Characterization of Hepatitis C Virus Subgenomic Replicon Resistance to Cyclosporine In Vitro

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Treatment of hepatitis C virus (HCV) infection has been met with less than satisfactory results due primarily to its resistance to and significant side effects from alpha interferon (IFN-α). New classes of safe and broadly acting treatments are urgently needed. Cyclosporine (CsA), an immunosuppressive and anti-inflammatory drug for organ transplant patients, has recently been shown to be highly effective in suppressing HCV replication through a mechanism that is distinct from the IFN pathway. Here we report the selection and characterization of HCV replicon cells that are resistant to CsA treatment in vitro, taking advantage of our ability to sort live cells that are actively replicating HCV RNA in the presence of drug treatments. This resistance is specific to CsA as the replicon cells most resistant to CsA were still sensitive to IFN-α and a polymerase inhibitor. We demonstrate that the resistant phenotype is not a result of general enhanced replication and, furthermore, that mutations in the coding region of HCV NS5B contribute to the resistance. Interestingly, a point mutation (I432V) isolated from the most resistant replicon was able to rescue a lethal mutation (P540A) in NS5B that disrupts its interaction with its cofactor, cyclophilin B (CypB), even though the I432V mutation is located outside of the reported CypB binding site (amino acids 520 to 591). Our results demonstrate that CsA exerts selective pressure on the HCV genome, leading to the emergence of resistance-conferring mutations in the viral genome despite acting upon a cellular protein.

Hepatitis C virus (HCV) infects more than 170 million people worldwide, leading to both acute and chronic liver diseases in patients. So far there is no prophylactic vaccine to prevent HCV infection. The current treatment, alpha interferon (IFN-α) in combination with ribavirin (RBV), is not satisfactory because of significant side effects and resistance. Even though the mechanism of this IFN resistance is not yet completely understood, both the virus and the host probably play important roles (10, 32, 33). On one hand, specific genetic backgrounds and/or the physiological statuses of nonresponding patients may account for the failure to achieve sustained virological responses. Factors such as race, gender, age, and obesity status have all been shown to modulate the outcome of IFN therapy (10). On the other hand, the greater likelihood of developing IFN resistance by patients infected with the predominant strain of the virus, genotype 1, indicates that viral factors also influence the success rate of therapy.

HCV populations in vivo exist in a quasispecies nature, likely due to the low fidelity of the RNA-dependent RNA polymerase and high turnover rate of the viral RNA. Not surprisingly, the genetic diversity of HCV in the in vitro model system, the replicon, is also very high. The majority of the HCV replicons contain cell culture-adapted mutations, and the mutation rate of long-term replicons has been estimated to be approximately 3.0 × 10⁻³ base substitutions/site/year (3, 14, 21). The replicon system has been used to evaluate the resistance profiles of many compounds considered for development as anti-HCV drugs. Well-defined mutations in viral RNA have been identified for a number of enzyme inhibitors targeting either the viral protease NS3 or the polymerase NS5B (15–17, 19, 22, 24, 25). These mutations typically map to the binding site of the inhibitors. The mechanisms of replicon resistance to nonspecific inhibitors such as IFN and RBV are less understood but are likely conferred by changes in the host cells (27, 34, 37, 45, 47). Recently, cyclosporine (CsA), a widely used immunosuppressive drug, has been shown to be highly effective in suppressing HCV both in vivo and in vitro through a mechanism distinct from the IFN pathway (11–13, 28, 29, 44). Several lines of evidence suggest that CsA suppresses HCV replication by binding to a cyclophilin and disrupting its interaction with the HCV polymerase NS5B, although it is not clear how many cyclophilins are involved or whether different genotypes use different cyclophilins as cofactors (13, 29, 44). Resistance to CsA was not previously characterized, and it is not known whether viral mutations can emerge to confer resistance to a compound that does not directly act on a viral target.

The replication of HCV in vitro can now be studied using either replicons or infectious particles (3, 7, 20, 21, 41, 46). We recently adapted a green fluorescent protein (GFP) replicon cell line and developed a flow cytometry-based assay for measuring HCV replication that is simple, fast, and unbiased against cell growth arrest (30). Here, we took advantage of our ability to sort live cells that actively replicate HCV RNA and selected HCV replicon clones resistant to CsA. We demonstrated that specific resistance to CsA was conferred by replicon RNA. By characterizing the resistant replicon, we identify a point mutation in the coding region of NS5B that not only confers resistance to the wild-type genotype 1b (GT1b) replicon but also rescues a lethal point mutation in NS5B that abolishes its interaction with cyclophilin B (CypB). NS5B pro-
tein from the resistant replicons bound strongly to RNA even when the interaction between NS5B and CypB was disrupted.

