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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 living 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 RNA, 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-

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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). GS5 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 various replicon cells were routinely maintained in Dulbecco's modified medium supplemented with antibiotics, 10% fetal bovine serum, and 500 μg/ml G418. GS5-cured cells were obtained by treating the GS5 cells with 100 U/ml 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 of G418 for an additional 3 weeks. Colonies that survived the double selection were pooled and expanded to give rise to the CsA-R cells. For cell sorting to derive RS1 and RS2 cells, 2×10^7 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 sorter (FACS) analysis, cells were fixed in 2% paraformaldehyde and then analyzed with a FACSCanto flow cytometer (BD Biosciences).

Single-cell cloning. Single-cell clones of the RS1 cells were obtained by a limited dilution method. We mixed 1.5×10^2 of RS1 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 of 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 RS2, 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 RS2 replicon cells was then introduced into 4×10^6 of the cured cells by electroporation with a Gene Pulsor 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-PCRs (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.

Plasmids and site-directed mutagenesis. Plasmid Rep1b (also named Rep1b-BB7) was provided by Charles Rice (Rockefeller University). A ClaI and XhoI fragment that contains the NS5B coding region was subcloned into pCR2.1-TOPO for mutagenesis. Site-directed mutagenesis was performed with a QuikChange kit (Stratagene, San Diego, CA). The mutagenesis primers were I432V sense, 5′-CTG ATG ACT CAT TTC TTC TCC GTC CTT CTA GCT CAG GAA C-3′; I432V antisense, 5′-GTT CCT GAG CTA GAA GGA CGG AGA AGA AAT GAG TCA TCA G-3′; P540A sense, 5′-CAA ACT CAC TCC AAT CGC GGC TGC GTC CCA GTT GG-3′; and P540A antisense, 5′-CCA ACT GGG ACG CAG CCG CGA TTG GAG TGA GTT TG-3′. The mutated NS5B sequences were then cloned back into the Rep1b plasmid by using ClaI and XhoI sites to generate the mutant replicons.

In vitro transcription, electroporation, and colony formation assay. Replicon plasmids were linearized with ScaI. 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 CTG-3′, and for RHA-reverse, 5′-AGA GGC CCC TAC CTC AGA ATT-3′.

Antibodies and Western blots. An anti-NS5A monoclonal antibody was purchased from Virogen (Boston, MA), and an anti-Ku80 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, and 0.5% sodium deoxycholate), and 25 μg 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 GGA ACC GGT GAG-3′, and for internal ribosome entry site reverse, 5′-CGG GTT GAT CCA AGA AAG 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′-AAGGTGGAGAGCACCAAGACA-3′. The primers for the CypB siRNA were 5′-GAACTAGTGGATCCGACGCC-3′ and 5′-ggcGGATCCAAAAAA ggtgagagcaccaagacaTCTCTTGAAtgtcttggtgctctcaccAAACAAGGCTTTTCT CCAAGGG-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 inserted into the NS5A region and display strong green fluorescence when analyzed by microscopy or flow cytometry. 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 3.32 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

