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Replication efficiency of chimeric replicon containing NS5A–5B genes derived from HCV-infected patient sera

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Abstract

A transient subgenomic replicon-based shuttle vector system has been developed to investigate how genetic heterogeneity affects HCV replication efficiency. Individual NS5A or NS5B genes or cassettes containing both NS5A and NS5B genes were amplified from "quasispecies" pools derived from HCV genotype 1a or 1b patient sera using RT-PCR and cloned into their respective shuttle vectors. All shuttle vectors containing NS5A or NS5A–5B genes were constructed with the S2204I "adaptive" mutation because replicons lacking the S2204I mutation replicated poorly. Gene sequences of the quasispecies pools within either genotype 1a or 1b patient samples ranged from 94 to 95% in identity. The replication capacity of 1b shuttle vectors containing patient-derived NS5A or NS5B genes averaged 67 and 75%, respectively, relative to the laboratory-optimized 1b replicon. In contrast, the replication efficiencies of both 1a and 1b shuttle vectors containing patient-derived NS5A–5B gene cassettes averaged around 2% relative to the respective laboratory-optimized replicon. All patient-derived replicons were tested in a transient assay for their sensitivity to either interferon-α (IFN-α) or to the polymerase inhibitor A-782759. Despite the differences in replication efficiency, IC_{50} values measured for most of the patient-derived replicons were equivalent to the respective values measured in the control laboratory strain replicons. These results demonstrate that patient sequence heterogeneity affects replication efficiency whenever patient-derived NS5A–5B genes are inserted into the laboratory-optimized replicon. The findings also demonstrate the utility of the shuttle vector system to test patient-derived gene sequences for sensitivity to IFN-α and to small molecule inhibitors.
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Keywords: HCV; NS5A; NS5B; Sub-genomic replicon; Quasispecies pools; Transient assay

1. Introduction

Hepatitis C virus (HCV) infection is estimated to afflict more than 170 million people worldwide. Chronic HCV infection is established in a majority of the cases but a small number of infected individuals resolve the initial infection. Chronic HCV infection can remain asymptomatic, however it can also result in serious long-term liver damage including fibrosis, cirrhosis and heptocellular carcinoma (Seeff, 1999; Lauer and Walker, 2001; Seeff, 2002; Hoofnagle, 2002). Pegylated interferon-alpha (IFN-α) in combination with ribavirin remains the most effective treatment option. These drugs, however, have potentially severe side effects, causing a significant number of genotype 1 patients to withdraw from treatment (Fried, 2002). In addition a significant proportion of patients fail to achieve a sustained virological response with IFN-α/ribavirin treatment (Fried and Hadziyannis, 2004). Poor tolerance of current treatment and low rate of treatment success point to a need for more specific, less toxic and more active antiviral therapies for HCV (Pawlotsky, 2000; Clarke, 2000).

The discovery of new antiviral agents has been hampered by a number of factors, including a lack of a well-established animal model and the lack of a permissive cell culture system allowing in vitro virus propagation. Recently progress has been made in the production of recombinant infectious virus in tissue culture (Lindenbach et al., 2005; Wakita et al., 2005;

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Zhong et al., 2005). However, to date this has been limited to a single genotype 2a isolate. Thus far, replication competent subgenomic replicon systems in which the HCV non-structural NS3–5B genes are expressed have been the driving force behind antiviral development (Lohmann et al., 1999; Blight et al., 2002). These subgenomic replicons rely on cell "adaptive" mutations for propagation and are limited to laboratory-optimized genotypes. HCV however, circulates within an infected host as a heterogeneous viral population containing genetically distinct, but closely related variants, known as quasispecies (Martell et al., 1992; Bukh et al., 1995). The sequence diversity that exists in the quasispecies can be a possible source of drug resistance, which the current subgenomic replicons are unable to address.

Previously this laboratory has developed a genotype-specific replicon-based shuttle vector system for cloning the NS5B gene from patient quasispecies pools and evaluating the clones for their susceptibility to polymerase inhibitors (Middleton et al., 2004). Generating additional genotype-specific replicon-based shuttle vectors for cloning HCV non-structural genes extends the utility of the shuttle vector here. This report investigates how the genetic heterogeneity affects HCV replication efficiency in a replicon-based transient assay and tests HCV-infected patient-derived gene sequences for sensitivity to IFN-α and to a small molecule inhibitor against NS5B.

2. Materials and methods

2.1. Genotype-specific plasmids

Two genotype-specific shuttle vector cassettes (1a and 1b) were designed to permit cloning of NS5A alone, NS5B alone or an NS5A–NS5B gene cassette. Genotype 1a shuttle vector components are depicted in Fig. 1A. The N-terminal 73 amino acids of NS3 were derived from genotype 1b con1 sequence; the remaining NS3–NS5B sequence is derived from 1a strain H77. These 73 amino acid are important for the replication of genotype 1a in Huh-7 cells (Yi and Lemon, 2004; Gu et al., 2003). Amino acid changes at Q1067R, E1202G, K1691R, and S2204I (numbered relative to the amino acid position in the viral

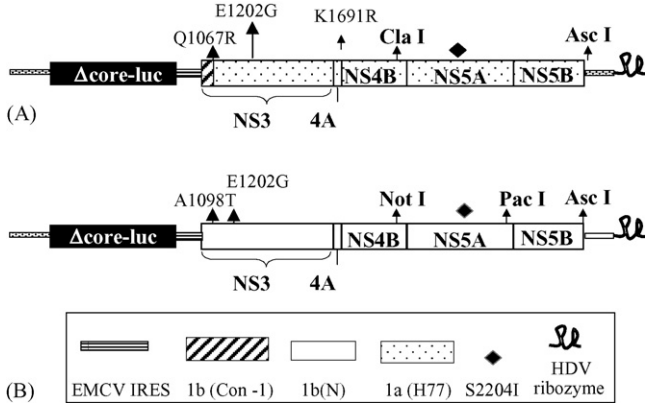

Fig. 1. (A and B) Genotype 1a and 1b genetic organization of the shuttle vector used for cloning NS5A and/or 5B from HCV-infected subjects.

open reading frame) were introduced for efficient replication (Yi and Lemon, 2004). The 5′- and 3′- non-translated regions (NTR) of the 1a shuttle vector were derived from genotype 1a strain H77. The genotype 1b shuttle vector (Fig. 1B), contains non-structural genes NS3 through NS5B from strain N, with adaptive mutations A1098T, E1202G and S2204I (Yi et al., 2002). The 5′ NTR of the 1b shuttle vector was derived from genotype 1a strain H77 whereas the 3′ NTR was derived from genotype 1b strain N. As described previously (Lu et al., 2004), the tat-2A-Neo cassette from the original vector was replaced by the first 12 amino acids of HCV core protein, which was fused to the luciferase gene.