MATERIALS AND METHODS

Compounds. Cyclosporine A was purchased from Alexis Corporation (San Diego, CA). 2′-C-methyl-adenosine (2CMA) was a gift from Steve Carroll (Merck, Inc.). IFN-α was purchased from Sigma-Aldrich.

Cell lines. GS4 cells have been described previously (30). GS4 cells were obtained by sorting and expanding the top 15% of the GS4 cells with the strongest GFP expression levels. Huh-7.5 cells were obtained from Apath, LLC and Charles Rice (Rockefeller University). The variable replicon cells were then maintained in Dulbecco’s modified medium supplemented with antibiotics, 10% fetal bovine serum, and 500 μg/ml G418. GS4-cured cells were obtained by treating the GS4 cells with 100 μM IFN-α for 4 weeks and were then maintained in culture medium without G418. For all Csa treatments, the appropriate amount of the Csa was added to the culture medium within 24 h of plating the replicon cells.

Selection of Csa-resistant replicon cells and flow cytometry. GS4 cells were treated with 1 μg/ml (0.83 μM) Csa for 3 days and cultured in double-selection medium containing 1 μg/ml (0.83 μM) Csa and 500 μg/ml G418 for an additional 4 weeks. Colonies that survived the double selection were pooled and expanded to give rise to the Csa-R cells. For cell sorting to derive R1 and R2 cells, 2 × 10^6 cells were treated with CsA for 4 days and then subjected to live cell sorting under sterile conditions with a FACSaria flow cytometer (BD Biosciences). The 10 to 15% of the cells with the strongest GFP signals after treatment were recovered and expanded. For routine fluorescence-activated cell sorting (FACS), GFP or RHA cells were fixed in 4% paraformaldehyde and then analyzed with a FACSCan flow cytometer (BD Biosciences).

Single-cell cloning. Single-cell clones of the R1 and R2 cells were obtained by a limited dilution method. We mixed 1.5 × 10^5 of R1 cells with 2 × 10^6 naïve Huh-7 cells and plated the cell mixture onto a 10-cm plate. The cells were then cultured in selection medium containing 500 μg/ml G418 for 4 weeks. Individual G418-resistant colonies were isolated with cloning rings and expanded for treatment with Csa.

Curing and remixing replicons. To obtain the IFN-cured cells of GS5 and R2, we treated these replicon cells for 4 weeks with 100 U/ml IFN-α. A portion of the cured cells were then treated with 500 μg/ml G418 for 3 weeks to ensure that no G418-resistant colonies could develop. Ten micrograms of total RNA from GS5 or R2 replicon cells was then introduced into 4 × 10^6 of the cured cells by electroporation with a Gene Pulser Xcell apparatus (Bio-Rad Laboratories) at 270 V and 950 μF. Stable replicon cells were selected with 500 μg/ml G418 for 3 weeks, and the G418-resistant colonies were expanded to obtain new replicon cells.

RT-PCR and DNA sequencing. Total RNA from replicon cells was isolated with TRIzol reagent (Invitrogen, San Diego, CA) and subjected to reverse transcription-PCR (RT-PCRs) to amplify overlapping fragments that covered the full length of the replicon. The PCR products were cloned into the TA cloning vector pCR2.1-TOPO for sequencing.

In vitro transcription, electroporation, and colony formation assay. HCV replicon RNAs were generated by in vitro transcription using a MEGAscript T7 kit (Ambion, TX). One microgram of the in vitro-transcribed RNA was used in the electroporation of 4 × 10^6 cells, which were then plated onto 10-cm plates. Stable replicon cells were selected with 500 μg/ml G418, and the G418-resistant colonies were stained with crystal violet for the colony formation assay. In the experiments with CsA treatment, we included 0.375 μg/ml of CsA in the selection medium.

Transient replication assay. To measure transient replication without selection, the total RNAs from transfected cells were isolated at 4 and 8 days post-electroporation in the absence of G418 selection and then subjected to RT-PCR analysis of HCV RNA. The primers used to detect HCV RNA in replicon cells have the following sequences: for NS5B-forward, 5′-TAC TCG ATG TCC TAC ACA TGG-3′, and for NS5B-reverse, 5′-AAC AGG ATG GCC TAT TG-3′. The RHA primers have the following sequences: for RHA-forward, 5′-GGT GAC GTT AAA AAT TTT CGT-3′, and for RHA-reverse, 5′-AGA GCC CCC TAC CTC AGA ATT-3′.

