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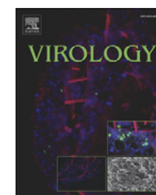

Non-genotype-specific role of the hepatitis C virus 5′ untranslated region in virus production and in inhibition by interferon

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The 5′ untranslated region (5′UTR) of hepatitis C virus (HCV) is structured into four domains (I–IV) with numerous genotype-specific nucleotides. It is unknown whether the polymorphisms confer genotype-specific functions to the 5′UTR. Using viable JFH1-based Core-NS2 recombinants, we developed and characterized HCV genotypes 1–7 recombinants with highly diverse 5′UTRs (genotypes 1a and 3a), 2a recombinants (J6/JFH1) with 5′UTR of genotypes 1–6 or with heterotypic chimeric (1a/3a and 3a/1a) 5′UTR domains I, II or III, and 1a recombinants with 5′UTR domain I of genotypes 1–6. All were fully functional in Huh7.5 cells; therefore, the 5′UTR apparently functions in a non-genotype-specific manner in HCV production *in vitro*. However, adenine at the 5′-terminus was required. We demonstrated that J6/JFH1 with 5′UTR of genotypes 1–6 responded similarly to interferon-α2b. This study provides novel insight into the role of the 5′UTR in the HCV life cycle and facilitates HCV basic research and testing of 5′UTR-targeting antivirals.

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Introduction

The hepatitis C virus (HCV) is classified in the genus *Hepacivirus* within the *Flaviviridae* family. The viral genome is a ~9.6 kb positive-sense single-stranded RNA consisting of a single open reading frame (ORF) flanked by 5′ and 3′ untranslated regions (UTR) (Moradpour et al., 2007). The 5′UTR is one of the most conserved regions of the HCV genome, consisting of ~341 nucleotides. The 5′UTR is predicted to form four major domains (I–IV) (Fig. 1A), with structures largely conserved among HCV genotypes, GB virus B (GBV-B), and pestiviruses (Brown et al., 1992; Bukh et al., 1992; Honda et al., 1999a). Domain I contains one stem-loop structure (SLI) (Honda et al., 1999a) and two adjacent microRNA 122 (miR-122) binding sites and their upstream nucleotides that were recently identified (Figs. 1A and B) (Jopling et al., 2005, 2008; Machlin et al., 2011). Domains II and III contain multiple stems and apical loops; domain IV extends up to 10 nucleotides downstream of the start codon AUG in the core coding sequence (Honda et al., 1999a). Domains I and II are essential for HCV RNA replication, which is likely to be regulated by domain III (Friebe et al., 2001; Kim et al., 2002; Luo et al., 2003; Reusken et al., 2003). Domains II, III and IV comprise the internal ribosome entry site (IRES) that initiates cap-independent translation of HCV RNA, which might be modulated by SLI, Core coding sequences and the 3′UTR (Honda et al., 1996; Reynolds et al., 1995; Song et al., 2006), to produce a single polyprotein of ~3000 amino acids (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The polyprotein precursor is cleaved co- and post- translationally by cellular signal peptidases and viral proteases into at least 10 proteins, including structural proteins Core, E1, and E2 at the N-terminus, followed by a small protein p7 and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Moradpour et al., 2007).

HCV has been classified into seven major genotypes and numerous subtypes, differing in approximately 31–33% and 20–25% of their genome sequence, respectively (Murphy et al., 2007; Simmonds et al., 2005). HCV genotype-specific responses were found in clinical interferon-alpha (IFN-α)-based therapy (Zeuzem, 2008), and *in vitro* regarding newly developed inhibitors (Gottwein et al., 2011; Scheel et al., 2011b) and neutralizing antibodies (Gottwein et al., 2009; Jensen et al., 2008; Prentoe et al., 2011; Scheel et al., 2008). Most of the previously developed JFH1-based HCV genotypes 1–7 specific Core-NS2 recombinants required adaptive mutations for efficient growth in Huh7.5 cells (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008, 2011a), which indicates that genotype-specific interactions exist between Core-NS2 and other regions of the HCV genome or between HCV RNA and cellular proteins. Further, using JFH1-based HCV culture systems, our research group recently demonstrated that the E2 hypervariable region 1 (Prentoe et al., 2011) and NS5A (Scheel et al., 2011b) apparently functioned in a genotype-specific manner in production of infectious HCV particles. We have recently demonstrated that JFH1-based HCV recombinants with genotypes 1–6 specific 5′UTR-NS2 were fully functional with

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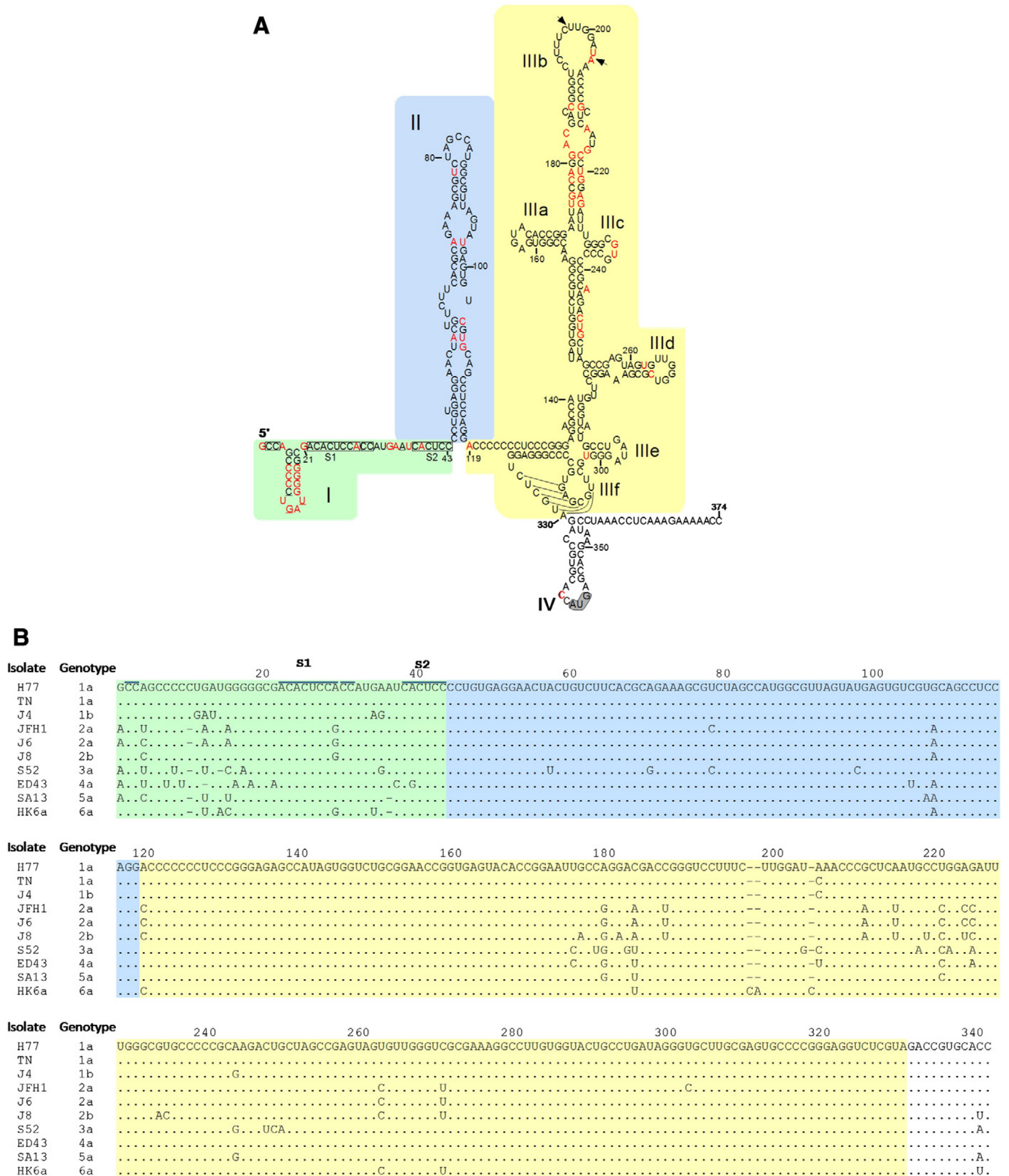


Fig. 1. Structure and sequences of the HCV 5′UTR. A) Secondary structure of the 5′UTR of HCV genotype 1a strain H77 as predicted by Honda et al. (Honda et al., 1999a). The nucleotides at positions with heterogeneity among HCV genotype isolates shown in panel B are in red; those with deletions are underlined. Arrows indicate the positions of insertion nucleotides of HCV genotype 6a. The microRNA miR-122 binding site 1 and 2 (S1 and S2) and their upstream nucleotides involved in this binding are boxed (Jopling et al., 2005, 2008; Machlin et al., 2011). The start codon AUG is highlighted. B) The 5′UTR sequences of HCV prototype strains of different genotypes. The entire 5′UTR sequence of genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a and 6a (Gottwein et al., 2010; Kato et al., 2001; Kolykhalov et al., 1997; Li et al., 2011; Sakai et al., 2007) are aligned and numbered according to the reference 1a strain H77 (GenBank accession number AF009606) (Kolykhalov et al., 1997). A dot indicates that the nucleotide is identical to H77; a dash indicates a gap. MicroRNA miR-122 binding sequences are indicated. In A) and B): Domains I, II and III are highlighted in colors.

no additional adaptive mutations (Li et al., 2011), which suggests that the 5′UTR has no genotype-specific interaction with NS3-3′UTR. However, whether the 5′UTR has genotype-specific functions or is involved in genotype-specific interactions with the Core-NS2 region could not be studied with these systems. We aimed at investigating this issue by developing novel JFH1-based recombinants with 5′UTR of different HCV genotypes.

Although the 5′UTR is highly conserved among HCV genotypes, the most distantly related isolates were found to differ in up to ~10% of their sequence; variable regions could be identified primarily in domains I, II and III (Bukh et al., 1992; Han et al., 1991; Li et al., 2011). A number of well-defined genotype-specific polymorphisms have been widely used for HCV genotyping (Ross et al., 2000; Simmonds et al., 2005). In addition, the 5′UTR was shown to be involved in long-range RNA–RNA interactions with coding sequences of Core (Honda et al., 1999b; Kim et al., 2003) and NS5B SL3.2 (also as SL9266) (Romero-Lopez and Berzal-Herranz, 2009) in *in vitro* translation and binding assays. Binding assays showed that interactions also existed between the 5′UTR domains (Lafuente et al., 2002). The 5′UTR was suggested to interact with the NS3 helicase, NS5A and NS5B in a genotype-specific manner, during positive-strand RNA synthesis using subgenomic 1b (strain Con1) and 2a (JFH1) replicon systems (Binder et al., 2007). However, it is unknown whether the heterogeneities in the 5′UTRs mediate genotype-specific functions important for production of infectious HCV particles.