Two unique restrictions sites *Cla* I and *Asc* I were created within the genotype 1a shuttle vector for cloning the NS5A–5B gene cassette. A *Cla* I site was introduced within the NS4B gene, 53 amino acids upstream of the start of NS5A, by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). The modified nucleotide sequence did not change the amino acid coding sequences. An *Asc* I site was inserted directly after the TGA stop codon, giving an insertion of GGCGCGCC at the 5′ end of the 3′ NTR. To prevent contamination of recombinant shuttle vectors with the parental NS5A–5B genes, the fragment between SnaBI and EcoRI within NS5A–5B was removed and end-filled with Klenow before religating the ends to create a deletion within the NS5A gene.

Three unique restriction sites *Not* I, *Pac* I, and *Asc* I were created in the genotype 1b shuttle vector. The *Not* I site was introduced within the NS4B gene, 30 amino acids upstream of the start of NS5A gene by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). The modified nucleotide sequence did not change the amino acid coding sequences. The *Pac* I site was introduced within the NS5A gene, eight amino acids ahead of the start of NS5B gene by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). This resulted in an insertion of the amino acid sequence Leu-Ile-Asn at this position. The *Asc* I site was inserted directly after the TGA stop codon, giving an insertion of GGCGCGCC at the 5′ end of the 3′ non-translated region. Both 1a and 1b shuttle vectors were tested after modification of the nucleotide sequences and no loss of replication efficiency and sensitivity to inhibitors was observed as compared to the unmodified vectors. To prevent contamination of recombinant shuttle vectors with the parental NS5A gene, the fragment between the *Blp* I-*Bsa*B I sites was removed and end-filled with Klenow before religating the ends to create a deletion within the NS5A gene.

2.2. RNA isolation and cDNA synthesis

Serum samples from patients chronically infected with HCV were obtained from the Gastroenterology Clinic of the University of Texas Medical Branch, Galveston, TX. Fig. 2A shows the overall strategy used for RT-PCR. Total RNA was isolated from HCV-containing sera by using the Qiagen Viral RNA kit, according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). The RNA was used as a template for the reverse transcriptase reaction (Superscript III RT; Invitrogen Life Tech-

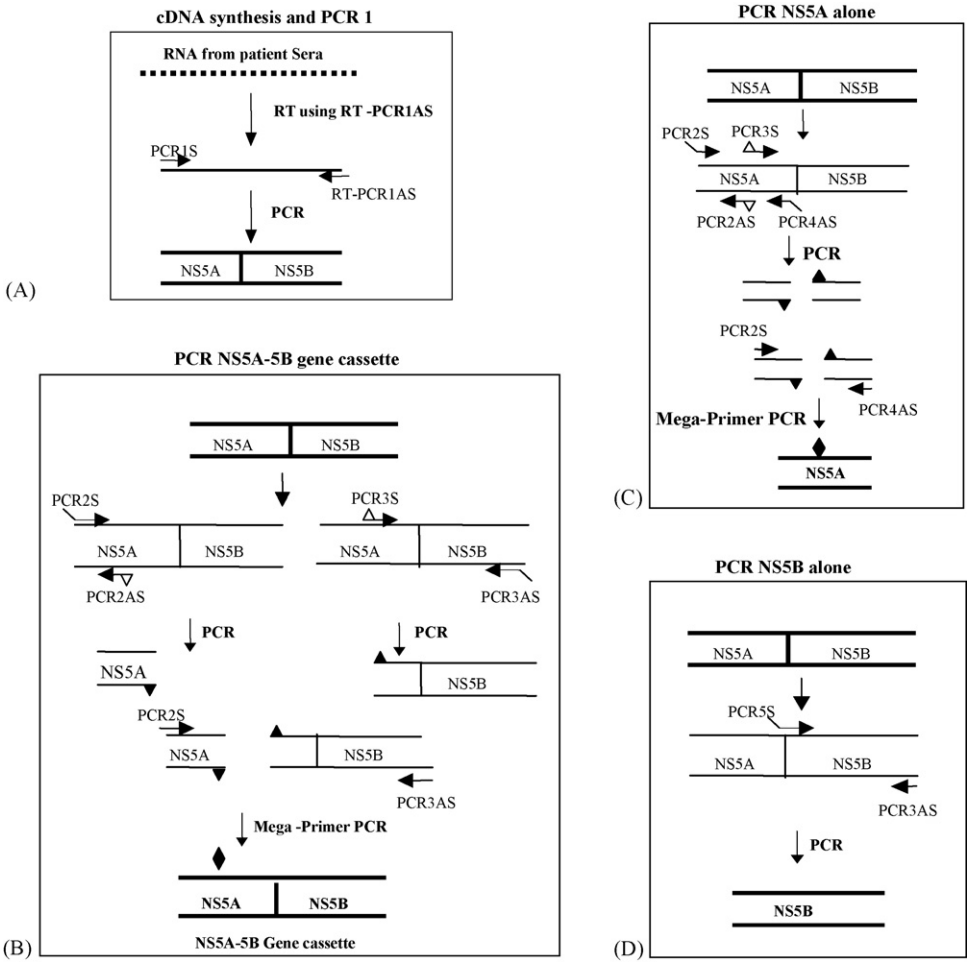

Fig. 2. (A) cDNA synthesis and PCR1. (B and C) Nested PCR combined with mega-primer PCR strategy used to generate NS5A–5B gene cassette containing S2204I using mega-primer PCR and NS5A gene alone. (D) Nested PCR strategy used to generate NS5B alone. See Section 2 for details.

nologies, Carlsbad, CA), which was primed with RT PCR1AS (Table 1) as directed by the manufacturer. Following reverse transcription, the reaction was incubated at 85 °C for 5 min to inactivate the enzyme and then digested with RNase H (Invitrogen Life Technologies) at 37 °C for 20 min to remove RNA prior to PCR.

