Enhancement of the replication of HCV replicons of genotypes 1-4 by manipulation of CpG and UpA dinucleotide frequencies and use of cell lines expressing SECL14L2 – application for antiviral resistance testing

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ABSTRACT

Treatment for hepatitis C virus (HCV) has improved greatly through the use of direct acting antivirals (DAAs). However, their effectiveness and potential for drug resistance development in non-genotype 1 variants of HCV remains relatively unexplored as in vitro assays to assess drug susceptibility are poorly developed and unsuited for a transient transfection format. In the current study, we have evaluated effects of dinucleotide frequency changes in the replicon and the use of a SEC14L2-expressing cell line on the replication of HCV of different genotypes and evaluated the resulting assay formats for susceptibility measurements to the DAA, Sofosbuvir. Removal of CpG and UpA dinucleotides from the luciferase gene used in HCV replicons of genotype 1b (Con1) and 2a (JFH-1) achieved between 10-100-fold enhancement of replication over wild type post transfection. Removal of CpG/UpA-high neomycin genes in replicons of genotype 3a (S52) and 4a (ED43) enhanced replication but phenotypic effects on altering luciferase gene composition were minimal. Further ten-fold replication enhancement of replicons from all four genotypes was achieved using a transgenic Huh7.5 cell line expressing SECL14L2, whose expression showed a dose-dependent effect of HCV replication that was reversible by siRNA knockdown of gene expression. Combining these strategies, the 100 to 1000-fold enhancement of replication allowed susceptibility to the RNA polymerase inhibitor, Sofosbuvir, in a transient transfection assay format to be robustly determined for all four genotypes. These methods of replication enhancement provide new tools for the monitoring of susceptibility and resistance of a wide range of HCV genotypes to DAAs.
INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense RNA virus of the Flaviviridae family first described in 1989 (1) and subsequently identified as the principal cause of non-A, non-B hepatitis in blood recipients and haemophiliacs, as well as widely infecting injecting drug users (2,3). Based on meta-analysis of serological data it is estimated that about 185 million people worldwide are infected with HCV (4). Currently there are 7 genotypes of HCV recognised of which genotype 1 accounts for 46.2% of all infections, followed by genotype 3 with 30.1% and genotypes 2, 4 and 6 for 22.8% (5). It is estimated that 70-80% of all new infections progress to chronicity that can ultimately lead to end-stage liver disease (6,7).

Until recently, HCV was treated by combination-therapy of pegylated interferon-α and the nucleoside inhibitor Ribavirin which is effective in genotypes 2 and 3 infections (approximately 80% sustained virological response), but less so in genotypes 1 and 4, where 50% or less achieve virus clearance (8-10). The phenotypic diversity of HCV genotypes, as manifested by these major differences in treatment response therefore requires the effectiveness of and resistance development to novel antiviral treatments to be evaluated separately for different HCV genotypes and subtypes. Such testing is generally performed using subgenomic replicons in which the structural genes are replaced by a luciferase or other reporter gene to allow replication to be rapidly quantified (11,12). However, most wild-type replicons replicate poorly in cell culture and generally restricted to the Huh7.5 cell line in which a number of cellular defence pathways are non-functional, including the cytosolic RNA receptor, RIG-I (13). However, under antibiotic selection for replicon-containing cells, stable cell lines can be selected in which replicons rapidly acquire cell culture adaptive mutations that enhance replication. For example, in the genotype 1b Con 1 replicon, adaptive mutations occur in the NS3, NS4B and NS5A genes (12,14,15) that may enhance HCV protein-protein interactions and viral morphogenesis, although the mechanisms remains poorly understood.
understood (16). Such mutants, however, show increases in viral RNA in transient replication assays without antibiotic selection. Although there now exist a wide range of stably transfected Huh7.5 cell lines containing replicons of different HCV genotypes, the existence of functionally poorly defined adaptive mutations has the potential to influence their susceptibility to directly acting antivirals (DAAs) and effects of DAA-associated mutations on antiviral susceptibility. While transient replication assays are clearly preferable for such testing, many of the currently available replicons do not have the required level of replication needed in this assay format to accurately estimate changes in replication levels in drugs inhibition studies.

