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13. We repeated the simulations illustrated in Fig. 3. Instead of using a constant-amplitude noise for all orientations and contrasts in the simulation, however, we used the values of Fig. 2G. The simulations resulted in contrast invariance indistinguishable from that shown in Fig. 3, E and F.
14. Spike threshold in the model, in some cases, was lowered below its measured value in order to obtain nonzero spike rates for predictions from averaged traces without added noise. In each cell, the same value for spike threshold was used for all three predictions.
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Efficient Initiation of HCV RNA Replication in Cell Culture

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Hepatitis C virus (HCV) infection is a global health problem affecting an estimated 170 million individuals worldwide. We report the identification of multiple independent adaptive mutations that cluster in the HCV nonstructural protein NS5A and confer increased replicative ability in vitro. Among these adaptive mutations were a single amino acid substitution that allowed HCV RNA replication in 10% of transfected hepatoma cells and a deletion of 47 amino acids encompassing the interferon (IFN) sensitivity determining region (ISDR). Independent of the ISDR, IFN-α rapidly inhibited HCV RNA replication in vitro. This work establishes a robust, cell-based system for genetic and functional analyses of HCV replication.

HCV (*1*) typically evades clearance by the host's immune system, allowing the establishment of a persistent infection in at least 70% of infected individuals. HCV-associated end-stage liver disease is now the leading cause of liver transplantation in the United States. Most patients treated with IFN alone either fail to respond or do not mount a sustained response. In Japanese patients, the amino acid sequence in a defined region of NS5A, designated the ISDR, appears to correlate with the effectiveness of IFN treatment. However, this association is substantially weaker or absent in patients infected with genotype 1a HCV strains or European patients infected with genotype 1b strains. Although considerable genetic heterogeneity exists among different HCV isolates, genotypes 1a and 1b are the most prevalent worldwide (*2*).

The single-stranded, positive-sense HCV RNA genome is ~9.6 kb in length and comprises a 5′ nontranslated region (NTR) that contains an internal ribosome entry site (IRES), a polyprotein coding region consisting of a single long open reading frame (ORF), and a 3′ NTR. Despite the availability of infectious cDNA clones [for examples, see (*3*, *4*)], efficient in vitro replication has not been observed. Recently, HCV replication was reported in the human hepatoma cell line, Huh7, after transfection of genotype 1b subgenomic RNA replicons expressing a selectable marker (*5*). These replicons contained (i) the HCV 5′ NTR fused to 12 amino acids of the capsid coding region; (ii) the neomycin phosphotransferase gene (Neo), which upon expression confers resistance to G418; (iii) the IRES from encephalomyocarditis virus (EMCV), which directs translation of HCV proteins NS2 or NS3 to NS5B; and (iv) the 3′ NTR. However, only 1 in 10^6 Huh7 cells supported HCV replication, which the authors attributed to low numbers of permissive cells (*5*).

To extend this system to other HCV genotypes, we constructed similar selectable replicons based on the HCV-H genotype 1a infectious clone (*3*). In contrast to the earlier study (*5*), we found that transfection of deoxyribonuclease (DNase)-treated RNA replicons (*6*, *7*) into multiple human hepatoma cell lines (*8*) failed to confer antibiotic resistance. The inability of the HCV-H–derived replicons to establish efficient HCV replication could indicate that the earlier success (*5*) was dependent on the particular genotype 1b consensus cDNA clone studied. As a positive control, we synthesized the genotype 1b replicon, I_{377}/NS3-3′ (*5*), using a polymerase chain reaction (PCR)–based gene assembly procedure (*9*) and transfected RNA transcripts into Huh7 cells (*6*, *7*). G418-resistant colonies supporting autonomous HCV replication (*10*) were observed at low frequency (*11*). In contrast, colonies were never observed for Huh7 cells electroporated in parallel with replicon RNA carrying a polymerase-defective lethal mutation in NS5B (pol$^-$) (*12*).

High-level replication may reflect a need for adaptation of the replicon to the host cell; hence, we sequenced uncloned reverse transcriptase (RT)–PCR products amplified from five independent G418-resistant cell clones (*13*). Each cell clone contained replicons with mutations in a defined region of NS5A; four clones contained replicons encoding amino acid substitutions upstream of the putative ISDR, and the fifth clone harbored a replicon encoding an in-frame deletion of 47 amino acids encompassing the ISDR. In addition to an amino acid change in NS5A, one replicon also encoded an amino acid substitution in NS3 and another encoded amino acid substitutions in both NS3 and NS4B (*14*). Sequence analysis of the NS5A coding region revealed that 12 of 17 additional cell clones had replicons with point mutations upstream of the ISDR (Fig. 1A). Thus, we identified nine different NS5A substitutions localizing to a region of about 30 amino acids, and a deletion of 47 amino acids (Fig. 1A).