HCV infects approximately 180 million people worldwide, and around 80% of infected patients develop chronic hepatitis with high risk of developing liver cirrhosis and hepatocellular carcinoma (Manns et al., 2007). No vaccine is available for hepatitis C. Vigorous efforts are ongoing to search for potential new therapeutics, including 5′UTR-targeting drugs (Lanford et al., 2010; Parsons et al., 2009; Paulsen et al., 2010). Silencing of miR-122 by antisense locked nucleic acids (LNA) SPC3649 was found to be a promising approach, as it was demonstrated to suppress HCV genotype 1 chronic infection in chimpanzees (Lanford et al., 2010) and to inhibit the infection of HCV genotypes 1–6 5′UTR-NS2 recombinants *in vitro* (Li et al., 2011). Nevertheless, IFN-α2 and ribavirin have long been the only approved therapy for HCV patients (Zeuzem, 2008), although recently the protease inhibitors Telaprevir and Boceprevir were licensed for use in combination with pegylated interferon and Ribavirin for the treatment of genotype 1 chronic hepatitis C patients. Combination therapy with IFN-α2 and ribavirin overall cures 40–80% of chronically infected individuals completing treatment (Alter and Seeff, 2000; Poynard et al., 2003). The outcome of this combination therapy is highly dependent on the HCV genotype; patients infected with HCV genotypes 2 and 3 show higher sustained viral response rates than those infected with genotypes 1 and 4 (Pawlotsky, 2003; Zeuzem, 2004). The mechanisms of HCV resistance to IFN-based treatment are poorly understood. Although various genome regions, such as E2 and NS5A, were thought to play a role in IFN resistance, the observations are still controversial [reviewed in (Gottwein and Bukh, 2008)]. Regarding the role of the 5′UTR in response to IFN treatment, differing results have also been reported (Koev et al., 2002; Lu et al., 1999; Soler et al., 2002; Takeda et al., 2004). Thus, studies of the genetic basis that confers resistance to IFN for various HCV genotypes are highly relevant.

Results

Apparent lack of genotype-specific interaction between the 5′UTR and other HCV genome regions in virus production in vitro

We and others previously developed JFH1-based HCV genotypes 1–7 Core-NS2 recombinants, in which the Core-NS2 region was from different genotypes 1–7 strains, and the 5′UTR and NS3-3′UTR were from genotype 2a strain JFH1 (Gottwein et al., 2007, 2009; Jensen et al., 2008; Lindenbach et al., 2005; Scheel et al., 2008, 2011a). The HCV 5′UTR was reported to be involved in long-range RNA–RNA interactions and RNA–protein interactions important for HCV RNA replication and translation [reviewed in (Shi and Lai, 2006)]. To study whether these potential functional interactions were genotype-specific and influenced virus production, here we developed (i) JFH1-based HCV genotypes 1–7 Core-NS2 recombinants with highly diverse 5′UTR of genotypes 1a and 3a, and (ii) HCV 2a Core-NS2 recombinants (J6/JFH1) (Lindenbach et al., 2005) with 5′UTR of genotypes 1–6 (see below). By determining virus spread, culture supernatant HCV infectivity titers and supernatant HCV RNA titers of these recombinants in transfected and infected Huh7.5 cells, we concluded that the 5′UTR did not appear to have genotype-specific functions and interactions with other HCV genome regions important for virus production in Huh7.5 cells.

1) JFH1-based HCV genotypes 1–7 Core-NS2 recombinants with heterologous 5′UTR of genotypes 1a and 3a strains were fully functional in vitro

We previously determined the 5′UTR sequence of HCV prototype genotypes 1b (strain J4), 2a (J6), 2b (J8), 3a (S52), 4a (ED43), 5a (SA13) and 6a (HK6a) using experimentally infected chimpanzee plasma pools (Bukh et al., 2010; Gottwein et al., 2010; Li et al., 2011). An alignment of these HCV 5′UTR sequences and the genotype 1a 5′UTR of reference strain H77 (GenBank accession number AF009606) (Kolykhalov et al., 1997) (Fig. 1B) showed that the 3a 5′UTR differed from the 1a 5′UTR by 27 nucleotides and 2 single nucleotide deletions. Of these differences, 13 nucleotides and 1 deletion were found to be unique to the 3a 5′UTR. Thus, the 3a 5′UTR represents a highly deviated 5′UTR among HCV genotypes. In addition, the 5′UTR of HCV genotype 3 was relatively conserved among subtype isolates (HCV databases), and the 5′UTR of 3a strain S52 should therefore be representative of most genotype 3 isolates. To study whether 1a and 3a-specific nucleotides mediated genotype-specific functions or interactions with other HCV genome regions, such as the Core-NS2 region, we generated novel JFH1-based HCV genotypes 1–7 Core-NS2 recombinants with heterotypic 1a or 3a 5′UTR, based on previously developed Core-NS2 recombinants that all contain the genotype 2a (strain JFH1) 5′UTR (Fig. 2A) (Gottwein et al., 2007, 2009; Jensen et al., 2008; Lindenbach et al., 2005; Scheel et al., 2008). The RNA transcripts of these recombinants were transfected into Huh7.5 cells; corresponding recombinants with JFH1 5′UTR or with genotype-specific 5′UTR (referred to as 5′UTR-NS2 recombinants) (Li et al., 2011) (Fig. 2A) were included in parallel for comparison. HCV positive cells were detected by immunostaining for HCV NS5A and estimated as percentage of infected cells to monitor virus spread. All JFH1-based HCV genotypes 1–7 Core-NS2 recombinants spread efficiently in transfected cultures and reached peak infection (estimated by anti-NS5A staining with ≥80% infected cells) within 5 to 8 days after transfection, irrespective of the genotype strain of the 5′UTR. The peak HCV infectivity titers of culture supernatants of the Core-NS2 recombinant viruses with heterotypic 5′UTR of 1a or 3a were similar to those of corresponding viruses with JFH1 5′UTR or with genotype-specific 5′UTR (Fig. 2B).

Next, we performed comparative growth kinetics studies for genotypes 2a and 3a Core-NS2 recombinant viruses with different 5′UTRs. We infected naïve Huh7.5 cells with transfection-derived viruses at a multiplicity of infection (MOI) of 0.003 focus forming units (FFU) per cell. The results showed that the viral growth kinetics and peak viral infectivity titers of the 2a Core-NS2 recombinant (J6/JFH1) viruses with heterotypic 1a or 3a 5′UTR were comparable to those of corresponding viruses with JFH1 5′UTR or with genotype-specific 5′UTR (Fig. 2C). The HCV RNA titers were also similar at the corresponding time points with peak titers reaching $10^{8.0}$–$10^{8.2}$ IU/mL. Likewise, the 3a Core-NS2 recombinant viruses with different 5′UTRs were comparable with respect to growth kinetics, peak HCV infectivity titers (Fig. 2C) and HCV RNA titers [$10^{8.4}$ international unit (IU)/mL]. Therefore, exchanging the 5′UTR from original 2a (JFH1) 5′UTR to 1a or 3a 5′UTR did not affect viability of JFH1-based HCV genotypes 1–7 Core-NS2 recombinants; the nucleotide polymorphisms between the 5′UTR of