In the current study, we have investigated the effectiveness of two novel approaches to enhance HCV replication in cell culture. The first approach is to reduce frequencies of CpG and UpA dinucleotides in the reporter gene of HCV replicons to enhance replication. The rationale is based on previous studies that demonstrate that lowering CpG and UpA frequencies in coding regions of echovirus 7 or the luciferase gene in a derived replicon substantially enhanced their replication over wild type virus (17,18). A second, separate approach is based upon the recent finding that expression of the SEC14L2 gene is a limiting factor in HCV cell culture replication and enhancement of HCV replication was achieved through the use of cell lines that overexpressed the SEC14L2 gene, increasing the number of colony forming units in antibiotic-selected non-transient replicon studies (19).

MATERIALS AND METHODS

Replicon construction, RNA in vitro transcription and translation. The Con1 subgenomic replicon was provided by R. Bartenschlager (11), SGR-JFH1 and SGR-JFH1/GND were provided by J. McLauchlan (20), S52/SG-Feo (Al1) and ED43/SG-Feo (VYG) were obtained from C. Rice (21). A synthetic DNA sequence used to replace the wild type (WT) luciferase sequence was specified using
the program Sequence Mutate in the SSE package (22) and was cloned in the various replicons using unique restriction sites at the 5’ end (Asci) and 3’ end (Pmei) of the luciferase coding sequence (Fig. 1).

Replicon plasmids were linearized with XbaI, Mung-bean nuclease treated, purified, quantified and used as template for in vitro RNA transcription (MEGAscript, Invitrogen). RNA was precipitated using LiCl, washed, resuspended in water, aliquoted and stored at -80°C.

Transcript RNAs (125 ng) were used in nuclease-treated rabbit reticulocyte lysate translation assays (Promega) to compare the translation speed of the different luciferase constructs according to the manufacturer’s instructions. Samples were taken after 30 minutes incubation and the luciferase expression quantified as described below.

Cell lines, electroporation and luciferase quantification. Huh7.5 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 4,500 mg/l glucose, 2 mM L-glutamine, 10% heat-inactivated foetal calf serum (FCS; Harlan Sera-Lab), nonessential amino acids, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C, 5% CO₂, and 100% relative humidity.

For electroporation, cells were washed 3x in cold PBS, counted and diluted to 10⁷ cells/ml. Using 4mm electroporation cuvettes, 400μl of the cell suspension was mixed with 1 μg of replicon RNA and electroporated (at 270V, 950μF) using the exponential setting in an electroporator (Bio-rad, Gene pulser XCell). Cells were immediately resuspended in warm, complete DMEM and transferred to the appropriate sized cell culture dishes. At the desired time points, medium was removed, the cells washed with PBS and lysed in passive lysis buffer (Promega) and the luciferase expression measured using the ‘Steady-glo’ assay system (Promega) and a luminometer (Promega, Glomax Multi detection system).
To obtain stable cell lines expressing SEC14L2, a lentiviral vector expressing this gene (Applied Biological Materials) was used to generate lentiviruses according to the manufacturer’s instructions. Huh7.5 cells were transduced with the lentivirus particles and selected with 5 μg/ml of puromycin. Single colonies were isolated and grown for further evaluation.

**Protein isolation and Western blotting.** Cell monolayers were washed with PBS before being lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton-X100), mixed with sample buffer (Sigma, Laemmli 2x concentrate) and boiled for 10 minutes. Protein samples were loaded on 10% gels (Bio-rad, mini-protean TGX), transferred by semi-dry blotting to Immobilon paper (Millipore) and blocked in 5% milk-powder in PBS. After washing in PBS-T (PBS with 0.05% Tween), blots were probed with anti-SEC14L2 (Santa Cruz Biotechnology) or anti β-tubulin (Abcam) as a loading control for one hour at room temperature, washed and probed with anti-mouse HRP or anti-rabbit HRP respectively, for one hour at room temperature, washed and developed using ECL (ECL prime, Amersham).

**siRNA transfection.** Knockdown of SEC14L2 was performed using the commercially available and validated esiRNA, EHU146781 (Sigma-Aldrich). The irrelevant siRNA sequences used as a control was obtained from the same manufacturer (EHUEGFP). To knock-down SEC14L2 expression in the stable cell line, a titration of SEC14L2-specific siRNA was performed by transfecting 0, 100, 200 and 400 ng of siRNA/ 24-well using Lipofectamine RNAIMAX transfection reagent (Invitrogen) using the manufacturer’s instructions. Mock treated cells received 400 ng of validated non-targeted control siRNA. After 48 hours, SEC14L2 expression levels were quantified by Western-blot and ImageJ software.