2.3. NS5A–5B, NS5A and NS5B genes from patient sera

Individual genes were amplified using a nested PCR approach as shown in Fig. 2. Primers used for all PCR reactions are shown in Table 1. Mega-primer PCR was used to introduce S2204I within the NS5A gene during the generation of the

Table 1
Sequence of primers used for cDNA and gene amplification

Primer	Description	Sequence
1b-RT-PCR1AS	RT and outside anti-sense	ATTGGCCTGGAGTGTTTAGCTCCC
1b-PCR1S	Outside sense	GCGTTCGCTTCGCGGGGTAACC
1b-PCR2S	Nested sense	GCCTGAGAGCGACGCGGCCGCGCGTGTCACTCAGGTCCTCTC
1b-PCR2AS	S2204I mutagenic anti-sense	GCGCAGACAACTGGATAGCTGAAGAGCTGGC
1b-PCR3S	S2204I mutagenic sense	GCCAGCTCTTCAGCTATCCAGTTGTCTGCGC
1b-PCR3AS	Nested anti-sense	GCGCATTAGGCGCGCCTCAYCGGTTGIGGAGCARGTAGATGCCTACC
1b-PCR4AS	Nested anti-sense	CCTCTTAATTAACTCCTCGCTCACGGTAGACCAAGACCC
1b-PCR5S	Nested sense	GCATAGGATTAATTAACGCTRGTGAGGACGTCGTCTGCTGCTCRATGTC
1a-RT-PCR1AS	RT and outside anti-sense	TGGCCTAWKAGSCYGGAGTGTTTA
1a-PCR1S	Outside sense	CGTTGGCCCGGGCGAGGGGGC
1a-PCR2S	Nested sense	TGGATGAATCGATTAATAGCCTTCGCCTCCCGGG
1a-PCR2AS	S2204I mutagenic anti-sense	GCAAGTTGCCTTGAGAGATGGAGCGGACAGCTGGATAGCCGAGGAGC
1a-PCR3S	S2204I mutagenic sense	GCTCCTCGGCTATCCAGCTGTCCGCTCCATCTCTCAAGGCAATTGC
1a-PCR3AS	Nested anti-sense	GCGCATTAGGCGCGCCTCATCGGTTGGGGASGAGGTAGATGCCTAC

NS5A alone and NS5A–5B gene cassette (Fig. 2B and C). PCR was performed with a High Fidelity PCR enzyme Platinum Pfx (Invitrogen Life Technologies) as directed by the manufacturer. The product from the first PCR was used as the template and was subjected to two separate rounds of PCR using either PCR2S–PCR2AS primers or PCR3S–PCR3AS primers. The two PCR products were subsequently combined along with the outside primers PCR2S and PCR3AS to generate the final NS5A–NS5B gene cassette. NS5A alone was obtained using a similar strategy, however it was first amplified using PCR2S–PCR2AS and PCR3S–PCR4AS primer pairs to generate the two PCR products. Finally, the outside primer pair PCR2S–PCR4AS was used to generate the final product. The NS5B gene (Fig. 2D) was generated using a nested PCR approach with PCR5S–PCR3AS primer pair. The manufacturer's recommended PCR conditions (Invitrogen Life Technologies) were used for all amplifications. The nested PCR primers were designed to incorporate unique restriction sites such that the individual gene products could be digested with appropriate restriction enzymes and ligated into the genotype-specific vector DNA. Genotype 1b NS5A–NS5B gene cassette PCR products were digested with *Not* I and *Asc* I and ligated to similarly digested genotype 1b vector. Genotype 1b NS5A genes or NS5B genes were cloned into their vectors using *Not* I and *Pac* I or *Pac* I and *Asc* I restriction enzymes, respectively. Genotype 1a NS5A–NS5B gene cassettes were cloned using restriction enzymes *Cla* I and *Asc* I. The ligated products were transfected into *E. coli* XL-10 strain (Stratagene, CA) and grown under antibiotic selection using both solid and liquid cultures. Colonies growing on antibiotic selection plates were used to determine the efficiency of transformation. The liquid medium was used to propagate the quasispecies pool. Plasmid DNA was extracted from the liquid culture and linearized with *Xba* I prior to in vitro transcription of RNA. RNA was synthesized using a T7 Megascript RNA synthesis kit (Ambion Inc., TX) according to the manufacturer's instructions. In vitro transcribed RNA was purified using the LiCl method as recommended by the manufacturer. Purified RNA was used for the transient replication assays.

2.4. Cell lines and transient replication assay

A Huh-7 cell line carrying a stably maintained replicon was cured of the replicon by treatment with interferon-α as described previously (Lu et al., 2004). Cells were grown to a density of $6–10 \times 10^4$ cells/cm^2 and maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies). Ten to twenty micrograms of template RNA was used to electroporate 3×10^6 cells in a 200 μl volume. Electroporation was done with a Gene Pulser II (Bio-Rad, CA) at 480 V, 25 μF, 200 Ω using two manual pulses. Transfected cells were diluted to 7.5×10^4 cells/ml and plated in 96 well plates at 7.5×10^3 cells per well in DMEM with 5% FBS and 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Technologies). Four hours 4 day post-transfection, one plate was harvested for luciferase measurement; this plate provided a measure of the amount of input RNA that can be translated, and thus of transfection efficiency. To the remaining plates, test compounds were added in DMSO (0.5% DMSO final concentration), and plates were incubated at 37 °C and 5% CO_2 for 4 days. Window is defined as the ratio of luciferase activity obtained from 0.5% DMSO treated cells versus the activity from cells treated with 500 nM BILN 2061 added in 0.5% DMSO.

