Supporting Information

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SI Materials and Methods

Construction of JFH1-Based HCV Genotypes 1–6 5′ UTR-NS2 Recombinants and Derived Mutants. The JFH1 5′ UTR of Core-NS2 recombinants pH77C/JFH1$_{V787A,Q1247L}$ (1), pJ4/JFH1$_{F886L,Q1496L}$ (2), pJ6/JFH (3), pJ8/JFH1 (2), pS52/JFH1$_{I793S,K1404Q}$ (2), pED43/JFH1-$\gamma_{T827A,T977S}$ (1), pSA13/JFH1$_{A1022G,K1119R}$ (4), and pHK6a/JFH1$_{F350S,N417T}$ (2) was replaced by strain-specific 5′ UTR to obtain 5′ UTR-NS2 recombinants pH77C$^{5'UTR\text{-}NS2}$/JFH1$_{V787A,Q1247L}$ (genotype 1a), pJ4$^{5'UTR\text{-}NS2}$/JFH1$_{F886L,Q1496L}$ (1b), pJ6$^{5'UTR\text{-}NS2}$/JFH1 (2a), pJ8$^{5'UTR\text{-}NS2}$/JFH1 (2b), pS52$^{5'UTR\text{-}NS2}$/JFH1$_{I793S,K1404Q}$ (3a), pED43$^{5'UTR\text{-}NS2}$/JFH1$_{T827A,T977S}$ (4a), pSA13$^{5'UTR\text{-}NS2}$/JFH1$_{A1022G,K1119R}$ (5a), and pHK6a$^{5'UTR\text{-}NS2}$/JFH1$_{F350S,N417T}$ (6a), respectively (Fig. 1). For recombinants with deletions, insertions, or point mutations, sequences were synthesized (GenScript) or amplified by PCR. The T7 promoter was added immediately upstream of the 5′ UTR to enable in vitro transcription. In cases with A at the 5′ terminus of the 5′ UTR, G was inserted immediately upstream to enhance in vitro transcription, unless otherwise stated. The final maxi-preparation of all constructs was confirmed by sequence analysis, including T7 promoter to the 3′-terminal nucleotide of the HCV genome (Macrogen).

Analysis of the 5′ UTR Sequences of HCV Genotypes 1–6 Prototype Strains. The 5′ UTR of HCV strain H77 (genotype 1a), J6 (2a), S52 (3a), and ED43 (4a) was amplified from full-length clones pCV-H77C (5), pJ6CF (6), pS52 (7), and pED43 (7), respectively. The 5′ UTR of J6, S52, and ED43 was further confirmed by the 5′ RACE procedure (see below) using plasma pools from experimentally infected chimpanzees (8). From the chimpanzee plasma pools, the 5′ UTR of strain J4 (1b), J8 (2b), SA13 (5a), and HK6a (6a) was first amplified by RT-PCR and further confirmed by the 5′ RACE procedure.