**Sofosbuvir susceptibility testing.** For the titration of Sofosbuvir (PSI-7977, Cayman chemical), cells were electroporated with replicon RNA and seeded at the required density. After 4 hours the medium was replaced with DMEM containing the desired concentration of Sofosbuvir dissolved in
ethanol or mock treatment control (ethanol) and refreshed every 24 hours to ensure a constant Sofosbuvir presence in the medium.

qRT-PCR. SEC14L2 mRNA expression levels were quantified by qRT-PCR using the forward primer: 5’-TGCAGTGATCTGGCATCTATG-3’ and reverse: 5’-TGAGGCTTTGTCTGGAAGCAG-3’. RNA was extracted using the RNeasy kit (Qiagen), DNAse-treated and reverse transcribed using GoScript reverse transcriptase (Promega). The qRT-PCR was performed in a Rotorgene real-time PCR cycler (Qiagen) using the SensiFAST sybr kit (Bioline) and GAPDH for normalization (Forward 5’-GAAATCCCATCACCATCTTCCAGG-3’; Reverse 5’-GAGCCCCAGCCTTCTCCATG-3’). RNA stability of the different replicons was determined by measuring luciferase-RNA levels at four hours post electroporation by qPCR (Forward 5’-CCCTGGTTCCTGGAACAATTGC-3’ and Reverse 5’-AAGAATTGAAGAGGTTCCTGC-3’).

RESULTS

Effect of CpG/UpA dinucleotide composition on the replication of HCV replicons. In its unmodified state, the genotype 2a replicon (JFH1) already shows a robust level of replication in a number of cell lines, without any need for cell-culture adaptive mutations (20, 23, 24). We used the JFH1 replicon expressing the firefly luciferase gene driven by the HCV 5’ UTR (20). The WT luciferase sequence shows a striking elevation of the observed to expected frequency of CpG dinucleotides (1.21; Table 1) compared to that in HCV (0.71 – 0.74 in genotypes 1-6) and in human mRNA sequences (mean 0.43). High frequencies of this dinucleotide and UpA substantially restricted the replication of echovirus 7 (17) and we reasoned this may also influence the replication capability of the HCV replicons in mammalian cell culture. We therefore replaced the wild type luciferase gene (L) in JFH1 with the CpG/UpA-low mutant (l) in both the replication-competent (GDD) and replication
incompetent background (GND). The modified luciferase sequence was mutated to remove all 100 of the CpG dinucleotides present in the native sequence and 64 of the 86 UpA dinucleotides, the maximum possible while retaining identical amino acid coding to the WT sequence (Table 1; Fig. 1A). The modified sequence, however, contained a similar G+C content, codon adaptation index (CAI) and codon pair bias (CPB) to the WT sequence.

Genotype 2a replicons with WT and CpG/UpA-low luciferase reporter genes (2a/l-GDD and 2a/L-GDD respectively) were transfected into Huh7.5 cells and luciferase expression quantified at different time points post-electroporation (Fig. 2A). To ensure equal amounts of RNA were delivered in the cells, an aliquot of cells were taken 15 minutes after electroporation, RNase-treated to remove extracellular RNA and the internalized luciferase -RNA quantified by qRT-PCR. No significant differences in amounts of RNA were found between the different mutants used was observed (data not shown). Despite this equal delivery of RNA, the replication kinetics of the 2a/L-GDD (original) and 2a/l-GDD (CpG/UpA-minimised) were distinct with an approximate hundred-fold (2-log) difference in luciferase expression at 48 hours and 96 hours. This difference was greater than the 5-fold-difference in luciferase gene expression 4 hours post-transfection and at earlier time points before significant replication of HCV had taken place (Fig. S1; Supplementary Data).