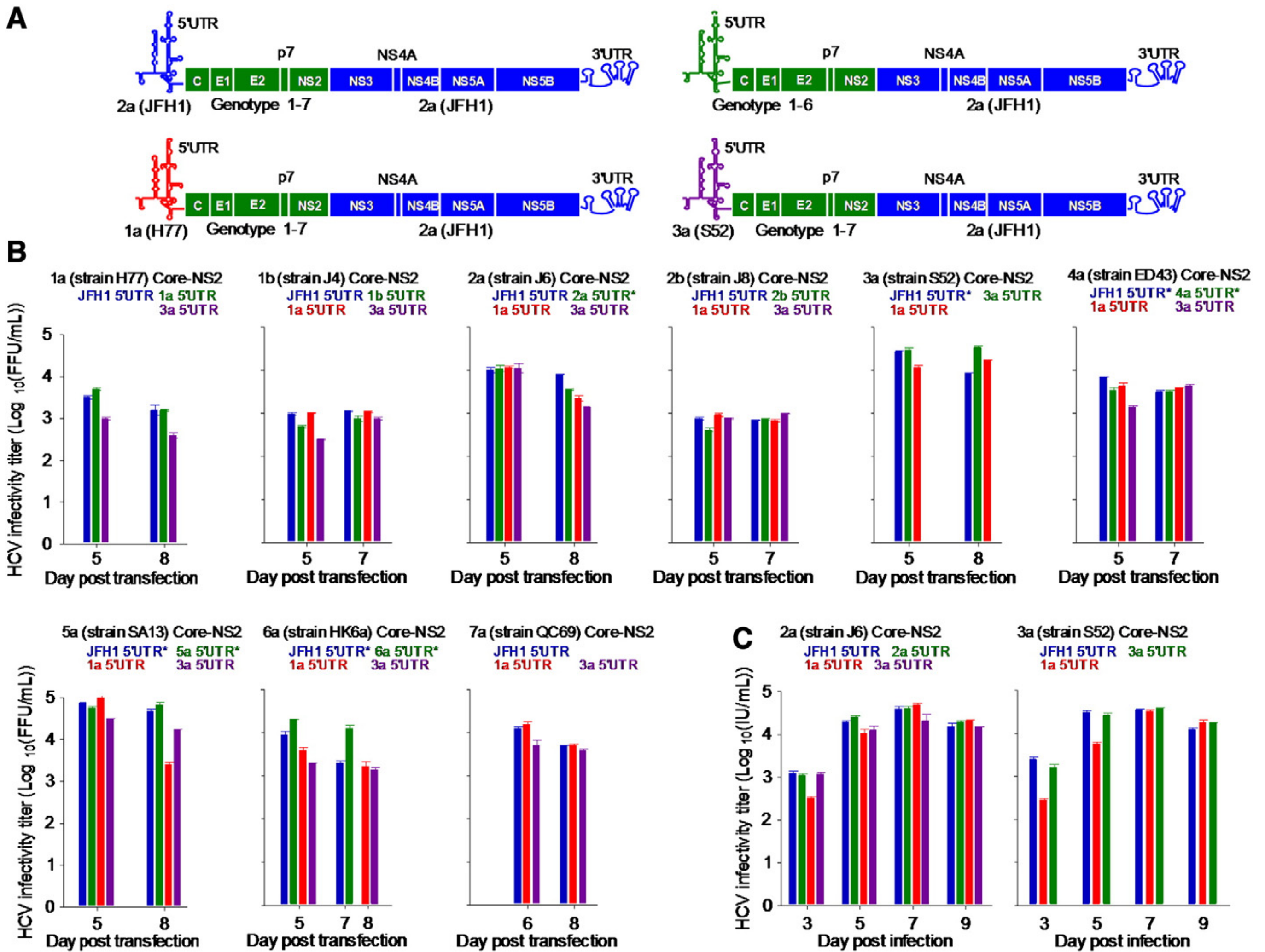


Fig. 2. JFH1-based genotypes 1–7 Core-NS2 recombinants with 5′UTR of genotypes 1a and 3a were fully viable in Huh7.5 cells. A) Schematic diagram of JFH1-based HCV recombinants. Using previously developed JFH1-based HCV genotypes 1–7 Core-NS2 recombinants, which contain JFH1 5′UTR and NS3-3′UTR (in blue) (Gottwein et al., 2007, 2009; Jensen et al., 2008; Lindenbach et al., 2005; Scheel et al., 2008), here we developed corresponding recombinants with heterotypic 5′UTR of genotypes 1a (strain H77) (in red) and 3a (S52) (in purple). The 3a 5′UTR used here has G at the 5′-terminus, different from the prototype 3a strain S52 from the chimpanzee plasma pool, which has A at the 5′-terminus (Fig. 1B) (Gottwein et al., 2010; Li et al., 2011). Previously developed JFH1-based HCV genotypes 1–6 Core-NS2 recombinants with genotype-specific 5′UTR (referred to as 5′UTR-NS2 recombinants) (Li et al., 2011) were included for comparison. B) Transfection of Huh7.5 cells with RNA transcripts from indicated recombinants. In RNA transfection, the transfection complexes were incubated with cells in DMEM medium supplemented with 10% FBS for ~16 h before subculture, and HCV infection and spread were detected by immunostaining for HCV NS5A (see Materials and methods). HCV infectivity titer of culture supernatants collected at peak infection (estimated by anti-NS5A staining, with ≥80% infected cells) was determined by focus forming units (FFU) assay and presented as mean ± standard error of the mean (SEM) of triplicate infections (see Materials and methods for details). *, the recombinant was from a separate transfection experiment. C) Comparative growth kinetics study. Naïve Huh7.5 cells were infected with transfection-derived 2a and 3a Core-NS2 recombinants with different 5′UTRs at MOI of 0.003 FFU/cell, cells were split every 2–3 days, and culture supernatant infectivity titers were determined (supernatant was not collected at day 1) and shown as mean ± SEM of triplicate infections.

genotypes 1a and 3a did apparently not mediate genotype-specific function or interactions important for production of infectious HCV *in vitro*.

To demonstrate whether exchange of the 2a (JFH1) 5′UTR with 1a or 3a 5′UTR required additional adaptive mutations in the genome other than those required for the original Core-NS2 genotype recombinant viruses, we selected the 2a, 3a and 5a Core-NS2 recombinants with 5′UTR of 1a and 3a for further analysis. Recombinant viruses derived from peak infection supernatant of first passage (Fig. 2C and not shown) required no additional mutations in the ORF and the 3′UTR, however, the 5′-terminal G of the 5′UTR was in all cases changed to A (Table 1), in line with previous observations for 5′UTR-NS2 recombinants (Li et al., 2011). The 5′-terminal nucleotide of prototype 3a strain S52 is A (Fig. 1) (Gottwein et al., 2010), however we used G in all recombinants with the 3a 5′UTR. Thus the G to A change in the 3a 5′UTR of these recombinants might have represented reversions to the original 3a sequence.

2) J6/JFH1 recombinants with heterologous 5′UTR of genotypes 1–6 were fully functional in vitro

To study whether the 5′UTR of HCV genotypes 1b, 2b, 4a, 5a and 6a mediated genotype-specific interactions important for production of infectious virus particles, we constructed J6/JFH1 recombinants with 5′UTR of these genotypes (Fig. 3A). Together with the previously developed 2a 5′UTR-NS2 recombinant (Li et al., 2011) and the J6/JFH1 recombinants with 5′UTR of genotypes 1a and 3a (Fig. 2A), we thus completed a panel of J6/JFH1 recombinants with 5′UTR of HCV genotypes 1–6 (Fig. 3A). This enabled us to perform functional analysis of the 5′UTR of HCV genotypes 1a (strain H77), 1b (J4), 2a (J6), 2b (J8), 3a (S52), 4a (ED43), 5a (SA13) and 6a (HK6a) in an identical Core-3′UTR genetic background. Transfection of Huh7.5 cells with RNA transcripts of these recombinants showed that all J6/JFH1 recombinants with 5′UTR of genotypes 1–6 reached peak infection at day 3 post-transfection, and produced infectious virus particles with similar

Table 1
Characterization of JFH1-based HCV genotypes 2a, 3a and 5a Core-NS2 recombinants with 1a or 3a-specific 5′UTR.

JFH1-based HCV recombinants		Transfection		First Passage			Mutation		
5′UTR (strain)	Core-NS2 (strain)	Day of ≥80% infected cells	Peak infectivity titer (Log_{10}(FFU/mL))	MOI	Day of ≥80% infected cells	Peak infectivity titer (Log_{10}(FFU/mL))	5′UTR*	Core-NS5B#	3′UTR¤
1a (H77)	2a (J6)	5	4.1	0.003	7	4.7	G1A	None	NCC
3a (S52)	2a (J6)	8	4.2	0.003	7	4.3	G1A	None	NCC
1a (H77)	3a (S52)	8	4.1	0.003	7	4.5	G1A	None	NCC
3a (S52)	3a (S52)	8	4.5	0.003	7	4.5	G1A	None	NCC
1a (H77)	5a (SA13)	3	4.5	n.d.	7	4.8	G1A	None	NCC
3a (S52)	5a (SA13)	8	4.5	0.015	11	4.7	G1A	None	NCC

The 2a, 3a and 5a Core-NS2 recombinant viruses with 5′UTR of genotypes 1a and 3a derived from infection supernatant taken at peak infection (estimated with ≥ 80% infected cells) were subjected to sequence analysis of the entire genome, including the 5′ and 3′UTRs. MOI, multiplicity of infection. n.d., MOI was not determined; 1 ml transfection supernatant was used for infection. *, the entire 5′UTR sequences were determined by rapid amplification of 5′ complementary DNA ends (5′RACE) procedure on HCV positive-strand RNA extracted from culture supernatant, and the second round PCR products were directly sequenced. Nucleotide (nt) G was used at 5′ terminus of 3a 5′UTR (prototype 3a strain S52 has A at 5′ terminus (Fig. 1B) (15, 34)). ¤, the 3′UTR sequences were determined by 5′RACE on HCV negative-strand RNA extracted from infected cells, and the second round PCR products were cloned for sequence analysis. No consensus change (NCC) was observed in the 3′UTR, however, poly(U/UC) tracts of different length were identified, without any particular pattern for a particular recombinant. #, in other independent experiment(s), mutations were identified in recombinant J6/JFH1 with 1a 5′UTR [in the 2nd experiment: NS5A, T6352A(F2004Y) and A6761A/G; in the 3rd experiment: E2, A1671G(T444A) and NS3, T5252C/t] and 5a Core-NS2 recombinant with 1a 5′UTR [NS2, T3334G(L998R) and NS5B, G8303T(M2654I)]. Although these genomes replicated efficiently, it is possible that these mutations represent putative adaptive mutations, but they could also be mutations resulting from errors introduced during RT-PCR.

infectivity titers ($10^{4.6}$–$10^{4.8}$ FFU/mL). The transfection culture supernatant collected at peak infection was passaged to naïve Huh7.5 cells with MOI of 0.003 FFU/cell. In these cultures, all J6/JFH1 viruses with 5′UTR of genotypes 1–6 spread comparably with increasing and equivalent HCV infectivity titers (Fig. 3B) and HCV RNA titers (reaching $10^{7.7}$–$10^{7.8}$ IU/mL) during days 3–9. Therefore, the 5′UTR of HCV genotypes 1–6 did not appear to have genotype-specific function or interactions with other HCV genome regions being of importance in production of infectious HCV *in vitro*.

Sequence analysis of the 5′UTR of the passaged viruses collected at day 7 post infection (Fig. 3B) revealed that the 5′-terminal G was changed to A in the 5′UTR of genotypes 1a, 1b, 2b, 3a and 6a; the 5′-terminal A of the 5′UTR of 2a, 4a and 5a was maintained, however,

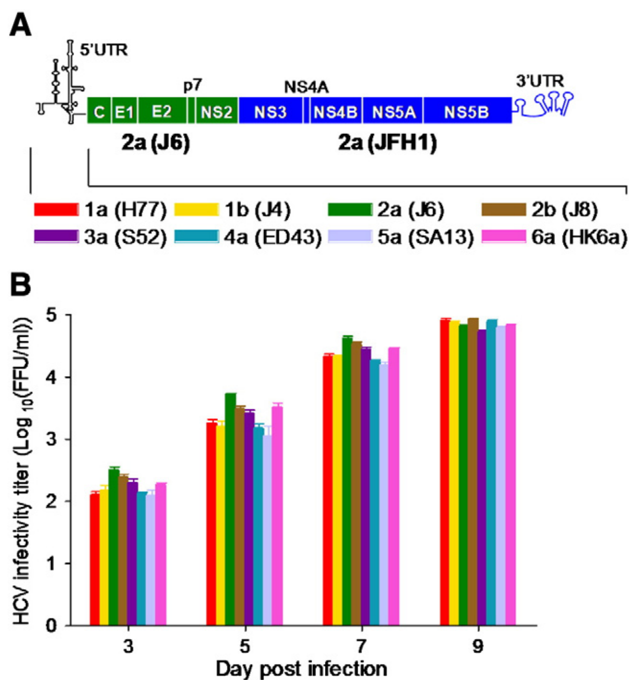


Fig. 3. J6/JFH1 recombinants with 5′UTR of HCV genotypes 1–6 were fully functional in Huh7.5 cells. A) Schematic diagram of HCV recombinants. The 5′UTR of HCV genotypes 1–6 was engineered into the corresponding region of the 2a recombinant J6/JFH1, which contains 2a J6 Core-NS2 and JFH1 NS3-3′UTR (Lindenbach et al., 2005). B) Comparative growth kinetics study. Naïve Huh7.5 cells were infected with transfection-derived 2a Core-NS2 recombinants with 5′UTR of genotypes 1–6 (MOI = 0.003 FFU/cell). Culture supernatant infectivity titers (mean ± SEM of triplicate infections) are shown.

the G inserted immediately upstream of the 5′-terminal A for enhancing *in vitro* transcription was deleted. The change at the 5′-terminus is consistent with our observations for Core-NS2 recombinants with 5′UTR of 1a or 3a (Table 1) and with our previous observations for 5′UTR-NS2 recombinants (Li et al., 2011).

