Efficient Replication of Genotype 3a and 4a Hepatitis C Virus Replicons in Human Hepatoma Cells

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Despite recent advances in the treatment of hepatitis C, the quest for pan-genotype, effective, and well-tolerated inhibitors continues. To facilitate these efforts, it is desirable to have in vitro replication systems for all major HCV genotypes. However, cell culture replication systems exist for only genotypes 1a, 1b, and 2a. In this study, we generated G418-selectable subgenomic replicons for prototype strains of genotypes 3a (S52) and 4a (ED43). Production of G418-resistant colonies by S52 and ED43 in HuH-7.5 cells required the amino acid substitutions S2210I and R2882G, respectively, cell culture adaptive mutations originally reported for genotype 1b replicons. RNA replication was confirmed by quantitative reverse transcription-PCR and detection of viral protein. Sequencing of multiple independent replicon clones revealed the presence of additional nonsynonymous mutations. Interestingly, all potentially adaptive mutations mapped to the NS3 protein. These mutations, when introduced back into original constructs, substantially increased colony formation efficiency. To make these replicons useful for high-throughput screening and evaluation of antiviral compounds, they were modified to express a chimeric fusion protein of firefly luciferase and neomycin phosphotransferase to yield stable replicon-expressing cells. Using these constructs, the inhibitory effects of beta interferon (IFN-β), an NS3 protease inhibitor, and an NS5B nucleoside polymerase inhibitor were readily detected by monitoring luciferase activity. In conclusion, we have established functional replicons for HCV genotypes 3a and 4a, important new additions to the armamentarium required to develop inhibitors with a pan-genotype activity.

According to estimates of the World Health Organization (WHO), hepatitis C virus (HCV) currently infects at least 130 million people worldwide, which is 2.2% of the global population (33). HCV infection becomes chronic in 60% to 80% of infected adults and can progress to hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma (HCC) (11). There is no vaccine against HCV infection, and the standard of care until last year, consisting of pegylated alpha interferon (IFN-α) and ribavirin, resulted in a sustained virological response (SVR) in only half of patients (43). The recent addition of the HCV protease inhibitors telaprevir and boceprevir has increased SVR rates to 70 to 80% (26, 48, 53). However, the efficacy of these inhibitors is limited by the emergence of resistance, challenging side effect management, and limited HCV genotype coverage (19, 37, 38, 50, 57, 58). Thus, there continues to be an urgent need to find better and more-broadly acting anti-HCV drugs. To promote this goal, it is important to establish in vitro replication systems for all HCV genotypes that can be used as preclinical tools for screening and optimization of new inhibitors.

HCV strains from different parts of the world show significant genetic heterogeneity, and on the basis of phylogenetic analysis, HCV has been classified into seven genotypes and a number of subtypes. HCV genotypes 1 (subtypes 1a and 1b) and 2 are the most prevalent in North America, parts of Europe, and Japan (32). For this reason, much of the HCV research over the last 2 decades has been focused on these genotypes. Recently, there is a growing interest in other HCV genotypes, which differ in their geographic distribution, pathogenesis, and treatment response. For example, combination therapy with interferon and ribavirin has a high success rate in genotype 2- and 3-infected patients, in contrast to the rate for those infected with genotypes 1 and 4 (35). The newly approved direct-acting antivirals (DAAs) telaprevir and boceprevir are less effective against genotype 3a (12, 17, 25). Similarly, hepatic steatosis has been specifically found in patients infected with genotype 3 (1, 24). These findings underscore the importance of studying the biology and pathogenesis of diverse HCV genotypes in addition to examining their sensitivity to approved antiviral inhibitors and those in the pipeline.