The same changes were also made in the genotype 1b background, using the Con1 subgenomic replicon (1b/L-GDD, 1b/l-GDD and replication defective counterparts, 1b/L-GND, 1b/l-GND; Fig. 2B). Unlike the 2a replicons, the WT (1b/L-GDD) replicon showed very poor replication levels in Huh7.5 cells (Fig. 2B) in the first 24 hours. RT-qPCR analysis confirmed that comparable amounts of RNA were electroporated in the cells at one hour (data not shown). Similarly, from 24 hours, their replication kinetics were distinct, where the low-CpG/UpA luciferase replicon showed a greater than 2-log higher luciferase levels at 96 hours post electroporation compared to 1b/L-GDD.

To investigate effects of dinucleotide composition changes for other genotypes, a range of replicons were constructed using genotype 3a (S52/SG-Feo(AII)) and 4a (ED43/SG-Feo(VYG)) backbones.
The original replicons expressed luciferase as a fusion with neomycin (N) which also possesses high frequencies of CpG and UpA dinucleotides (Fig. 1B). Mutants were constructed in which luciferase was replaced with the CpG/UpA-low sequence (3a/IN-GDD and 4a/IN-GDD), those with deletion of the neomycin gene (3a/L-GDD and 3a/L-GND) and a combination of CpG/UpA-low luciferase sequences and neomycin deletion (3a/l-GDD and 4a/l-GDD).

Both of the original replicons, 3a/LN-GDD and 4a/LN-GDD, of genotypes 3 and 4 replicated poorly in transient replication assays compared to genotype 2a (Figs. 1C, 1D). In contrast to previous experiments using 1b and 2a-based replicons, replacing the luciferase sequence in genotype 3 (3a/IN-GDD) did not enhance replication. However, deletion of the neomycin sequence in the 3a/L-GDD increased replication compared to 3a/LN-GDD, although this effect was not reproduced in the low luciferase version of the neomycin-deleted construct (3a/l-GDD). For genotype 4, deletion of the neomycin gene (4a/L-GDD) and introduction of low-CpG/UpA luciferase (4a/IN-GDD) both enhanced replication over the original replicon (4a/LN-GDD), effects that were synergistic at early time points (4a/l-GDD). For all genotype 3 and 4 replicon mutants, quantification of transfected RNA at one hour demonstrated that comparable amounts of RNA were electroporated into the cells (data not shown).

**Effect of SEC14L2 expression on HCV replication.** To investigate whether the reported enhancement of replication by expression of SEC14L2 (19) could also be achieved in a transient transfection replication assay, a number of clonal cell-lines stably expressing SEC14L2 were made. Based on SEC14L2 expression levels measured by RT-qPCR, several lines were selected and tested for SEC14L2 proteins expression levels by Western blot (Fig. 3A). All five cell lines selected constitutively expressed detectable but variable levels of SEC14L2 protein while it was undetectable in the parental Huh7.5 cell line (labelled “P”).

Three out of five cell lines supported enhanced replication of 1b/L-GDD replicon (Fig. 3B), with cell line #23 showing the highest increase in replication compared to the parental cell line.
To verify this replication enhancement arose directly from increased expression of SEC14L2 and was not an artefact introduced the transduction and consequent single cell selection, siRNA was transfected in #23 cells to knock-down SEC14L2 protein expression (Fig. 3C). The effect of SEC14L2 knockdown on HCV replication was investigated in cells transfected with a siRNA concentration that achieved a 75% knock-down of expression (Fig. 3D). These cells were electroporated with 1b/I-GDD, and luciferase expression measured compared to mock-treated #23 cells (Fig. 3D). Knock down SEC14L2 decreased replicon replication by approximately 4-fold at 72 hours, confirming the involvement of SEC14L2 in the control of replicon replication.

Replication of HCV genotypes 1-4 in SEC14L2-expressing cells. Replicons with all four genotype backbones and corresponding CpG/UpA-low luciferase mutants were electroporated into the SEC14L2 expressing cell line #23 and replication compared to that of the parental Huh7.5 cells (Fig. 4). Replication of the 2a/L-GDD replicon was increased approximately 17-fold in the SEC14L2 expressing cell line at the 72 h.p.e. time point; a slightly lower enhancement was observed in the 2a/I-GDD (CpG/UpA-low) construct (Fig. 4A). Replication enhancement was synergistic, with a 340-fold increase in luciferase expression in 2a/I-GDD in #23 cells compared to 2a/L-GDD in Huh7.5 cells. Consistent with a role for SEC14L2 expression in enhancing replication, luciferase expression from both 2a/L-GND and 2a/I-GND defective replicons was comparable between cell lines (Fig. 4A).