Rapid Amplification of 5′ cDNA Ends (5′ RACE). The 5′ RACE system (rapid amplification of cDNA ends; Invitrogen) with dC or dA tailing technology was used to analyze the 5′ UTR and 3′ UTR of HCV. For the 5′ UTR, RNA was extracted from 200 μL of virus-containing culture supernatant or from 200 μL plasma pools of experimentally infected chimpanzees using TRIzol LS Reagent (Invitrogen). The RNA was denatured at 70 °C for 10 min, and cDNA was synthesized at 42 °C for 30 min with SuperScript II RT (Invitrogen) and HCV genotype-specific antisense primers in the core coding region: 1a2a4a5a6a7aR443, 5′-CCCCTGCGCGGCAACAAGTA-3′; 1bR443, 5′-CCCCTGCGCGGCAACAGGTA-3′; 2bR443, 5′-CCCCTGCGCGGCAGCAAGTA-3′; 3aR443, 5′-CCCCTGCGCGGCAACACGTA-3′. After cDNA purification and tailing, the first round PCR was performed with Abridged Anchor Primer (Invitrogen) or Oligo dT-anchor primer (5′/3′ RACE Kit, second generation; Roche) and genotype-specific core antisense primers: 1a2b6aR415, 5′-CCAACGATCTGACCGCCACCC-3′; 1b5aR418, 5′-ACTCCACCAACGATCTGACCACCG-3′; 3aR415, 5′-CCACCAACGATCTGTCCGCCACCC-3′; 4aR415, 5′-ACCAACGATCTGGCCACCACCC-3′; 2aR397, 5′-CCGCCCGGAAACTTAACGTCTTGT-3′, followed by nested PCR with bridged Universal Amplification Primer (Invitrogen) or PCR anchor primer (5′/3′ RACE Kit, second generation; Roche) and individual genotype-specific core antisense primers: 1a1b4aR352, 5′-GTGTTACGTTTGGTTTTTCTTTGAGGTTTAGGA-3′; 2a2b3a5aR352, 5′-GTGTTTCTTTTGGTTTTTCTTTGAGGTTTAGGA-3′; 6aR352, 5′-GTGTTTCTTTTGGTTTTTCTTTGGGGTTTTGGA-3′. The PCR products were directly sequenced or cloned into pCR2.1-TOPO (Invitrogen) for subsequent sequence analysis. To determine the 3′ UTR sequence, total RNA was extracted from cells infected with HCV using TRIzol Reagent (Invitrogen). The 5′ RACE procedure was performed on HCV RNA negative-strand using the 5′ RACE system (Invitrogen) or 5′/3′ RACE Kit, second generation (Roche), following the manufacturer's instructions. JFH1 NS5B-specific primers –JFH1R427, 5′-GCGGTGAAGACCAAGCTCAAACTC-3′, were used for reverse transcription; –JFH1R314, 5′-CGCCCGACCCCGCTCATTACTCTT-3′, for first-round PCR; and –JFH1R294, 5′-TCTTCGGCCTACTCCTACTTTTCGT-3′, for nested PCR. The PCR products were purified and cloned into pCR2.1-TOPO (Invitrogen) and 5–7 clones were subsequently sequenced. The consensus sequence was considered to reflect the 3′ UTR sequence of recovered viruses.

Focus-Forming Units (FFUs) Assay. The FFU assay was performed to determine the infectivity titer of recovered viruses. Naïve Huh7.5 cells were seeded in 96-well Nunc optical bottom plates (6×10^3 cells/well) in 200 μL of complete growth medium for ∼16 h, infected with 100 μL of twofold or higher serial virus dilutions in triplicates, and incubated for 48 h. The cells were fixed with methanol (−20 °C) and stained for HCV-infected cells using anti-HCV NS5A monoclonal antibody 9E10 (a gift from C. M. Rice, The Rockefeller University, New York), as previously described (1–3, 9). The number of FFUs was counted manually under the light microscope (2) or by automated counting with ImmunoSpot Series 5 UV Analyzer with customized software (CTL Europe) (10). For automated counting, the mean FFU counts of 3–6 negative wells were always <15, and this number was subtracted from FFUs in experimental wells. Limit of detection was set to the mean of negative wells + 3 SDs + 3. Counts of up to 200 FFUs/well were in the linear range of test dilution series and comparable to manual counts.

Biological Software. Sequencher (Gene Codes), Vector NTI (Invitrogen), and free software GENEDOC were used for sequence analysis, alignments, and analysis of restriction sites. Web-based software Mfold was used to analyze RNA secondary structure (11). HCV sequences were retrieved from the Los Alamos HCV Sequences Database and the European HCV Database.