HCV, a member of the family Flaviviridae, is an enveloped virus with a single-stranded, positive-sense RNA genome. The viral genome consists of a single open reading frame encoding a polyprotein which is co- and posttranslationally cleaved by host and viral proteases (18, 21) to yield three structural (core, E1, and E2) and seven nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (4). The open reading frame is flanked by 5′ and 3′ untranslated regions (UTRs) (2). The 5′ UTR contains four domains (8, 9, 22, 23), the first of which is essential for virus replication (13, 23, 42); the remaining three domains, along with the first few nucleotides of the core protein region, constitute the internal ribosomal entry site (IRES), which directs the translation of the polyprotein with the help of the 3′ UTR (23, 49, 55). NS3 through NS5B are essential for viral RNA replication, and this pathology is common in patients infected with genotype 3 (1, 24). However, the efficacy of these inhibitors is limited by the emergence of resistance, challenging side effect management, and limited HCV genotype coverage (19, 37, 38, 50, 57, 58). Thus, there continues to be an urgent need to find better and more-broadly acting anti-HCV drugs. To promote this goal, it is important to establish in vitro replication systems for all HCV genotypes that can be used as preclinical tools for screening and optimization of new inhibitors.

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coding region and the 5′ and 3′ UTRs are the components typically present in HCV subgenomic RNA replicons (41). Replicons are often bicistronic constructs, with neomycin phosphotransferase (NPTII) translation driven by the HCV IRES, followed by the HCV NS3-3′ UTR under the control of a second IRES derived from encephalomyocarditis virus (EMCV). When in vitro-transcribed RNA from such constructs is introduced into Huh-7 or its highly permissive sublines (e.g., Huh-7.5) (7) and selected with G418, replicon-containing cell clones can be generated with persistently replicating HCV RNA. These replicons have been very useful in understanding the viral and host factors involved in HCV replication as well as for screening and evaluation of replication inhibitors. To date, HCV replicon systems have been successfully established for only genotypes 1a, 1b, and 2a (5, 27, 41).

Here, we report the generation of robust replicon systems for prototype isolates of HCV genotypes 3 and 4. We identified novel cell culture adaptive mutations that markedly increased RNA replication efficiency and modified the replicons to express a convenient reporter to facilitate inhibitor screening and evaluation.

MATERIALS AND METHODS

Cells, antibodies, and chemicals. Huh-7.5 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids (NEAA), and penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Huh-7.5 cells harboring selectable subgenomic replicons were grown in medium containing 750 μg/ml G418 (Sigma). Mouse monoclonal antibody 9E10 against HCV NSSA protein has been described previously (39). IFN-β and danoprevir (RG7227/ITMN-191) were purchased from PeproTech Inc. (NJ) and Selleck (TX), respectively, while 2′/C-MeA was obtained from Merck Research Laboratories (PA).

Construction of replicons. Full-length cDNA clones of genotypes 3a (S52) and 4a (ED43) have been described (16). The neomycin-selectable subgenomic replicons S52/SG-neo and ED43/SG-neo were generated by replacing amino acids 20 to 1,032 and 20 to 1,026 of S52 (GenBank accession number GU814264) and ED43 (GenBank accession number GU814266), respectively, with a cassette containing the NPTII gene and the EMCV IRES. The NPTII gene of SGR-JFH1 (27), S52/SG-neo, and ED43/SG-neo was replaced with a chimeric gene encoding the firefly luciferase protein fused in-frame with NPTII to synthesize JFH1/SG-Neo, S52/SG-Neo, and ED43/SG-Neo, respectively, as described elsewhere (56). Mutations were introduced by PCR-based mutagenesis.

RNA synthesis and transfection of cultured cells. Plasmids were linearized with XbaI and purified with the MinElute PCR purification kit (Qiagen Sciences, MD). One microgram of template DNA was transcribed using the T7 RiboMAX Express large-scale RNA production system (Promega, WI). Template DNA was removed by an additional on-column treatment with RNase-free DNase at room temperature for 15 min; RNA quality was assessed by agarose gel electrophoresis. RNA was electroporated into 5 million Huh-7.5 cells using a BTX Electro Square Porator as described previously (7). Transfected cells were suspended in cell culture medium and transferred to 10-cm-diameter dishes. For selection of colonies, G418 (final concentration of 750 μg/ml) was added to the culture medium at 48 h postelectroporation. Cells were fed every third day with fresh medium containing 750 μg/ml G418. Three weeks after transfection, G418-resistant cell colonies were either isolated and expanded for further analysis or fixed with 7% formaldehyde and stained with crystal violet. Colony formation efficiency was measured as described previously (7). Briefly, transfected cells were plated in 10-cm-diameter dishes at multiple densities (between 1 × 105 and 2 × 105 cells) together with cells transfected with replication-defective RNA transcripts such that the total number of cells in each dish was maintained at 2 × 105 cells. G418 selection was performed as described above. Four weeks postelectroporation, colonies were stained with crystal violet and colony formation efficiency was calculated by measuring the ratio between the number of colonies/plate and the number of cells (electroporated with replication-competent constructs) plated after electroporation.