Antibodies and Western blots. An anti-NS5A monoclonal antibody was purchased from Virogen (Boston, MA), and an anti-Cy508 monoclonal antibody was purchased from Sigma. Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate), and 25 μl of total protein was loaded onto a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The membrane was first probed to detect NS5A and then stripped and reprobed for Ku80 to normalize protein loading.

RNA extraction and Northern blots. Total RNAs from Csa-treated cells were isolated 48 h postaddition of the drug. Equal amounts of RNA (10 μg per sample) were loaded onto formaldehyde-containing agarose gels for electrophoresis. Northern blotting and HCV RNA detection were performed as previously described (30). The quantification of band intensity of the Northern blot was performed with Quantity One software on a Bio-Rad ChemiDoc gel documentation system.

Quantitative RT-PCR. Real-time RT-PCR was performed with an SYBR green PCR kit (Applied Biosystems) according to the manufacturer’s instructions. The HCV-specific primers used in the real-time RT-PCR had the following sequences: for internal ribosome entry site forward, 5′-GTC TGC GAC AGG GAT GTG-3′, and for internal ribosome entry site reverse, 5′-GGG GGT GAT CCA AAG AAC GAC-3′.

Poly(U) RNA binding assay. The in vitro RNA binding assay was performed as described previously (44). Briefly, the cell lysates of replicon cells were incubated with poly(U) Sepharose beads in a binding buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40) for 24 h at 4°C. The bound proteins were then analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and immunostaining with anti-NS5B (Virogen, Boston MA) and anti-CypB (ABR, Golden, CO) antibodies.

Delivery and expression of siRNA using a lentiviral vector. The pHIV-7/Puro vector and short hairpin RNA targeting luciferase have been described previously (42). The target sequence for CypB short interfering RNA (siRNA) was 5′-AAGTTGGGAGGACCCAAGAC-3′. The primers for the CypB siRNA were 5′-GAACTGAGTGGTACCCGCCC-3′ and 5′-ggggGATCCAAAAAAGtggtgaggacccaataCTTCCTTGAAtgttggggttcaccaAAACAGGTTTCTTCCAAAGG-3′. (The lowercase letters indicate the siRNA sequence.) Vesicular stomatitis virus G-pseudotyped lentivirus was packaged using a lentivirus support kit (Invitrogen). Huh-7.5-based replicon cells were transduced with standard methods.

RESULTS

Selection of Csa-resistant HCV replicon cells in vitro. We recently optimized a FACS-based HCV replicon assay using GFP as a surrogate marker for viral replication and expression (30). GS4 cells harbor an active GT1b replicon with GFP as a surrogate marker for viral replication and expression. When treated with 1 μg/ml (0.83 μM) Csa, the GFP signal and the HCV RNA in these cells were inhibited by more than 90% (30). To isolate Csa-resistant replicon cells in vitro, we used a combined approach of antibiotic selection and live cell sorting. A diagram of the selection scheme is shown in Fig. 1A. In brief, we first selected derivatives of GS4 cells that survived G418 selection in the presence of continuous (more than 3 weeks) Csa treatment and designated these as Csa-R cells. When later treated with inhibitors, the Csa-R cells were significantly more resistant to CsA but maintained their sensitivities to IFN-α and 2CMA (Fig. 1B to D). Higher concentrations of CsA (2 to 4 μg/ml/1.66 to 3.23 μM), however, still efficiently suppressed viral expression in these cells. To obtain cells that were even more resistant to CsA, we treated Csa-R cells with 2 μg/ml (1.66 μM) of CsA for 3 days and subjected the cells to flow cytometry analysis. Cells that remained GFP positive were termed CsA-R cells.
(~15% of the total population) after the treatment were recovered by live cell sorting and expanded. We designated these as RS1 cells. This process was repeated with RS1 cells and 4 μg/ml (3.32 μM) CsA to obtain RS2 cells. The IFN and CsA sensitivities of these cells were also examined. As a control, we used GS5 cells that were obtained by similarly sorting the top 15% of GFP-expressing GS4 cells in the absence of CsA treatment. Like the GS4 cells, the NS5A-GFP expression of the GS5 cells was effectively inhibited by CsA with a 50% inhibitory concentration of 0.25 μg/ml (0.21 μM). In contrast, the RS1 and RS2 cells were largely resistant to levels as high as 2 μg/ml (1.66 μM) of CsA (Fig. 1B). Importantly, there was no correlation between the level of resistance to CsA and any measurable resistance to IFN-α and 2CMA for these CsA-resistant cells (Fig. 1C to D), indicating specific resistance to CsA. In addition, the high level of NS5A expression in RS2 cells persisted for up to 10 days, long after NS5A expression in GS5 cells was effectively suppressed (Fig. 1E). These data further suggest that the observed resistance is not a result of enhanced replication.