A comparable 10-fold enhancement of the replication of the Con1 1b/L-GDD and 1b/I-GDD replicons was observed in #23 cells, with similarly no effect on their replication defective counterparts (Fig. 4B). SEC14L2 expression and lowering CpG/UpA frequencies had a synergistic effect, leading to an overall 1200-fold replication enhancement compared to the original replicon in Huh7.5 cells. Replicons based on genotype 3a and 4a backbones (3a/L-GDD and 4a/L-GDD) showed approximately 1-log increased luciferase expression. A much larger cell line-dependent increase was observed in the Neomycin-deleted 3a replicon, 3a/I-GDD (Figs. 3C, 3D).
Mechanism of replication enhancement. The increased replication of replicons with alterations to dinucleotide composition may have originated from differences in the efficiency of translation of the luciferase gene through associated alterations in codon usage or codon pair bias (25-27). Alternatively, as demonstrated for E7, changes in CpG and UpA frequency may influence the cellular response to infection and induce greater restriction of replication (17). To investigate effects of CpG and UpA dinucleotide frequency changes on translation, replicons from all four genotypes containing the original (L) or modified (l) luciferase genes were assayed for translation efficiency in an in vitro translation assay (Fig. 5). Despite the large differences in codon usage and codon pair bias between the insect-derived luciferase gene and the CpG/UpA-minimised mutant sequence (Table 1), expression of the original and mutant forms of the luciferase gene in all four replicons of genotypes 1-4 was similar. The two forms of luciferase gene showed at most 2-fold differences in translation efficiency but with no evidence for any consistent greater expression of the CpG/UpA-low luciferase sequences over wild type (Fig. 5). To ensure the assay was not saturated with RNA that narrowed differences in expression, the assay was repeated using different RNA transcript amounts. Transfecting 4 times more and four times less RNA confirmed that the read-outs for the assay concentrations used were in the linear range (Fig. S2; Supplementary Data). Replicons containing modified luciferase gene sequences showed comparable stability post-transfection. In the absence of replication, RNA levels of both genotype 1 and 2a replicons showed a comparable at 4 hours post-transfection (Fig. S3; Supplementary Data).

As the restriction in replication engendered by increased frequencies of CpG and UpA dinucleotides composition was not mediated through differences in translation efficiency or greater RNA instability, we next investigated whether the inhibition of replication in replicons expressing native (high CpG/UpA) luciferase genes was mediated on the replicon containing the gene sequence (in cis) or induced a global change in the cell in permissivity to HCV replication (in trans). 1b/L-GDD and 1b/l-GDD (containing WT- and CpG/UpA-low luciferase gene sequences respectively) were co-
electroporated into Huh7.5 cells (Fig. 6A). The presence of 1b/L-GDD RNA minimally reduced the
replication of 1b/l-GDD (approximately 50%) compared to expression levels of 1b/l-GDD
electroporated alone. The experiment was repeated in the SEC14L2 expressing cell line, where no
effect on the expression of luciferase compared to electroporation on 1b/l-GDD alone (Fig. 6B).
These findings provide evidence that effects of dinucleotide composition are mediated locally (in cis)
on the RNA molecule possessing the altered dinucleotide composition rather than such sequence
inducing a whole cellular restriction on replication (eg. mediated through induction of interferon-β).