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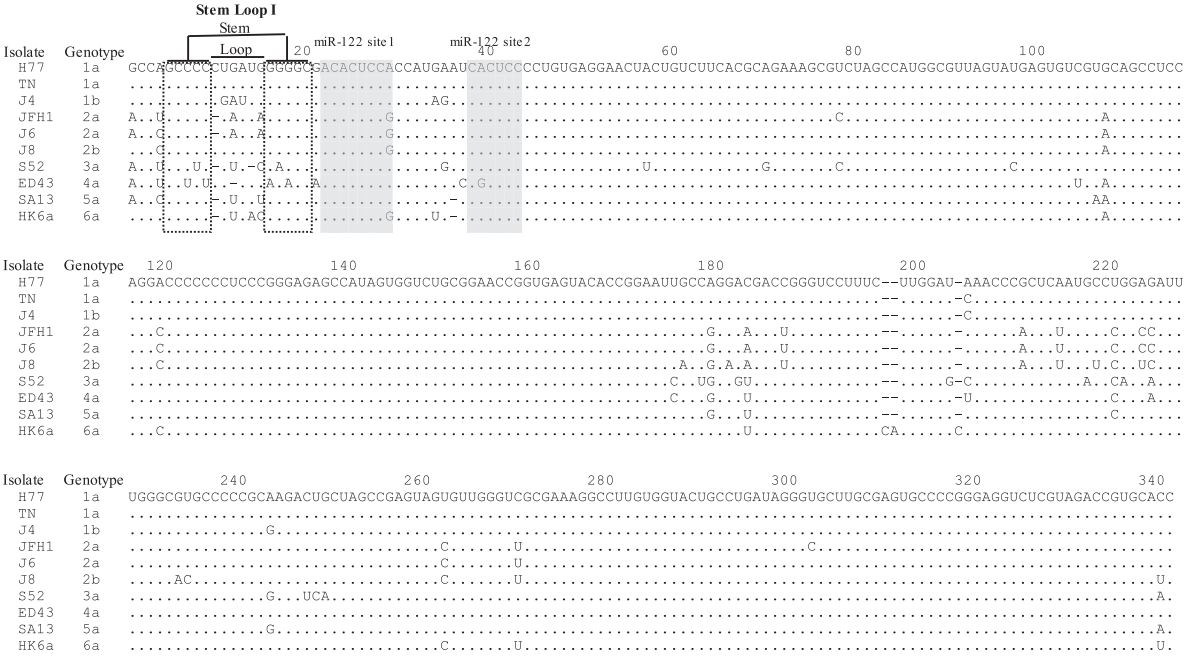

Fig. S1. Determination of the entire 5′ UTR sequence of HCV prototype isolates of genotypes 1–6 using plasma pools from experimentally infected chimpanzees. HCV RNA of prototype genotypes 1b, 2a, 2b, 3a, 4a, 5a, and 6a HCV strains was extracted from infected chimpanzee plasma pools generated previously (1), and the 5′ UTR sequences were determined by 5′ RACE procedure (*SI Materials and Methods*). The 5′ UTR sequences are aligned to the H77 isolate; the position numbering is according to the H77 reference sequence (GenBank accession no. AF009606) (2). A dot indicates that the nucleotide is identical to H77; a dash indicates a gap. The nucleotides forming the SLI structure is indicated, and the miR-122 binding sites are shaded. The 5′ UTR of HCV isolates TN (genotype 1a; EF621489) (3) and JFH1 (genotype 2a; AB047639) (4) were included for comparison. We recently reported the 5′ UTR sequences of the 3a and 4a strains (5).

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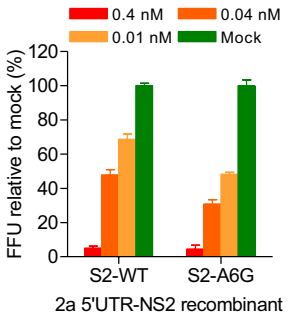

Fig. S2. MiR-122 antagonism efficiently suppresses the infection of HCV recombinant virus with mutation in the miR-122 binding site 2 (S2). Huh7.5 cells were transfected with LNA SPC3649 at 0.4, 0.04, and 0.01 nM, and infected with 2a 5′ UTR-NS2 recombinant viruses with wild-type S2 (S2-WT) and mutated S2 nucleotide [A to G at position 6 (S2-A6G)]. The number of FFU was determined 48 h postinfection and presented as a percentage relative to respective infection in SPC3649-free mock-transfected controls (100%). The U3 insertion mutant Cell-U3 was included in parallel in the experiment and showed 94% infection (Fig. 6). Mean of triplicates ± SEM is shown.

Table S1. HCV infectivity and HCV RNA titers of JFH1-based recombinants with HCV genotypes 1–6 specific 5′ UTR-NS2