Apparent lack of genotype-specific interaction of HCV 5′UTR domains I, II and III with influence on virus production in vitro

The nucleotide heterogeneities found in the 5′UTR are primarily located in domains I, II and III [Fig. 1B and (Bukh et al., 1992)], and we therefore investigated whether these heterogeneous nucleotides mediate genotype-specific interactions among 5′UTR domains. We constructed and characterized (i) HCV 1a 5′UTR-NS2 recombinants with 5′UTR domain I of genotypes 1–6, and (ii) J6/JFH1 recombinants with heterotypic chimeric 5′UTR, in which 5′UTR domains I, II or III of 1a and 3a were exchanged (see below). By characterization of these recombinants in transfection and infection of Huh7.5 cells, we concluded that the interactions, if any, among HCV 5′UTR domains I, II and III did not appear to be genotype-specific and essential for HCV production *in vitro*.

1) JFH1-based HCV 1a 5′UTR-NS2 recombinants with 5′UTR domain I sequences of genotypes 1–6 were fully functional in Huh7.5 cells

The 5′UTR domain I (nucleotides 1–43) is essential for HCV RNA replication (Friebe et al., 2001). This domain contains SLI that is important for *in vitro* HCV RNA replication (Luo et al., 2003) and virus production (Li et al., 2011). A cluster of nucleotide heterogeneities was found in domain I of the 5′UTR, primarily within SLI (nucleotides 5–20); genotypes 2a (strain J6), 3a (S52), 4a (ED43), 5a (SA13) and 6a (HK6a) contain deletions in this region (Fig. 1B). We thus studied whether these heterogeneous nucleotides mediate genotype-specific functions or interactions in virus production. We developed 1a (strain H77) 5′UTR-NS2 recombinants with heterotypic 5′UTR domain I of genotypes 1–6 (Fig. 4A), using the previously developed 1a 5′UTR-NS2 recombinant (Li et al., 2011). RNA transfection of Huh7.5 cells showed that these recombinants spread efficiently in transfected cultures and produced infectious virus particles with peak HCV infectivity titers ($10^{3.5}$–$10^{3.8}$ FFU/mL) similar to 1a 5′UTR-NS2 recombinant virus ($10^{3.5}$ FFU/mL). Viruses in supernatant from peak HCV infection were further characterized by comparative growth kinetics studies by infection of naïve Huh7.5 cells with identical virus doses (MOI = 0.003 FFU/cell) (Figs. 4B and C). The results showed that the HCV growth kinetics monitored by supernatant HCV infectivity titers (Figs. 4B and C) and peak HCV RNA titers ($10^{8.1}$–$10^{8.5}$ IU/mL) of these

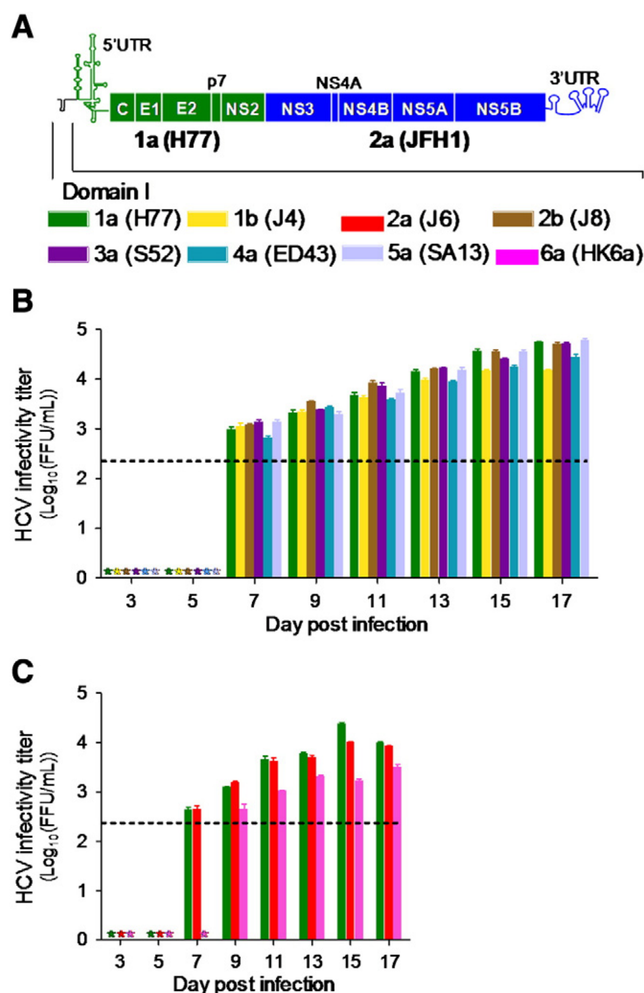


Fig. 4. JFH1-based 1a 5′UTR-NS2 recombinants with HCV genotypes 1–6 specific domain I sequences were fully viable in Huh7.5 cells. A) Schematic diagram of 1a (strain H77) 5′UTR-NS2 recombinants (Li et al., 2011) with 5′UTR domain I (nucleotides 1–43) of HCV genotypes 1b, 2a, 2b, 3a, 4a, 5a and 6a. B and C) Comparative growth kinetics study. Naïve Huh7.5 cells were infected with transfection-derived 1a 5′UTR-NS2 recombinants with 5′UTR domain I of HCV genotypes 1–6 (MOI = 0.003 FFU/cell). Culture supernatant HCV infectivity titers (mean ± SEM of triplicate infections) are shown. *, indicates that the HCV infectivity titer was below the detection limit of 2.3 Log_{10} (FFU/mL), as shown by a broken line. In a separate experiment, the peak HCV infectivity titer of the 1a 5′UTR-NS2 recombinant virus with 5′UTR domain I of 6a were comparable to that of the virus with wild-type 1a 5′UTR (data not shown).

heterotypic 5′UTR domain I viruses were comparable to those of the 1a 5′UTR-NS2 recombinant virus.

Sequence analysis of the 5′UTR of first passage virus revealed that the heterotypic 5′UTR domain I was maintained in all viruses. However, the 5′-terminal G of genotypes 1b, 3a and 6a was changed to A, while the 5′-terminal G of genotype 2b was changed to AC (in 3 out of 3 clones analyzed). The 5′-terminal A of 2a, 4a and 5a domain I was maintained, however, the G inserted immediately upstream of the 5′-terminal A for enhancing *in vitro* transcription was deleted. Taken together, the 5′UTR domain I of genotypes 1b, 2a, 2b, 3a, 4a, 5a and 6a had no genotype-specific functions or interactions with other HCV genome regions essential for production of infectious HCV in Huh7.5 cells.

2) HCV recombinant J6/JFH1 with heterotypic chimeric genotype 1a/3a or 3a/1a 5′UTR domains I, II and III were fully viable in Huh7.5 cells

Since 5′UTR nucleotide heterogeneities are primarily found in domains I, II and III (Fig. 1B) (Bukh et al., 1992), we further investigated whether these nucleotides mediate genotype-specific interactions among 5′UTR domains. The 3a 5′UTR varied the most among HCV genotypes 1–6 strains, differing from the 1a 5′UTR by 7, 4 and 15 nucleotides in domains I, II and III, respectively. In addition, the 3a 5′UTR domain I had two single nucleotide deletions (Fig. 1B). We thus generated J6/JFH1 recombinants with heterotypic 5′UTR with 1a/3a or 3a/1a combination of domains I, II and III (Fig. 5A). Transfection of Huh7.5 cells with RNA transcripts from these recombinants showed that all six J6/JFH1 recombinants with chimeric 1a/3a or 3a/1a 5′UTRs spread efficiently in the culture and produced infectious viruses with HCV infectivity titers comparable to corresponding viruses with the complete 1a or 3a 5′UTR (~$10^{4.5}$ FFU/mL). Viruses in culture supernatants derived from the peak of HCV infection were further characterized by comparative growth kinetics studies. In naïve Huh7.5 cells infected with identical MOI (0.003 FFU/cell), the HCV growth kinetics determined by supernatant HCV infectivity titers (Fig. 5B), as well as the peak HCV RNA titers ($10^{7.2}$–$10^{7.6}$ IU/mL) of the viruses with chimeric 5′UTR were similar to those of corresponding viruses with complete 1a or 3a 5′UTR. Sequence analysis of the entire 5′UTR of the viruses recovered at day 7 post-infection (Fig. 5B) revealed that the chimeric 5′UTR domains were maintained. However, the 5′-terminal G was in all cases changed to A, in line with observations with other JFH1-based recombinants [Table 1 and (Li et al., 2011)]. Hence, the heterogeneities in domains I, II and III of the 5′UTR of genotypes 1a and 3a did not appear to mediate genotype-specific functions or interactions among 5′UTR domains essential for production of infectious HCV in Huh7.5 cells.