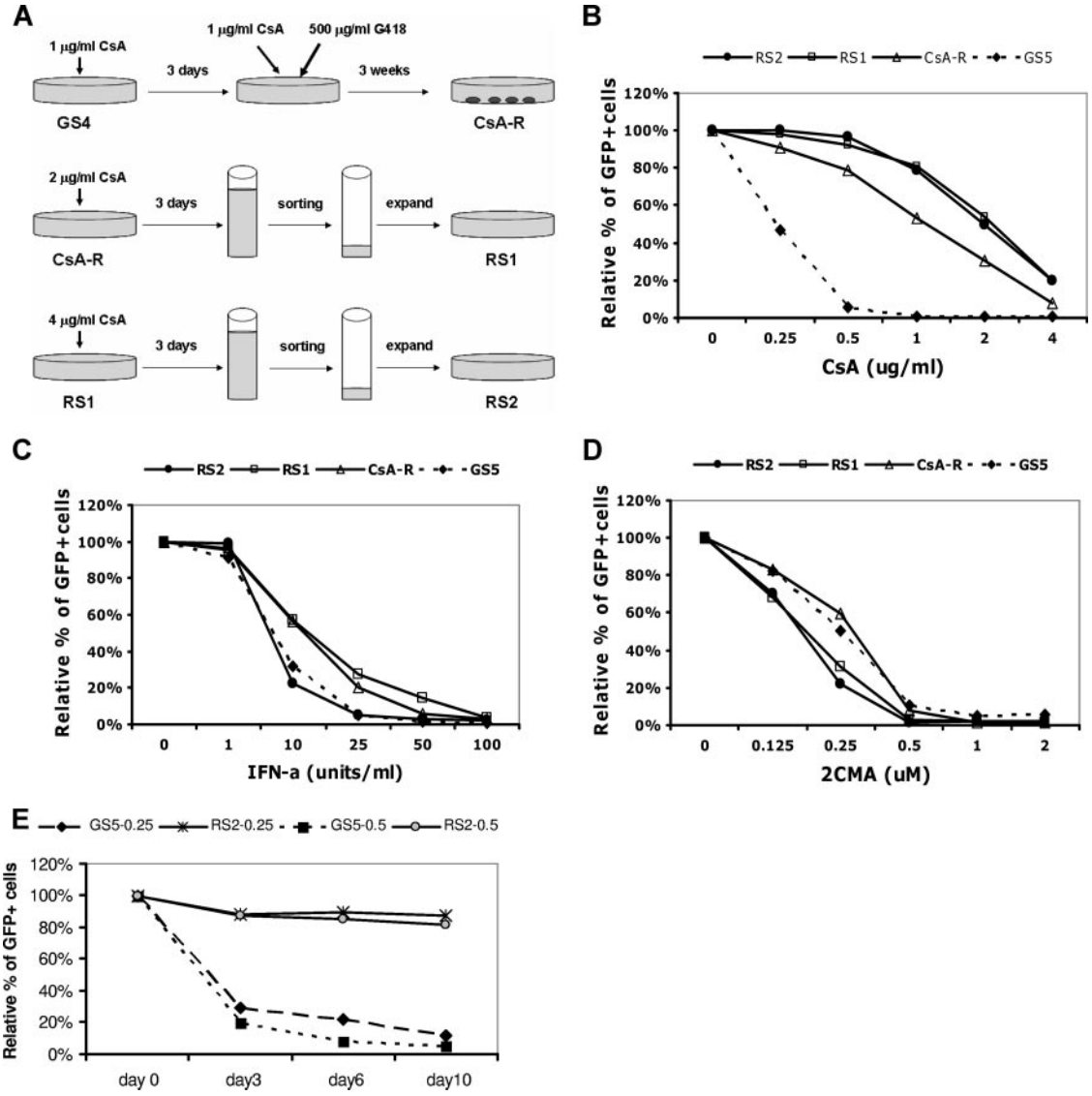

FIG. 1. Selection and characterization of CsA-resistant HCV replicon cells in vitro. (A) Diagram of the selection scheme which combines antibiotic selection and live cell sorting. Note that the double selection with CsA and G418 was carried out for 3 weeks to obtain colonies, and the subsequent sorting of resistant cells was performed 3 days posttreatment. (B) CsA resistance profiles of the various replicon populations. GS5 cells, a population consisting of the top 15% of GFP-expressing GS4 cells sorted in the absence of CsA treatment, were used as the negative control. The CsA-resistant cells remained susceptible to IFN-α (C) and 2CMA (D). The GFP-based replicon cells were routinely treated with the indicated drugs for 4 days before being fixed for FACS analysis. The percentage of GFP-positive cells at the end of the treatments is normalized to the value of the untreated sample (set at 100%). (E) RS2 cells were resistant to long-term treatment with CsA. GS5 and RS2 cells were treated with 0.25 and 0.5 μg/ml CsA for 10 days, and cells were fixed for FACS analysis at days 3, 6, and 10 after the addition of the drug.

(~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

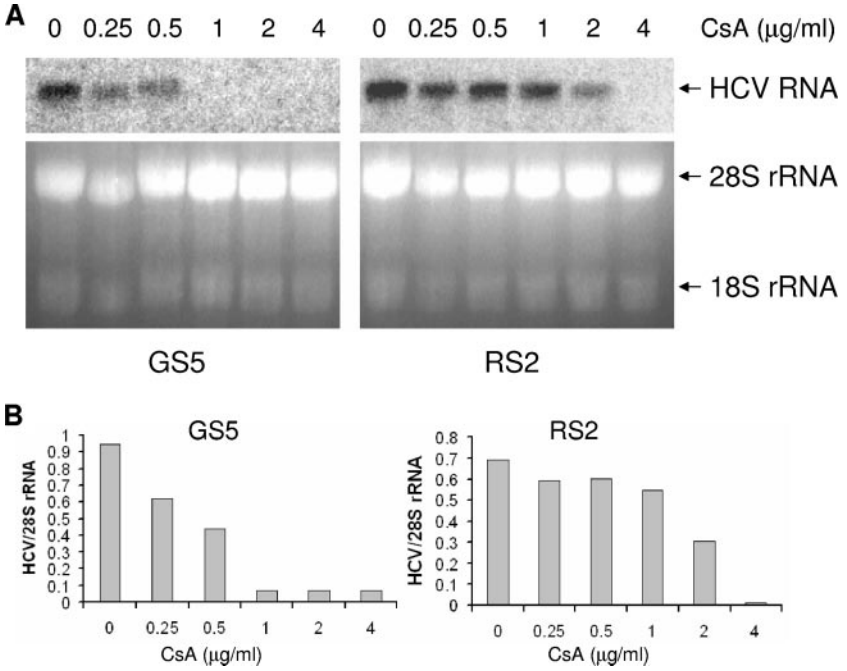

FIG. 2. Quantification of CsA resistance at the RNA level. (A) A representative Northern blot. The GS5 and RS2 cells were treated with the indicated amounts of CsA for 48 h. Total RNA from the replicon cells was then extracted and subjected to Northern analysis with a radioactively labeled probe that corresponded to the NS5B sequence of the GT1b HCV (29). A photograph of the RNA electrophoresis gel before Northern transfer showed that equivalent amounts of RNA were loaded for each sample. (B) Quantification of the ratio of HCV RNA to 28S rRNA using band densities from panel A. The Northern blot bands were analyzed with a PhosphorImager (Molecular Dynamics; Storm 860 scanner), and the rRNA bands were analyzed with a Bio-Rad ChemiDoc system. The ratio (plotted) serves as an indicator of the HCV RNA steady-state levels in the various samples after normalization to total RNA loaded.

