereas mutants containing either CSFV subdomain IIIb (chimera 4) or CSFV domain III (chimera 6) showed a stronger reduction to 20 and 5 %, respectively. In all cases, these colonies were, on average, smaller in size (data not shown).

Characterization of chimeric replicon cell lines

To verify that the selected G418-resistant colonies contained replicating viral RNA and not integrated DNA, we performed a [^{3}H]uridine labelling in the presence of actinomycin D on cell lines generated from isolated clones of chimeras 1, 3 and 4 and analysed the resulting radiolabelled RNAs (Fig. 3c). Synthesis of cellular RNAs was properly blocked, while replication of HCV RNA was shown to be resistant to actinomycin D. The radiolabelled replicon RNAs all had the correct size. It has been shown that the efficiency of colony formation is determined primarily by the initial level of RNA replication upon transfection and that similar levels of viral RNA are obtained in selected cell lines regardless of this initial replication efficiency (Krieger *et al.*, 2001). Therefore, the RNA synthesis observed for these established colonies cannot be linked to the differences in colony formation (Fig. 3 b).

To verify that the replicating RNA present in the selected colonies contains the chimeric 5′NTR sequence, total RNA was isolated from the cell lines of chimeras 1, 3 and 4 and the corresponding cDNA was sequenced. The replicating RNAs did contain the chimeric 5′NTR sequence (data not shown).

DISCUSSION

The 3′-termini of RNA templates of RNA viruses usually contain promoter elements for the initiation of complementary strand synthesis. Consequently, an overlap between *cis*-acting sequences involved in translation and replication could exist in the 5′NTR of the genomic strand, especially for viruses with an IRES-mediated expression of their genome, which usually covers the majority of the 5′NTR. By studying the HCV/CSFV chimeric 5′NTRs in translation and replication assays, we observed an overlap between HCV RNA elements involved in these processes. Domain II, which is part of the HCV IRES element, appears to be absolutely required for HCV RNA replication. The domain II chimeras with an IRES activity ranging from 70 to 110 % of the wild-type IRES failed (chimeras 2 and 7) or were severely impaired (chimera 1) in G418-resistant colony formation. While domain III forms the essential core of the HCV IRES element, it appears to have a modulating role in replication. Domain III chimeras with IRES activity ranging from 30 to 90 % (chimeras 3, 4 and 6) of the wild-type IRES were replication-competent, albeit with a reduced efficiency in colony formation and growth.

The translation activity of the chimeric 5′NTRs in which stem–loops II (chimera 2), III (chimera 6) or II and III of

HCV (chimera 7) were replaced by corresponding domains of the CSFV 5′NTR demonstrates clearly that IRES activity is regulated by higher order structures rather than primary sequence. Furthermore, the observations with the different stem–loop III chimeras in which only subdomains of stem–loop III were replaced by CSFV sequences (chimeras 3, 4 and 5) suggest that interactions within stem–loop III are important for IRES activity. These substitutions could have resulted in subtle structural changes, thereby destabilizing/stabilizing the RNA tertiary structure or influencing the interaction with essential translation factors like the 40S small ribosome subunit and eIF3 (Hellen & Sarnow, 2001; Rijnbrand & Lemon, 2000). Chimeras 7 and 8 confirmed the requirement of the pseudoknot structure for a functional IRES (Wang *et al.*, 1995).

The reduced translation observed with chimeras 2–4, 6 and 7 is not the only possible explanation for the observed reduction in replication efficiency. The expression of the viral replicase complex is independent of these chimeric 5′NTRs, as it is driven by the EMCV IRES. Furthermore, while the IRES activities of chimeras 2 and 3 are similar, chimera 2 is completely replication incompetent and chimera 3 forms colonies up to 60 % of the wild-type level. Moreover, although the IRES activity of chimera 4 is only one-third of that of chimeras 6 and 7, it forms more colonies than these two chimeras.

There are several possible explanations for the observed effects on RNA replication. First, the primary sequence of domains II and/or III, which is altered as a consequence of the domain exchanges, could be an important determinant for replication. The sequences of HCV and CSFV 5′NTRs differ significantly from each other. Only a few short stretches of high sequence identity can be found (reviewed by Rijnbrand & Lemon, 2000). At least some flexibility at the nucleotide level is allowed, since the genotype 1b replicon in which the 5′NTR was replaced by the genotype 1a 5′NTR is almost as viable as the original replicon. This is consistent with an earlier observation that transcripts of a chimeric cDNA that encodes a genotype 1b polyprotein flanked by 3′ and 5′NTRs of genotype 1a was infectious in chimpanzees (Yanagi *et al.*, 1998). Interestingly, there are no differences between genotype 1a and 1b sequences within the crucial domain II. Second, the structure of the positive-strand promoter could be disrupted by the domain swops, thereby influencing its activity. Whereas the chimeras were designed to maintain IRES structure, the structure of the positive-strand promoter was not taken into account. Recently, two studies showed by chemical and enzymatic probing in combination with computer predictions that the negative-strand 3′NTR does not fold into a mirror image of the positive-strand 5′NTR (Schuster *et al.*, 2002; Smith *et al.*, 2002). Both studies predict an identical structure for the 3′-terminal 220 nt of the negative strand (Fig. 4), which is folded into five consecutive stem–loops (A–E) and contains the core of the positive-strand promoter. The predicted structural differences between the genomic 5′NTR and its