Analysis of G418-resistant replicon-containing cells. Independent G418-resistant colonies were isolated using 8-mm cloning cylinders (Corning, NY) and expanded until they reached 80% to 90% confluence in P100 dishes. For quantification of HCV RNA, total RNA was extracted from the replicon cells when the first P100s were split and then another isolation was done two passages later. Total RNA was quantified using a NanoDrop (NanoDrop Products, Wilmington, DE), and quantitative reverse transcription-PCR (qRT-PCR) was performed using the LightCycler 480 real-time PCR system (Roche Applied Sciences) and MultiCode-RTx Analysis Software v.1.1.5d from EraGen (EraGen Biosciences, Madison, WI) on both RNA samples, in duplicate, and the four data points used to calculate the mean HCV RNA copy number per μg of total RNA. Viral antigen was detected by flow cytometry as described previously (44). Briefly, cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at 4°C. Fixed cells were washed twice in PBS and permeabilized for 15 min at room temperature in Cytofix/Cytotoxperm (BD Biosciences, CA). Cells were washed once in Cytoperm/Cytowash and stained for 30 min at room temperature with an Alexa-647-conjugated monoclonal antibody specific for HCV NSSA (9E10). Stained cells were washed twice in Cytoperm/Cytowash and resuspended in PBS prior to analysis using FACScalibur (BD Biosciences).

Identification of mutations. Two micrograms of total RNA was subjected to reverse transcription using SuperScript III (Invitrogen, Carlsbad, CA) and random hexamers. The cDNAs were amplified with Ex Taq DNA polymerase (TaKaRa Bio, Kyoto, Japan) as five overlapping fragments spanning the 5′ UTR and NS3-NS5B region. The sequence of each amplified DNA was determined by direct sequencing.

Firefly luciferase assay. To measure firefly luciferase activity, replicon cells were washed twice with PBS and lysed with 1× cell culture lysis reagent (Promega) according to the manufacturer’s recommendations. Luciferase activity was measured with the luciferase assay system (Promega) using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wild- bad, Germany).

RESULTS

Subgenomic replicons of genotype 3a strain S52. The consensus full-length cDNA clone of S52 has been described (16). Whereas RNA transcribed from this clone was infectious in vivo, demonstrating that all viral elements are functional, it failed to replicate when introduced into Huh-7.5 cells. Here, we generated a G418-selectable subgenomic derivative of S52 (S52/SG-neo) by replacing the core-NS2 region with a cassette containing the NPTII gene followed by the EMCV IRES such that the HCV IRES directs the expression of NPTII and the HCV NS3-NS5B region is driven by the EMCV IRES. We synthesized two replicons, S52/SG-neo containing the wild-type sequence and S52/SG-neo(1) containing an S2210I substitution in NS5A (corresponding to S2204I in genotype 3a strain H77; GenBank accession number AF009606) that has been shown to be highly adaptive for genotype 1 replicons (5, 6). In vitro-transcribed RNA was introduced into Huh-7.5 cells by electroporation, and the cells were subjected to G418 selection (750 μg/ml) 48 h later. After 3 weeks, no colonies were observed for the wild-type replicon, whereas a few colonies were visible for the cells transfected with S52/SG-neo(1) (see Fig. S1A in the supplemental material). From two independent electroporations with S52/SG-neo(1), eight colonies were isolated and expanded. HCV RNA levels in these replicon cell clones were measured by qRT-PCR, and the NSSA protein was detected by flow cytometry. HCV RNA copies ranged from 1.9 × 107 to 5.5 × 107 copies/μg RNA, corresponding to approximately 150 to 400 copies of HCV
RNA/cell (Fig. 1A). Flow cytometric analysis showed that the percentage of cells with detectable HCV NS5A ranged from 55 to 88% (Fig. 1B). Furthermore, when total RNA extracted from the G418-selected colonies was introduced into naïve Huh-7.5 cells by electroporation followed by G418 selection, significantly higher numbers of colonies were obtained in these cells than in S52/SG-neo(I)-electroporated cells. This suggested that the HCV RNA might have acquired additional adaptive mutations that increased replication efficiency (see Fig. S1B in the supplemental material). To examine this possibility, total cellular RNA was extracted from the replicons containing the indicated mutations and sequenced. While the S2210I mutation was intact in S52/SG-neo(I)-electroporated cells, the HCV RNA might have acquired additional adaptive mutations that increased replication efficiency (see Fig. S1B in the supplemental material).
TABLE 1 Nonsynonymous mutations observed in S52/SG-neo(I) replicon clones