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-

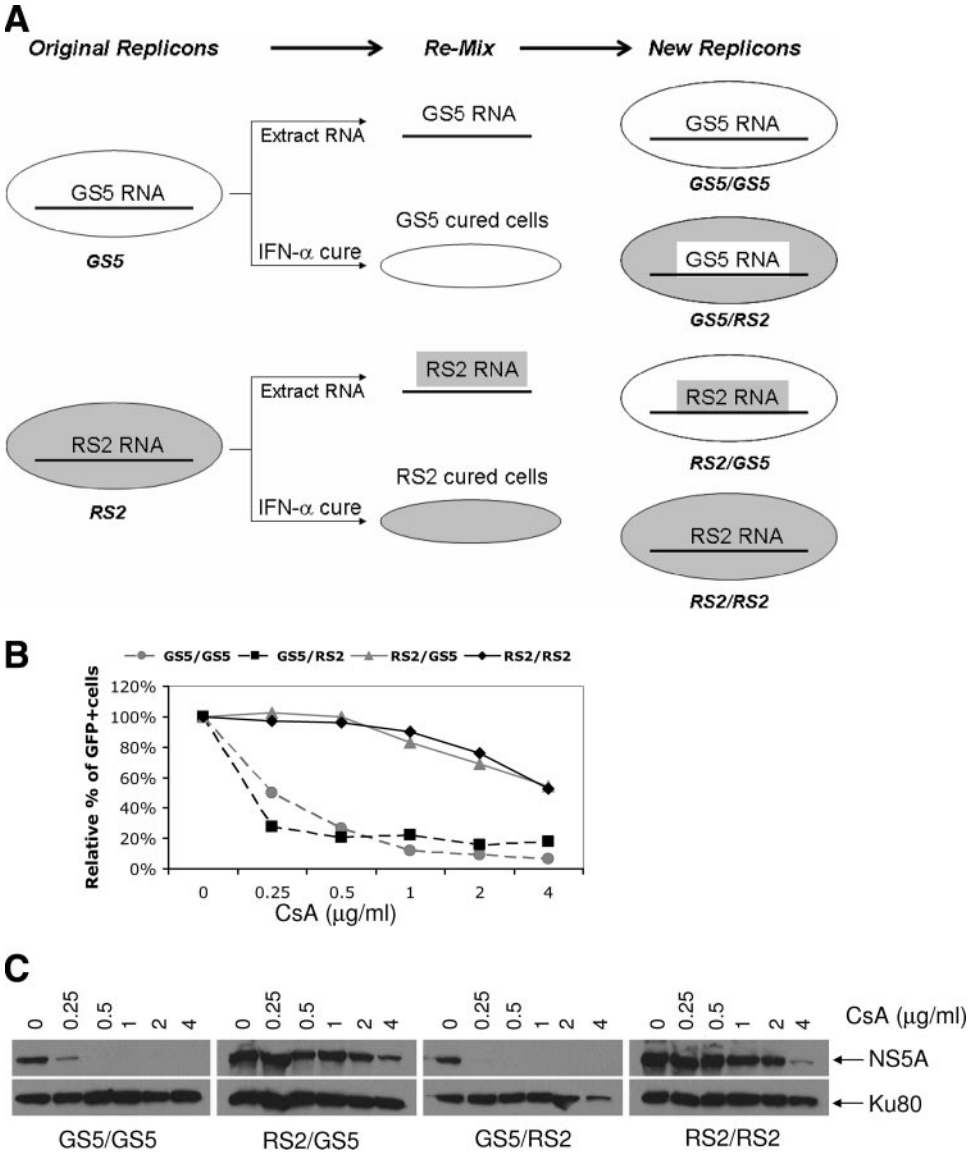

FIG. 3. Replicon RNA, not the host cell, was responsible for the CsA resistance of RS2 cells. (A) Experimental design for mapping the resistance to the replicon RNA or the host cell. We separated the viral RNA and host cells for both GS5 and RS2 replicons and remixed them to generate all four possible 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.

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 μM) 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.

TABLE 1. Mutations in NS5B identified in CsA-resistant GT1b replicons

Replicon name	Cell line description	IC_{50} (μM)	Mutation(s) in NS5B
GS5	Control	0.22 ± 0.04	NA
RS2	Resistant cell pool	2.4 ± 0.8	I432V, K535N
RS1-2	Resistant single clone	2.2 ± 0.8	I432V

IC_{50}, 50% inhibitory concentration; NA, not applicable.