Use of enhanced transient transfection assays to measure susceptibility to DAAs. For testing the
inhibitory capacity of HCV antiviral agents in transient replication assays, achieving sufficient
replication levels to quantify degrees of inhibition is essential. We therefore investigated whether
the enhanced replication achieved by replacing the wild-type luciferase or culture in SEC14L2-
expressing cell line facilitated the evaluation the NSSB inhibitor Sofosbuvir in genotypes 1b, 3a and
4a. Cells were electroporated with each replicons in the presence of a range of Sofosbuvir
concentrations spanning the previously established IC_{50} concentration (28,29). Luciferase expression
levels were measured at time points 48, 72 and 96 hours post electroporation. Non-replicating
replicons were included as baseline levels of luciferase expression. The 1b/L-GDD and 4a/L-GDD
replicons replicated at such low levels in the Huh7.5 cell lines that replication inhibition by
Sofosbuvir could not be detected (Fig. 7, left two panels). However, performing the assay in SEC14L2
expressing cells improved the replication and enabled a concentration-dependent inhibition of
replication by sofosbuvir to be detected (Fig. 7A/7C, panels 7-9). A major enhancement was
observed using the 1b/I-GDD replicon in Huh7.5 cells which improved even more when using
SEC14L2 expressing cells. This same pattern, but to a lesser extent was observed in genotype 4a.
Although the wild type genotype 3a replicon already shows usable titration data in Huh7.5 cells,
removal of Neomycin and especially the use of SEC14L2 expressing cells improved overall replication
and enabled a robust estimation of IC_{50} values for sofosbuvir for this genotype (Table 2).
Despite only reproducing the intracellular replication steps of the HCV lifecycle, replicons are a valuable tool in HCV research, especially in drug discovery programs and have been instrumental in the discovery of the first direct-acting antivirals (DAAs) (30), including Sofosbuvir (31), Simeprevir (32,33) and Boceprevir (34). In addition to susceptibility testing, in vitro systems play an essential role in monitoring the phenotypic effects of resistance-associated mutations that arise during DAA treatment on drug susceptibility (35,36). Resistance mutations are frequently genotype-specific and for their effects to be quantified, inhibition assays require that these mutations should be tested in the same genotypic background in vitro. DAA susceptibility and resistance testing should also be performed without the confounding effect of unpredictable cell-culture adaptive mutations that are likely to arise with antibiotic selection.

To date however, most information on susceptibility and resistance testing has been based on in vitro assays using cell lines stably expressing HCV replicon-RNA, typically H77 (genotype 1a) and Con1 (genotype 1b) (36). Selection of stable cell lines prior to testing is also normally used in the analysis or selection of resistance mutations (38-40). However, construction of stably transformed cell lines is a time-consuming procedure and which is also likely to introduce additional cell culture adaptive mutations that may also influence DAA susceptibility, complicating the comparison with the wild-type replicons. Therefore the use of a transient expression assay with a range of genotypes would be highly advantageous in terms of speed, simplicity and avoidance of cell culture-induced artefacts. Although some of these have been used in the analysis of, for example, several resistance-associated mutations to Sofosbuvir (29), the low level of replication generally achieved means that such studies are problematic to extend for a wider range of HCV strains and genotypes.

To improve the replication of replicons in transient expression assays we first replaced the wild-type luciferase gene with a mutated version in which all CpG dinucleotides were removed and as many
UpA dinucleotides as possible while retaining the same coding sequence and similar codon usage. In both genotypes 1b (Con1) and 2a (JFH1), this replacement resulted in 2-log improved replication rates compared to replicons containing the original wild type luciferase sequence (Figs. 1A and 1B). This enhancement of replication was comparable to that exhibited in the echovirus replicon with similar replacement of the luciferase gene (17). How this enhancement of both initial gene expression and subsequent increased replication is mediated remains uncertain, although observation in the current study can rule out some mechanisms. Firstly, the absence of any consistent difference in translation efficiency of original and modified luciferase genes in different replicon constructs clearly demonstrates that the enhancement of replication of CpG/UpA-low replicons was not mediated though a translational mechanism. These findings are consistent with previous studies demonstrating comparable translation efficiency of mutants of E7 with regions of genome with altered dinucleotide frequencies and codon pair bias (18), and of poliovirus in which relatively small differences in translation rates between PV-Min, WT and PV-Max mutants differing in codon pair usage / dinucleotide frequencies were not predictive of their replicative ability (26). A detailed investigation of a range of compositional variables on the replication of poliovirus reported that CpG and UpA dinucleotide frequencies primarily influenced the replication of poliovirus, and was unaffected by variation in codon usage, codon pair bias and other metrics of predictive of translational optimisation, such as CAI (27). Modification of codon pair bias and CpG/UpA dinucleotide frequencies in echovirus 7 similarly indicated the primary influence of dinucleotide frequencies on virus replication (18). In a broader context, differences in expression of the luciferase gene mediated purely through translational effects cannot contribute to the replication fitness of HCV replicons as its purpose is simply to act as reporter gene. The enhancement of replication of replicons containing CpG/UpA-low luciferase coding sequences must therefore be mediated though alternative mechanisms.

