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Development of a robust luciferase reporter 1b/2a hepatitis C virus (HCV) for characterization of early stage HCV life cycle inhibitors

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ABSTRACT

The development of JFH1 based intergenotypic recombinants that exploit the unique replication characteristics of JFH1 has made it possible to study infectious hepatitis C virus (HCV) encoding the structural genes of additional HCV genotypes. To facilitate the study of 1b structural proteins, we aimed to develop a robust 1b/2a chimera encoding a humanized *Renilla* luciferase reporter gene (1b/2a hRluc). The unadapted genome replicated efficiently but produced very low titers of infectious virus. Adaptation by continuous passage over a novel Huh-7 Lunet clone improved viral titers approximately 100-fold but caused an unexpected decline in luciferase activity, limiting the utility of the reporter-containing virus. Genotypic analysis revealed 17 adenosine to guanosine (A to G) nucleotide mutations in the luciferase gene and two potential adaptive mutations. To overcome the problems of low viral titers and editing of the luciferase gene during viral adaptation, six adaptive mutations previously identified in a non-reporter 1b/2a HCV genome were introduced into the 1b/2a hRluc genome. This resulted in the immediate production of high-titer viral stocks (approximately 1000-fold greater than the parental virus) that could efficiently infect naïve cells and generate robust luciferase signals. The improved sensitivity of the luciferase reporter also facilitated time of addition studies validating the utility of this system for characterizing the early steps of HCV infection. Thus, the development of the 1b/2a hRluc reporter virus described here provides a versatile tool for discovery of inhibitors targeting the early steps of the viral life cycle and genotype 1b structural genes.

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1. Introduction

Genotype 1 is the most prevalent hepatitis C virus (HCV) genotype, accounting for about 60% of global HCV infections; it is also the least responsive to interferon-α plus ribavirin treatment (Gravitz, 2011). Because interferon-α plus ribavirin is not effective at eliminating HCV infection in a significant proportion of the patient population, more effective drugs need to be developed. Recently, two new antivirals targeting the NS3 protease of HCV were approved by the FDA, marking the beginning of a new paradigm for HCV treatment.

The addition of the protease inhibitors telaprevir or boceprevir to interferon-α plus ribavirin has increased the cure rate from <50% to ~70%, with the additional benefit of a potentially shortened treatment duration (Burney and Dusheiko, 2011; Gravitz, 2011; Schlutter, 2011). Despite this breakthrough in treatment, HCV is prone to the development of viral resistance which is frequently observed during telaprevir or boceprevir treatment failures (Hiraga et al., 2011; Susser et al., 2009). Thus, to more effectively treat HCV combination therapy using inhibitors against different viral and host targets are being pursued (Chen and Tan, 2005; Pawlotsky, 2012).

The HCV genome is 9.6 kb and encodes a polyprotein of about 3000 amino acids. Proteolytic processing of the polyprotein by host and viral proteases yield 10 mature viral proteins. The first three N-terminal proteins, core, E1, and E2, are structural proteins that make up the extracellular virion (Gentzsch et al., 2011). The other seven are the non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. NS3–NS5B are replicase proteins that are necessary and sufficient for viral RNA replication. In contrast, p7 and NS2 are not predicted to be major components of the virion, nor are they required for RNA replication. The functions of p7 and NS2 are not well defined, but evidence suggests that they may function at an early stage of virion morphogenesis, prior to the assembly of infectious virus (Jones et al., 2007). To date, much of what is known about HCV comes from in vitro cell culture systems

Abbreviations: ADAR1, adenosine deaminases acting on RNA; DMEM, Dulbecco's modified Eagle medium; EC_{50}, 50% effective concentration; FMDV, foot and mouth disease virus; GAPDH, glutaraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HCVcc, hepatitis C virus in cell culture; IFN-α, interferon-α; MAb, monoclonal antibody; MOI, multiplicity of infection; PVDF, polyvinylidene fluoride; S:B, signal:background; $TCID_{50}$, 50% tissue culture infective dose.

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that recapitulate only replication and translation parts of the viral life cycle, i.e. HCV subgenomic replicons (Scheel et al., 2011). However, the development of infectious cell culture systems based on the genotype 2a isolate JFH1 enabled the study of other important steps in the HCV life cycle such as viral entry and assembly for other HCV genotypes.

To date, no genotype 1 HCV isolates have been identified that support robust replication and production of high titer infectious virus particles in cell culture. Recently, intergenotypic HCV chimeras were developed that encode the nonstructural genes of JFH1, with structural genes of other genotypes (Gottwein et al., 2007, 2009; Pietschmann et al., 2006; Scheel et al., 2008; Yi et al., 2007; Zhang et al., 2008). Although these chimeras allow the study of non-genotype 2 structural proteins, they typically produce low viral titers, which limit their utility. To facilitate the study of genotype 1 structural proteins we sought to develop a robust 1b/2a HCV chimera encoding a humanized *Renilla* luciferase reporter gene (1b/2a hRluc). Two approaches were pursued: (1) adaptation by passaging over Huh-7 Lunet derived cells which resulted in selection of adaptive viral variants with increased viral titers but a hypermutated luciferase gene with diminished luciferase activity and (2) introduction of previously identified adaptive mutations (Chan et al., 2012) which resulted in the immediate increase of viral titers by >3 logs and robust luciferase activity compared to the wild-type.

2. Materials and methods

2.1. Cell culture

497-5 Lunet cells were derived from Huh-7 Lunet cells (Friebe et al., 2005) as previously described (Chan et al., 2012). Cells were grown in Dulbecco's modified Eagle medium (DMEM) with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml of penicillin (Invitrogen), 100 μg/ml of streptomycin (Invitrogen), and 10 mM non-essential amino acids (Invitrogen) and maintained in humidified incubators at 37 °C and 5% CO_2.