CsA was shown to suppress HCV expression by reducing the viral RNA level of the replicon cells (28, 43). Resistance to CsA is therefore also likely to occur at the RNA replication level, even though the expression of a viral protein was used as
a marker for isolation of the resistant cells. To confirm this result, we performed Northern blotting to detect HCV RNA in CsA-treated replicon cells. When GS5 cells were treated with CsA, HCV RNA decreased steadily with increasing concentrations of the drug, falling below detection at levels of 1 μg/ml (0.83 μM) CsA and above (Fig. 2A, left panel). The replicon RNA in RS2 cells, however, remained detectable until the drug concentration reached 4 μg/ml (3.32 μM) of CsA (Fig. 2A, right panel). The quantification of the intensities of the bands revealed that the ratio of steady-state level HCV RNA versus 28S rRNA in RS2 cells is slightly lower than the ratio in GS5 cells (Fig. 2B).

**HCV RNA, but not the host cells, confers CsA resistance.** To distinguish the contribution by the host cell from that of the virus, we separated the viral RNA from its host cells for the GS5 and RS2 replicons and subsequently remixed them together, this time with all of the possible combinations of RNA and host cells. GS5- and RS2-cured cells were obtained by prolonged treatment with 100 U/ml of IFN-α (Fig. 3A) (see Materials and Methods). Total RNA from GS5 and RS2 cells, which contain GS5 and RS2 replicon RNA, were then introduced into the cured cells by electroporation to generate the new replicon cells. We then treated these replicons with CsA to test for resistance. Only the cells that received RS2 RNA became resistant again, regardless of the host cell origin (Fig. 3B and C). On the other hand, the cured cells generated from the RS2 replicon were not able to confer any resistance to GS5 RNA. These results suggest that the resistant phenotype we observed for RS2 cells was conferred by the replicon RNA, presumably containing mutations. We performed similar analyses for CsA-R and RS-1 cells and obtained the same results (data not shown).

**A single amino acid change in NS5B can confer CsA resistance to HCV replicons.** We analyzed the mutations associated with the resistant phenotype by determining the sequences of the replicon RNA from the resistant cells and then comparing them to that of the nonresistant replicon sequence represented by the GS5 RNA. After RT-PCR and cloning, multiple DNA clones of the RS2 replicon were sequenced to obtain a consensus sequence. Two RS2-specific mutations were identified in the NS5B gene, the proposed viral target for CsA-mediated suppression of HCV replication. These mutations resulted in two predicted changes in the amino acid sequence of the polymerase protein: a change from isoleucine to valine at position 432 (I432V) and a change from lysine to asparagine at position 535 (K535N). The I432V mutation was also identified in a single-cell clone of the RS1 replicon (RS1-2) that had a resistance profile similar to that of RS2 (Table 1). The K535N mutation was not found in RS1-2.

To validate the potential contribution of the I432V mutation to the resistant phenotype of the RS2 replicon, we engineered it back into a replicon background with the wild-type NS5B sequence via site-directed mutagenesis. K535N and the double mutant of I432V/K535N were also created in a similar fashion. Rep1b-BB7, which contains no GFP insertion (3), was chosen as the wild-type replicon into which the mutations were inserted. This controls for the possibility that what we observed was somehow related to GFP expression. We generated repli-
con cells harboring the mutations, applied CsA treatments to these replicon cells, and then examined the HCV expression level by Western blotting using an NS5A-specific antibody. The Rep1b wild-type replicon, which was used as a control, was inhibited by CsA in a dosage-dependent manner (Fig. 4A, left panel), while the replicon containing the I432V mutation was more resistant to CsA treatment than was Rep1b, as strong replication persisted with 0.375 μg/ml (0.31 M) CsA (Fig. 4A, lanes 4 and 9). In addition, K535N alone did not confer any resistance and combining it with I432V did not further increase resistance (data not shown). These data are consistent with the fact that K535N was not found in the resistant single-cell clone (Table 1, last row) containing I432V.