Replication efficiency was calculated using the following equation, $[100 \times 4\ \text{day}_{\text{patient}}]/[(4\ \text{h}_{\text{patient}}/4\ \text{h}_{\text{HCV-wt}}) \times 4\ \text{day}_{\text{HCV-wt}}]$. IC_{50} values and standard error values of test compounds were calculated by nonlinear regression using the Prism 3.0 program. Signal to noise window was determined as the ratio of luciferase activity from mock-treated cells (addition of 0.5% DMSO only) versus activity from cells treated with 100 nM BILN 2061 (Lamarre et al., 2003) added in 0.5% DMSO.

2.5. Luciferase assay

Cell culture medium was removed and wells were washed with 100 μl phosphate-buffered saline. To each well Passive Lysis buffer (Promega, WI) was added and the plates were incubated for 15 min with rocking to lyse cells. Luciferin solution (50 μl, Promega, WI) was added, and luciferase activity was measured with a Victor II luminometer (Perkin-Elmer).

2.6. Compounds

The polymerase inhibitor A-782759 was synthesized at Abbott Laboratories. The structure of A-782759 is shown in Fig. 3. The biological profile including IC_{50} and resistance data of A-782759 has been reported by Mo et al. (2005). The macrocyclic protease inhibitor BILN 2061 (Lamarre et al., 2003) was also synthesized at Abbott Laboratories. Interferon-α was purchased from Sigma and diluted in media. All other compounds were dissolved in DMSO prior to use.

3. Results

3.1. Construction of genotype-specific vectors

Two genotype-specific shuttle vectors were designed for cloning NS5A and/or NS5B genes from HCV-infected subjects as shown in Fig. 1A and B. The strain 1a H77 subgenomic replicon was used for cloning NS5A–5B from genotype 1a infected subjects. The region encoding the first 73 amino acid of NS3 of the genotype 1a vector was derived from the genotype 1b con1

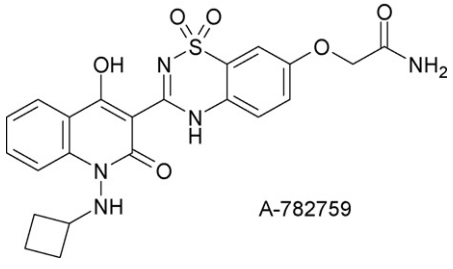

Fig. 3. Structure of A-782759.

sequence while the rest was derived from the 1a H77 strain. Additionally, the genotype 1a vector contained three adaptive mutations for replication in Huh-7 cells. Two unique restriction sites, *Cla* I within NS4B and *Asc* I within the 3′ non-translated (NTR) region, were created for cloning the NS5A–5B gene cassette. 1b strain N subgenomic replicon was used for cloning NS5A and/or NS5B from HCV genotype 1b subjects. The 3′ NTR of the 1b replicon was derived from 1a strain H77 while the remaining vector was derived from the 1b HCV-N strain. The 1b replicon also contained two adaptive mutations for replication in Huh-7 cells. Three unique restriction sites were introduced for cloning NS5A and/or NS5B. The *Not* I site within NS4B and *Pac* I within NS5A allowed for NS5A cloning whereas the *Pac* I site plus an *Asc* I restriction site within the 3′ NTR allowed for cloning of the NS5B gene. NS5A gene cloning from either genotype 1a or 1b samples included the incorporation of the adaptive mutation S2204I for replication in Huh-7 cells.

3.2. Strategy for cloning of NS5A, NS5B or NS5A–NS5B genes from the sera of HCV-infected subjects

The overall strategy for cloning NS5A and/or NS5B genes from the sera of HCV-infected subjects is described in Fig. 4. RNA from sera of HCV-infected subjects was extracted and cDNA was synthesized using genotype-specific primers. NS5A and/or NS5B gene products were generated using nested PCR. The PCR products were sequenced to determine the population sequence of a given sample. The clonal population of the gene products after insertion into the vector was sequenced and no differences from the original PCR product were found. The individual gene products were digested with appropriate restriction enzymes and ligated into genotype-specific vector DNA. Ten percent of the transformation reaction was plated on antibiotic selection plates to determine the efficiency of transformation and the remaining sample was added directly to liquid media to propagate the quasispecies pool. Plasmid DNA was extracted from the liquid culture and linerized prior to in vitro transcription of RNA. Transfection of cured Huh-7 cells was carried out using cells that were plated in a 96-well plate in the presence or absence of inhibitor. Luciferase activities after 4 h and 4 days post transfection were used to determine replication efficiencies, window and IC_{50} values.

3.3. Replication efficiencies of NS5A, NS5B or NS5A–NS5B from 1b subjects

To investigate how genetic heterogeneity affects HCV replication efficiency in a replicon-based transient assay, subge-