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-

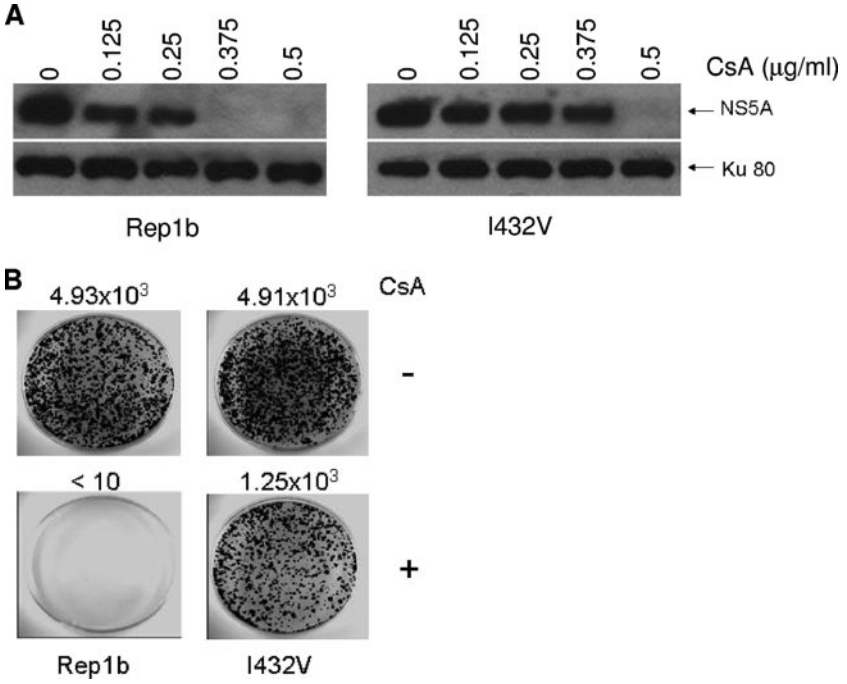

FIG. 4. A point mutation at amino acid 432 of NS5B contributes to the CsA resistance of RS2 cells. (A) Rep1b and a derivative replicon containing the I432V mutation were treated with increasing amounts of CsA for 4 days before being subjected to Western blotting with an anti-NS5A antibody. Concentrations 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.

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

TABLE 2. Sequence covariance between P540A and I432V in HCV isolates

Mutation in NS5B	No. of NS5B sequences retrieved for indicated HCV database[a]:		
	LANL[b]	Japan HCV[c]	euHCVdb[d]
P540A	12	12	54
I432V	12	14	55
P540A/I432V	11	11	51
Total	150	173	382

[a] The *P* value of covariance ($df = 1$) was ≤0.001 for the three databases.
[b] http://hcv.lanl.gov/content/hcv-db/index.
[c] http://s2as02.genes.nig.ac.jp/.
[d] http://euhcvdb.ibcp.fr/euHCVdb/.

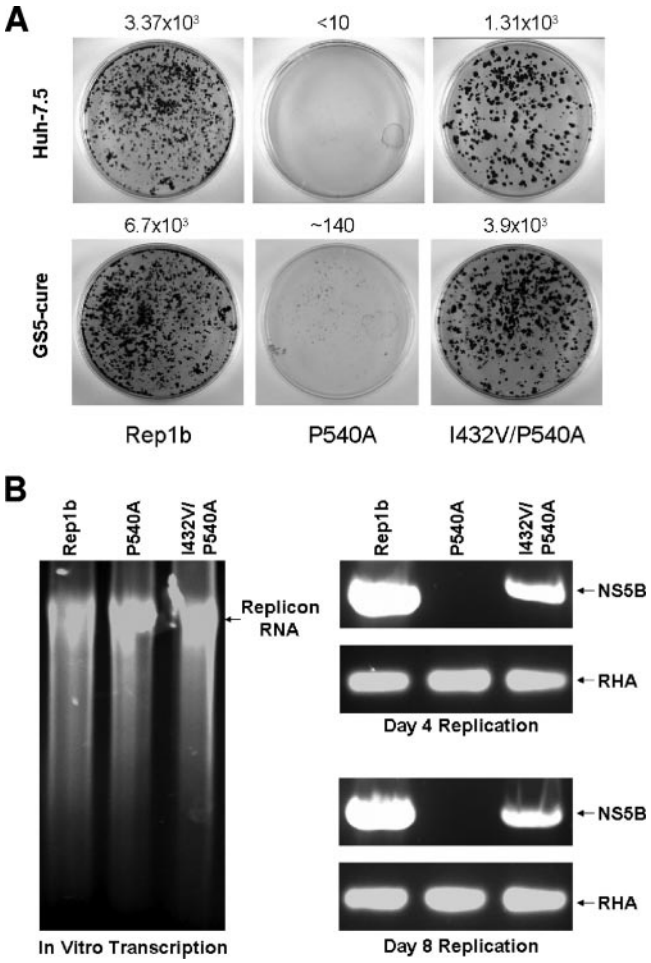

FIG. 5. I432V rescues replication of a GT1b replicon from the lethal effect of P540A mutation. (A) Colony formation assay for Rep1b and derivative replicons. One microgram of the replicon RNA generated by in vitro transcription was electroporated into either Huh-7.5 or GS5 IFN-cured cells. G418-resistant cells were selected with 500 μg/ml of G418 for 3 weeks. Cell colonies were stained with crystal violet at the end of the selection. (B) Transient replication assay. Equivalent amounts (1 μg) of the RNA of the three replicons generated by in vitro transcription (left) were used in the electroporation of Huh-7.5 cells. Total RNA was then extracted 4 and 8 days postelectroporation and subjected to RT-PCR for the detection of HCV RNA. Primers that amplify the NS5B coding region of GT1b were used to detect HCV RNA, and the cDNA for RNA helicase A (RHA), a cellular gene, was amplified as a control for the input of total cellular RNA.

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 RS1-2 cells (Fig. 7B). The addition of CsA to the binding reaction significantly suppressed the binding of NS5B to RNA for the GS5 replicon. Similarly, the association between NS5B and CypB was disrupted. However, the NS5B protein from RS1-2 cells still bound to poly(U) RNA in the presence of CsA even though cyclophilin B was no longer present in the pull-down complex. The relative CsA resistance of the RS1-2 replicon measured by the NS5B-poly(U) RNA interaction was approximately 3.2-fold (Fig. 7C).