Adenine at the 5′-terminus of the HCV genome is required for JFH1-based recombinants in vitro

Since all JFH1-based HCV recombinant viruses recovered from cultures, either in the present study (Table 1 and above) or in our previous

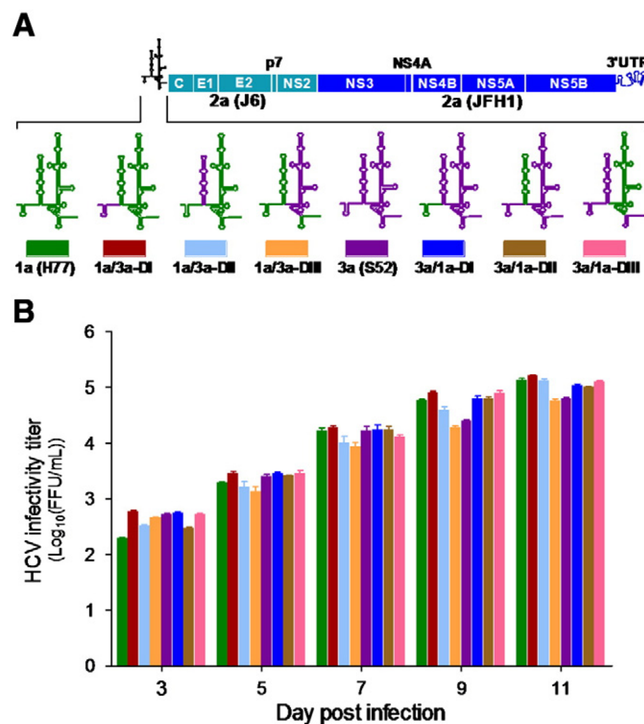


Fig. 5. Fully functional JFH1-based HCV 2a Core-NS2 recombinants with heterotypic 1a/3a or 3a/1a chimeric 5′UTR domains. A) Schematic diagram of 2a (strain J6) Core-NS2 recombinants with 1a/3a chimeric 5′UTRs. The domains I, II or III of the 5′UTR of genotypes 1a and 3a were exchanged and engineered into the 2a Core-NS2 recombinant. The 2a Core-NS2 recombinants with wild-type 5′UTR of genotypes 1a and 3a were included for comparison. B) Comparative growth kinetics study. Naïve Huh7.5 cells were infected with transfection-derived 2a Core-NS2 recombinants with heterotypic chimeric 5′UTRs (MOI = 0.003 FFU/cell). Culture supernatant HCV infectivity titers are shown (mean ± SEM of triplicate infections).

studies with genotypes 1–6 5′UTR-NS2 recombinants (Li et al., 2011), had A at the 5′-terminus, we speculated that the A at the 5′-terminus is required for efficient growth of JFH1-based HCV recombinants in Huh7.5 cells, irrespective of the genotype strain of the 5′UTR. We thus mutated the 5′-terminal G of 1a (strain H77) 5′UTR-NS2 recombinant to A, U and C, designated 1a-G1A, 1a-G1U and 1a-G1C, respectively (Fig. 6A) and the 5′-terminal A of 2a 5′UTR-NS2 recombinant into G, U and C, designated 2a-A1G, 2a-A1U and 2a-A1C, respectively (Fig. 6B). In addition, we deleted the 5′-terminal A of the 2a 5′UTR-NS2 recombinant to generate 2a-A1Δ (Fig. 6B). To study the authenticity of the RNA transcripts used for transfection, an aliquot of the *in vitro*-transcribed RNA transcripts was subjected to 5′RACE procedures to determine the entire 5′UTR sequence, while another aliquot of the RNA transcripts was used for transfection of Huh7.5 cells. For all constructs except 2a-A1C the correct sequences of the RNA transcripts were identified (Figs. 6A and B). However, in the clones with C at the 5′-terminus additional cytidines were identified in the RNA transcripts (Figs. 6A and B). In RNA transfection of Huh7.5 cells, all 5′-terminal nucleotide mutants spread efficiently and produced HCV with peak viral infectivity titers comparable to corresponding virus with the wild-type 5′-terminal nucleotide (Figs. 6A and B). The transfection-derived recombinant virus could be passaged to naïve Huh7.5 cells. Sequence analysis of the 5′UTR of such first passage viruses revealed that A was invariably identified at the 5′-terminus of 1a and 2a 5′UTR-NS2 recombinant viruses, regardless of whether A, G, U or C were present at the 5′-terminus of the RNA transcripts (Figs. 6A and B). In a separate independent experiment, in which RNA transcripts of these nine recombinants were not confirmed by 5′RACE procedure, all the recovered viruses also contained A at 5′-terminus (data not shown). Hence, A at the 5′-terminus is apparently required for efficient growth of the JFH1-based recombinants in Huh7.5 cells.

In first passage-recovered viruses of 2a-A1C, 2a-A1U or 2a-A1Δ, insertions of one or two cytidines were observed in 5′-proximal sequences between nucleotide 1 and 5 of several clones (Fig. 6B and data not shown from the independent experiment, where the RNA transcripts were not sequenced). These insertions might have resulted from insertions introduced during synthesis of the RNA transcripts (Fig. 6B). The cytidines at position 2 and 3 of the 5′UTR sequences were believed to be involved in binding to the miR-122 molecules that bind to miR-122 binding site 1 (Fig. 1) (Machlin et al., 2011). We recently demonstrated that introducing mutations in miR-122 binding site 1 could confer viral resistance to the inhibitory effect of SPC3649-mediated miR-122 antagonism (Li et al., 2011). Thus, we constructed a 2a 5′UTR-NS2 recombinant with insertion of two cytidines (2a-Ins2C) to test its sensitivity to miR-122 silencing. In RNA transfection of Huh7.5 cells, the viability of this recombinant was comparable to that of the wild-type 5′UTR 2a recombinant (data not shown). However, in first passage-recovered virus only one C was maintained (2a-Ins1C). The infection of the 2a-Ins1C virus in SPC3649-transfected Huh7.5 cells was efficiently suppressed in two experiments, to the level of inhibition observed for the wild-type 5′UTR 2a recombinant virus, whereas a resistant mutant Cell-U3 (Li et al., 2011) was not inhibited by the treatment (Figs. 7A and B). Thus, the data indicated that insertion of one C at the 5′-proximal sequence (between nucleotides 1 and 5) did not confer viral resistance to the inhibitory effect of miR-122 antagonism.

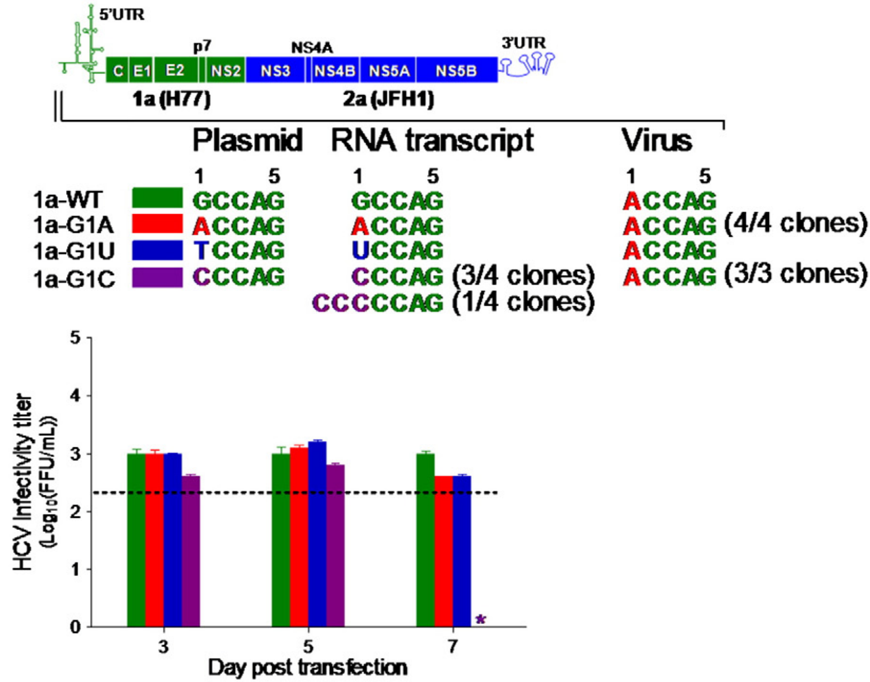
The J6/JFH1 recombinants with 5′UTR of genotypes 1–6 responded similarly to interferon-α2b

Although it has been suggested that the 5′UTR could be a target of IFN (Dash et al., 2005; Hazari et al., 2005) and that the IRES might contribute to IFN resistance (Koev et al., 2002), no suitable culture systems were available to test the role of the HCV 5′UTR in response to IFN in a genotype-specific manner. Development of viable J6/JFH1 recombinants with 5′UTR of HCV genotypes 1–6 (Fig. 8A) provides a unique opportunity to study the 5′UTR of HCV major genotypes in viral responses to IFN treatment. We infected Huh7.5 cells with first passage recombinant viruses, and then treated with IFN-α2b. Median effective concentration (EC50) was determined to be 0.2–1.2 IU/mL for J6/JFH1 recombinant with JFH1 5′UTR (Gottwein et al., 2011; Scheel et al., 2011b). Treatment of infected Huh7.5 cells with 40 IU/mL and 60 IU/mL in two independent experiments showed that virus infections were suppressed, with ~20% infection relative to the respective infection in mock-treated controls, while treatment with 0.4 IU/mL and 0.6 IU/mL showed 60–80% infection. The IFN doses used were previously shown not to have a cytotoxic effect (Gottwein et al., 2009; Scheel et al., 2011b). All the recombinant viruses with different 5′UTRs responded to IFN treatment in a dose dependent manner, and no major difference was observed between the viruses with different 5′UTRs (Figs. 8B and C). Thus, the 5′UTR of HCV genotypes 1–6 did not appear to confer a genotype-specific response to IFN-α2b treatment in Huh7.5 cells.

Discussion

The functional importance and the conserved nature of the HCV 5′UTR attract great interest in exploring this genome region as a therapeutic target against HCV infection. However, current knowledge on the role of the 5′UTR of HCV was mainly gained from the *in vitro* HCV replicon systems and translation assays (Shi and Lai, 2006), which did not permit studies of the complete HCV life cycle. Also, it has not been possible to study the 5′UTR of different HCV genotypes, primarily due to the unavailability of entire 5′UTR sequences and suitable culture systems. In this study, we for the first time demonstrated that the 5′UTR of HCV genotypes 1–6 functioned in a non-genotype-specific manner in production of infectious HCV particles *in vitro*, by developing and characterizing a large panel of novel viable JFH1-based recombinants. We also demonstrated that the HCV 5′UTR of genotypes 1–6 had no genotype-specific functions or interactions with other genome regions, with special emphasis on the Core-NS2 sequences included in JFH1-based recombinants. We also found that despite significant sequence heterogeneity, there are apparently no genotype-specific interactions among the 5′UTR internal domains of importance for the complete HCV life cycle *in vitro*. We further demonstrated by reverse genetic studies that A at the 5′-terminus was required for JFH1-based HCV recombinants in Huh7.5 cells. The developed HCV genotypes 1–7 Core-NS2 recombinants (Fig. 2A), as well as J6/JFH1 recombinants (Fig. 3A), with heterotypic 5′UTRs provided optimal platforms for studying the interactions that the 5′UTR may be involved with in a genotype-specific manner. These systems could also facilitate the development of novel 5′UTR-based antivirals against HCV. The HCV recombinants with chimeric 5′UTR domain(s) (Figs. 4A and 5A) further enable the analysis of interactions among 5′UTR domains or future screening of 5′UTR domain-specific drugs, for example, domain II-targeting benzimidazole derivatives (Parsons et al., 2009; Paulsen et al., 2010).