					Infectivity titer		HCV RNA titer		Specific infectivity	
5′ UTR	Core-NS2	Recombinant	Day p.i.	Infected cells, %	EC $\log_{10}$, FFU/mL	IC $\log_{10}$, FFU/10^5 cells	EC $\log_{10}$, IU/mL	IC $\log_{10}$, IU/10^5 cells	EC FFU/IU	IC FFU/IU
JFH1	1a	H77C/JFH1$_{V787A,Q1247L}$	9	90	4.0	2.5	7.7	7.0	1/5,000	1/31,600
1a	1a	H77$^{5'UTR-NS2}$/JFH1$_{V787A,Q1247L}$	9	90	4.1	2.6	7.9	7.5	1/6,300	1/79,400
JFH1	1b	J4/JFH1$_{F886L,Q1496L}$	13	90	3.5	2.4	7.4	7.0	1/7,900	1/39,800
1b	1b	J4$^{5'UTR-NS2}$/JFH1$_{F886L,Q1496L}$	13	80	3.0	2.0	7.3	7.1	1/20,000	1/125,900
JFH1	2a	J6/JFH	7	95	4.6	2.4	8.2	7.6	1/4,000	1/158,500
2a	2a	J6$^{5'UTR-NS2}$/JFH1	7	90	4.6	2.4	7.7	7.5	1/1,300	1/125,900
JFH1	2b	J8/JFH1	9	80	3.4	2.4	7.2	7.2	1/6,300	1/63,100
2b	2b	J8$^{5'UTR-NS2}$/JFH1	9	80	3.5	2.4	7.4	7.1	1/7,900	1/50,100
JFH1	3a	S52/JFH1$_{I793S,K1404Q}$	7	90	4.6	2.4	8.4	7.4	1/6,300	1/100,000
3a	3a	S52$^{5'UTR-NS2}$/JFH1$_{I793S,K1404Q}$	7	95	4.6	2.6	8.4	7.9	1/6,300	1/199,500
JFH1	4a	ED43/JFH1$_{T827A,T977S}$	11	80	3.5	2.1	7.6	6.7	1/126,000	1/39,800
4a	4a	ED43$^{5'UTR-NS2}$/JFH1$_{T827A,T977S}$	11	80	3.3	1.7	7.3	6.9	1/10,000	1/158,500
JFH1	5a	SA13/JFH1$_{A1022G,K1119R}$	9	90	5.1	3.5	7.7	8.3	1/400	1/63,100
5a	5a	SA13$^{5'UTR-NS2}$/JFH1$_{A1022G,K1119R}$	9	90	4.9	3.3	7.9	8.3	1/1,000	1/100,000
JFH1	6a	HK6a/JFH1$_{F350S,N417T}$	7	80	4.2	2.7	7.6	7.2	1,2500	1/31,600
6a	6a	HK6a$^{5'UTR-NS2}$/JFH1$_{F350S,N417T}$	7	80	4.0	2.8	7.3	7.1	1/2,000	1/20,000

In comparative growth kinetics studies, Huh7.5 cells were infected with JFH1-based genotypes 1–6 5′ UTR-NS2 recombinants with an MOI of 0.003 FFU/cell (Fig. 1*B*). Peak HCV infectivity and HCV RNA titers were determined for infection supernatants [extracellular titer (EC)] as well as for cells [intracellular titer (IC)]. The specific infectivity was calculated by the ratio of HCV infectivity/HCV RNA titers (FFU/IU). The respective virus with JFH1 5′ UTR (genotype 2a) was included for comparison. IU, international units; p.i., postinfection.

Table S2. Characterization of JFH1-based HCV recombinants containing genotypes 1–6 specific 5′ UTR-NS2

		Transfection			First passage				Mutation	
5′ UTR-NS2	Recombinant	Day	Infected cells, %	Infectivity titer, $\log_{10}$, FFU/mL	MOI	Day	Infected cells, %	Infectivity titer, $\log_{10}$, FFU/mL	5′ UTR	Core-3′ UTR*
1a	H77$^{5'UTR-NS2}$/JFH1$_{V787A,Q1247L}$	5	90	3.5	0.01	10	90	4.0	G1A	None
1b	J4$^{5'UTR-NS2}$/JFH1$_{F886L,Q1496L}$	8	90	2.5	0.002	15	90	3.0	G1A	None
2a	J6$^{5'UTR-NS2}$/JFH1	8	90	4.5	0.006	5	80	4.8	None	None
2b	J8$^{5'UTR-NS2}$/JFH1	7	90	3.9	n.d.	10	90	4.2	G1A	None
3a	S52$^{5'UTR-NS2}$/JFH1$_{I793S,K1404Q}$	8	90	4.5	0.003	7	90	4.5	G1A	None
4a	ED43$^{5'UTR-NS2}$/JFH1$_{T827A,T977S}$	5	90	3.2	0.015	11	80	3.5	None	None
5a	SA13$^{5'UTR-NS2}$/JFH1$_{A1022G,K1119R}$	5	90	4.8	0.06	5	90	4.9	None	None
6a	HK6a$^{5'UTR-NS2}$/JFH1$_{F350S,N417T}$	7	90	4.0	n.d.	8	90	4.5	G1A	None