We next engineered each change back into the I_{377}/NS3-3′ replicon. After transfection of mutant RNA transcripts into Huh7 cells and G418 selection (*6*, *7*), each construct established replication in 0.2 to 10% of transfected cells (Fig. 1A), as compared with 0.0005% for the original I_{377}/NS3-3′ replicon. Hence, each of the 10 mutations in NS5A enhanced the ability of HCV replicons to replicate. Engineering the Δ47aa and S1179I mutations into the HCV-H genotype 1a–derived replicon did not result in detectable replication in Huh7 cells. This suggests that different or additional adaptive mutations may be required for RNA replication of other isolates.

To examine the ability of these mutant constructs to replicate over a shorter time scale in the absence of G418 selection, we measured HCV-specific RNA amplification at different times after transfection. Replicons with the highest transduction efficiency displayed the greatest level of HCV RNA accumulation in this first cycle assay (Fig. 1B). For the replicon S1179I, HCV RNA levels increased to ~20-, 300-, and 400-fold relative to the pol$^-$ negative control at 24, 48, and 96 hours, respectively (Fig. 1B).

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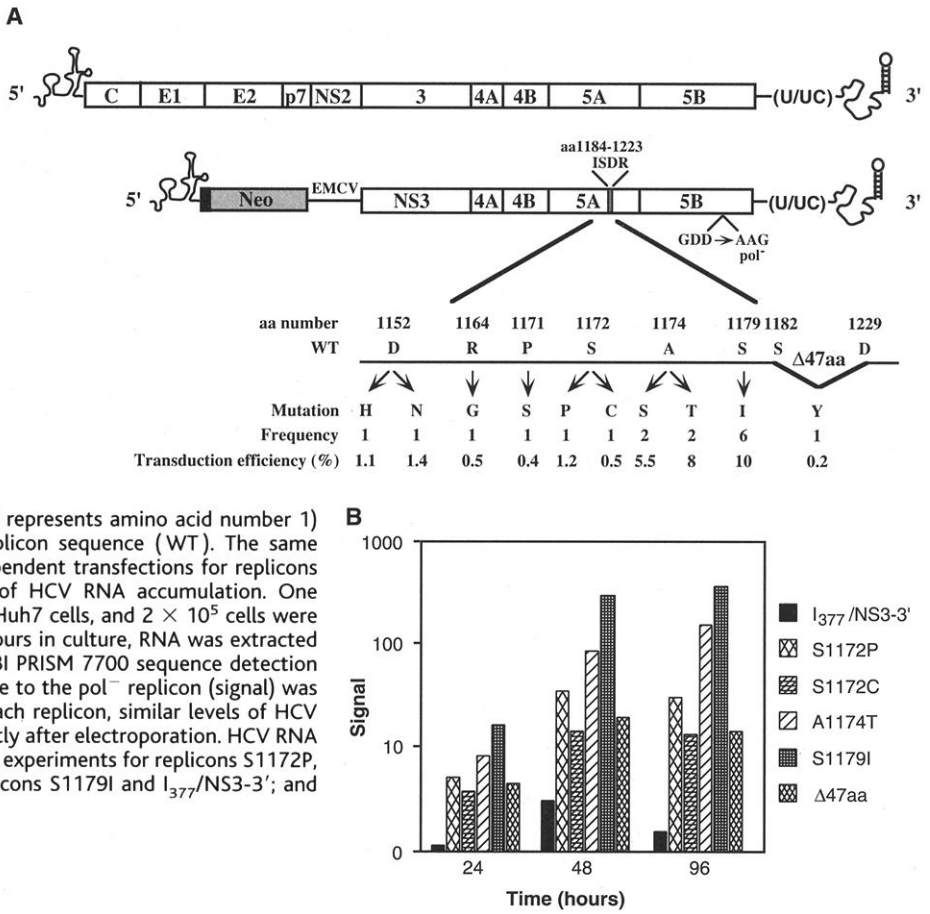

Fig. 1. Features and relative replication efficiencies of HCV-selectable replicons. (**A**) (Top) Schematic of the complete HCV genome. The 5′ and 3′ NTRs flank the ORF (open box) with the structural proteins located in the NH_2-terminal portion of the polyprotein, and the remainder encodes the nonstructural proteins (NS2 to NS5B). (Middle) Structure of the selectable replicon, I_{377}/NS3-3′, indicating the 5′ NTR fused to a small portion of the capsid coding region (solid box), the Neo gene (shaded box), EMCV IRES (solid line), and NS3 to NS5B coding region (open box), followed by the 3′ NTR structure. The locations of the ISDR (hatched box) and the lethal mutation in NS5B (pol$^-$) are illustrated. (Bottom) The positions, frequencies, and relative transduction efficiencies (*30*) of the adaptive mutations identified in NS5A. Overlapping RT-PCR products were sequenced (*13*) and the mutations observed in NS5A are illustrated, along with the frequency with which each arose. The amino acid number (Met at the beginning of NS3 represents amino acid number 1) is indicated above the wild-type I_{377}/NS3-3′ replicon sequence (WT). The same transduction efficiency was obtained in five independent transfections for replicons S1179I and Δ47aa. (**B**) Graphic representation of HCV RNA accumulation. One microgram of replicon RNA was transfected into Huh7 cells, and 2×10^5 cells were plated into 35-mm dishes. After 24, 48, and 96 hours in culture, RNA was extracted and HCV-specific RNA was measured with the ABI PRISM 7700 sequence detection system (*12*). The fold increase of HCV RNA relative to the pol$^-$ replicon (signal) was plotted against the time after transfection. For each replicon, similar levels of HCV RNA were detected in cellular RNA extracted directly after electroporation. HCV RNA accumulation was comparable in two independent experiments for replicons S1172P, S1172C, and A1174T; seven experiments for replicons S1179I and I_{377}/NS3-3′; and four repetitions for the Δ47aa replicon.