The in vitro binding results raised the possibility that the

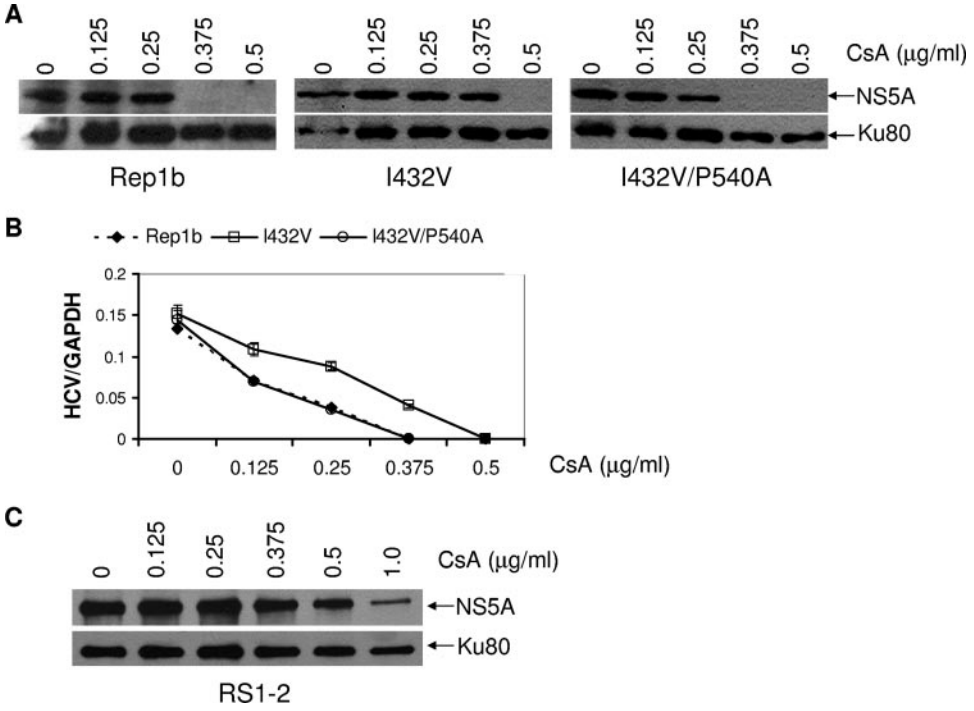

FIG. 6. The I432V/P540A replicon and the Rep1b replicon are equally sensitive to CsA. (A) We treated the indicated replicon cells with increasing amounts of CsA for 72 h and then detected HCV NS5A expression. (B) RNA analysis. The total RNA in the CsA-treated replicon cells was extracted 72 h posttreatment and then analyzed with real-time RT-PCR to detect HCV RNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA simultaneously. The ratios of HCV RNA versus GAPDH RNA were calculated through the relative cycle threshold values. (C) RS1-2 is more resistant than the I432V replicon. The RS1-2 replicon, which contains a single I432V mutation in NS5B and many other mutations elsewhere in the genome, was treated and analyzed in the same manner as the Rep1b-based replicon cells shown in panel A.

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 GS5 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

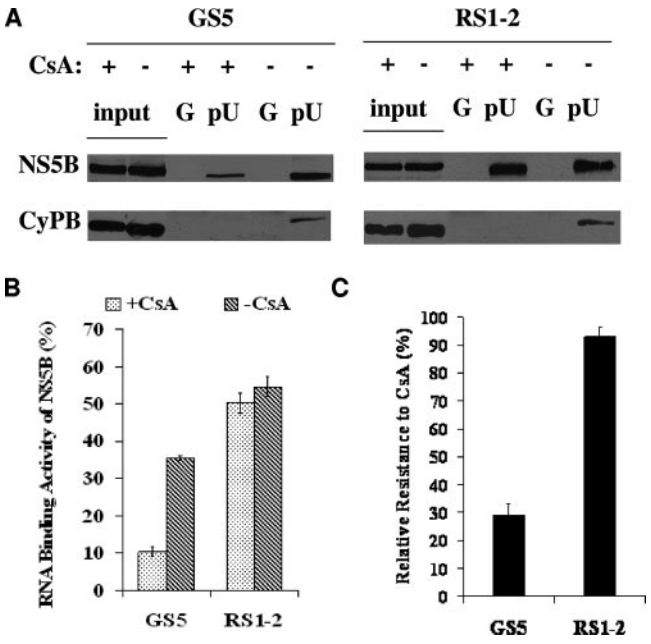

FIG. 7. RNA binding activity of NS5B is enhanced in CsA-resistant replicon cells. (A) An in vitro RNA-NS5B binding assay was conducted as described in Materials and Methods. Cell lysates of CsA-treated (8 μg/ml) or untreated GS5 or RS1-2 replicon cells were incubated with poly(U)-Sepharose or protein G-Sepharose beads in the presence or absence of 32 μg/ml of CsA. Precipitates were separated by SDS-PAGE and immunoblotted with anti-NS5B and anti-CypB antibodies. Twofold amounts of cell lysate were used for "input" in the RNA binding assay. "pU" represents poly(U), and "G" represents protein G-Sepharose beads. −, absence of; +, presence of. (B) The percentage of the NS5B bound to RNA was calculated by quantifying the band intensities. The ratio of "bound" versus "input" is plotted with the twofold difference in loading taken into consideration. (C) Direct comparison of the effect of CsA on the RNA binding activity of NS5B from GS5 and RS1-2 replicons. The percentage of bound RNA in the presence or absence of CsA is calculated and shown for both GS5 and RS1-2 cells. Error bars indicate standard deviations.