The culture supernatant from peak infection of the transfection experiment was passaged to naïve Huh7.5 cells, and the supernatant at peak infection was collected and subjected to sequence analysis of the entire HCV genome, including the 5′ UTR (by 5′ RACE on positive-strand HCV RNA extracted from infection supernatant), ORF (by direct sequencing of second round PCR products of 12 overlapping fragments from viral genomes recovered from infection supernatant), and the 3′ UTR (by 5′ RACE on negative-strand HCV RNA recovered from cells). The results showed that in recovered first-passage virus, the 5′-terminal nucleotide (nt) G of the 5′ UTR of genotypes 1a, 1b, 2b, 3a, and 6a was changed to A [3a (strain S52) 5′ UTR from chimpanzee plasma pool had A at the 5′ terminus]; the nucleotide G, inserted immediately upstream of the 5′-terminal A of the 5′ UTR of 2a, 4a, and 5a (to enhance in vitro transcription) was deleted, and the 5′-terminal A was maintained. MOI, multiplicity of infection expressed as FFU per cell; n.d., MOI was not determined; one milliliter of transfection supernatant was used for infection.

*No mutation was acquired in the ORF; no consensus change was observed in the 3′ UTR; however, the length of the poly(U/UC) tract differed among the various clones. In other independent experiment(s), mutations were identified in the ORF of the 5′UTR-NS2 recombinants of genotype 2a, 2b, and 6a. In the 2a recombinant J6$^{5'UTR-NS2}$/JFH1, mutations were in NS2 [C3015C/T (I892T) and T3149T/C (Y937H)] and NS3 (C3613C/T). In the 2b recombinant J8$^{5'UTR-NS2}$/JFH1, mutations were in NS2 [C3397C/T (A1019V)] and NS3 [A3690A/T (S1117C) and T5207T/A]. In the 6a recombinant HK6a$^{5'UTR-NS2}$/JFH1$_{F350S,N417T}$, mutations were in E2 [A1570A/G (N410D) and G2542G/T (D734Y)], NS2 [T3017T/C (F892S)], and NS5A [A6424G/a (T2028A), G6865A/g (D2175N), T7024T/C (Y2228H), and T7157T/C (I2272T)]. Two capital letters separated by a slash indicate the presence of 50/50 quasispecies; a capital letter separated by a slash from a lowercase letter indicates quasispecies with a predominant vs. a minor nucleotide. The position corresponds to the respective genome; resulting amino acid changes are shown in parentheses.

Table S3. Identification of viable HCV with insertion of host cellular or downstream viral RNA sequences in 5′ UTR domain I