Previous studies showed that exchange of the genotype 2a (JFH1) 5′UTR with 1a (H77) or 1b (Con1) 5′UTR reduced the replication of a JFH1 subgenomic replicon with firefly luciferase reporter, while exchange with the 2a (J6) 5′UTR did not affect replication (Binder et al., 2007). Thus, the data suggested a genotype-specific role of the 5′UTR in HCV RNA synthesis. Here we demonstrated that genotypes 1–7 Core-NS2 recombinants with heterotypic 5′UTR of genotypes 1a and 3a were fully viable in Huh7.5 cells, with viral infectivity titers similar to corresponding viruses with JFH1 5′UTR (Gottwein et al., 2007, 2009; Jensen et al., 2008; Lindenbach et al., 2005; Scheel et al., 2008), or with matching genotype-specific 5′UTR (Li et al., 2011) (Figs. 2A and B). Further, the viability of J6/JFH1 recombinants with 5′UTR of HCV genotypes 1–6 was similar (Fig. 3). Thus, our data indicated that the 5′UTR had no genotype-specific functions or interactions that were critical for efficient production of infectious HCV *in*



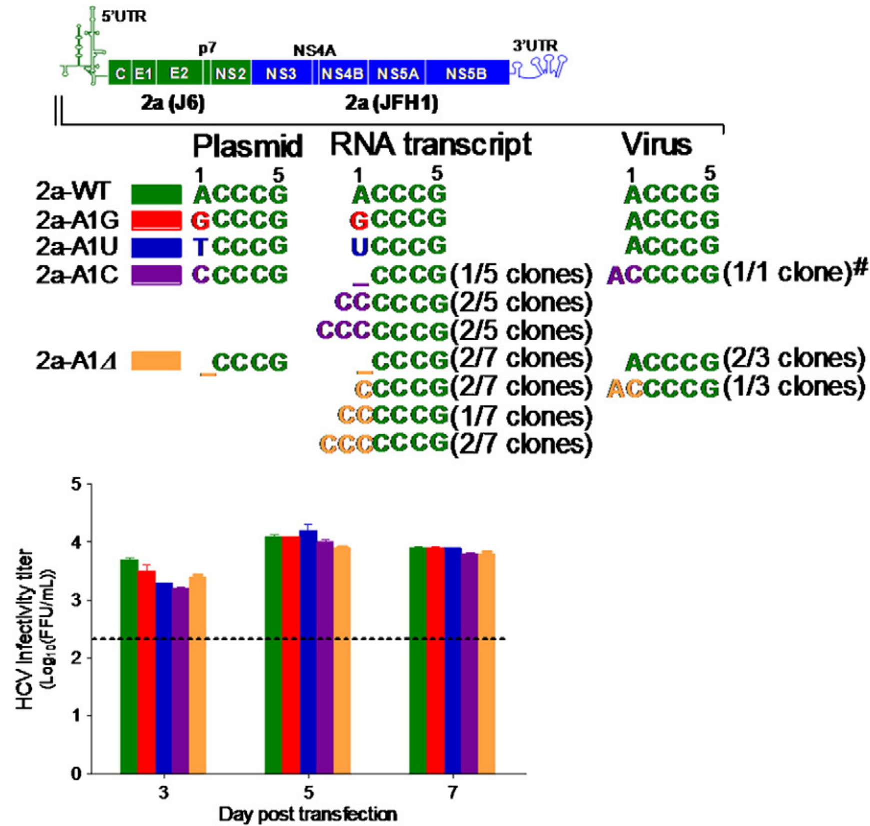


Fig. 6. Mutagenesis studies of the 5′-terminal nucleotide of JFH1-based HCV recombinants in Huh7.5 cells. The 5′-terminal nucleotide of 1a (A) and 2a (B) 5′UTR-NS2 recombinant viruses were mutated to other nucleotides as indicated. In addition, the 5′-terminal A of the 2a 5′UTR-NS2 recombinant was deleted (2a-A1Δ). RNA transcripts of the recombinants were generated using T7 RNA polymerase and divided into two aliquots. The HCV 5′UTR sequence of the RNA transcripts was analyzed by 5′RACE procedures using one aliquot of the RNA transcripts, and the 5′-terminal sequences are indicated (direct consensus sequencing or, if required, clonal analysis presented as the number of clones with indicated sequence/total clones analyzed); no changes were observed in the remainder of the 5′UTR. The other aliquot of the RNA transcripts was used for transfection of naïve Huh7.5 cells. HCV infectivity titers of transfection culture supernatants are shown (mean ± SEM of triplicate infections). *, indicates that the infectivity titer was below detection limit of 2.3 $\log_{10}$(FFU/mL), as shown by a broken line. Transfection culture supernatants collected at day 7 were passaged to naïve Huh7.5 cells and the 5′UTR sequence of the recovered viruses were analyzed by 5′RACE procedures (direct consensus sequencing or, if required, clonal analysis); no changes were observed in the remainder of the 5′UTR. Throughout nucleotides identical to the wild-type sequence are in green. #, only one clone with full-length 5′UTR was obtained; however the direct sequencing of the 5′RACE amplicons clearly indicated the presence of ACCCG and ACCCCG.

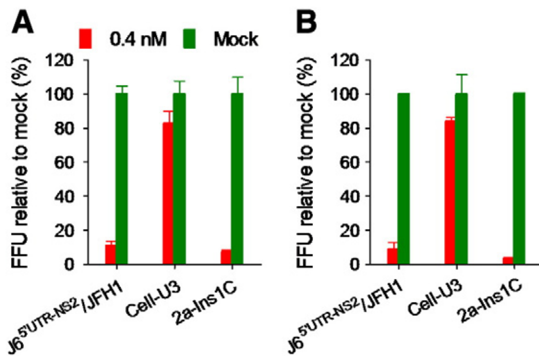


Fig. 7. A 2a 5′UTR-NS2 virus with one cytidine insertion between nucleotides 1 and 5 of the 5′UTR was inhibited by miR-122 antagonism. Huh7.5 cells were transfected with miR-122 antisense LNA SPC3649 at concentration of 0.4 nM (Li et al., 2011), and then infected with first passage 2a-Ins1C virus for 48 h. The FFU counts were determined and presented as percentage relative to the respective infection in SPC3649-free mock transfection controls (100%); values are means of triplicate infections ± SEM (see Material and methods). A 2a 5′UTR-NS2 recombinant with wild-type 5′UTR and a resistant mutant Cell-U3 which contains U3 small nucleolar RNA insertion replacing 5′UTR SLI and miR-122 binding site 1 (Li et al., 2011) were included for comparison. A and B; two independent experiments.

vitro. The discrepancies in the role of the 5′UTR observed in two culture systems may be attributed to different RNA replication efficiency, as in general full-length HCV RNA replication was slightly lower than subgenomic RNA replication [reviewed in (Blight and Norgard, 2006)], which may lead to a systematic difference. In addition, production of HCV in cultured cells might be influenced by multiple functions in the HCV life cycle. The replicon system only allows for studies of RNA replication, while the functional significance of these genotype-specific signals in the complete HCV life cycle could not be studied. Therefore, the different replication efficiency observed between 5′UTR of genotypes 1a (H77) and 2a (JFH1 and J6) (Binder et al., 2007) might not lead to significant differences in production of infectious HCV particles in Huh7.5 cells. Future studies with parallel comparison of various genotype 5′UTRs in the context of replicon systems may be required to further address these differences.

The 5′UTR is involved in long-range RNA-RNA interaction with coding sequences and with the 3′UTR of the HCV genome, regulating viral RNA replication and translation (Honda et al., 1999b; Kim et al., 2003; Romero-Lopez and Berzal-Herranz, 2009). The viability and genetic stability of our previously developed JFH1-based HCV genotypes 1–6 5′UTR-NS2 recombinants, which all contained NS3-3′UTR of JFH1, indicated that the 5′UTR did not interact with the NS3-3′UTR region in a genotype-specific manner important for virus production. This notion was further confirmed here by the fact that J6/JFH1 recombinants with the 5′UTR of genotypes 1–6 were fully functional (Fig. 3). Importantly, full viability of J6/JFH1 recombinants with 5′UTR of genotypes 1–6 (Fig. 3) and HCV genotypes 1–7 Core-NS2 recombinants with 5′UTR of 1a and 3a (Fig. 2) also indicated that the 5′UTR did not interact with the Core-NS2 region in a genotype-specific manner essential for HCV production. Indeed, the nucleotides involved in the interaction between 5′UTR IIId and the internal loop of NS5BSL3.2 (or SL9266) in *in vitro* binding assays (Romero-Lopez and Berzal-Herranz, 2009) were highly conserved among HCV genotypes. Previous reports described that the translation efficiency of a 1a 5′UTR was lower (Buratti et al., 1997) or similar (Collier et al., 1998; Motazakker et al., 2007) to that of a 3a 5′UTR in *in vitro* reporter systems. Full viability of HCV recombinants with the 5′UTR of 1a or 3a suggested that the potential differences in translation efficiency between the 1a and 3a 5′UTR did not affect the production of JFH1-based HCV recombinant viruses (Figs. 2 and 3). Overall, it appears that *in vitro* the nucleotide heterogeneities among the 5′UTR of different genotypes do not result in detectable biological differences, while the conserved regions of the 5′UTR