To exclude the possibility that the observed resistance to CsA by the I432V replicon is a result of generally enhanced replication, we performed colony formation assays to compare the replication efficiency between I432V and Rep1b. When equal amounts of RNA from two replicons were electropo-

![Diagram](image)

**FIG. 3.** Replicon RNA, not the host cell, was responsible for the CsA resistance of RS2 cells. (A) Experimental design for mapping the resistance to the replicon RNA or the host cell. We separated the viral RNA and host cells for both GS5 and RS2 replicons and remixed them to generate all four possible combinations of new replicons (see Materials and Methods for details). (B) Only the RS2 RNA was able to confer CsA resistance to the new replicons. The new replicons containing GS5 RNA, regardless of the host cell origin, retained the same level of CsA sensitivity as did GS5 cells, while the RS2 RNA was able to confer resistance to host cells originated from the GS5 cells. (C) Western blot analysis results of an independent experiment confirming the FACS results in panel B. Concentrations of CsA are indicated above each lane.

<p>| Table 1. Mutations in NS5B identified in CsA-resistant GT1b replicons |
|--------------------------|-----------------|-----------------|--------------------------|</p>
<table>
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<tr>
<th>Replicon name</th>
<th>Cell line description</th>
<th>IC_{50} (μM)</th>
<th>Mutation(s) in NS5B</th>
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<tr>
<td>GS5</td>
<td>Control</td>
<td>0.22 ± 0.04</td>
<td>NA</td>
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<tr>
<td>RS2</td>
<td>Resistant cell pool</td>
<td>2.4 ± 0.8</td>
<td>I432V, K535N</td>
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<tr>
<td>RS1-2</td>
<td>Resistant single clone</td>
<td>2.2 ± 0.8</td>
<td>I432V</td>
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IC_{50}, 50% inhibitory concentration; NA, not applicable.
rated into Huh-7.5 cells, equivalent numbers of G418-resistant colonies were formed for the two replicons in the absence of any CsA treatment, indicating that the replication capacity of I432V is comparable to that of Rep1b (Fig. 4B, top panels). A dramatically different result was obtained when CsA treatment was applied during the G418 selection process. The Rep1b RNA was effectively inhibited by the treatment and lost its ability to form colonies, while the I432V RNA resisted the treatment and formed a large number of colonies (Fig. 4B, lower panels). Taken together, these results indicate that a single amino acid change at position 432 of the NS5B can render the HCV replicon resistant to CsA treatment, while the K535N mutation does not contribute to the resistance.

**I432V rescues a lethal mutation in NS5B.** Using a GT1b replicon, Watashi et al. identified CypB as an essential cofactor for NS5B (44). They further mapped the CypB binding site to the C terminus of NS5B, extending from amino acids 521 to 591, and identified a lethal mutation (P540A) that disrupts the NS5B-CypB interaction. Although amino acid 535 is located in the general vicinity of P540 in both the primary sequence and the three-dimensional structure of the NS5B polymerase (2, 6, 18), the identified lysine-to-asparagine change did not have any contribution to the resistance. In contrast, even though amino acid 432 is located outside the reported CypB binding site, the I432V mutation was able to confer resistance. Based on this result and some interesting sequence covariance between I432V and P540A that we uncovered in the HCV sequence database (Table 2) (see Discussion), we set out to determine whether the I432V can rescue the lethal phenotype of P540A in the GT1b replicon background. We engineered the P540A mutation into either the wild-type Rep1b or the I432V background and tested the resultant replicon RNA for its ability to replicate. In the colony formation assay, replicon RNA carrying the P540A mutation had a significant reduction in the number of G418-resistant colonies formed in both Huh-7.5 and GS5-cured cells, consistent with the previous report (44). Under the same conditions, the I432V mutation rescued the defect in the colony-forming efficiency of P540A (Fig. 5A). When large amounts (10 μg) of replicon RNA were used for electroporation, we could sometimes obtain G418-resistant cells with replicon RNA harboring the P540A mutation as well, even though these cells were later shown to contain no HCV RNA or protein (data not shown). This happened only when

![FIG. 4. A point mutation at amino acid 432 of NS5B contributes to the CsA resistance of RS2 cells. (A) Rep1b and a derivative replicon containing the I432V mutation were treated with increasing amounts of CsA for 4 days before being subjected to Western blotting with an anti-NS5A antibody. Concentrations of CsA are indicated above each lane. The detection of Ku80 served as a loading control. (B) Replication of I432V is resistant to CsA in a colony formation assay. In the CsA-treated group, 0.375 μg/ml CsA was included in the selection medium, which also contained 500 μg/ml of G418. •, not treated with CsA; +, treated with CsA.](http://jvi.asm.org/content/jvi/81/7/5834/F4)