	Transfection			First passage							
5′ UTR-NS2 recombinant	NS5A detected, day	Peak infection day	Infected cells, %	Peak infection day	Infected cells, %	Infectivity titer, log_{10}, FFU/mL	RNA titer, log_{10}, IU/mL	Clone*	Point mutation in 5′ UTR†	Sequence of 5′ UTR domain I of recovered virus	Description
1aWT	1	3	90	10	90	4.0	7.9		G1A	ACCAGCCCCCUGAUGGGGGCGAC ACUCCACCAUGAAUCACUCC	
1aΔLoop	5	19	90	3	90	4.0	7.2	4/4	G1A	ACCAGCCCCGGGGGCGCCAG CCCCGGGGGCGACACUCCACC AUGAAUCACUCC	Repeat from loop-deleted SLI of 1a 5′ UTR
1bWT	1	5	90	13	80	3.2	7.2		G1A	ACCAGCCCCCGAUUGGGGGCGACACU CCACCAUAGAUCACUCC	
1bΔStem	19	42	90	9	80	3.2	7.5		G1A	ACCCAUGUCACGAACGACUGC UCCAACUCAAGCAUUGUGUA UGAGGCAGCGGACGUGAUCAU GACACUCCACCAUAGAUCACUCC‡	Repeat from HCV 1b E1 sequence
1bΔLoop	12	43	90	15	80	3.4	7.6	3/3	G1A	ACCAGCCCCGGGGCGCCAGCCC CGGGGCGACACUCCACCAUAGAUC ACUCC	Repeat from loop-deleted SLI of 1b 5′ UTR
2aWT	1	3	90	6	90	4.8	7.8		None	ACCCGCCCCUAAUAGGGGCG ACACUCCGCCAUGAAUCACUCC	
2aΔSLI	23	56	90	7	80	3.9	7.3	1/4	None	ACCC**GG**CACUCCGCCAUGAAU UCACGCAGAAAGCGUCUAGCCA UGGCGUUAG**CCGA**CACUCCG CCAUGAAUCACUCC‡,§	Repeat from domain I and II of HCV 2a 5′ UTR
								1/4	None	ACC**GG**CACUCCGCCAUGAAUCU UCACGCAGAAAGCGUCUAGCCA UGGCGUUAG**CCGA**CACUCCG CCAUGAAUCACUCC	Repeat from domain I and II of HCV 2a 5′ UTR
								1/4	None	ACCC**GG**CACUCCGCCAUGAAU AGCCAUGGCGUUAG**CCGA**CAC UCCGCCAUGAAUCACUCC	Repeat from domain I and II of HCV 2a 5′ UTR
								1/4	None	ACCC**GG**CACUCCGCCAUGAAU CUAGCCAUGGCGUUAG**CCGA** CACUCCGCCAUGAAUCACUCC	Repeat from domain I and II of HCV 2a 5′ UTR
2aΔSLI (nt7–17)	10	22	90	8	90	3.5	7.0		C4U/C	ACC(C/U) GCGCAAGGAUGACACGCAAAUU CGUGAAGCGUUCCAUAUUUUU **GC**GCGACACUCCGCCAU GAAUCACUCC‡	Insertion from host noncoding RNA U6 snRNA (NR_002752)
2aΔStem	3	28	90	7	80	3.4	7.0		C4-, A46U	ACCUAAUAGACACUCCGCCAU GAAUCACUCC	
3aWT	1	5	90	8	90	4.6	7.7		G1A	ACCUGCCUCUUACGAGGCGACACUCC ACCAUGGAUCACUCC	

Table S3. Cont.

5′ UTR-NS2 recombinant	Transfection: NS5A detected, day	Transfection: Peak infection day	Transfection: Infected cells, %	First passage: Peak infection day	First passage: Infected cells, %	First passage: Infectivity titer, log_{10}, FFU/mL	First passage: RNA titer, log_{10}, IU/mL	First passage: Clone*	First passage: Point mutation in 5′ UTR†	First passage: Sequence of 5′ UTR domain I of recovered virus	First passage: Description
3aΔStem	12	44	90	5	90	4.2	7.0	4/4	G1A	ACUGAGGUCCUUUAAGGACCGU ACACUCCACCAUGGAUCACUCC‡	Insertion from the 5′ UTR of ATPase family, AAA domain-containing 1 (ATAD1; NM_032810)
3aΔLoop	1	12	90	7	80	3.8	7.4	1/3	G1A	ACCUGCCUCGAGGCGACACU CCACCAUGGAUCACUCC	
								1/3	G1A	ACCUGCCUCGA**A**GGCGACACU CCACCAUGGAUCACUCC	One nucleotide insertion
								1/3	None	GCCUGCCUCGAGGCGA**UAGAG GCGA**CACUCCACCAUGGA UCACUCC	Short insertion of unknown origin
5aWT	1	4	90	6	90	5.0	8.1		None	ACCCGCCCCUUAUUGGGGCGACACU CCACCAUGAUCACUCC	
5aΔSLI	17	61	80	11	80	2.8	7.6		G226A	ACCUAUACUUUCAGGGAUCAUUUC UAUAGUGUGUUACUAGAGAAGUU UCUCUGAACGUGUAGAGCACCGAU UGAUCACUCC‡	Insertion from host noncoding U3 snoRNA (NR_006881)
5aΔLoop	17	43	90	8	90	4.7	7.8		C9U, G10A	ACCCGCCCUAGGGCGACACUCCA CCAUGAUCACUCC	