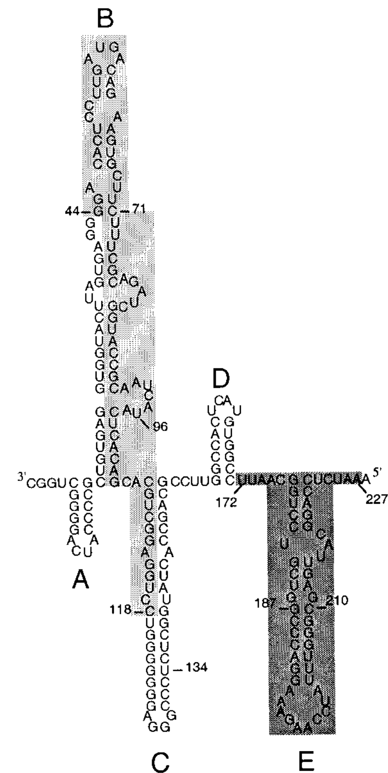

Fig. 4. Predicted secondary structure of the HCV genotype 1a negative-strand 3′-terminal 228 nt, as proposed by Smith *et al.* (2002) and Schuster *et al.* (2002). Numbers refer to positions fused to CSFV sequences in the chimeric genomic 5′NTRs (see Table 1). Structural domains are labelled A–E. Nucleotides shaded in light grey are complementary to domain II and nucleotides shaded in dark grey are complementary to domain IIIb in the genomic 5′NTR.

complement could explain why swops, as in chimeras 1 and 2, which are minor with respect to IRES structure but disrupt a major stem–loop in the core promoter structure, have minimal effect on translation but abolish replication. Furthermore, the observation with the domain III exchanges, the efficiency of colony formation decreases with an increase in the presence of CSFV-derived sequences in the HCV 5′NTR, could reflect their various effects on negative-strand structure. This is expected to be minor for chimera 3 and substantial for chimera 6.

The observation that 16 codons of core-encoding sequence need to be retained in the replicon for replicon activity suggested that a direct fusion of the neomycin-resistance marker to the HCV 5′NTR results in a decrease in IRES activity to the point where insufficient gene expression occurs to allow formation of HCV replicon-containing cell lines. However, our translation assay of HCV IRES-mediated translation in the absence of the first 16 core codons showed similar activity (96 %, $n=3$) to the comparable IRES construct containing this sequence (data not shown). These data indicate that the core-derived

sequence is not important for translation and suggest that the complement of this sequence forms, or is part of, a RNA signal that is important for HCV replication. This issue is currently under investigation.

Nevertheless, we realize that it cannot be excluded that the replication determinants reported here are not part of the actual replication signal but rather influence a proper folding of this signal. In addition to this, it has to be kept in mind that in the HCV replicon, the expression of the neomycin selection marker is regulated by the chimeric 5′NTRs.

The involvement of domains II and III in the replication of the HCV replicons was also documented recently in two other studies (Friebe *et al.*, 2001; Kim *et al.*, 2002). In both studies, *cis*-acting signals for replication were separated from signals for translation by fusing various parts of the HCV 5′NTR to the poliovirus IRES. It was shown that domains I and II are sufficient for a low level of replication, which is strongly enhanced by the presence of domains III and IV. While in these studies truncated NTRs fused to the poliovirus IRES were analysed, we studied the various domains as part of a functional IRES structure.

Although the 5′NTRs of HCV and pestiviruses are conserved in their higher order structure and use an identical mechanism of IRES-mediated translation, the determinants for replication appear to be more complex for HCV than for the pestiviruses, for which it was shown in studies with bovine viral diarrhoea virus (BVDV), BVDV/HCV and BVDV/EMCV 5′NTR chimeric viruses that only the 5′-terminal 4 nt are required to direct specific replication and only domains Ia and Ib are important for efficient replication (Frolov *et al.*, 1998). When studied in the context of a subgenomic BVDV replicon, mutations in domain Ia were also found to effect replication (Yu *et al.*, 2000). An overlap between translation and replication signals was also shown for other positive-stranded RNA viruses with IRES-mediated translation, such as poliovirus (Borman *et al.*, 1994; Ishii *et al.*, 1999).

In summary, we observed that replication and translation signals overlap in the HCV 5′NTR and that domain II is crucial for replication. Future studies will focus on the effect of the HCV/CSFV sequence exchanges on RNA–RNA and RNA–protein interactions involved in HCV translation and/or replication. Furthermore, it will be interesting to define the *cis*-acting replication signals in more detail and to analyse the sequence of chimeras that were impaired, but viable, to look for compensating mutations.

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