<table>
<thead>
<tr>
<th>Replicon clone</th>
<th>Nucleotide mutation</th>
<th>Amino acid substitution</th>
<th>NS protein</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1879 (3539) T→C</td>
<td>I1067 V→A</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3663 (5323) A→C</td>
<td>I1662 T→P</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2143 (3803) C→G</td>
<td>I1155 T→R</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3663 (5323) A→G</td>
<td>I1662 T→A</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2355 (4015) C→T</td>
<td>R1226 P→S</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2988 (4648) G→C</td>
<td>P1437 D→H</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2301 (3961) A→C</td>
<td>I1208 S→R</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3886 (5546) A→G</td>
<td>E1736 E→E/G</td>
<td>NS4B</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2041 (3701) C→T</td>
<td>I1121 P→L</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1929 (3589) A→G</td>
<td>M1084 M→V</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1863 (3523) A→G</td>
<td>I1062 T→A</td>
<td>NS3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2983 (4643) C→T</td>
<td>T1435 T→I</td>
<td>NS3</td>
<td></td>
</tr>
</tbody>
</table>

*Positions within the S52 subgenomic replicon and within the full-length S52 genome (with the latter given in parentheses) are given.

**The position within the full-length S52 polyprotein is given.

***NS, nonstructural.

all replicon clones, one or two nonsynonymous mutations were present in the NS3 coding region of all clones (Table 1). Mutations were found in both the serine protease and helicase NS3 domains. In one clone, a nonsynonymous mutation where NS4B residue 19 was a mixture of Glu and Gly was also identified in NS4B. To analyze their possible adaptive value, the mutations, individually or in combination, were introduced into the S52/SG-neo(I) backbone and compared for G418 transduction efficiency in Huh-7.5 cells. All mutations except the one found in NS4B conferred various degrees of adaptation, in the range of 2- to 1,400-fold higher than that of the S52/SG-neo(I) parental clone (Fig. 1C; see also Fig. S2A in the supplemental material). Next, we asked whether the de novo mutations were sufficient for replication of S52/SG-neo in the absence of the S2210I mutation. The six most highly adaptive mutations were analyzed, and in all cases, elimination of the S2210I substitution diminished colony formation efficiency, indicating that S2210I was indeed important for the overall adaptive phenotype (see Fig. S2B in the supplemental material).