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 RNA was able to rescue the lethal phenotype of the P540A mutation, suggesting a previously unidentified relationship between residues Ile-432 and Pro-540. This potential interaction is also supported by a significant sequence covariation of the P540A and I432V among natural HCV variants. We examined the NS5B sequences of the HCV isolates retrieved from three HCV databases (Table 2). The proline at position 540 of the NS5B protein is quite conserved, and the P540A variation exists in only a small percentage of the natural isolates (12/150, 12/173, and 54/382 from the LANL, Japan HCV, and euHCVdb databases, respectively). The same also holds true for the isoleucine at position 432, as the I432V variation exists in a small percentage of the isolates (12/150, 14/173, and 55/382 from the LANL, Japan HCV, and euHCVdb databases, respectively). Nearly all the NS5B sequences with the P540A variation also have the I432V variation (11/12, 11/12, and 51/54 from the LANL, Japan HCV, and euHCVdb databases, respectively). This highly significant covariance between P540A and I432V in the natural isolates greatly complements our experimental data, showing that I432V can counteract the negative effect of the P540A mutation and rescue replication.

The mechanism by which I432V rescues HCV replication and, presumably, NS5B function of P540A GT1b replicon is currently unclear. Ile-432 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 has cyclophilin binding but no I432V change); the I432V replicon is more resistant to CsA since it has both elements, and the I432V/P540A reverts to the wild type because it only has one element (I432V but no cyclophilin binding), like the wild type. Consistent with this hypothesis, the increase of hydrophobicity brought about by the change from Pro to Ala is exactly the same as the decrease of hydrophobicity resulting from an Ile-to-Val change.

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 I423V 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 replicons 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 I/5A-GFP (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-

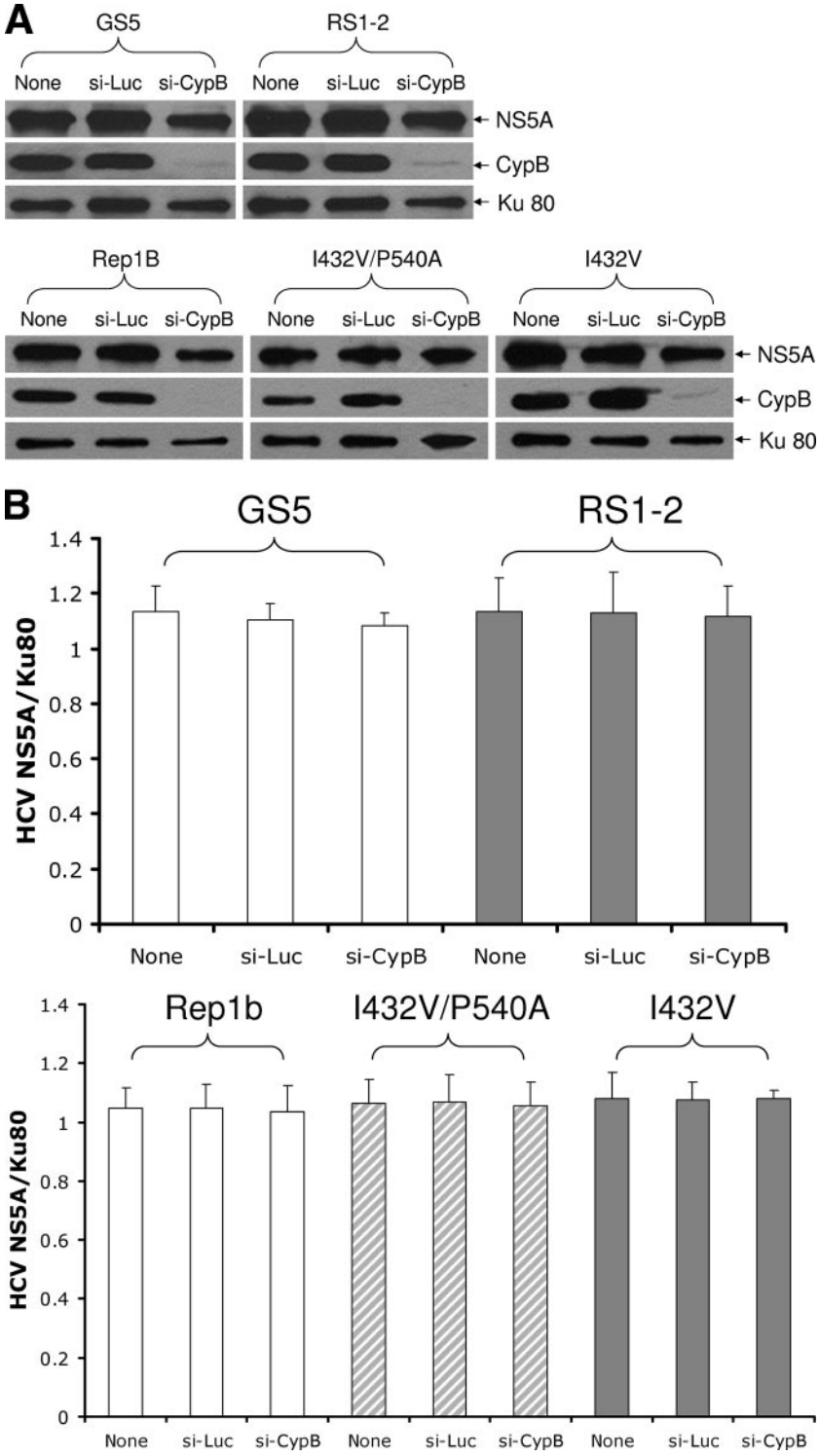

FIG. 8. Cyclophilin B knockdown in replicon cells had no significant effect on NS5A expression. (A) Various wild-type and CsA-resistant replicon cells were transduced with lentiviral vectors expressing either a luciferase siRNA (si-Luc) or a CypB siRNA (si-CypB) for 7 days before being lysed for protein 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 GS5-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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REFERENCES