<table>
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<th>Mutation in NS5B</th>
<th>LANL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Japan HCV&lt;sup&gt;b&lt;/sup&gt;</th>
<th>euHCVdb&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>P540A</td>
<td>12</td>
<td>12</td>
<td>54</td>
</tr>
<tr>
<td>I432V</td>
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<td>14</td>
<td>55</td>
</tr>
<tr>
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<td>51</td>
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<td><strong>Total</strong></td>
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<td><strong>173</strong></td>
<td><strong>382</strong></td>
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<sup>a</sup> The P value of covariance (df = 1) was ≤0.001 for the three databases.
<sup>b</sup> [http://hcv.lanl.gov/content/hcv-db/index](http://hcv.lanl.gov/content/hcv-db/index).
<sup>c</sup> [http://s2as02.genes.nig.ac.jp/](http://s2as02.genes.nig.ac.jp/).
<sup>d</sup> [http://euhcvdb.ibcp.fr/euHCVdb/](http://euhcvdb.ibcp.fr/euHCVdb/).
the amount of input RNA was very high, and cells became confluent before G418 was added.

Sequencing of the NS5B region of the I432V/P540A replicon confirmed that both mutations were maintained in the rescued replicon cells. However, since the G418 selection lasted three to 4 weeks, it was possible that mutations in the other parts of the genome may have emerged to compensate for the defect caused by P540A. To examine whether I432V could rescue the replication of P540A shortly after electroporation without G418 selection, we performed transient replication assays where replication was measured without G418 selection. Equivalent amounts of in vitro-transcribed replicon RNA for Rep1b, P540A, and I432V/P540A were electroporated into Huh-7.5 cells, and total RNA was isolated at various time points postelectroporation for the detection of HCV RNA with RT-PCR. I432V rescued RNA replication of a P540A mutation as early as 4 days postelectroporation without any G418 selection (Fig. 5B). These results confirm the lethal effect of the P540A mutation on the GT1b replicon and identify I432V as a compensatory mutation outside the CypB binding site.

The I432V/P540A double mutant and the wild-type replicon are equally sensitive to CsA. We were interested in determining whether the I432V/P540A double mutant retained the resistance profile of the I432V mutant. We treated replicon cells containing these mutations, along with wild-type replicons, and compared their sensitivities to CsA. In both Western blot and real-time RT-PCR analyses, the I432V/P540A double mutant showed the same level of sensitivity as that of the wild-type replicon, while the I432V replicon exhibited resistance (Fig. 6A and B). Colony formation assays also confirmed these results (data not shown), which suggest that the valine at position 432 and the proline at position 540 contribute additively to NS5B function.

Despite the ability to rescue the replication of the P540A mutant and to directly confer a certain level of resistance by itself, the I432V mutation did not restore the full range of resistance observed in the RS2 or the RS1-2 cells. Although both replicons contained only one mutation (I432V) in the NS5B region, the RS1-2 replicon was significantly more resistant than the I432V replicon (Fig. 6C). Two possibilities, among others, could explain this difference. First, mutations elsewhere in the genome could be contributing to the higher level of resistance in RS1-2 cells. Indeed, sequencing results revealed that RS1-2, obtained by prolonged passage in culture, contained numerous additional mutations in other parts of the genome. Second, reversible changes in the selected cells, which disappear when the replicon cells are cured with IFN, could also be responsible for the difference. The results in Fig. 3 do not completely eliminate the possibility that some reversible changes in the RS2 or RS1-2 cells, either caused by mutations in the replicon or working in combination with the mutations, contributed to the higher levels of resistance in the CsA-selected cells.

Enhanced RNA binding by NS5B correlates with CsA resistance. To investigate the molecular mechanism of CsA resistance, we studied the in vitro binding of RNA and cyclophilin B by the NS5B proteins from the wild-type (GS5) and resistant (RS1-2) replicon cells. As reported previously (44), poly(U) RNA could pull down NS5B and cyclophilin B from the replicon cell lysates (Fig. 7A). We quantified the percentage of the input NS5B that could be bound to an excess amount of poly(U) RNA and found a modest increase (1.5-fold) for the input NS5B that could be bound to poly(U) RNA with RT-PCR. I432V rescued RNA replication of a P540A mutation as early as 4 days postelectroporation without any G418 selection (Fig. 5B). These results confirm the lethal effect of the P540A mutation on the GT1b replicon and identify I432V as a compensatory mutation outside the CypB binding site.