Subgenomic replicon of genotype 4a strain ED43. RNA transcribed from the full-length cDNA clone of ED43 has been shown to be infectious in a chimpanzee, but it failed to replicate in Huh-7.5 cells (16). We generated two subgenomic derivatives of ED43, ED43/SG-neo containing the wild-type sequence and ED43/SG-neo(K), respectively (see Fig. S3B in the supplemental material). Since combining mutations in NS3 with those in other genes of HCV has been shown to increase adaptation (6, 29, 40), we made another set of replicons in which the above-described mutations were combined with S1280I, a mutation found to be highly adaptive for the Con1 subgenomic replicon (29), to create ED43/SG-neo(S+I), ED43/SG-neo(g+I), ED43/SG-neo(H+I), ED43/SG-neo(G+I), and ED43/SG-neo(K+I). All mutations except R2882G alone or in combination with S1280I failed to produce G418-resistant colonies in Huh-7.5 cells. A few colonies were visible for ED43/SG-neo(G), while higher numbers of colonies were observed for ED43/SG-neo(G+I), thus suggesting a cooperative effect of mutations in NS5B and NS3 (see Fig. S3B in the supplemental material). Six colonies were picked from two independent electroporations with ED43/SG-neo(G); RNA levels were measured by qRT-PCR, and NS5A protein was detected by flow cytometric analysis. HCV RNA copies ranged from 2.3×10^6 to 1.7×10^7 copies/μg of cellular RNA, corresponding to approximately 20 to 130 HCV RNA copies/cell (Fig. 2A). Flow cytometric analysis showed that 63% to 83% of cells in different replicon cell clones had detectable HCV NS5A (Fig. 2B). When total RNA extracted from G418-selected colonies was introduced into naïve Huh-7.5 cells by electroporation and the cells were selected with G418, significantly higher numbers of colonies were visible in these cells than in ED43/SG-neo(G)-transfected cells, indicating that viral RNAs present inside the replicon cells had higher replication efficiency (see Fig. S3C in the supplemental material). To examine whether this increased efficiency was due to additional adaptive mutations in the viral genome, the NS3-NS5B coding region was amplified by RT-PCR and sequenced. While the R2882G mutation was conserved, all clones had at least one additional nonsynonymous mutation; all mutations were in the NS3 helicase domain (Table 2). To analyze the possible adaptive potential of these mutations, they were individually introduced into ED43/SG-neo(G) and replication efficiency was measured by titrating the colony formation. Most mutations conferred various degrees of adaptation, ranging from ~120- to 345-fold higher than that of ED43/SG-neo(G) (Fig. 2C; see also Fig. S4A in the supplemental material). The mutation A1309P existed as a mixture of alanine and proline and was the least adaptive, increasing G418-resistant colony formation by only 1.75-fold. Colony formation efficiency was drastically decreased when the R2882G substitution was eliminated, showing that this mutation was important for the adaptive value of de novo mutations (see Fig. S4B in the supplemental material).

Synthesis and characterization of HCV-Feo replicons. To optimize replicons for high-throughput screening and evaluation of antiviral compounds, we generated Feo replicons from JFH-1 (JFH/SG-Feo), S52 (S52/SG-Feo), S52/T1062A+T1435I [S52/SG-Feo(A1)], S52/P1226S+D1437H [S52/SG-Feo(SH)], ED43 (ED43/SG-Feo), ED43/T1369K [ED43/SG-Feo(K)], and ED43/D1431Y [ED43/SG-Feo(Y)]. Huh-7.5 cells were electroporated with RNA transcribed from the Feo replicons and selected with G418 (750 μg/ml), resulting in a large number of drug-resistant colonies in the cases of JFH/SG-Feo, S52/SG-Feo(A1), and S52/SG-Feo(SH) but small numbers of colonies for ED43/SG-Feo(K) and ED43/SG-Feo(Y). As expected, the cells transfected with S52/SG-Feo and ED43/SG-Feo did not survive at this concentration of G418. The HCV RNA level in G418-selected cells, as measured by qRT-PCR, was highest for the JFH1 replicon (1.4×10^6 copies/μg cellular RNA), followed by the genotype 3a replicons (~2×10^5 copies/μg cellular RNA), whereas the cells harboring genotype 4a replicons exhibited HCV RNA levels in the range of 3×10^4 to 6×
10⁶ copies/μg cellular RNA (Fig. 3A). By flow cytometric analysis, HCV NS5A protein was detected in 82 to 97% of cells (Fig. 3B). In agreement with the results obtained with qRT-PCR, the mean fluorescence intensity (MFI) of NS5A was higher in the cells carrying the JFH1 replicon, confirming the higher replication level of JFH1 subgenomic RNA (Fig. 3B). Expression of firefly luciferase showed a similar trend (Fig. 3C), indicating that the luciferase assay can be used for quantitative analysis of RNA replication.