1. **Aberham, C., S. Weber, and W. Phares.** 1996. Spontaneous mutations in the human immunodeficiency virus type 1 *gag* gene that affect viral replication in the presence of cyclosporins. J. Virol. **70:**3536–3544.
2. **Ago, H., T. Adachi, A. Yoshida, M. Yamamoto, N. Habuka, K. Yatsunami, and M. Miyano.** 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure **7:**1417–1426.
3. **Blight, K. J., A. A. Kolykhalov, and C. M. Rice.** 2000. Efficient initiation of HCV RNA replication in cell culture. Science **290:**1972–1974.
4. **Braaten, D., C. Aberham, E. K. Franke, L. Yin, W. Phares, and J. Luban.** 1996. Cyclosporine A-resistant human immunodeficiency virus type 1 mutants demonstrate that Gag encodes the functional target of cyclophilin A. J. Virol. **70:**5170–5176.
5. **Braaten, D., and J. Luban.** 2001. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. EMBO J. **20:**1300–1309.
6. **Bressanelli, S., L. Tomei, A. Roussel, I. Incitti, R. L. Vitale, M. Mathieu, R. De Francesco, and F. A. Rey.** 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc. Natl. Acad. Sci. USA **96:**13034–13039.
7. **Cai, Z., C. Zhang, K. S. Chang, J. Jiang, B. C. Ahn, T. Wakita, T. J. Liang, and G. Luo.** 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J. Virol. **79:**13963–13973.
8. **Damaso, C. R., and S. J. Keller.** 1994. Cyclosporine A inhibits vaccinia virus replication in vitro. Arch. Virol. **134:**303–319.
9. **Di Marco, S., C. Volpari, L. Tomei, S. Altamura, S. Harper, F. Narjes, U. Koch, M. Rowley, R. De Francesco, G. Migliaccio, and A. Carfi.** 2005. Interdomain communication in hepatitis C virus polymerase abolished by small molecule inhibitors bound to a novel allosteric site. J. Biol. Chem. **280:**29765–29770.
10. **Gao, B., F. Hong, and S. Radaeva.** 2004. Host factors and failure of interferon-α treatment in hepatitis C virus. Hepatology **39:**880–890.
11. **Inoue, K., K. Sekiyama, M. Yamada, T. Watanabe, H. Yasuda, and M. Yoshiba.** 2003. Combined interferon α2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. J. Gastroenterol. **38:**567–572.
12. **Inoue, K., and M. Yoshiba.** 2005. Interferon combined with cyclosporine treatment as an effective countermeasure against hepatitis C virus recurrence in liver transplant patients with end-stage hepatitis C virus related disease. Transplant Proc. **37:**1233–1234.
13. **Ishii, N., K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, and K. Shimotohno.** 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. **80:**4510–4520.
14. **Kato, N., T. Nakamura, H. Dansako, K. Namba, K. Abe, A. Nozaki, K. Naka, M. Ikeda, and K. Shimotohno.** 2005. Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture. J. Gen. Virol. **86:**645–656.
15. **Kukolj, G., G. A. McGibbon, G. McKercher, M. Marquis, S. Lefebvre, L. Thauvette, J. Gauthier, S. Goulet, M. A. Poupart, and P. L. Beaulieu.** 2005. Binding site characterization and resistance to a class of non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase. J. Biol. Chem. **280:**39260–39267.
16. **Le Pogam, S., W. R. Jiang, V. Leveque, S. Rajyaguru, H. Ma, H. Kang, S. Jiang, M. Singer, S. Ali, K. Klumpp, D. Smith, J. Symons, N. Cammack, and I. Najera.** 2006. In vitro selected Con1 subgenomic replicons resistant to 2′-C-methyl-cytidine or to R1479 show lack of cross resistance. Virology **351:**349–359.
17. **Le Pogam, S., H. Kang, S. F. Harris, V. Leveque, A. M. Giannetti, S. Ali, W. R. Jiang, S. Rajyaguru, G. Tavares, C. Oshiro, T. Hendricks, K. Klumpp, J. Symons, M. F. Browner, N. Cammack, and I. Najera.** 2006. Selection and characterization of replicon variants dually resistant to thumb- and palm-binding nonnucleoside polymerase inhibitors of the hepatitis C virus. J. Virol. **80:**6146–6154.
18. **Lesburg, C. A., M. B. Cable, E. Ferrari, Z. Hong, A. F. Mannarino, and P. C. Weber.** 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. **6:**937–943.
19. **Lin, C., K. Lin, Y. P. Luong, B. G. Rao, Y. Y. Wei, D. L. Brennan, J. R. Fulghum, H. M. Hsiao, S. Ma, J. P. Maxwell, K. M. Cottrell, R. B. Perni, C. A. Gates, and A. D. Kwong.** 2004. In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. J. Biol. Chem. **279:**17508–17514.
20. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623–626.
21. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113.
22. **Lu, L., T. J. Pilot-Matias, K. D. Stewart, J. T. Randolph, R. Pithawalla, W. He, P. P. Huang, L. L. Klein, H. Mo, and A. Molla.** 2004. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro. Antimicrob. Agents Chemother. **48:**2260–2266.
23. **Matsuda, S., F. Shibasaki, K. Takehana, H. Mori, E. Nishida, and S. Koyasu.** 2000. Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation. EMBO Rep. **1:**428–434.
24. **Migliaccio, G., J. E. Tomassini, S. S. Carroll, L. Tomei, S. Altamura, B. Bhat, L. Bartholomew, M. R. Bosserman, A. Ceccacci, L. F. Colwell, R. Cortese, R. De Francesco, A. B. Eldrup, K. L. Getty, X. S. Hou, R. L. LaFemina, S. W. Ludmerer, M. MacCoss, D. R. McMasters, M. W. Stahlhut,**