The minimal or absent interference on replication of the 1b/L-GDD by the wild type 1b/L-GDD replicon similarly argues against a global restriction in permissivity for viral replication that would be
expected in interferon-primed cells. The lack of interference between replicons is however compatible with the hypothesis advanced previously to explain the restriction of CpG/UpA-high mutants of E7. In this account, possession of high CpG and UpA dinucleotides may induce a localised stress response in the cell that influences the ability of viruses to establish replication complexes (17). The HCV replicon system established in the current study provides a valuable tool for future dissection of the restriction mechanisms associated with altered dinucleotide frequencies.

The clear replication enhancement observed in the genotype 1b and 2a replicons containing CpG/UpA-low luciferase sequence was not fully reproduced in genotypes 3a and 4a, but showed a more subtle and complicated pattern. The original versions of both replicons expressed luciferase as a fusion protein with neomycin (21). Removal of neomycin in genotype 3a increased replication although in contrast to other replicons, replacement of the luciferase component of the fusion protein with the CpG/UpA-low sequence negatively impacted replication of the genotype 3a replicon (Fig. 2C) for reasons that remain undetermined. In the genotype 4a replicon, removal of the neomycin gene similarly resulted in enhanced replication at late time points but in this case the further introduction of the low CpG/UpA luciferase further increased replication to a similar degree to that observed in genotype 1b and 2a. Understanding how the restrictions in replication mediated though dinucleotide composition interact with the limited or complete inability of many genotype or strains of HCV to replicate in cell culture will require a much better understanding of the cellular pathways that mediate these replication phenotypes and their potential for interaction.

As a further manifestation of the complexity of the restriction of HCV replication in vitro, substantial increases in the replication of replicons with all four genotype backgrounds were achieved in cells over-expressing SEC14L2. SEC14L2 was originally reported to enhance the replication of non-cell culture adapted isolates of HCV that are not resistant to lipid peroxidation (19). We investigated whether same positive effect on replication could be achieved for replicons in a transient expression assay format. Transduction of parental Huh7.5 cells and consequent selection yielded colonies with
varying levels of SEC14L2 mRNA- and protein expression (Fig. 3A). Knock-down of SEC14L2 by siRNA was used to confirm the involvement of this gene and not an artefact of transduction or selection (Figs. 2C and 2D). Transient expression assays of HCV replicons showed a broadly consistent ten-fold increase in replication in cells expressing high levels of SEC14L2 compared to the parental Huh7.5 cell line with some higher (genotype 3a, neomycin-low CpG/UpA) and lower (genotype 4a, low CpG/UpA) exceptions (Figs. 4A-C). Expression of SEC14L2 had no effect on the expression of luciferase in replication-incompetent (GND) replicons, suggesting that SEC14L2 directly influenced replication rather than RNA stability and/or translation. However, in contrast to previously reported results (19), we observed a consistent and substantial increase in the replication in the lipid-peroxidation resistant genotype 2a JFH1 replicon, whose replication was not enhanced in stably-transfected cell lines. Differences in assay systems may have contributed to this difference; firstly our experiments were performed in a cell line with SEC14L2 expressed from a transgene that is not under the same regulatory control as the native gene. Secondly, we electroporated the cells instead of using transfection; this may deliver RNA at a different location and efficiency and influence the efficiency of initial translation and gene expression and consequent replication efficiency.

Irrespective of the likely complex mechanisms underlying the restriction of HCV replication in cell culture, this study achieved its original goal of improving the replication of replicons that enable their use in transient expression assays. This enabled a pilot study of the susceptibility of different genotypes to the NS5B inhibitor Sofosbuvir (Fig. 7). The enhancement of replication in SEC14L2-expressing cells and changing to the low CpG/UpA luciferase reporter gene enabled robust measurement of IC50s for genotypes 1-4 (Table 2), generating values that were comparable to those reported previously from other assay systems (28,29).

In summary, we have shown that reducing the number of CpG and UpA dinucleotides in HCV subgenomic replicons can greatly enhance replication levels but with some variability between genotypes. Combined with further increases in HCV replication in cell expressing SEC14L2, the 30-
1000-fold increases in replication achieved across all four genotypes will greatly facilitate susceptibility and resistance mutation testing in a convenient and rapid transient expression assay format.