**Suppression of HCV RNA replication by antiviral inhibitors.** To evaluate the use of the developed replicon systems for antiviral screening, cells expressing JFH/SG-Feo, S52/SG-Feo(AI), and ED43/SG-Feo(Y), henceforth called 2a/SG-Feo, 3a/SG-Feo, and 4a/SG-Feo, respectively, were treated with various concentrations of IFN-β, and the inhibitory effect on HCV replication was determined by measuring firefly luciferase activity. IFN-β reduced expression of the luciferase in a dose-dependent manner (Fig. 4A, upper panel). To demonstrate that the decrease in luciferase expression correlated with suppression of subgenomic RNA replication, HCV RNA was quantified in IFN-β-treated cells. qRT-PCR showed a dose-dependent decrease in subgenomic RNA copies (see Fig. S5A in the supplemental material). Similar results were obtained when the effect of IFN-β on RNA replication was examined by NS5A staining of cells (see Fig. S5B in the supplemental material). These results indicated that luciferase expression can be used as a measure of RNA replication to evaluate the effects of different inhibitors. To examine the inhibitory effect of direct-acting antivirals (DAAs), we exposed the replicons to various concentrations of danoprevir, an NS3 protease inhibitor, and 2′CMeA, an NS5B nucleoside polymerase inhibitor. Danoprevir and 2′CMeA are highly potent against genotype 1b replicons, with reported 50% effective concentrations (EC50s) of 1.8 nM and 0.2 to 0.5 μM, respectively (20, 52, 54). Our results showed that genotype 2a and 4a replicons, with average EC50s of 65 and 1.2 nM, respectively, were sensitive to danoprevir, whereas the EC50 for genotype 3a replicons was 900 nM, suggesting a lower inhibitory effect of danoprevir against genotype 3a (Fig. 4B, upper panel). On the other hand, 2′CMeA inhibited the replication of all genotypes with comparable efficiency, and average EC50s were 1.34 μM, 0.78 μM, and 0.15 μM for genotype 2a, 3a, and 4a replicons, respectively (Fig. 4C, upper panel). Finally, treatment with these drugs had little effect on cell viability and proliferation, indicating that suppression of the HCV replication was due to the specific action of these drugs (Fig. 4, lower panels). These data show that HCV-

![FIG 2 Replication of ED43-derived subgenomic replicons in Huh-7.5 cells. (A) HCV RNA copies in replicon cell clones, as measured by qRT-PCR. Results are the means and SDs. C, clone. (B) Staining of cells with anti-NS5A antibodies, followed by flow cytometric analysis. (C) Colony formation efficiency of the mutants was measured as described in Fig. 1. The names of the original replicon clones in which the indicated mutations were observed are mentioned in parentheses. Results are the means and SDs of two independent electroporations.](http://aac.asm.org/)
Feo replicon cells can be used for high-throughput screening and evaluation of anti-HCV inhibitors.

**DISCUSSION**

The HCV subgenomic replicon system was first reported in 1999 (41), and since then, it has been used as a valuable tool to study the viral and host factors associated with HCV and to screen antiviral inhibitors (reviewed in reference 3). However, replicons have been reported so far for only genotypes 1 and 2. In this study, we generated subgenomic replicons for genotypes 3a and 4a that successfully replicated in Huh-7.5 cells, as evidenced by the presence of HCV RNA and the viral protein NS5A (Fig. 1A and B and 2A and B). Furthermore, transfection of naïve Huh-7.5 cells with total RNA isolated from replicon cells transmitted G418 resistance (see Fig. S1B and S3B in the supplemental material). Adaptive mutations which, when introduced, increased the colony formation by several orders of magnitude (Fig. 1C and 2C; see also Fig. S2A and S4A in the supplemental material). These mutations conferred the adaptive phenotype in Feo replicons as well (Fig. 3). The replication was sensitive to treatment with anti-HCV inhibitors (Fig. 4).

Although they were infectious in chimpanzees, RNA transcripts from full-length cDNA clones of S52 and ED43 did not lead to productive replication in Huh-7.5 cells. As the ability to establish infection in vivo indicates that these clones were fully functional, the inability to grow in human hepatoma cells may be due to deficiency of some critical host factor(s) in all or most of these cells, so that few, if any, cells in the population supported viral replication. Thus, replication of unmodified clones may be too low to be detected by traditional assays, such as immunostaining and qRT-PCR. Second, a cytopathic effect of HCV may impart a growth disadvantage on cells supporting the viral replication, eventually leading to the elimination of such cells from the population. Some of these restrictions have been overcome by constructing neomycin-selectable replicons that permit the survival of only those cells which actively support the viral replication. This prompted us to examine whether Huh-7.5 cells are able to support the replication of subgenomic replicons derived from S52 and ED43. Initial efforts to select stable cell colonies after transfection of cells with S52/SG and ED43/SG were unsuccessful. In the past, efforts to grow an H77-derived subgenomic replicon in these cells had also proven unsuccessful until the inclusion of S2204I, a highly adaptive mutation that was identified in NS5A of the Con1 replicon (5). We speculated that introducing this mutation in the background of S52 (S2210I) and ED43 (S2204I) might confer upon them the ability to replicate. A serine-to-isoleucine substitution at this position in S52, but not in ED43, resulted in a small number of G418-resistant colonies in Huh-7.5 cells. However, ED43/SG containing another adaptive mutation, R2882G, that was also identified in the Con1 genetic background (7) yielded a few colonies. These results indicate that in spite of broad intergenotypic heterogeneity among HCV genotypes, cell culture adaptive mutations can have global effects.