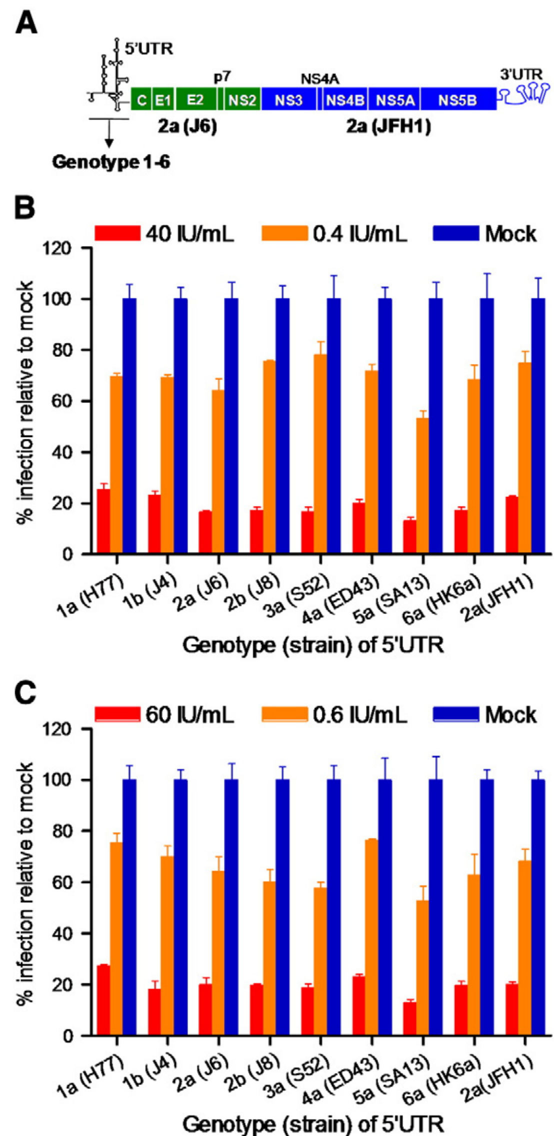


Fig. 8. HCV 2a recombinant J6/JFH1 with 5′UTR of HCV genotypes 1–6 responded similarly to interferon (IFN)-α2b treatment in Huh7.5 cells. A) Schematic diagram of JFH1-based 2a Core-NS2 recombinant viruses with 5′UTR of HCV genotypes 1–6. B and C) IFN treatment. Huh7.5 cells were infected with first passage virus, treated with IFN-α2b at different concentration at 24 and 48 h post infection, and fixed at 72 h to determine the number of infected cells (see Materials and methods). The data are shown as the mean ± SEM of infected cells from triplicate infections in the experiment, relative to respective infection in mock-treated controls (100%).

may be evolutionarily selected with important function for all HCV genotypes and isolates.

Previous *in vitro* competition binding assays showed that interactions existed between 5′UTR domains II and IV, as well as domains II and IIIabcd (Lafuente et al., 2002). With fully functional JFH1-based HCV culture systems, we demonstrated that J6/JFH1 recombinant virus with heterotypic 5′UTR of chimeric domains I, II or III of genotypes 1a and 3a were fully viable (Fig. 5); similarly, exchanging 5′UTR domain I with genotypes 1–6 did not affect the viability of the 1a 5′UTR-NS2 recombinant virus (Fig. 4). Thus, the 5′UTR domain I, II or III has no genotype-specific functions or interactions that significantly influence the viability of HCV recombinant viruses in Huh7.5 cells.

The 5′UTR of HCV was reported to bind numerous cellular proteins [reviewed in (Shi and Lai, 2006)], including retinoic acid inducible gene I (RIG-I), an interferon-inducible cellular DExD/H box RNA

helicase (Sumpter et al., 2005). Although the entire 5′UTR may be a weak inducer, RIG-I interaction with HCV pathogen-associated molecular patterns, such as HCV RNA 5′ triphosphate and 3′UTR polyU/UC tracts, triggers intracellular signaling cascades to drive IFN production and the expression of IFN-stimulated genes, thus forming innate antiviral immune defenses to limit HCV infection [(Saito et al., 2008) and reviewed in (Horner and Gale, 2009)]. However, unlike original Huh7 cells, derivative Huh7.5 cells are defective in the RIG-I pathway, which may contribute to its high permissiveness to HCV replication (Sumpter et al., 2005). Previous studies have shown that the spread of JFH1 infection was impaired in Huh7 cells (Wakita et al., 2005; Zhong et al., 2005); no infectivity could be detected in the supernatant of infected Huh7 cells up to 6 days after inoculation (Zhong et al., 2005). Nonetheless, it would also be interesting in future studies to investigate whether the differences in replication and translation efficiency of the 5′UTRs observed previously (Binder et al., 2007; Buratti et al., 1997; Honda et al., 1999b) could affect the ability of the JFH1-based HCV recombinants with different genotype 5′UTRs (Fig. 3) to establish infections and if these 5′UTR recombinant viruses induce IFN response in Huh7 cells in a genotype specific manner. In addition to interaction with cellular factors, the 5′UTR also interacts with HCV proteins, for instance, the sequences in 5′UTR domains I (Tanaka et al., 2000) and III (Hwang et al., 1995; Tanaka et al., 2000; Yu et al., 2009) have been shown to bind to the HCV Core protein. The Core-NS2 recombinants with the 5′UTR of different genotypes (Fig. 2A), especially the panel of J6/JFH1 recombinants with the 5′UTR of genotypes 1–6 (Fig. 3A), provide a unique tool to study potential interactions of the 5′UTR and viral or host factors in a genotype-specific manner and in the setting of the complete HCV life cycle.

Synthesis of the HCV RNA positive strand is supposed to be directed by an intermediate minus strand through the function of RNA-dependent RNA polymerase (RdRp), within the NS5B protein (Moradpour et al., 2007). *De novo* initiation is believed to be the mechanism for HCV replication *in vivo*, since authentic 5′ and 3′-terminal nucleotides are produced (Chinnaswamy et al., 2010; Luo et al., 2000; Zhong et al., 2000b). In *de novo* RNA synthesis assays performed with recombinant HCV NS5B, nucleotides A and G were favorable initiation nucleotides (Luo et al., 2000; Zhong et al., 2000a, 2000b), and both, A and G, were found at the 5′-terminus of HCV clinical isolates (HCV databases and (Cai et al., 2004)). However, we observed that genomes from culture-recovered JFH1-based recombinant viruses, generated in this study or in a previous study (Li et al., 2011), all had A at the 5′-terminus. Further reverse genetics studies demonstrated that A at the 5′-terminus was required for JFH1-based HCV recombinants in Huh7.5 cells (Fig. 6). The change of the 5′-terminal nucleotide also seems to be a highly likely event, as all the recombinant viruses with mutated 5′-terminal nucleotide spread efficiently after RNA transfection (Figs. 2 and 6), suggesting that HCV possesses an efficient terminus-repairing mechanism. Similar observations were previously reported in the Con1 (1b) replicon system, in which the 5′-terminal G was changed to A upon multiple rounds of replication, while A was stably maintained (Cai et al., 2004). However, we previously found that the HCV genotype 1a H77C genome recovered from acute phase of infection in an intrahepatically transfected chimpanzee maintained the 5′-terminal G (Li et al., 2011; Yanagi et al., 1997). The discrepancy in the 5′-terminal nucleotide observed for *in vitro*- and *in vivo*-derived HCV might be due to the structural differences of HCV RdRp NS5B between HCV isolates. The JFH1-based recombinants tested all contained JFH1 RdRp, and prototype JFH1 strain has A at the 5′-terminus (Kato et al., 2001). Thus, the JFH1 RdRp might preferably select A as initiation nucleotide for JFH1-based recombinants during replication cycles. Alternatively, A at the 5′-terminus could be an *in vitro* effect, as the cultured hepatoma cells constitute a population with relatively low complexity, which may restrict the selection of the initiation nucleotide. In contrast, *in vivo* HCV replication may take place in various cell types in the liver or other tissues with different nucleotide concentrations, or with certain host proteins required for incorporating a guanine at the 5′ end. Thus, both A and G could be selected to initiate the RNA synthesis *in vivo*. Nevertheless, parallel analysis of the 5′ G-containing JFH1-based HCV recombinants derived from *in vitro* and *in vivo* systems, such as the genotype 2b (J8) 5′UTR-NS2 recombinant that did not require adaptive mutations (Gottwein et al., 2009; Li et al., 2011), would help to further clarify this issue. A recent report speculated that G or A at the 5′-terminus of HCV was selected due to the requirement of base-pairing with U at position 17 of the miR-122 molecule by forming a G-U wobble or a canonical A-U base pair (Machlin et al., 2011). However, this hypothesis could not directly explain why this change occurred in culture-derived JFH1-based HCV recombinants, but not in chimpanzee infection-derived full-length HCV (Li et al., 2011; Yanagi et al., 1997). Given that the original Huh7 cells were shown to produce less miR-122 than produced in liver tissue (Chang et al., 2004; Jopling et al., 2005), it could be further speculated that A at the 5′-terminus facilitate the A-U base-pair, which could compensate for the lack of *in vivo* host factors beneficial for HCV replication in a 5′ G-U base pair dependent manner, to adapt to a low miR-122 environment in cultured Huh7.5 cells.

To date, the mechanism of HCV genotype-dependent IFN resistance is unclear. Development of JFH1-based HCV recombinants with specific HCV genome regions of various genotypes allowed us to test its role for IFN treatment in a genotype-specific manner. We have previously demonstrated that Core-NS2 (Gottwein et al., 2009), the NS3/NS4A protease (Gottwein et al., 2011) and NS5A (Scheel et al., 2011b) were not linked to differential responses to IFN-α2b treatment in Huh7.5 cells. Through IFN-α2b treatment of the cultures infected with J6/JFH1 recombinant viruses with 5′UTR of genotypes 1–6, here we demonstrated that the 5′UTR of HCV genotypes 1–6 did not confer genotype-specific responses to IFN-α2b treatment (Fig. 8). The data suggested that the 5′UTR may not confer a genotype-specific IFN resistance. It remains to be determined whether other viral elements might be involved in the genotype-specific IFN resistance observed in patients. However, it is possible that IFN resistance required the presence of multiple elements, thus, it will be interesting to combine the potential IFN resistant elements, such as genotype-specific 5′UTR and NS5A, in a JFH1-based recombinant in the treatment. Alternatively, however, our short-term IFN treatment assay might not be suitable to reveal an IFN resistance phenotype.

In summary, we have demonstrated a non-genotype-specific role of the 5′UTR in production of infectious HCV *in vitro*. We also demonstrated that the 5′UTR might not account for the HCV genotype-specific responses to IFN treatment. The developed HCV recombinants with genotypes 1–6 specific 5′UTR would be optimal systems to study genotype-specific interactions of the 5′UTR with cellular proteins or testing of novel 5′UTR-based antivirals. This study advances our understanding of the 5′UTR of different genotypes in the complete HCV life cycle and contributes valuable information for future HCV basic research and HCV drug development.