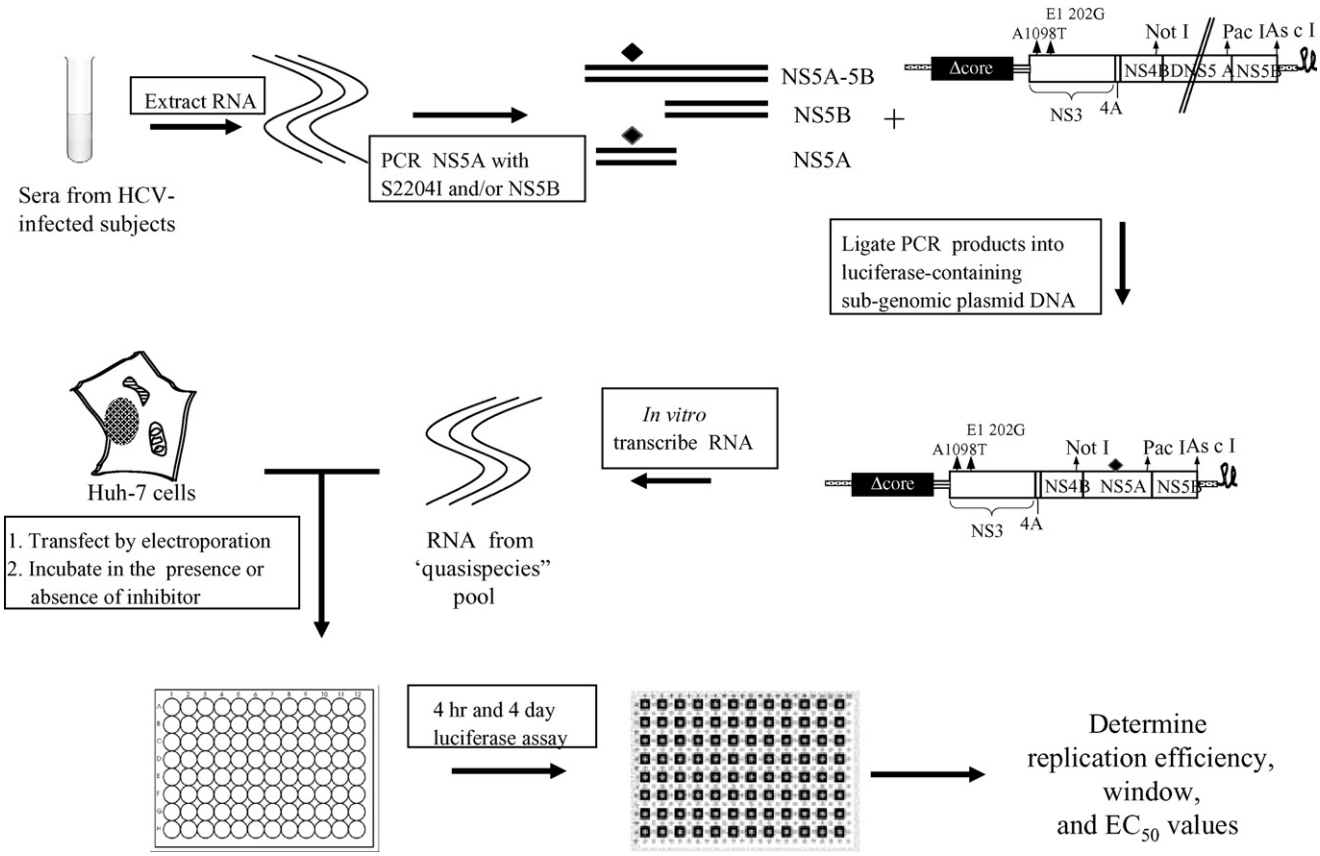

Fig. 4. Overall strategy for cloning NS5A and/or 5B into genotype-specific replicon-based shuttle vector. RNA was extracted from HCV-infected subject and cDNA generated. Mega-primer PCR strategy was used to generate adaptive mutation S2204I within the NS5A gene. Individual PCR products from NS5A, NS5B or NS5A–5B gene cassette were digested and ligated into genotype-specific shuttle vector. DNA was extracted from transformed *E. coli* as a pool and linearized with *Xba* I. RNA was transcribed in vitro and used to transfect Huh-7 cells using electroporation. Four hour and 4 day luciferase assays were used to determine replication efficiency, window and IC_{50} values.

Table 2
Replication window and efficiencies of NS5A, NS5B or NS5A–5B gene isolated form sera of genotype 1b subjects

Subject ID	NS5A–5B		NS5A		NS5B	
	Replication window[a]	Replication efficiency[b] (%)	Replication window[a]	Replication efficiency[b] (%)	Replication window[a]	Replication efficiency[b] (%)
1b (N)	2129 ± 320	100.0	2129 ± 320	100.0	2129 ± 320	100.0
#1	2 ± 0.5	0.3 ± 0.01	456 ± 56	21 ± 2.4	786 ± 36	17 ± 3.0
#2	191 ± 24	3 ± 2.5	2461 ± 394	120 ± 36.0	965 ± 35	177 ± 27.0
#3	448 ± 147	6 ± 0.7	710 ± 71	31 ± 3.0	123 ± 27	94 ± 6.0
#4	25 ± 4	2 ± 1.9	339 ± 30	24 ± 2.0	481 ± 19	98 ± 17.0
#5	3.0 ± 0.3	0.09	1283 ± 43	61 ± 19.5	1838 ± 162	36 ± 19.0
#6	30 ± 6	3 ± 2.0	1042 ± 100	51 ± 5.0	74 ± 24	17 ± 3.0
#7	1.5 ± 0.1	0.14	1448 ± 87	146 ± 7.0	1117 ± 117	96 ± 6.0

[a] Window: This is measured by the ratio of the 4-day luciferase value of the untreated cells and the cells treated with replicon inhibitor, BILN 2061. This value is an indication of the signal to noise ratio.
[b] Replication efficiency: cured Huh-7 cells were transfected with replicons containing lab-strain or subject NS5A–5B. The luciferase values were measured after 4 h and 4 days. The 4-h luciferase value measures the integrity of the input RNA and allows normalization between samples. The replication efficiency is defined as the ratio of the 4-day luciferase value of the subject replicons and the lab-strain, normalized by the 4-h luciferase values.

nomic replicons containing individual NS5A or NS5B genes, or NS5A–5B gene cassettes from HCV-containing serum samples were used to transfect Huh-7 cells and the luciferase assay was used to measure the replication efficiency. The replication efficiency is defined as the ratio of the 4-day luciferase value of the subject replicons and the laboratory strain, normalized by the 4-h luciferase value. Window is determined by measuring the ratio of the 4-day luciferase value of the untreated cells and the cells treated with replicon inhibitor, BILN 2061. This value is an indication of the signal to noise ratio. Table 2 shows the replication efficiencies and windows from genotype 1b subjects. The replication efficiencies of 1b shuttle vectors containing subject-derived NS5A–5B were only ~2% whereas that of vectors containing NS5A or NS5B alone averaged 67 and 75%, respectively, relative to the laboratory-optimized 1b replicon. This suggests that the NS5A–5B gene cassette derived from the quasispecies pool of 1b viral isolates replicates poorly in the 1b HCV-N strain background. NS5A–5B "quasispecies" population sequences from genotype 1b isolates were aligned with the 1b HCV-N strain reference sequence. The amino acid identities ranged from 94–95% against the reference sequence and between patient isolates (alignment not shown).