2.2. Plasmid construction

2.2.1. pCon1/JFH1 (1b/2a HCVcc)

The plasmid pCon1/JFH1, which contains genotype 1b Con1 (AJ238799) sequence from core to the first 33 amino acids of NS2 fused between the 5′UTR of genotype 2a JFH1 (AB047639) and the rest of NS2 through the remaining non-structural genes to the 3′UTR of JFH1, was constructed as previously described (Chan et al., 2012; Zhang et al., 2008).

2.2.2. pCon1/JFH1 hRluc (1b/2a hRluc HCVcc)

The reporter hRluc gene fused to the 2A protease of foot and mouth disease virus (FMDV) was inserted into pCon1/JFH1 as described by Zhang et al. (2008) to construct pCon1/JFH1 hRluc. EcoR1-5′UTR-hRluc-FMDV2a-1bcore-E1-BglII was synthesized by GenScript Inc. (Piscataway, NJ) and cloned using traditional molecular cloning methods into pCon1/JFH1. All amino acid substitutions were introduced by site-directed mutagenesis using a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). All final constructs were verified by DNA sequencing (Elim Biopharmaceuticals Inc., Hayward, CA).

2.3. RNA transcription

RNA was synthesized with T7 MEGAScript reagents (Ambion, Austin, TX) following the manufacturer's suggested protocol and RNA was purified using an RNA easy kit (Qiagen) in accordance with the manufacturer's protocol.

2.4. Electroporation of HCV RNAs

497-5 Lunet cells were electroporated with RNA transcripts using a Gene Pulser system (Bio-Rad, Hercules, CA) and re-cultured as described previously (Chan et al., 2012).

2.5. Adaptation of 1b/2a hRluc HCVcc to 497-5 Lunet Cells

497-5 Lunet cells were electroporated as described above with 10 μg 1b/2a hRluc wild-type RNA and seeded into 75 cm^2 flasks. After 96 h post transfection, cells were transferred into larger 162 cm^2 flasks. Cells were serially passaged at a dilution of 1:3 every 3 days and monitored for presence of HCV in cells by NS5A immunostaining and in supernatants by 50% tissue culture infective dose ($TCID_{50}$) assay at the indicated time points. When the cells were highly infected with HCV, virus-containing supernatants were used to infect naïve 497-5 Lunet cells followed by further serial cell passaging until sufficient virus spread and infectivity was achieved by the adapted virus.

2.6. Indirect immunofluorescence staining for HCV NS5A protein

Infected cells were stained for HCV NS5A protein (NS5A monoclonal antibody 9E10, Apath, Brooklyn, NY), and the percentage of infected cells was determined using an ImageXpress Micro (Molecular Devices, Sunnyvale, CA) with MetaExpress 2.0 software as described previously (Chan et al., 2012).

2.7. Determination of virus titers in cell culture supernatants

497-5 Lunet cells were infected with one-to-four serial dilutions of virus-containing cell culture supernatant as described previously (Chan et al., 2012). Infected cells were stained for NS5A as described above and virus titer was determined by calculating the 50% tissue culture infectious dose ($TCID_{50}$/ml) according to the method of Reed and Muench (1938).

2.8. Extraction and isolation of HCV RNA for genotypic analysis

HCV RNA was isolated from cells infected with virus or cell culture supernatant using RNeasy and QiaAmp Viral RNA kits, respectively (Qiagen, Valencia, CA). First-strand cDNA synthesis from isolated RNA followed by PCR was performed as described previously (Chan et al., 2012).

2.9. Infectivity assay

497-5 Lunet cells were seeded in 96-well plates at a density of 5000 cells per well. After overnight incubation, cells were infected with 100 μl of viral inocula. Following 3 days of incubation, cell culture medium was removed, and cells were lysed and assayed for NS3-4A protease activity or luciferase activity as a marker for intracellular HCV replication levels. Intracellular NS3-4A protease activity was measured by time-resolved fluorescence (VICTOR^{3}™ V Multilabel Counter, Perkin Elmer) using a Europium labeled NS3-4A protease substrate as described previously (Chan et al., 2012). Luciferase activity was measured with *Renilla* luciferase assay buffer and substrate (Promega), following the manufacturer's protocols, using a VICTOR3™ V Multilabel Counter (Perkin Elmer, Shelton, CT).

2.10. Antiviral inhibitors

2'-C-Methyl adenosine (2'CMA) and BILN-2061 were purchased from Acme Bioscience (Belmont, CA). EI-1 was purchased from ChemBridge (San Diego, CA). Anti-CD81 monoclonal antibody (MAb) JS-81 was purchased from BD Pharmingen, San Jose, CA.

2.11. Antiviral and neutralization assays

497-5 Lunet cells were infected with viral inocula at a multiplicity of infection (MOI) of 0.3 as described previously (Chan et al., 2012). Final concentrations of drug ranged from 2.5 to 50,000 nM for 2'CMA, E1-I and BILN-2061. Anti-CD81-MAb was serially diluted in DMEM to yield final concentrations from 1 ng/ml to 2500 ng/ml. Following 3 days of incubation, NS3 protease activity or luciferase activity was used to quantify intracellular HCV replication levels as described above.