Materials and methods

Construction of JFH1-based HCV Core-NS2 recombinants with 5′UTR of different genotypes and derivative mutant recombinants

The 5′UTR sequences of HCV genotypes 1a (strain H77), 1b (J4), 2a (J6), 2b (J8), 3a (S52), 4a (ED43), 5a (SA13) and 6a (HK6a) were obtained as previously described (Li et al., 2011). The JFH1 5′UTR of previously developed JFH1-based Core-NS2 recombinants H77C/JFH1$_{V787A,Q1247L}$ (1a) (Scheel et al., 2008), J4/JFH1$_{F886L,Q1496L}$ (genotype 1b) (Gottwein et al., 2009), J6/JFH (2a) (Lindenbach et al., 2005), J8/JFH1 (2b) (Gottwein et al., 2009), S52/JFH1$_{I793S,K1404Q}$ (3a) (Gottwein et al., 2009), ED43/JFH1-$\gamma_{T827A,T977S}$ (4a) (Scheel et al., 2008), SA13/JFH1$_{A1022G,K1119R}$ (5a) (Jensen et al., 2008), HK6a/JFH1$_{F350S,N417T}$ (6a)

(Gottwein et al., 2009) and QC69/JFH1 (7a) (Gottwein et al., 2009) was replaced with the 5′UTR of genotypes 1a (strain H77) or 3a (strain S52) to generate corresponding recombinants with heterotypic 5′UTR of 1a or 3a (Fig. 2A). The JFH1 5′UTR of 2a Core-NS2 recombinant (J6/JFH1) (Lindenbach et al., 2005) was also replaced by the 5′UTR of genotypes 1b (strain J4), 2b (J8), 4a (ED43), 5a (SA13) and 6a (HK6a) (Fig. 3A).

To construct JFH1-based HCV recombinants containing heterotypic 5′UTR domains I, II or III the 5′UTR fragments with heterotypic domains were chemically synthesized (GenScript, USA) and cloned into appropriate recombinants. The HCV recombinants with 5′-terminal nucleotide mutations were constructed by site-directed PCR mutagenesis. A T7 promoter sequence TAATACGACTCACTATA was added immediately upstream of the 5′UTR to enable *in vitro* transcription. A guanine was inserted between the T7 promoter and the 5′UTR in the cases when the 5′-terminal nucleotide of the 5′UTR was adenine, unless otherwise stated. The entire HCV sequence for final plasmid constructs tested in Figs. 2, 3 and 6 was confirmed by sequence analysis (except 2a-A1Δ in Fig. 6, see below) (Macrogen, Korea). The final maxi preparations of other plasmids used in the study and the 2a-A1Δ were confirmed by sequencing the T7 promoter and the entire 5′UTR sequence.

Transfection and infection of Huh7.5 cells

The human hepatoma cell line Huh7.5 was cultured as previously described (Gottwein et al., 2007; Lindenbach et al., 2005). For transfection, 10 μg of recombinant plasmid DNA was linearized with *Xba*I (New England Biolabs) and treated with Mung Bean Nuclease (New England Biolabs). RNA transcripts were generated from purified DNA using T7 RNA Polymerase (Promega) and used for transfection of Huh7.5 cells plated in 6-well plates ~24 h before transfection using Lipofectamine 2000 (Invitrogen). The transfection complexes were incubated with cells in OptiMEM medium (Invitrogen) for ~16 h, unless otherwise stated. The cells were then sub-cultured every 2–3 days; the supernatant was collected, filtered (0.45 μm) and stored at −80 °C. To passage viruses, naïve Huh7.5 cells at ~80% confluence in T25 flask were incubated with 1 ml cell-free virus-containing transfection-derived supernatant. For comparative growth kinetics studies, naïve Huh7.5 cells were infected with virus with an MOI of 0.003 FFU/cell.

Determination of virus infection and HCV infectivity titers

HCV infections in transfected or infected cultures were detected by immunostaining for HCV NS5A. Infected cells were grown in chamber slides, fixed with acetone (−20 °C) and incubated with primary monoclonal antibody anti-NS5A, 9E10 (Lindenbach et al., 2005), at 1:250 dilution in PBS with 1% BSA. The secondary antibody was Alexa Fluor 594 goat anti-mouse IgG (H+L) (Invitrogen) diluted in PBS with 0.1% Tween-20 at 1:500. Hoechst 33342 (Invitrogen) was used at a 1:10,000 dilution for staining of cell nuclei. The percentage of HCV infected cells was examined by confocal microscopy and used to follow the status of HCV infection in the culture.

For titration of the virus infectivity, 6×10^3 naïve Huh7.5 cells per well were grown in polylysine coated Nunc 96 Well™ Optical Bottom Plate (Nunc, Denmark) for 24 h in 200 μL complete growth medium. The cells were then infected with 100 μL of 2-fold or higher serial dilution of the virus-containing supernatants in triplicate for each dilution and incubated for 48 h. The cells were fixed with methanol (−20 °C), and HCV infected cells were detected by immunostaining for NS5A. The primary antibody anti-NS5A 9E10 (Lindenbach et al., 2005), was used at a 1:1000 dilution in PBS with 0.1% Tween-20; the secondary antibody ECL horseradish-peroxidase-linked anti-mouse immunoglobulin G (GE Healthcare) was diluted 1:300 in PBS with 0.1% Tween-20. Horseradish-peroxidase substrate (DAB Substrate Kit, Dako) was used for focus forming unit (FFU) stainings. The number of FFU was manually counted using a light microscope or automatically counted by an ImmunoSpot Series 5 UV Analyzer with customized software (CTL Europe GmbH) (Gottwein et al., 2010). In automated counting, the mean FFU count of 3–6 negative wells was always below 15 and was subtracted from FFU counts in each experimental well. Detection cutoff was set to the mean FFU count of negative control wells plus 3 standard deviations (SD) plus 3. Counts of up to 200 FFU/well were in the linear range of test dilution series and comparable to manual counts. The viral infectivity titer is presented as FFU/mL of supernatant averaged from three independent dilutions.

Determination of HCV RNA titers

HCV RNA was purified from 200 μl cell-free supernatant using the Total Nucleic Acid Isolation Kit (Roche Applied Science). HCV RNA titers were determined using real time RT-PCR TaqMan assay as previously described (Gottwein et al., 2007) and are given as international units per milliliter (IU/mL) of supernatant.

Sequence analysis of the ORF of recovered HCV

Virus RNA extracted from first passage supernatant was subjected to nested RT-PCR followed by direct sequencing of twelve overlapping second round PCR products as previously described (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008; Yanagi et al., 1997) or direct sequencing of four overlapping second round PCR products. To generate four overlapping PCR products the reverse transcription of HCV RNA was performed as previously described (Gottwein et al., 2007, 2009; Jensen et al., 2008; Scheel et al., 2008; Yanagi et al., 1997), the first round PCR was done using forward primers 5′UTRF40, 5′-CTCCCCTGTGAGGAACTACTGTCTTCACGC-3′, specific to 5′UTR of genotypes 1a, 1b, 2a, 2b, 4a, 5a and 6a, or primer 3a5′UTRF38, 5′-CTCCCCTGTGAGGAACTTCTGTCTTCACGC-3′, for genotype 3a 5′UTR and all with previously described reverse primer 9470R(24)_JFH1, 5′-CTATGGAGTGTACCTAGTGTGTGC-3′ (Gottwein et al., 2007). The second round PCR generating four overlapping amplicons was done using primers 5′UTRF74, 5′-GCGTCTAGCCATGGCGTTAGTATGAGTGT-3′, specific to the 5′UTR of genotypes 1a, 1b, 2a, 2b, 4a, 5a and 6a, or 5'UTRF74_3a, 5′-GCGCCTAGCCATGGCGTTAGTACGAGTGT-3′, specific to the 3a 5′UTR, and reverse primer JR3952, 5′-AAGTGGGAGACCTTGTAACAACGTCGAGT-3′, for amplicon one; primers JF3703, 5′-CAAGTCTTTGGAGCCGTGCAAGTGT-3′, and JR7356, 5′-AGTTGCTGGAGGGCTTCTGATATGG-3′, for amplicon two; previously described primers 6862S_JFH1, 5′-TGGGCACGGCCTGACTACAA-3′, and 7848R_JFH1, 5′- GGCCATTTTCTCGCAGACCCGGAC-3′, for amplicon three (Gottwein et al., 2007); primer 7741S_J6 5′-ATGGCCAAAAATGAGGTGTTCTGC-3′ (Gottwein et al., 2007), and JR9446 5′-CAGTTAGCTATGGAGTGTACCTAGTGTGTGCC-3′, for amplicon four.

Rapid amplification of 5′ complementary DNA ends (5′RACE)

The 5′RACE System for Rapid Amplification of cDNA Ends (Invitrogen) with dA or dC tailing technology was used to determine the 5′UTR and 3′UTR sequences of recovered HCV as previously described (Li et al., 2011). To determine the 5′UTR of the virus, 200 μl virus-containing culture supernatant was used for RNA extraction using TRIzol LS Reagent (Invitrogen). The 5′RACE with dA-tailing procedure was performed on the HCV RNA positive strand. The second round PCR products were sequenced directly or cloned into pCR2.1-TOPO (Invitrogen) for sequencing. For sequence analysis of the 3′UTR, total RNA was extracted from HCV infected cells using TRIzol Reagent (Invitrogen). The 5′RACE with dC tailing technology was conducted on the HCV RNA negative strand. The PCR products were purified and cloned into pCR2.1-TOPO (Invitrogen) for sequencing. The consensus sequence was considered to reflect the 3′UTR sequence of recovered HCV.

To determine the 5′UTR of RNA transcripts, one aliquot of RNA transcription products used for transfection was purified by RNeasy Mini Kit (Qiagen), reverse transcribed, and the cDNA was tailed with dA for the transcripts with A, G, C, and U at the 5′-terminus, and in addition with dC for the transcripts with U at the 5′-terminus. Second round PCR products were directly sequenced to determine the entire 5′UTR sequence; in case of unclear direct sequencing reads, the PCR products were cloned for sequence analysis to determine the 5′UTR sequence of the RNA transcripts.

LNA transfection and virus infection

Transfection of Huh7.5 cells with miR-122 antisense LNA and subsequent determination of virus infection were performed as previously described (Li et al., 2011).

Interferon treatment

Huh7.5 cells were seeded in 96-well Nunc 96 Well™ Optical Bottom plates (Nunc) at a density of 5×10^3 cells/well in 200 µl growth medium. After 24 h, the cells were infected with 190 µl virus dilutions that had been pre-warmed at 37 °C for 1 h, and incubated for 24 h. The cultures were treated with 190 µl growth medium containing IFN-α2b at specified concentrations 24 and 48 h after infection. After another 24 h the cultures were fixed with methanol (−20 °C). The number of single infected cells was visualized by immunostaining for HCV NS5A (see above) and counted by ImmunoSpot Series 5 UV Analyzer with customized software (Scheel et al., 2011b).

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