3.4. Susceptibility of HCV 1b subject-derived NS5A, NS5B or NS5A–NS5B genes to IFN-α or polymerase inhibitor A-782759

The HCV-infected subject-derived gene sequences were tested for sensitivity to interferon-α (IFN-α) and to a small molecule inhibitor of HCV polymerase to demonstrate the utility of the vector. A-782759 is a potent HCV polymerase inhibitor and has been described previously (Mo et al., 2005). The IC_{50} against genotype 1b and 1a replicons are reported in Tables 3 and 4. The compound is not cytotoxic and has a TD_{50} value of 63 ± 17 μM in genotype 1b subgenomic replicon stable cell lines. Table 3 shows the IC_{50} values determined from genotype 1b HCV-infected subjects. IFN-α IC_{50} values measured from samples from seven HCV-infected subjects were equivalent to those measured in the control laboratory strain. IC_{50} values for polymerase inhibitor A-782759 was equivalent to or slightly higher than the values measured in the control laboratory strain replicons. The dose–response curves for IFN-α and polymerase inhibitor A-782759 against HCV replicons containing subject-derived NS5A or NS5B are shown in Fig. 5. The potency differences may reflect the heterogeneity of the subject-derived quasispecies pools. IC_{50} values were successfully determined

Table 3
Susceptibility of HCV 1b subject-derived NS5A and/or NS5B genes to IFN-α or the polymerase inhibitor A-782759

	IC_{50} NS5A–5B		IC_{50} NS5A		IC_{50} NS5B	
	IFN (U/ml)	A-782759 (nM)	IFN (U/ml)	A-782759 (nM)	IFN (U/ml)	A-782759 (nM)
1b (N)	0.07 ± 0.21	6 ± 3.0	0.07 ± 0.21	6 ± 3.5	0.07 ± 0.21	6 ± 3.5
#1	ND	ND	0.13 ± 0.007	4.6 ± 2.4	0.146 ± 0.01	6.12 ± 0.9
#2	0.179 ± 0.01	11.03 ± 0.2	0.18 ± 0.07	1.7 ± 0.4	0.118 ± 0.01	3.76 ± 1.5
#3	0.186 ± 0.09	7.82 ± 1.0	0.18 ± 0.03	4.7 ± 0.2	0.216 ± 0.2	19.98 ± 9.5
#4	0.114 ± 0.11	8.98 ± 1.1	0.07 ± 0.01	3 ± 1.9	0.081 ± 0.05	7.83 ± 2.8
#5	ND	ND	0.26 ± 0.12	5.7 ± 1.2	0.137 ± 0.01	4.67 ± 0.2
#6	0.095 ± 0.08	7.69 ± 0.2	0.09 ± 0.02	11.3 ± 6.3	0.064 ± 0.02	10.45 ± 7.3
#7	ND	ND	0.09 ± 0.004	4.6 ± 0.002	0.095 ± 0.006	3.13 ± 1.5

ND: Not determined due to very poor replication efficiency.

Table 4
Replication efficiencies and susceptibility of HCV 1a subject-derived NS5A–5B genes to IFN-α or a polymerase inhibitor

Subject ID	NS5A–5B		IC_{50} NS5A–5B	
	Replication window[a]	Replication efficiency[b] (%)	IFN-α (U/ml)	A-782759 (nM)
1a (H77)	666 ± 385	100.0	0.08 ± 0.03	10 ± 2
#1	1145 ± 60	15 ± 5	0.17 ± 0.03	45 ± 23
#2	34.3 ± 21	6 ± 0.5	0.18 ± 0.04	30 ± 6
#3	68 ± 12	12 ± 3.5	0.15 ± 0.07	57 ± 25
#4	230 ± 44	43.3 ± 0.2	0.07 ± 0.03	15 ± 9
#5	607 ± 207	105.7 ± 35.4	0.08 ± 0.03	27 ± 19

[a] Window: This is measured by the ratio of the 4-day luciferase value of the untreated cells and the cells treated with replicon inhibitor, BILN 2061. This value is an indication of the signal to noise ratio.

[b] Replication efficiency: Cured Huh-7 cells were transfected with replicons containing lab-strain or subject NS5A–5B. The luciferase values were measured after 4 h and 4 days. The 4-h luciferase value measures the integrity of the input RNA and allows normalization between samples. The replication efficiency is defined as the ratio of the 4-day luciferase value of the subject replicons and the lab-strain, normalized by the 4-h luciferase values.

for all patient samples tested, although the replication efficiencies ranged from 23–150% relative to the laboratory optimized reference replicon. The results suggest that the shuttle vector system can be used to test genes from genetically heterogeneous populations for sensitivity to small molecule inhibitors targeted to HCV non-structural genes.