S52/SG-neo(I) and ED43/SG-neo(G) needed additional mutations for efficient replication in Huh-7.5 cells. In the case of S52/SG-neo(I), two additional adaptive mutations, R2882G and T720A, were required for efficient replication. Similarly, ED43/SG-neo(G) needed two additional mutations, R2882G and T720A, for efficient replication. These results indicate that in spite of broad intergenotypic heterogeneity among HCV genotypes, cell culture adaptive mutations can have global effects.
SG-neo(I), the mutations were distributed across the length of the NS3 protein. Each of these mutations, when combined with NS5A S2210I, led to various degrees of adaptation. Mutations in the NS3 protein in the presence of the S2204I substitution have been reported for H77-derived subgenomic replicons (6). In fact, the combination of a mutation in NS5A with one or two mutations in NS3 has been shown to have strong effects on HCV replication in genotype 1a- and 1b-derived replicons (6, 29). Although we were able to select G418-resistant colonies for some mutations in the absence of S2210I, the extremely low efficiency of colony formation with these transcripts suggests that additional adaptations may be required. In the case of ED43/SG-neo(G), additional mutations were identified exclusively in the helicase domain of NS3 protein. This domain has been proposed to play a role in HCV replication by unwinding RNA secondary structures and/or double-stranded RNA intermediates (45). An active NS3 helicase is essential for replication of subgenomic replicons (31). Although it will be interesting to examine whether the mutations in the helicase domain increased the enzymatic activity of this protein, given the fact that these mutations were unable to confer a high degree of adaptation in the absence of the NS5B mutation, we speculate that they might have acted by improving the physical interaction between NS3 and NS5B. Furthermore, NS3 helicase and NS5B RNA-dependent RNA polymerase have been reported to stimulate each other’s enzymatic activity (46, 60). The mutations identified in this study may also be useful for probing the interaction among viral proteins required for efficient RNA replication of different HCV genotypes.

HCV genotype 3 is widespread, with about half of the cases of hepatitis C associated with this genotype in some European countries. Furthermore, this genotype has high prevalence in Australia and many countries of Asia and South America (15). Genotype 4a is prevalent in the Middle East and North Africa, with an increasing number of cases reported from Central and Northern Europe (10). Up to 10 to 20% of the general population is infected with HCV genotype 4a in Egypt (15), clearly indicating the urgent need to develop effective antiviral agents against this genotype. However, the lack of cell culture systems for these genotypes has hampered studies of their pathogenesis and responsiveness to anti-HCV inhibitors. For example, all the cell-based testing of an HCV NS3 protease inhibitor (PI), telaprevir, one of the only two direct-acting antivirals (DAAs) approved by the FDA to treat HCV genotype 1 patients, was conducted on genotype 1-derived subgenomic replicons; less is known about its efficacy against other genotypes (30, 50). These problems have been partially overcome by the use of chimeric systems expressing one or multiple genotype-specific proteins in the backbone of genotype 1- or 2-based replicons or the infectious genotype 2a clone JFH-1 (14, 17, 34, 47, 51). Although such systems facilitate the evaluation of broadly acting antivirals, altered or diminished cross-genotypic interactions between proteins can potentially affect the results. Replicons for genotypes 3a and 4a may therefore be more relevant for testing the inhibitors for efficacy against these genotypes. To facilitate such studies, we modified subgenomic replicons of JFH1, S52, and ED43 to express a chimeric protein of Fluc and NPTII. These Feo constructs enable the selection of stable replicon cells and facilitate the easy quantification of HCV replication levels by measuring the luciferase activity. Feo replicons have previously been used as efficient tools for quantification of HCV replication in cells treated with small interfering RNAs or anti-HCV inhibitors (28, 56, 59). By using this system, we showed that the sensitivity of genotype 3a replicons to the macrocyclic protease inhibitor danoprevir was