The in vitro binding results raised the possibility that the
NS5B protein from the resistant cells could bind to RNA in the absence of any appreciable cyclophilin B binding. One prediction from this was that the CsA-resistant replicons might be able to replicate independently of cyclophilin B. We then attempted to directly examine the effect of the cyclophilin B knockdown on the replication of GSS and RS1-2 cells by using RNA interference. Significant suppression of CypB expression with siRNA was readily achieved; this knockdown appeared to have an inhibitory effect on the NS5A level of the replicons at first (Fig. 8A); however, after careful normalization to an internal control protein (Ku80), no specific effect on HCV protein was seen even with the wild-type replicons GS5 and Rep1b (Fig. 8B). These results are at odds with the initial report on CypB (44) but consistent with a subsequent report that the dependence on CypB for replication may vary with different replicons (13). In any case, our results indicate that the wild-type replicons that we used could replicate well in the absence of CypB, precluding any conclusive study on the involvement of this protein in the observed CsA resistance.

**DISCUSSION**

HCV replicon cells resistant to various treatments have been isolated. For inhibitors that act directly on viral targets, such as protease and polymerase, resistance is conferred by viral mutations that map to the binding site of the inhibitors (15–17, 19, 22, 24, 25). For nonspecific treatments, such as IFN and RBV, that act indirectly through host cells, changes in host cell environment have been shown to be largely responsible for the resistant phenotype, with viral mutations possibly playing a minor role (27, 34, 37, 45, 47). In this study, we demonstrate that mutant viral RNA is the sole source of resistance to CsA, a drug that most likely acts upon a cellular factor to achieve viral inhibition.

The mechanism of action for CsA to suppress HCV replication is only beginning to be understood. At the cellular level, CsA binds to a group of proteins called cyclophilins (CyPs). A closely related compound, FK506, interacts with FK506 binding proteins (FKBPs). CyPs and FKBPs belong to a family of proteins named immunophilins because of their involvement in the immunosuppressive functions of the CsA and FK506. Even though the interactions between the immunosuppressant and its target inactivate the peptidyl-prolyl cis/trans isomerase activity of the immunophilins, this inhibition does not seem to be important for the immunosuppressive functions of the compounds. Rather, the CsA-cyclophilin A (CypA) and FK506-FKBP complexes abolish the phosphatase activity of calcineurin, which is critical for the expression of the cytokines and their receptors in T cells, thus blocking T-cell activation (23). The calcineurin pathway and the immunosuppressive function overall, however, do not appear to be involved in the CsA-mediated suppression of HCV as FK506 does not have any inhibitory effect and CsA derivatives without immunosuppressive function can still inhibit HCV replication very effectively (31).

Recent evidence supports the hypothesis that one or more
cyclophilins are involved in the replication of HCV in vitro by serving as a cofactor for NS5B, the viral RNA-dependent RNA polymerase (13, 29, 44). In particular, CypB has been shown to bind to the NS5B of HCV and this interaction can be disrupted by CsA treatment. For GT1b, the binding of CypB to NS5B increased the RNA binding affinity of the polymerase and an HCV mutant (P540A) that failed to bind to CypB also failed to replicate in vitro (44). Remarkably, the point mutation (I432V) that we identified in the NS5B gene of the CsA-resistant replicon did not retain the CsA resistance but had the same level of CsA sensitivity as did the wild-type replicon. We interpret this result as an indication that the NS5B conformation changes caused by cyclophilin binding and the I432V mutation are two independently contributing elements that function in an additive pathway, leading to stronger binding of RNA. At least one of these is needed to bind RNA and replicate normally (the wild-type 1b replicon infectivity of a nonbinding lethal mutant (4)).

The mechanism by which I432V rescues HCV replication and, presumably, NS5B function of P540A GT1b replicon is currently unclear. Ile-452 is not part of the CypB binding domain but instead locates to a recently identified allosteric site on the surface of the thumb domain to which a group of nonnucleoside inhibitors of the polymerase bind (9), raising the intriguing possibility that the cofactor function of cyclophilin may be related to this regulatory site. A parallel observation has been reported for the mutations conferring CsA resistance to human immunodeficiency virus (HIV). These mutations were also located outside the cyclophilin binding site of the HIV capsid (CA) protein, and they, too, rescued the infectivity of a nonbinding lethal mutant (4).