3. Results

3.1. Generation of cell culture adapted 1b/2a hRluc chimeric viruses

To establish a robust, luciferase-encoding 1b/2a virus, we constructed the chimera originally described by Pietschmann et al. (2006) but also incorporated a humanized *Renilla* luciferase reporter as described by Zhang et al. (2008) (Fig. 1A). To adapt the 1b/2a hRluc wild-type virus to cell culture, we used a novel HCV-permissive cell line derived from Huh-7 Lunet cells, referred to as 497-5 Lunet (Chan et al., 2012). In vitro-transcribed 1b/2a hRluc RNA was transfected into 497-5 Lunet cells and the cells were maintained by serial passaging. At each passage, cells were immunostained for NS5A to monitor the degree of infection (Fig. 1B); while supernatants were collected to determine infectious viral titer (Fig. 1B) and to quantify luciferase reporter activity following infection (Fig. 1C).

Within 4 days of transfection, NS5A positive cells (11% of culture) and titers (3.16×10^1 $TCID_{50}$/ml) were detected, providing evidence that the 1b/2a hRluc genome could replicate and form viral particles, although at low levels (Fig. 1B). Thereafter, the number of NS5A-positive cells decreased from 11% to 3%, while viral titers became undetectable for the first six cell passages. A rise in infected cells was observed at day 21 post transfection (15% NS5A-positive cells) and this increased to 40% infected cells by day 38. Correspondingly, viral titer also increased to 3.45×10^2 - $TCID_{50}$/ml (Fig. 1B); luciferase activity also peaked at this time (Fig. 1C).

Viral supernatants from infected cells at day 38 were passaged onto naïve 497-5 Lunet cells. Infected cultures were passaged until they were again highly positive for NS5A at day 58 with a corresponding 1-log increase in viral titers to 2.02×10^3 $TCID_{50}$/ml (Fig. 1B). The supernatant from these cells was then used to re-infect naïve 497-5 Lunet cells and cells were passaged five more times until cells were highly positive for NS5A. The viral titers did not improve significantly from day 58 to day 78 during the last cycle of cell passaging, achieving final viral titers of 1.4×10^3 - $TCID_{50}$/ml (Fig. 1B). In contrast to the rising viral titer and virus spread, luciferase activity declined significantly over time, indicating that the reporter was not stably expressed from the virus over long periods of culture (Fig. 1C).

3.2. Genotypic analysis of the adapted 1b/2a hRluc virus

To identify potential mutations responsible for enhanced replication and lower luciferase expression in the adapted 1b/2a hRluc virus, we determined the consensus sequence of viral genomes isolated from culture supernatants collected at days 27, 38, 51 and 78 of the adaptation (Fig. 2). Genotypic analysis revealed two adaptive mutations, V364A in E1 and Y834C in NS2. The mutation Y834C in NS2 emerged first as it was observed in supernatant collected at day 27 during the first cycle of cell passaging and thereafter. The mutation V364A in E1 was observed after the first supernatant passage at day 51.

Interestingly, 17 A to G mutations were observed in the luciferase gene during the course of the adaptation (Fig. 2). This mutation pattern was specific to the luciferase gene and not detected in the other regions of the HCV genome by population sequence. At day 38 after the first cycle of cell passaging the detection of A to G mutations was rare and luciferase activity was detectable at sufficient levels (signal:background [S:B] >300, Fig. 1C). During the first and second supernatant passages when luciferase signal began to decline significantly, A to G mutations became more prevalent. At day 51 where only modest levels of luciferase activity remained detectable (S:B = 94), 11/17 positions were mutated with G as the dominant nucleotide and a mixture of A and G nucleotides was detected at the other six positions by population sequencing. At the time the adaptation was stopped, day 78, luciferase activity was nearly undetectable (S:B < 10) while viral titers were at their peak, and the G mutation was dominant at all 17 positions.

3.3. Previously identified adaptive mutations confer enhanced titer and luciferase expression to the reporter 1b/2a hRluc chimera

The adaptive mutations in E1 (V364A) and NS2 (Y384A) were inserted into the parental 1b2a hRluc construct to determine their individual effect on the production of infectious virus particles and virus spread (Fig. 3A). Transcribed RNA was transfected into 497-5 Lunet cells along with the parental 1b/2a hRluc construct and virus spread (Fig. 3B) and production (Fig. 3C) were monitored. The mutation in NS2 Y834A had the most significant effect producing viral titers of 4.3×10^3 $TCID_{50}$/ml and enhancing viral spread compared to wild-type. The mutation in E1 had minimal effect on increasing viral titers but improved replication as indicated by the increased virus spread compared to the wild-type observed over 7 days post transfection. As an outcome of improved viral titers and replication that was afforded by the mutations in E1 and NS2, luciferase activity also increased proportionally compared to wild-type (Fig. 3D). These data indicate that both mutations combined afforded viral titers (4.8×10^3 $TCID_{50}$/ml) similar to that of the cell culture adapted virus confirming that these mutations were responsible for the observed tissue culture adaptation but not the loss in luciferase activity.

To improve viral titers further and overcome the problem of luciferase gene mutation during adaptation, we engineered previously identified adaptive mutations into the 1b/2a hRluc genome. Specifically, we included six mutations that had been identified in a non-reporter 1b2a virus: A150 V in core, L839S in NS2, V1065G and I1312V in NS3, M2388I in NS5A, and V2937A in NS5B (Chan et al., 2012). The final construct was referred to as 1b2a hRluc-All 6 (Fig. 3A). After transfection the 1b/2a hRluc-All 6 genome spread throughout 497-5 Lunet cultures much more effectively and produced the highest viral titer stocks (Fig. 3B and C, respectively). In fact, 4 days after transfection the 1b/2a hRluc-All 6 virus achieved viral titers at 2×10^5 $TCID_{50}$/ml, 40-fold higher than the hRluc adapted virus and 1000-fold higher than the parental wild-type virus (Fig. 3C). The ability to detect infectivity of 1b/2a hRluc-All 6 supernatants by luciferase reporter assays was also assessed (Fig. 3D). Compared to wildtype and the 1b2a-hRluc viruses with mutations in E1_V364A and NS2_Y384A, the 1b/2a hRluc-All 6 virus was the most efficient virus to infect naive cells and generate robust luciferase signals, producing signal to background ratios >1000-fold and signals in excess of those produced by the well-established Jc-1 Rluc virus.