FIG 4 Suppression of HCV replication by anti-HCV inhibitors. HCV-Feo replicon-containing cells were seeded in 96-well plates at a density of 1 × 10⁴ cells/well. Twenty-four hours later, cells were exposed to the indicated concentrations of IFN-β (A), danoprevir (B), and 2’CMeA (C). After a 72-hour treatment with inhibitors, cells were harvested and firefly luciferase activity was measured. Results are the means and SDs of three independent experiments (upper panels). PrestoBlue cell viability reagent (Invitrogen, CA) was used to examine the cytotoxic effect of inhibitors according to the manufacturer’s recommendations. Data are the means and SDs of three independent experiments (lower panels).
significantly lower than that of genotype 2a and 4a replicons. These results are in accordance with a previous report in which Gottwein et al., by using chimeric systems expressing genotype-specific NS3 protease and NS4A (NS3P/NS4A) proteins in the backbone of genotype 2a/2a recombinant J6/JFH1 virus, showed that the genotype 3a protease was 16-fold less sensitive to danoprevir than was the genotype 2a protease (17). However, in spite of efforts, this group was unable to establish such systems for genotype 4a. The reduced sensitivity of the genotype 3a replicon to danoprevir is not likely a consequence of adaptive mutations present in the NS5 region, as the EC_{50} observed in this study is consistent with that reported by Gottwein et al. By exposing Huh-7.5 cells transfected with the Jc1 genotype 2a genomes expressing genotype-specific NS3P/NS4A to protease inhibitors (PIs), Imhof et al. showed that replication of the chimeric genomes encoding genotype 3a-specific protease was >100-fold less sensitive to danoprevir and BILN2061, the prototypic macrocyclic PI, than replication of those encoding genotype 4a-specific protease (25). NS3 amino acid residues 41, 43, 155, 156, and 168 are associated with danoprevir-resistant genotype 1b (36, 57). With the exception of position 168, all of these amino acids are the same in S52 as they are in genotype 1b. S52 residue 168 is glutamine, whereas it is aspartic acid for genotype 1b. Although the genotype 1b substitutions D168Y/T/A/H confer resistance to danoprevir, there is no information regarding possible resistance of D168Q. Taken together, our results highlight the importance of testing DAAAs against a diverse set of HCV genotypes. To improve anti-HCV therapy, it is important to understand the genetic basis for the differential sensitivity of HCV genotypes to PIs.

In conclusion, we have established subgenomic replicons for genotypes 3a and 4a which will be useful research tools for understanding the replication, pathogenesis, and persistence of these genotypes. In addition, these replicons will be instrumental in the search for more-effective antiviral agents and in vitro characterization of resistance mutations. To facilitate the development of broad-acting, pan-genotypic inhibitors, replicon systems are also needed for HCV genotypes 5 and 6. HCV research is limited by the numbers of isolates available for each genotype; therefore, efforts should be made to expand the repertoire of in vitro replication systems for major HCV genotypes.

ACKNOWLEDGMENTS

We thank J. Sable, E. Castillo, and A. Webson for laboratory support. This work was supported in part by NCI R01 CA057973 (C.M.R.) and in part by the Pakistan-U.S. Science and Technology Cooperation Program of the National Academy of Sciences and the U.S. Department of State. Additional funding was provided by the Greenberg Medical Research Institute, the Starr Foundation, and the Ronald A. Shellow, M.D., Memorial Fund (C.M.R.). L.B.D. and S.M. were supported by NIH R01 AI089957. T.K.H.S. is the recipient of a Ph.D. stipend from the Faculty of Health Sciences, University of Copenhagen, and a postdoctoral fellowship from the Danish Council for Independent Research, Medical Sciences. J.B. was supported by research grants from Lundbeck Foundation.

The opinions, findings, conclusions, or recommendations expressed in this article are those of the authors and do not necessarily reflect the views of the U.S. Department of State or the National Academy of Sciences.

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