3.5. Replication efficiencies and susceptibility of NS5A–5B from 1a subjects to IFN-α or a polymerase inhibitor

Only the NS5A–5B gene cassette from genotype 1a HCV positive sera was cloned into the 1a replicon-based shuttle vector. The results of the replication efficiencies and susceptibility

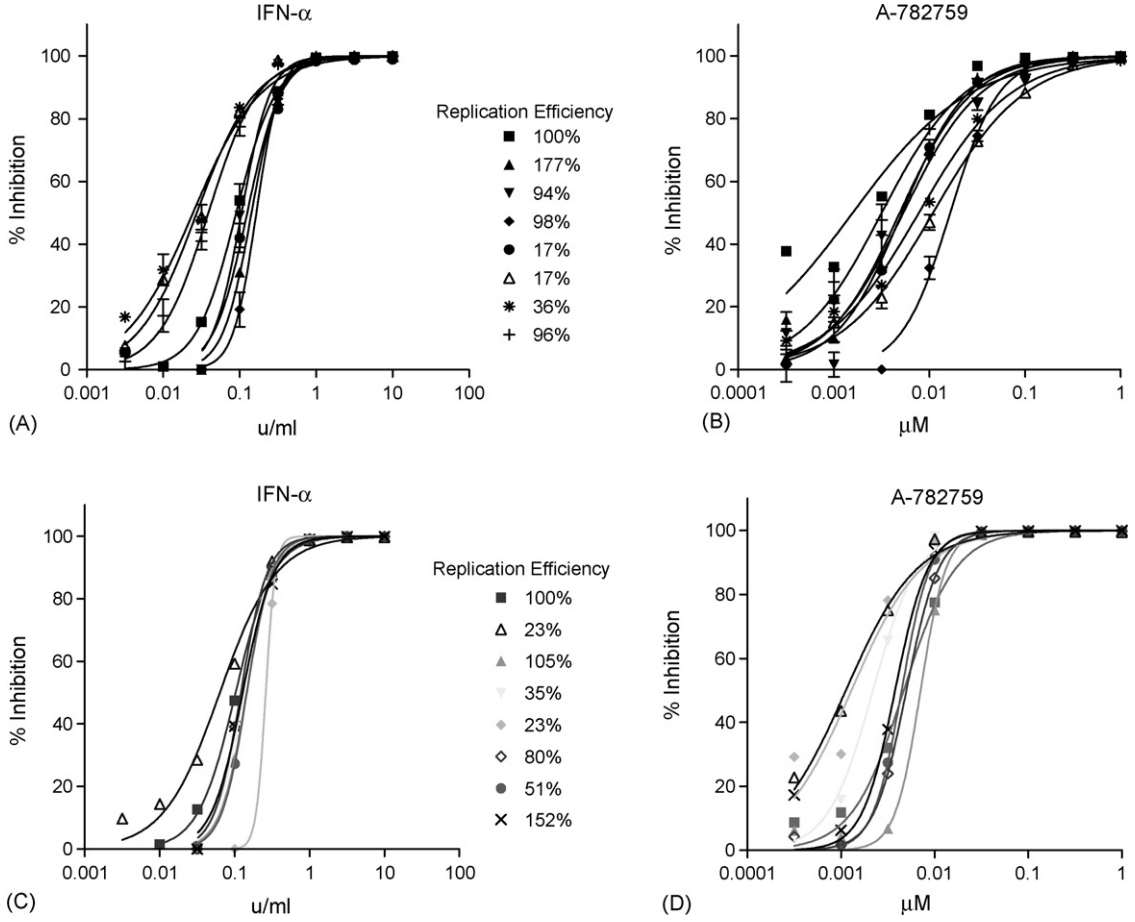

Fig. 5. Dose–response of IFN-α and a polymerase inhibitor A-782759 against HCV replicon containing subject-derived NS5A or NS5B. The replicons containing NS5A and/or NS5B derived from HCV-infected subjects were transfected into cured Huh-7 cells. The cells plated into 96-well plates, were subsequently treated with interferon-α or A-782759 for 4 days. The figure is a typical example of the dose–response curves of the replicon inhibitors. Replicons with replication capacities ranging from 20–100% of the lab-strain have sufficient window to allow for IC_{50} determinations. (A and B) Genotype 1b replicons containing NS5B alone. (C and D) Genotype 1b replicons containing NS5A alone.

to IFN-α and A-782759 of samples from five separate HCV-infected subjects are shown in Table 4. Replication efficiencies of the NS5A–5B gene cassette derived from genotype 1a HCV-infected subjects averaged around 36% relative to NS5A–5B from H77. This value is considerable higher than the efficiency of the NS5A–5B cassette derived from genotype 1b HCV-infected subjects. The IC_{50} values of IFN-α were equivalent to the laboratory strain and were slightly higher than the laboratory strain for the polymerase inhibitor.

4. Discussion

This laboratory previously described the construction of a genotype 1b shuttle vector that allowed us to compare phenotypes of NS5B genes from HCV patient isolates after modifying existing replicons (Middleton et al., 2004). The utility of such a system was demonstrated by generating a functional replicon using a quasispecies pool of sequences from the NS5B genes of HCV-infected subjects. This study reports the construction of two additional shuttle vectors for cloning NS5A alone or NS5A–5B genes from both genotypes 1a and 1b and development of an efficient system to study how genetic heterogeneity affects HCV replication efficiency. The design and application of the shuttle vector system will be useful in the study of individual genes such as NS5A, NS5B, or NS5A–NS5B together from HCV-infected subjects. Its utility can be extended to assess the phenotype of drug development candidates using targets from infected subjects and for phenotypic and genotypic characterization of patient isolates during clinical trials. Ludmerer et al. (2005) described a transient cell-based assay to evaluate clinical NS5B isolates for their replication fitness, and their sensitivities to NS5B polymerase inhibitors. Their study used sera from patients and chimpanzees to clone the NS5B gene. Individual clones harboring the polymerase genes from the samples were cloned in their chimeric replicons. The work reported here differs in that the entire patient quasispecies pools of sequences have been cloned into the replicon.

Two genotype-specific replicon-based shuttle vectors were developed to include unique restriction sites, which would allow for cloning of NS5A, NS5B or NS5A–NS5B genes from HCV-infected subjects. The genotype-specific system described here is designed to allow for cloning of genes from either 1a or 1b HCV-infected subjects. To ensure cloning of the quasispecies pool, a mega-primer PCR approach was used to introduce a replicon-specific adaptive mutation S2204I within the NS5A gene. When several genotype 1b NS5A–5B gene cassettes lacking the adaptive mutation were inserted into the 1b shuttle vector and assayed in the transient replicon assay there was no replication window, demonstrating the necessity of the adaptive mutation. Cloning the quasispecies pool is important, not only to maintain the in vivo population heterogeneity of a gene present in an HCV-infected subject, but also to allow for detection of the presence of any pre-existing drug resistant quasispecies within the population.