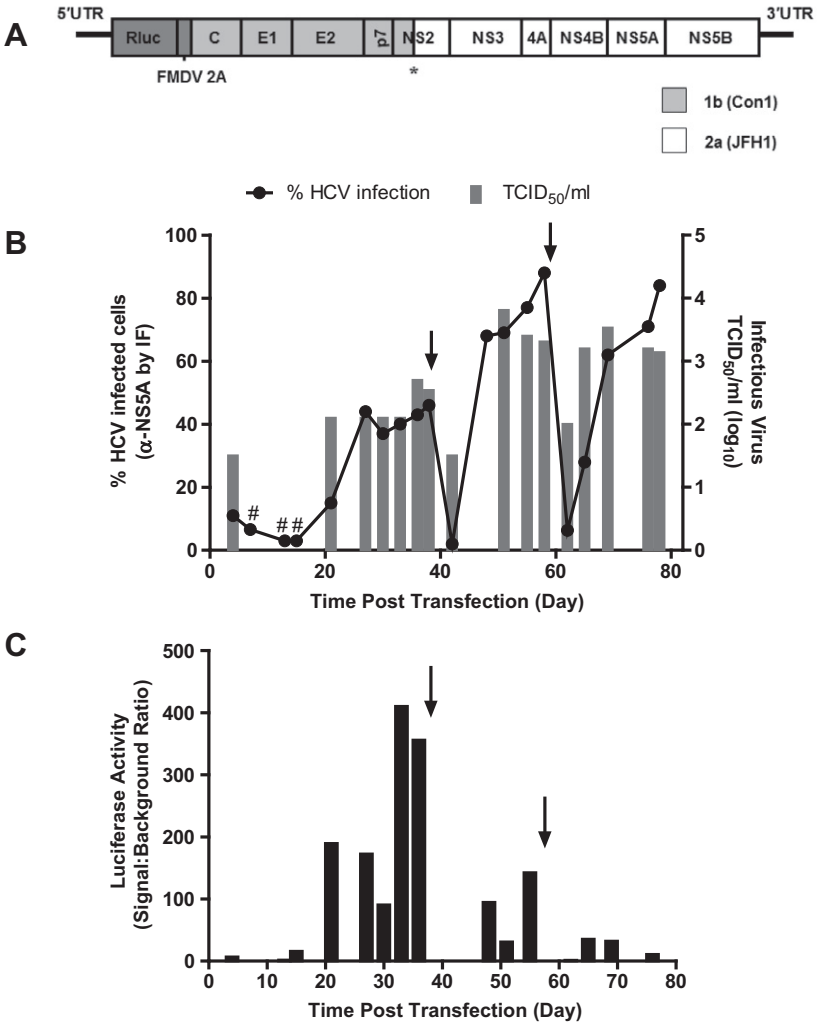

Fig. 1. (A) Schematic of genotype 1b/2a chimeric HCV with humanized *Renilla* luciferase reporter (Zhang et al., 2008). The symbol *, indicates the junction site between genotype 1b and 2a segments, which is at the first 33 amino acids of NS2 (Pietschmann et al., 2006). (B) Cell culture adaptation of chimeric 1b/2a hRluc genome. 497-5 Lunet cells were transfected with in vitro-transcribed 1b/2a hRluc RNA. Cells and supernatants were serially passaged for cell culture adaptation of the virus. Transfected cells and supernatants were harvested at the indicated time points post transfection to monitor virus spread within the cells and virus production. Percentage of HCV positive cells were determined by NS5A immunostaining (black line). Virus production was determined by $TCID_{50}$ assay (gray bars). (C) Naïve 497-5 cells were infected with collected cell culture supernatants, and luciferase activity was measured 72 h after infection. Mean values represent at least four independent wells. Cell passages are indicated by the symbol ●; viral supernatant passages onto naïve 497-5 cells are indicated by the symbol ↓; # indicates that no titer was detected.

Gene	*Renilla* luciferase																	E1	NS2
nucleotide position *1b/2a hRluc*	477	504	936	608	617	624	732	733	812	959	960	984	999	1009	1014	1020	1031	2487	3897
	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	T	A
Passage	Sequence of recovered virus																		
Cp#9, day 27	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	T	G
Cp#13, day 38	A	A	A	A	A	A	A	A	A	A/g	A	A	A/g	A	A	A	A	T	G
Sp#1, Cp#2, day 51	A/G	A/G	A/G	G	a/G	A	G	G	G	G	G	A/G	G	A/G	G	G	G	C	G
Sp#2, Cp#5, day 78	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	C	G

Amino acid subsitition	N29S	Y38C	K42R	R73G	I76V	D78G	K114R	I141V	K90G	E198G	Y203C	P206P	K208R	K210R	R214G	V364A	Y834C

Fig. 2. Identification of mutations in 1b/2a hRluc viruses recovered from cell culture adaptation. A capital letter represents a single or dominant species with ⩾50% presence in mixed sequences. A lowercase letter represents a minor sequence with <50% presence in mixed sequences. *Sp*, indicates supernatant passage; *Cp*, indicates cell passage.