The rescued replicon, I432V/P540A, did not retain the CsA resistance but had the same level of CsA sensitivity as did the wild-type replicon. We interpret this result as an indication that the NS5B conformation changes caused by cyclophilin binding and the I432V mutation are two independently contributing elements that function in an additive pathway, leading to stronger binding of RNA. At least one of these is needed to bind RNA and replicate normally (the wild-type 1b replicon infectivity of a nonbinding lethal mutant (4)).

The mutation that we identified in the NS5B region of the resistant replicon did not restore the full range of resistance when engineered back into a wild-type background. The RS2 cells were approximately 10 times more resistant than the GS5 cells, while the I432V cells were only about twofold more resistant than the Rep1b cells. Both viral mutations and reversible cellular changes could contribute to this difference. In particular, the genetic differences between the GS5 and the Rep1b replications may play a major role. We examined the replication efficiency of total RNA from various replicon cells and found that the replication level of GS5 or RS2 is at least 50 times higher than that of Rep1b or the original GFP replicon (data not shown). In fact, numerous mutations were found in the GS5 replicon (and, thus, the RS2 replicon) relative to the Rep1b sequence, which also represents the wild-type sequence of I/5A-GFP (26, 30; data not shown). These mutations were likely the result of the multiple rounds of sorting and enrichment for highly active replicon cells that were performed to derive the GS5 and RS2 cells from the I/5A-GFP cells (30). It is possible then that these mutations, though by themselves unable to render the GS5 cells resistant to CsA, contributed to the high level of resistance exhibited by RS2 cells when combined with the NS5B mutation. Experi-
FIG. 8. Cyclophilin B knockdown in replicon cells had no significant effect on NS5A expression. (A) Various wild-type and CsA-resistant replicon cells were transduced with lentiviral vectors expressing either a luciferase siRNA (si-Luc) or a CypB siRNA (si-CypB) for 7 days before being lysed for protein analysis. After SDS-PAGE separation, NS5A, CypB, and Ku80 were detected by sequential probing and stripping of these same membranes. (B) Quantitative analysis of the results shown in panel A. After normalization to the loading control Ku80, no specific effect on HCV expression was observed for the CypB knockdown. Error bars indicate standard deviations.
ments to examine the potential contribution of these G5S-specific mutations to the enhanced replication and overall level of CsA resistance of RS2 cells are currently underway.

The NS5B protein from the CsA-resistant replicon exhibited stronger RNA binding activity than did the wild-type NS5B. More importantly, while CsA effectively suppressed the RNA binding activity of wild-type NS5B, it was much less effective in doing so to the RS1-2 NS5B. This result indicates that enhanced RNA binding in the presence of CsA could, at least in part, explain the CsA resistance of the RS1-2 replicon. Two lines of evidence suggest that CypB is not directly involved in the CsA resistance. First, although we could detect an association of NS5B and CypB in vitro, the interaction between CypB and the RS1-2 NS5B was as sensitive to CsA as that between CypB and wild-type NS5B, in contrast to the situation with NS5B and RNA interaction. Second, knocking down CypB had a minimal effect on the replication level of any of the replicons studied here, consistent with a previous report stating that CypB is not universally required for HCV replication in vitro (13). However, the wild-type replicons used in this study are still efficiently inhibited by CsA, suggesting that other members of the large cyclophilin family may be involved in CsA resistance.

CsA has also been shown to inhibit other human pathogenic viruses, including HIV-1, herpes simplex virus, and vaccinia virus (8, 40). In particular, the mechanism by which CsA modulates HIV replication has been the subject of intensive studies that have led to the realization that CypA in target cells can regulate viral replication by virtue of its interaction with the CA protein of the incoming virus (5, 35, 36, 38, 39). Mutations around the CypA binding site of the HIV CA protein were identified in viruses that were selected for their abilities to replicate normally in the presence of CsA, and these mutations alleviated the regulation exerted by CypA on HIV replication (1, 4). The role of CypA and other members of the cyclophilin family in HCV replication is not clear. Two research groups reported contradictory results on the effect of knocking down CypA on HCV replication, and another report indicated that GT2a replication may be independent of CypB but dependent on another unidentified cyclophilin (13, 29, 44). Further studies are needed to clarify the potential cofactor functions of distinct cyclophilin family members and any genotype-specific usage of the cyclophilins by the HCV polymerase. The CsA-resistant replicon cells and the NS5B mutations reported here can serve as valuable tools for such studies.

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