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Author Contributions: JW and MMG carried out the laboratory work described in the study. PS and JW conceived the study and designed the vectors and experimental design and wrote the manuscript.

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TABLE 1  
Composition and coding parameters of WT and CpG / UpA-low luciferase sequences

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Symbol</th>
<th>Subs.</th>
<th>C+G%</th>
<th>CpG</th>
<th>ΔCpG</th>
<th>O/E</th>
<th>UpA</th>
<th>ΔUpA</th>
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<td>0</td>
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<td>0</td>
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<td>64</td>
<td>0.17</td>
<td>0.804</td>
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- *Number of sequence changes from WT sequence
- bChange in the numbers of CpG and UpA dinucleotides
- *Observed to expected frequencies of CpG and UpA dinucleotides
- dCalculated using the website [http://genomes.urv.es/CAIcal/](http://genomes.urv.es/CAIcal/) (41)
- *Calculated as previously described (26,42)
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<th>72 h.p.e.</th>
<th>96 h.p.e.</th>
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<td>-</td>
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a: Not determined (insufficient replication)

b: IC50 values are the concentration of Sofosbuvir that inhibits the replicon of the HCV replicon by 50%
FIGURE LEGENDS

Fig. 1. Structure of sub-genomic replicons and mutants. Diagrammatic representations of the replicons used in the current study, with changes to the luciferase gene (L, I), deletion of the neomycin gene (N) and replacement of the GDD motif in NS5B with GND to make it replication defective labelled.

Fig. 2. Replication of unmodified replicons and mutants with low CpG/UpA luciferase. Replication of genotype (A) 2a, (B) 1b, (C) 3a and (D) 4a replicons after electroporation into Huh7.5 cells. Luciferase activity of was measured at four time points (x-axis) and plotted as the absolute values (y-axis). Error bars represent standard deviations.

Fig. 3. Expression of SEC14L2 in different cell lines and effect of HCV replication. (A) Quantification of SEC14L2 expression by western blot of cell lysates from different cell lines. P: parental Huh7.5 cell line. Samples were normalized to total amount of protein loaded; a control immunoblot using β-tubulin is shown below (B) luciferase expression in different cell lines at different time points after electroporation of 1b/I-GDD. The values are normalised to luciferase expression of the 1b/I-GDD replicon in the parental Huh7.5 cell line at each time point. (C) Effect of transfection of SEC14L2-specific siRNA in line #23 on SEC14L2 expression levels as determined by western blot (D) Replication of 1b/I-GDD in siRNA-treated and irrelevant siRNA-treated #23 cells. Error bars depict standard deviations.

Fig. 4. Comparison of the replication of replicons from genotypes 1-4 in Huh7.5 and #23 cell lines. Replication of replicons from genotypes 1-4 generated in the study in Huh7.5 and #23 cell lines; right hand panels show luciferase expression from the replication incompetent control. Error bars depict standard deviations.
Fig. 5. *In vitro* translation assay of wild type and low CpG/UpA luciferase replicons. Measurement of luciferase activity of translation products of WT and CpG/UpA-low HCV replicons of genotypes 1-4 after a 30 minute incubation time. Bars show the mean of two replicates; error bars show standard deviations.

Fig. 6. Co-electroporation of CpG/UpA-low and WT luciferase containing replicons. Luciferase expression in (A) Huh7.5 cells and (B) #23 cells after electroporation of CpG/UpA-low, WT or both replicons; y-axis scale is normalised to luciferase expression by 1b/l-GDD (100%). Error bars depict standard deviations.

Fig. 7. Susceptibility testing to Sofosbuvir of original and modified replicons. Inhibition of replication of CpG-low and WT luciferase replicons (A) 1b/l-GDD and 1b/L-GDD, (B) 3a/LN-GDD and 3a/L-GDD and (C) 4a/LN-GDD and 4a/l-GDD to differing concentration of Sofosbuvir. The experiment was performed in Huh7.5 and #23 cell lines. Error bars depict standard deviations.
A) Genotype 2a (pSGR-JFH-1) and 1b (pSGR-Con1) replicons

B) Genotype 3a (S52/SG-Feo) and 4a (ED43/SG-Feo) replicons
A) Huh7.5 cells

B) #23 cell line

Relative luciferase expression (to low luciferase 0.5)

Hours post electroporation