D. B. Olsen, D. J. Hazuda, and O. A. Flores. 2003. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication in vitro. J. Biol. Chem. **278:**49164–49170.
25. **Mo, H., L. Lu, T. Pilot-Matias, P. Pithawalla, R. Mondal, S. Masse, T. Dekhtyar, T. Ng, G. Koev, V. Stoll, K. D. Stewart, J. Pratt, P. Donner, T. Rockway, C. Maring, and A. Molla.** 2005. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor in vitro. Antimicrob. Agents Chemother. **49:**4305–4314.
26. **Moradpour, D., M. J. Evans, R. Gosert, Z. Yuan, H. E. Blum, S. P. Goff, B. D. Lindenbach, and C. M. Rice.** 2004. Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. J. Virol. **78:**7400–7409.
27. **Naka, K., K. Takemoto, K. Abe, H. Dansako, M. Ikeda, K. Shimotohno, and N. Kato.** 2005. Interferon resistance of hepatitis C virus replicon-harbouring cells is caused by functional disruption of type I interferon receptors. J. Gen. Virol. **86:**2787–2792.
28. **Nakagawa, M., N. Sakamoto, N. Enomoto, Y. Tanabe, N. Kanazawa, T. Koyama, M. Kurosaki, S. Maekawa, T. Yamashiro, C. H. Chen, Y. Itsui, S. Kakinuma, and M. Watanabe.** 2004. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem. Biophys. Res. Commun. **313:**42–47.
29. **Nakagawa, M., N. Sakamoto, Y. Tanabe, T. Koyama, Y. Itsui, Y. Takeda, C. H. Chen, S. Kakinuma, S. Oooka, S. Maekawa, N. Enomoto, and M. Watanabe.** 2005. Suppression of hepatitis C virus replication by cyclosporin A is mediated by blockade of cyclophilins. Gastroenterology **129:**1031–1041.
30. **Nelson, H. B., and H. Tang.** 2006. Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. J. Virol. **80:**1181–1190.
31. **Paeshuyse, J. A. Kaul, E. De Clercq, B. Rosenwirth, J. M. Dumont, P. Scalfaro, R. Bartenschlager, and J. Neyts.** 2006. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro. Hepatology **43:**761–770.
32. **Pavio, N., and M. M. Lai.** 2003. The hepatitis C virus persistence: how to evade the immune system? J. Biosci. **3:**287–304.
33. **Pawlotsky, J. M.** 2003. Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C. Antivir. Res. **59:**1–11.
34. **Pfeiffer, J. K., and K. Kirkegaard.** 2005. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. J. Virol. **79:**2346–2355.
35. **Sayah, D. M., E. Sokolskaja, L. Berthoux, and J. Luban.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430:**569–573.
36. **Sokolskaja, E., D. M. Sayah, and J. Luban.** 2004. Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity. J. Virol. **78:**12800–12808.
37. **Sumpter, R., Jr., C. Wang, E. Foy, Y. M. Loo, and M. Gale, Jr.** 2004. Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. J. Virol. **78:**11591–11604.
38. **Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and H. G. Gottlinger.** 1994. Functional association of cyclophilin A with HIV-1 virions. Nature **372:**363–365.
39. **Towers, G. J., T. Hatziioannou, S. Cowan, S. P. Goff, J. Luban, and P. D. Bieniasz.** 2003. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat. Med. **9:**1138–1143.
40. **Vahlne, A., P. A. Larsson, P. Horal, J. Ahlmen, B. Svennerholm, J. S. Gronowitz, and S. Olofsson.** 1992. Inhibition of herpes simplex virus production in vitro by cyclosporin A. Arch. Virol. **122:**61–75.
41. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
42. **Waninger, S., K. Kuhen, X. Hu, J. E. Chatterton, F. Wong-Staal, and H. Tang.** 2004. Identification of cellular cofactors for human immunodeficiency virus replication via a ribozyme-based genomics approach. J. Virol. **78:**12829–12837.
43. **Watashi, K., M. Hijikata, M. Hosaka, M. Yamaji, and K. Shimotohno.** 2003. Cyclosporine A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology **38:**1282–1288.
44. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell **19:**111–122.
45. **Young, K. C., K. L. Lindsay, K. J. Lee, W. C. Liu, J. W. He, S. L. Milstein, and M. M. Lai.** 2003. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. Hepatology **38:**869–878.
46. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection *in vitro*. Proc. Natl. Acad. Sci. USA **102:**9294–9299.
47. **Zhu, H., D. R. Nelson, J. M. Crawford, and C. Liu.** 2005. Defective Jak-Stat activation in hepatoma cells is associated with hepatitis C viral IFN-alpha resistance. J. Interferon Cytokine Res. **25:**528–539.