To investigate how genetic heterogeneity affects HCV replication efficiency in a replicon-based transient assay, individual NS5A, NS5B or NS5A–5B gene cassettes were cloned from HCV-infected subjects. Alignment of the population sequence of the NS5A–5B quasispecies pool revealed identities of 94–95% within a given sub-genotype. The replication efficiencies of replicons containing genotype 1b NS5A or NS5B alone were comparable to the laboratory-optimized replicon. Substituting individual NS5A or NS5B genes within the laboratory-optimized background was well tolerated. However, the replication efficiency was poor when an NS5A–5B cassette was co-shuttled into either genotype 1a or 1b replicons. Although NS5A–5B gene cassette replicons replicated poorly, it is interesting to note that the chimeric NS5A–5B genes seem to be better tolerated with the genotype 1a cassette system (36% average replication efficiency) compared to genotype 1b (2% average replication efficiency). Statistical analysis by *t*-test to assess whether the mean replication efficiency of genotype 1a and 1b carrying NS5A–5B gene cassette are statistically different confirmed that the difference between the means for the two groups is significant ($p < 0.05$). One possibility to explain these differences is that both NS5A and NS5B may have significant strain-specific interactions with other parts of the replicase (excluding NS5A and NS5B) that are essential for RNA replication, and that the loss of fitness that occurs with substitution of one (NS5A or NS5B), due to impairments in these interactions with other parts of the replicase, is additive when both NS5A–5B are co-shuttled. It is possible that these strain-specific interactions are better tolerated in genotype 1a, therefore leading to improved replication efficiency as compared to genotype 1b. It was also observed that the correlation between the replication window and replication efficiency is high for some but not for others samples. This may be due to the fact that the replication efficiency is dependent on both the 4 h and 4-day luciferase values of the input RNA. The quality and quantity of RNA can vary the 4-h luciferase value thereby affecting the replication window and leading to the difference in correlation between replication window and efficiency.

Although there was roughly 95% amino acid identity between patient-derived 1b or 1a sequences and either 1b strain N or 1a strain H77, respectively, important amino acid substitutions might prevent a critical interaction between either cellular or viral proteins when NS5A–5B is co-shuttled from patient samples. Previously, Shirota et al. (2002) reported a direct interaction between NS5A and NS5B through two binding regions in NS5A. Using either NS5A expressed in mammalian cells or as purified recombinant protein they showed that NS5A could modulate the activity of NS5B polymerase through direct interactions. Recently, Shimakami et al. (2004) reported that interactions between NS5A and NS5B are critical for HCV RNA replication in the HCV subgenomic replicon system by introducing several internal deletion mutations within NS5A. Their results clearly show evidence that NS5A is indispensable for HCV RNA replication due at least in part through its interaction with NS5B. Protein–protein interactions have been identified between polymerase and other HCV non-structural proteins (Lin et al., 1997; Ishido et al., 1998; Yamashita et al., 1998; Shirota et al., 2002). Loss of binding affinity for any of the interactions

when NS5A–5B are co-shuttled could lead to incompatibility and thus to poor replication capacity. RNA secondary structure motifs in the 5′- and 3′ non-translated region and the carboxy-terminal region of the NS5B gene have been demonstrated to be essential for replication (Blight and Rice, 1997; Friebe et al., 2001; You et al., 2004). Strain and genotype-specificity could also arise from interactions either between non-structural protein and the secondary structures at the 5′ or 3′ end, which may contribute to loss of replication capacity when patient-derived NS5A–5Bs are co-shuttled. For example, Gates et al. (2004) constructed a panel of chimeric replicons containing non-structural (NS) proteins and 3′NTR sequences from different HCV strains or types and examined the requirements for stable replication in Huh-7 cells. They demonstrated that the optimal interactions between 3′NTR and NS proteins are critical in determining functionality of subgenomic replicons. Further experiments will have to be conducted to address these observations.

The replicon-based shuttle vector offers a powerful tool in screening for compounds with antiviral activity. It is important to assess the efficacy of novel compounds not only in laboratory-optimized replicon systems but also against a heterogeneous population that exists in HCV-infected subjects. To define the utility of the newly designed shuttle vectors, the HCV-infected subject-derived gene sequences were tested for sensitivity to IFN-α and to the small molecule HCV polymerase inhibitor A-782759. IC_{50} values were accurately measured against both protein targets using replicons with replication efficiencies as low as 3% relative to the laboratory-optimized reference. IC_{50} values for IFN-α against NS5A (Table 2) were comparable to the laboratory-optimized strain and comparable to the values reported by other groups (Blight et al., 2000; Frese et al., 2001; Tanabe et al., 2004). These IFN-α values, which differ by less than three-fold among the patient isolates tested, serves as reference for the susceptibility of the patient's NS5B protein to inhibition by A-782759. Susceptibility to inhibition by A-782759 targeting NS5B polymerase was similarly comparable to both laboratory-optimized genotype 1a and 1b replicons and comparable to values reported by Mo et al. (2005). However, the IC_{50} values in Fig. 5 exhibited a seven-fold range, reflecting the heterogeneity of the subject-derived quasispecies pools. None of the patient-derived samples contained the A-782759 identified resistance mutations. The ability to use a heterogeneous population in the shuttle vector system will not only aid in our understanding of antiviral activity, but it can also help identify pre-existing mutations within the infected subject that are resistant to antiviral compounds. Understanding and assaying for the presence of pre-existing mutants will become more important as new antiviral drugs are discovered and marketed.

In conclusion, the shuttle vectors described here extend the utility of HCV subgenomic replicons by permitting rapid genotypic and phenotypic characterization of large number of samples from HCV-infected patients. This report demonstrated the utility of the shuttle vector by characterizing the NS5A and/or NS5B genes, but the approach can be generally applicable to any other segments of HCV the genome that are necessary for replicon function.

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