3.4. A to G hypermutations in luciferase gene diminishes reporter activity

The observed A to G hypermutations at day 51 (11 A–G) and day 78 (17-A–G) of the adaptation were engineered into the luciferase gene of the robust 1b2a hRluc-All 6 construct to verify the effect of these mutations on the down regulation of luciferase activity and virus fitness (Fig. 4A). The mutated luciferase constructs were transcribed and transfected into 497-5 cells along side a non-mutated 1b/2a hRluc-All 6 virus. Four-days after transfection virus spread

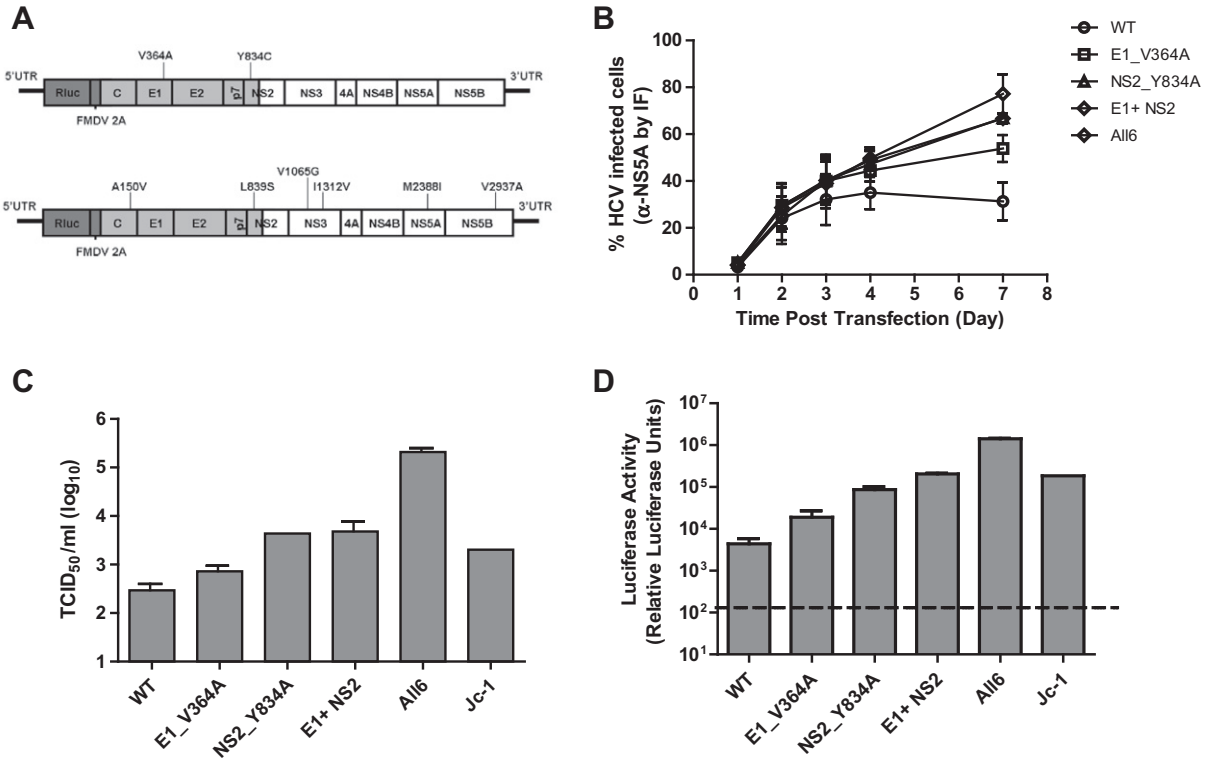

Fig. 3. Adapted mutations enhance the production of infectious 1b/2a hRluc HCVcc. (A) Location of adapted mutations inserted in 1b/2a hRluc. Top: mutations identified in 1b2a hRluc adaptation, E1_V364A and NS2_Y834C. Bottom: six previously identified adaptive mutations "1b2a hRluc – All 6". (B) RNA transcripts were transfected into 497-5 Lunet cells, the percentage of HCV positive cells was determined by NS5A immunostaining at the indicated time points. (C) The supernatants 96 h post-transfection were collected to determine virus production by $TCID_{50}$ assay and (D) luciferase activity of released virus particles. All values represent the mean of two independent experiments. Dotted line indicates background level of assay from naive 497-5 Lunet cells.

(Fig. 4B) and virus titer (Fig. 4C) were measured. The extent of virus spread and viral titers produced by the mutated luciferase viruses were comparable to the parental non-mutated 1b/2a hRluc-All 6 virus, confirming that the A to G hypermutations did not impact virus fitness. Luciferase activity, however, decreased significantly and proportionally to the number of A to G mutations present in the gene (Fig. 4D). The 11-A–G mutated virus diminished luciferase activity moderately similar to the day 51 adapted virus, while the 17-A–G mutated virus had almost undetectable luciferase levels similar to the day 78 adapted virus and non-luciferase containing 1b/2a-All 6 (no-luc) virus. These results confirm that the decline in luciferase reporter activity observed during serial passaging of the virus was due to A to G hypermutations present in the luciferase gene.

3.5. Evaluation of HCV inhibitors using infectious 1b2a hRluc All 6 reporter virus

To evaluate the 1b/2a hRluc-All 6 virus for antiviral inhibitor screening, we assessed the response of this virus to known HCV inhibitors. 2′-C-methyl adenosine (2′CMA), a nucleoside replication inhibitor, BILN-2061, a protease inhibitor, α-CD81 monoclonal antibody, a host-targeted entry inhibitor, and EI-1 (Baldick et al., 2010), a small molecule genotype 1-specific entry inhibitor, were tested in a 96-well antiviral assay using the 1b/2a hRluc-All 6 virus and the non-reporter version 1b/2a-All 6 virus for comparison.