The RNAs from JFH1-based 5′ UTR-NS2 recombinants of HCV genotypes 1–6 with deletions of entire SLI (ΔSLI) or partial SLI [ΔSLI(nt7–17)], 5-bp stem nucleotides (ΔStem) or loop nucleotides (ΔLoop; Fig. 2) were transfected into Huh7.5 cells. In the 4aΔloop recombinant, only nucleotides 10–14 were deleted to avoid generating an additional XbaI site (Fig. 2). Transfection supernatant was collected at peak infection and passaged to naïve Huh7.5 cells. The domain I sequence of recovered viruses is shown. Host cellular RNA insertion sequences are shaded; sequences originating from domain I of the 5′ UTR are underlined; sequences from other regions of the HCV genome are boxed. Inserted sequences (or nucleotides) of unknown origin are shown in bold. The domain I sequence of recombinant with WT 5′ UTR recovered from infection culture was included for comparison. Two capital letters separated by a slash indicates nucleotide quasispecies (50/50). A dash indicates a deletion.

*Clones with sequence identified/total clones; otherwise, the sequence was determined by direct sequence analysis of second round PCR product from the 5′ RACE procedure.

†G inserted immediately upstream of the 5′-terminal A of the 5′ UTR of 2a and 5a recombinants was deleted.

‡The inserted sequence was studied by reverse genetics (Fig. 3).

§The EcoRI site (GGATTC) in the insertion sequence was modified to GGATCC in the reverse genetic studies (Fig. 3).

Table S4. Consensus sequence analysis of Core-3′ UTR of first passage HCV 5′ UTR-NS2 recombinants with deletions in 5′ UTR SLI

HCV genome	C	C	E1	E1	E2	E2	E2	E2	p7	NS3	NS3	NS3	NS3	NS3	NS3	NS3	NS3	NS3	NS3	NS3
Nucleotide position																				
H77 reference (AF009606)	731	773	938	1416	1491	1960	2447	2456	2656	3978	4004	4370	4976	5035	5077	5099	5102	5150	5179	5210
SLI deletion recombinant																				
Position	719	761	920	1399	1479	1954	2443	2450	2652	3964	4000	4356	4976	5029	5067	5093	5096	5150	5175	5200
Nucleotide	T	A	A	G	G	A	T	A	T	A	G	A	G	T	C	A	A	C	G	T
Virus																				
1aΔLoop						A/C		G						T/G						
1bΔStem															G					T/C
1bΔLoop*																A/G	A/G			
2aΔSLI*			A/G						T/C		G/a								G/A	
2aΔStem																				
2aΔSLI (nt7-17)†	T/C												G/A					T/c		
3aΔStem		G/a			G/A															
3aΔLoop																				
5aΔSLI*				T						T		G								
5aΔLoop							G/t													
Amino acid position																				
H77 reference (AF009606)	130	144	199	359	384	540	702	705	772	1213	1221	1343	1549	1565	1579	1586	1587	1603	1613	1623
SLI deletion recombinant	130	144	199	359	384	540	703	705	776	1214	1225	1344	1549	1565	1579	1586	1587	1607	1617	1623
Change	•	•	T > A	A > S	E > K	N > T	•	•	F > S	N > Y	•	•	•	L > R	A > G	•	•	•	R > Q	•

Table S4. Cont.

HCV genome	NS4A	NS4A	NS4B	NS4B	NS4B	NS5A	NS5A	NS5A	NS5A	NS5A	NS5A	NS5A	NS5A	NS5A	NS5A	NS5B	NS5B	NS5B	NS5B
Nucleotide position																			
H77 reference (AF009606)	5355	5420	5576	5589	5984	6488	6653	6740	6934	7138	7339	7347	7376	7380	7491	7974	8243	8561	9224
SLI deletion recombinant																			
Position	5361	5407	5572	5585	5980	6482	6639	6746	6934	7122	7323	7341	7350	7369	7487	8024	8293	8605	9274
Nucleotide	G	G	C	A	T	T	A	T	A	T	T	A	G	C	T	G	C	A	G
Virus																			
1aΔLoop						T/C													
1bΔStem																		A/G	
1bΔLoop*																			
2aΔSLI*			C/T								T/C				T/C	A			G/a
2aΔStem														C/T					
2aΔSLI (nt7-17)†									A/T										
3aΔStem	G/T							T/C				A/G							
3aΔLoop																			
5aΔSLI*		G/A					T						G/A						
5aΔLoop				A/G	T/C					T/C							C/T		
Amino acid position																			
H77 reference (AF009606)	1672	1693	1745	1750	1881	2049	2104	2133	2198	2266	2333	2336	2345	2347	2384	2545	2634	2740	2961
SLI deletion recombinant	1678	1695	1749	1751	1882	2049	2105	2139	2202	2263	2333	2338	2342	2347	2388	2567	2653	2758	2983
Change	A > S	V > I	•	M > V	•	•	•	•	E > V	L > P	•	S > G	•	•		D > N	•	•	•