497-5 Lunet cells were infected with 100 μl of supernatant from either virus resulting in an MOI of approximately 0.3. Compounds were added immediately after and incubated for 72 h, after which luciferase activity or NS3-4A protease activity were measured to determine virus replication levels. Fifty percent effective concentrations (EC_{50} values) are shown in Table 1. All inhibitor EC_{50} values were comparable for both viruses, indicating that inclusion of the luciferase gene does not alter antiviral susceptibility of the virus. These results confirm that the 1b/2a hRluc-All 6 virus is sensitive to replication inhibitors and can be used as a tool to identify inhibitors that target genotype 1b structural proteins.

3.6. Evaluation of early stage viral inhibitors

HCV chimeric infectious systems make it possible to characterize the early steps of HCV infection because they can recapitulate the whole viral life cycle. We used the 1b/2a hRluc-All 6 chimeric virus to compare entry inhibitors with those that inhibit different stages of the HCV cycle, including viral assembly, translation, and RNA replication (classified as non-entry). To distinguish entry inhibitors from non-entry inhibitors, we investigated the inhibitory capacity of different agents when added at different time intervals in a "time of addition" assay (Fig. 5A). In this assay, an inhibitor was either added during HCV infection or 4 h post infection after cells were washed to remove unbound virus and re-fed with fresh cell culture medium. Under these conditions, entry inhibitors should block virus infection if present during the infection, but not when added after virus infection of the cells. Conversely, inhibitors that act post entry will block virus activity only when present in cells after the infection. To avoid multiple rounds of infection and cell-to-cell virus spread from affecting the assay, luciferase activity was measured 24 h post infection.

As shown in Fig. 5B, luciferase reporter activity was a far more sensitive for detecting HCV replication at 24 h after a single round of infection compared with the non-reporter virus assay that relies on the virus's NS3-4A protease activity. HCV inhibitors of entry, α-CD81 monoclonal antibody and EI-1, and the replication inhibitor BILN-2061 were evaluated in the time of addition assay to confirm

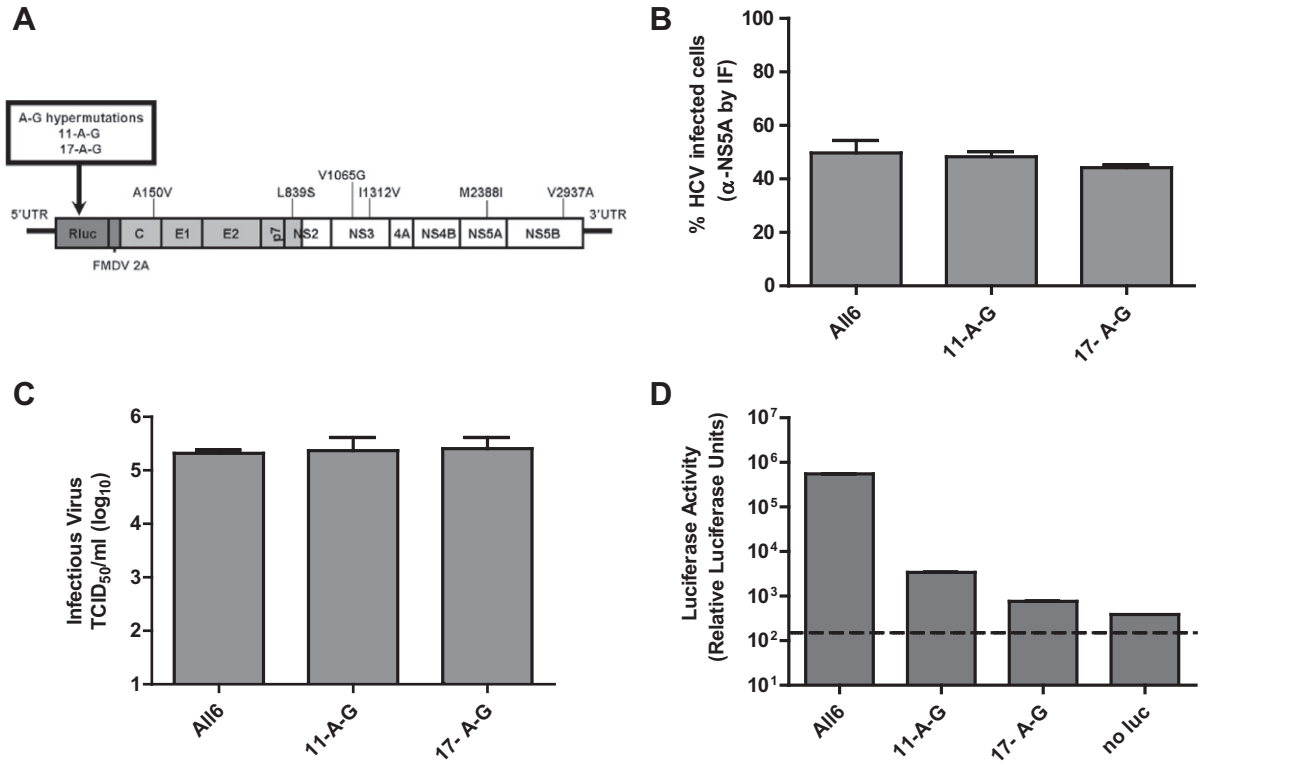

Fig. 4. A to G hypermutations diminish luciferase activity. (A) Schematic diagram of the A to G mutations identified at day 51 (11-A–G) and day 78 (17-A–G) of the adaptation inserted into the luciferase gene of the robust 1b2a hRluc-All 6 virus. (B) RNA transcripts were transfected into 497-5 Lunet cells. 96 h post-transfection the percentage of HCV positive cells was determined by NS5A immunostaining. (C) The supernatants 96 h post-transfection were collected to determine virus production by $TCID_{50}$ assay and (D) luciferase activity of released virus particles. All values represent the mean of two independent experiments. Dotted line indicates background level of assay from naive 497-5 Lunet cells.

their effects as entry or post-entry (replication) inhibitors. As expected, BILN-2061, a protease inhibitor, inhibited virus activity when added 4 h after cells were already infected with virus (Fig. 5C). However, no antiviral effect occurred when BILN-2061 was present only during the initial 4 h infection period. In contrast, the entry inhibitors α-CD81 and EI-1 inhibited virus activity only when present during the first 4 h of HCV infection, and had no effect when added after cells are already infected with HCV (Fig. 5C). These results demonstrate the utility of a luciferase reporter virus distinguishing entry and non-entry inhibitors.

Table 1
Evaluation of antiviral activity of HCV inhibitors.

Inhibitor	Inhibitor class	EC_{50}[a]		
		1b/2a All 6 hRluc	1b/2a All 6 Non-Luc	Cytotoxicity CC_{50}
E1-1 (nM)	1b-Specific entry inhibitor	8 ± 0.7	4 ± 2	>50,000
Anti-CD81 (ng/ml)	Host receptor binding antagonist	40 ± 4	90 ± 30	>2500
2′CMA (nM)	Nucleoside NS5B inhibitor	260 ± 5	350 ± 160	>50,000
BILN-2061 (nM)	Protease inhibitor	10 ± 2	8 ± 7	>50,000

[a] EC_{50} values are expressed as mean ± standard deviation from at least two independent experiments.

4. Discussion

To generate a robust, cell culture-adapted 1b/2a chimeric HCVcc encoding luciferase, we first attempted to adapt a previously reported luciferase chimera (Zhang et al., 2008). In vitro passaging of this virus for almost 80 days resulted in modest, but significantly improved titers compared with the starting virus. Genotypic analysis of the adapted virus identified two potential adaptive mutations: V364A in E1 and Y834C in NS2. The residues at both positions are highly conserved (78% for V364 in E1 and 99% for Y834C) across all HCV genotypes listed in the EU HCV database with their corresponding substitutions present at lower frequencies. The mutation Y834C was the most critical for the adapted phenotype. This mutation was previously reported to augment virus production of a 1b/2a luciferase reporter virus (Zhang et al., 2008). Furthermore, similar mutations in NS2 were also observed by us in 1b/2a chimeras (Chan et al., 2012) as well as in other intergenotypic chimeras including 1a/2a and 4a/2a chimeras; collectively, this suggests that mutation of NS2 is central to tissue culture adaptation of intergenotypic JFH-1 HCV chimeric genomes regardless of genotype. However, while this adapted virus produced improved titers in 497-5 Lunet cells (~10^3 $TCID_{50}$/ml), it lost luciferase expression (due to mutational inactivation) during the course of adaptation and thus did not achieve our goal of a robust luciferase-expressing chimera.

In parallel, we pursued an alternate approach to generate high-titer, luciferase-encoding 1b/2a HCV. Specifically, six cell culture adaptive mutations previously identified in an adapted 1b/2a virus that did not encode luciferase (Chan et al., 2012) were introduced to the luciferase-encoding virus. The resulting virus, 1b/2a hRluc-All 6, produced infectious titers 1000-fold higher than the parental virus. This increase in titer was not as pronounced as we observed for the non-luciferase virus in previous studies (10,000-fold), suggesting that the luciferase gene attenuates virus production by 10-fold. This is consistent with previous reports where insertion of reporter genes into the HCV genome affected RNA replication