First-passage virus of viable JFH1-based 5′ UTR-NS2 recombinants of HCV genotypes 1a, 1b, 2a, 3a, and 5a with deletions in SLI of the 5′UTR (Fig. 2) were subjected to sequence analysis. For Core-NS5B, 12 overlapping RT-PCR products were directly sequenced. Nucleotide (nt) and amino acid positions of the original constructs are listed; the corresponding position of H77 reference sequence (AF009606) is given. Two capital letters separated by a slash indicate positions with nucleotide quasispecies (50/50); a capital letter separated by a slash from a lowercase letter indicates quasispecies with a predominant vs. a minor nucleotide. Filled circle (•) indicates no amino acid change. The 3′ UTR sequence was determined by sequencing the clones of second-round PCR products from the 5′RACE procedure on negative-strand HCV RNA from cells. No consensus change was observed; however, the length of the poly(U/UC) tract varied among clones. The analyzed 5′UTR sequences of these viruses are shown in Table S3.

*HCV infection was only detected in one of two transfection experiments.

†The 3′ UTR sequence was not determined.

Table S5. Mutagenesis study of the HCV 5′ UTR S1

Virus	S1 sequence, nucleotides from 8 to 1	Transfection		Recovered virus			
		Infected cells at day 1, %	Infected cells of ≥80%, day	Experiment (day)	S1 sequence	Peak infectivity titer, log_{10}, FFU/mL	HCV RNA titer, log_{10}, IU/mL
Wild type	ACACUCCG	80	3	First passage (7)	ACACUCCG	4.3	8.2
mA8G	GCACUCCG	1	9	First passage (14)	ACACUCCG	4.4	7.9
mC7G	AGACUCCG	<1	28	Transfection (28)	ACACUCCG[†]	4.2	8.4
mA6G	ACGCUCCG	20	6	First passage (7)	ACACUCCG	4.2	8.3
mC5U	ACAUUCCG	20	5	First passage (10)	ACACUCCG	4.3	7.8
mU4C	ACACCCCG	40	3	First passage (7)	ACACCCCG	3.6	7.9
mC3G	ACACUGCG	30	3	First passage (7)	ACACUGCG	3.3	7.5
mC2A	ACACUCAG	30	3	First passage (7)	ACACUCAG	3.4	7.7
mG1U	ACACUCCU	80	3	First passage (5)	ACACUCCU	4.5	n.d.
M43	ACACCGCG	10	6	First passage (11)	ACACCGCG	3.3	7.0
M42	ACACCCAG	5	6	First passage (11)	ACACCCAG	3.4	7.3
M32	ACACUGAG	5	6	First passage (11)	ACACUGAG	3.7	7.2
M432	ACACCGAG	40	3	First passage (7)	ACACCGAG	3.5	7.7
M84321	GCACCGAU	<1	16	First passage (8)	ACACCGAU[‡]	3.8	7.8
M84321+CCA*	GCACCGAU	1	14	First passage (8)	GCACCGAU[§]	3.6	7.5

The nucleotides of S1 of the JFH1-based 2a 5′ UTR-NS2 recombinant were substituted singly or in combination; mutated nucleotides are underlined. For all constructs tested, G was not inserted immediately upstream of 5′-terminal A of the 2a 5′ UTR; all had efficient in vitro transcription. n.d., not determined.

*Downstream CCA of S1 were deleted.

[†]G at position 20 of this recovered virus genome was changed to U.

[‡]Recovered virus was designated as M4321 and used in SPC3649 treatment experiment (Fig. 6).

[§]5′-terminal A was changed to AC and AU (one clone for each), and the C at position 4 was changed to U (two clones; see Fig. 2 for domain I sequences).