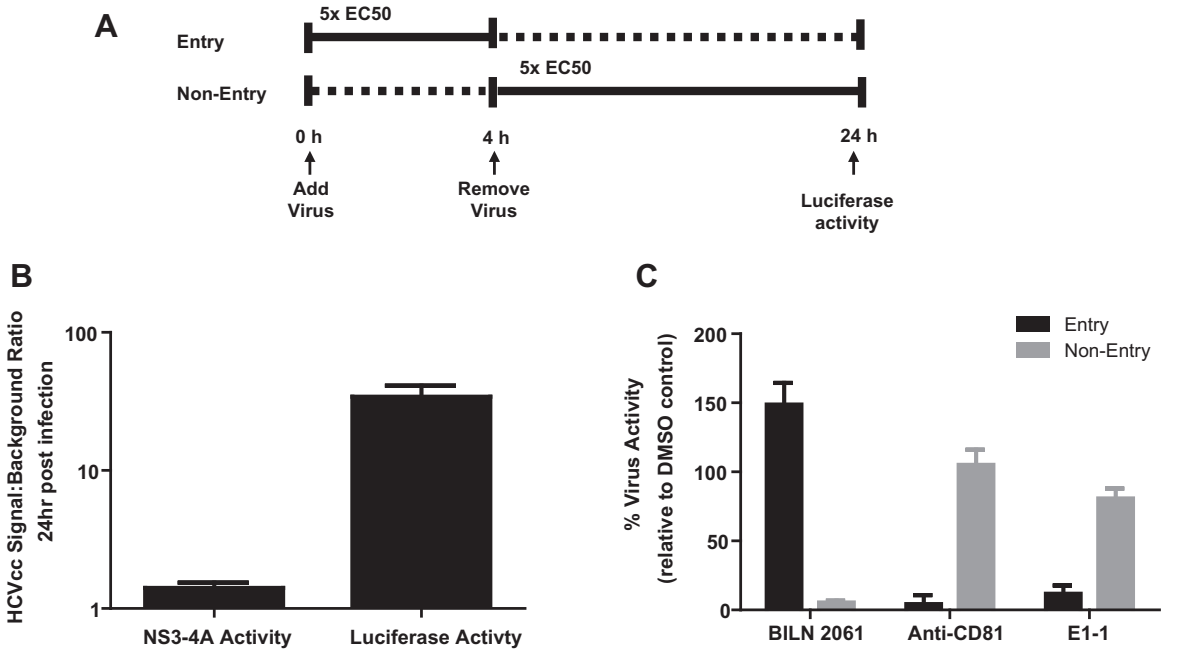

Fig. 5. Utility of luciferase reporter HCVcc for identification of early stage inhibitors. (A) "Time of addition" assay schematic for determination of entry or post entry stage HCV inhibitors. Solid lines indicate time interval compound is present. (B) Infection efficiency and signal intensity of luciferase reporter versus non-reporter 1b/2a viruses at 24 h after a single round of infection. (C) Evaluation of inhibitory activity of HCV entry inhibitors anti-CD81, E1-1 versus replication inhibitor, BILN-2061, in a time of addition assay.

(Koutsoudakis et al., 2006). Nevertheless, the 1b/2a hRluc-All 6 virus consistently replicated robustly (to titers of 10^4–10^5) and produced strong luciferase signals after infection.

Inclusion of the *Renilla* luciferase reporter greatly facilitates quantification of HCV replication through measurement of luciferase activity in cell lysates (Schmitt et al., 2011). Indeed, our studies demonstrated that using luciferase activity is much more sensitive than NS3 protease activity for detecting viral replication after a short single round of infection (e.g. at 24 h, Fig. 5B). Detection of a reliable signal from the NS3-4A protease assay required incubation of virus with cells for at least 48 h. However, harvesting cells 48 h post infection allows cell-to-cell spread and additional rounds of infection, which may confound some assays (e.g. "time of addition" assays). Multiple rounds of infection in a 48 h assay can increase the apparent potency of entry inhibitors and may also cause the incorrect classification of entry versus non-entry inhibitors. Thus, the luciferase reporter virus provides a preferred tool for studies on the early steps of the viral life cycle.

We also demonstrated that inclusion of luciferase did not alter the sensitivity of the 1b/2a virus to antivirals. In fact, we tested four different classes of inhibitors including two replication inhibitors and two entry inhibitors and, in all cases, we observed less than 2.5-fold differences in EC_{50} values generated with the luciferase and non-luciferase viruses. Thus, the luciferase 1b/2a virus offers a more facile and robust way to profile compounds without significant changes in antiviral activity. Inclusion of the genotype 1-specific E2 inhibitor, EI-1 (Baldick et al., 2010), in these studies also allowed us to confirm that the 1b/2a hRluc-All 6 virus was susceptible to genotype 1b-active compounds that specifically inhibit HCV at the entry stage of infection (Fig. 5C).

An interesting observation made during our first attempt to adapt the luciferase virus was the inactivation of the luciferase gene by A to G hypermutation. HCV replicates through an error prone process that supports the evolution of genetic variants. However, extensively mutated viruses would not be expected to replicate and survive. The inclusion of the luciferase gene that is non-essential for virus survival may have facilitated this rare detection of A to G hypermutations in our study. This specific pattern of A to G mutations led us to speculate that a host enzyme might be progressively and specifically mutating the viral genome. Based on the literature, ADAR1 (adenosine deaminases acting on RNA) is a logical candidate host protein. ADAR1 converts adenosine (A) to inosine (I) in double-stranded RNA; inosine residues are subsequently transcribed and translated as guanosine (G) leading to mutations (Samuel, 2011). In the context of HCV, it has been suggested that ADAR1 may act as an antiviral factor (Welzel et al., 2009). However, only limited mechanistic studies have been able to provide direct evidence of this possibility. Recently (Taylor et al., 2005), showed that silencing of ADAR1 in replicon cells stimulated the expression of HCV RNA and reduced the amount of inosine recovered from RNA in replicon cells. They also reported early evidence for editing in IFN-α treated replicon cells, where they isolated one clone containing A to G mutations in the HCV internal ribosome entry site. We found, by Western blotting, that two ADAR1 isoforms (p150 and p110) were expressed constitutively in infected and uninfected 497-5 Lunet cells, including at the time when the luciferase hypermutation occurred (Supplementary Fig. 1). Based on this observation, the current studies suggest that ADAR1 could be responsible for the observed hypermutation. Further investigation will be required to understand this phenomenon and the potential antiviral role of host editing enzymes like ADAR1 in modulating HCV replication.

5. Conclusions

Overall, our results demonstrate that a robust chimeric virus encoding the 1b structural region and 2a nonstructural region along with a luciferase reporter can be achieved by the inclusion of six adaptive mutations. Engineering these mutations into the parental chimeric construct avoids the need to adapt the virus de novo, which is time consuming and ultimately resulted in the loss of luciferase expression; interestingly, the loss of luciferase expression during adaptation was the result of A to G hypermutation, in

the luciferase gene. This virus encoding the six adaptive mutations (1b/2a hRluc-All 6) facilitates both routine antiviral screening as well as mechanistic assays (e.g. "time of addition" studies) that rely on generating robust replication signals in short periods of time (e.g. 24 h or less). The fact that this virus can replicate to titers up to 10^5 $TCID_{50}$/ml also facilitates the amplification of viral stocks for large scale applications.

Disclosure statement

All authors were employed by Gilead Sciences during the duration of the study. No competing interests exist for any author.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.01.005.

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