The clustering of mutations in a defined region of NS5A was unexpected. The NS5A protein is phosphorylated primarily on serine residues by an unknown cellular kinase (*15*, *16*). At least two distinct forms of phosphorylated NS5A, p56 (basal phosphorylated form) and p58 (hyperphosphorylated form), have been observed. Hyperphosphorylation of NS5A, but not basal phosphorylation, usually requires NS3-5A expression in cis (*17*, *18*). In addition, NS5A is thought to modulate the IFN-stimulated antiviral response, possibly through a direct interaction between the IFN-stimulated, double-stranded, RNA-dependent protein kinase (PKR) and the ISDR (*19*). NS5A also binds to several cellular proteins (*20*–*22*), but the functional significance of these interactions is unclear. Because most of the replicons contained a mutation in NS5A, we investigated the expression level and phosphorylation pattern of NS5A in G418-resistant cells with the engineered adapted replicons. Cell monolayers, radiolabeled in the presence of ^{35}S-methionine and ^{35}S-cysteine or [^{32}P]orthophosphate, were lysed and subjected to immunoprecipitation with antiserum to NS5A. Differences in the level of expression (*23*) and the degree of phosphorylation were apparent (Fig. 2). Three conserved serines, Ser^{1172}, Ser^{1177}, and Ser^{1179}, have been identified as putative sites of hyperphosphorylation (*24*). In our study, Ser^{1172} and Ser^{1179} were sites of adaptive mutation, resulting in a reduction or the disappearance of p58, respectively (Fig. 2), suggesting that in this context NS5A hyperphosphorylation is not essential for HCV replication.

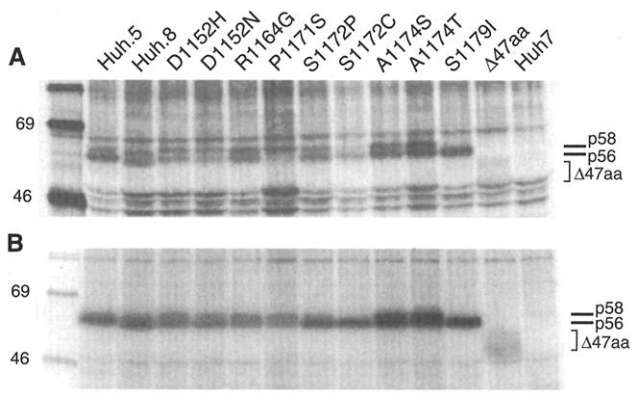

Fig. 2. NS5A expression levels and phosphorylation patterns in cell lines supporting HCV replication. Dishes (150 mm) containing stable colonies harboring autonomously replicating adapted replicons were trypsinized and maintained as population cell lines in media containing G418 (0.75 mg/ml). Two cell clones containing replicons with wild-type NS5A (Huh.5 and Huh.8), the 10-cell populations, and the parental Huh7 cells were incubated with 70 μCi of Expre^{35}S^{35}S protein labeling mix (NEN) (**A**) or 200 μCi of [^{32}P]orthophosphate (**B**) for 8 hours. Cell monolayers were lysed in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% SDS and 80 μg of phenylmethylsulfonyl fluoride per milliliter, and 80 μg of each lysate was immunoprecipitated with NS5A-specific antiserum. Immunoprecipitates were separated on SDS–10% polyacrylamide gels and visualized by autoradiography. Positions of the molecular size standards (in kilodaltons) are shown on the left, and p56 and p58 on the right. The diffuse and weak signal of NS5A expressed from the Δ47aa replicon may be influenced by antibody reactivity to determinants within the ISDR.

Chronic hepatitis C infection is treated with IFN, but response rates are variable. Mutations within the ISDR have been associated with resistance to IFN, and the I_{377}/NS3-3′ replicon has an ISDR characteristic of IFN nonresponders (*25*). Consequently, we assessed the sensitivity of HCV replication to IFN-α treatment in a cell clone harboring a replicon without any mutation in NS5A. In contrast to the predicted resistance, within 24 hours of treatment, IFN inhibited HCV replication, and HCV RNA levels declined over time (Fig. 3A) (*26*). To determine whether the deletion encompassing the ISDR

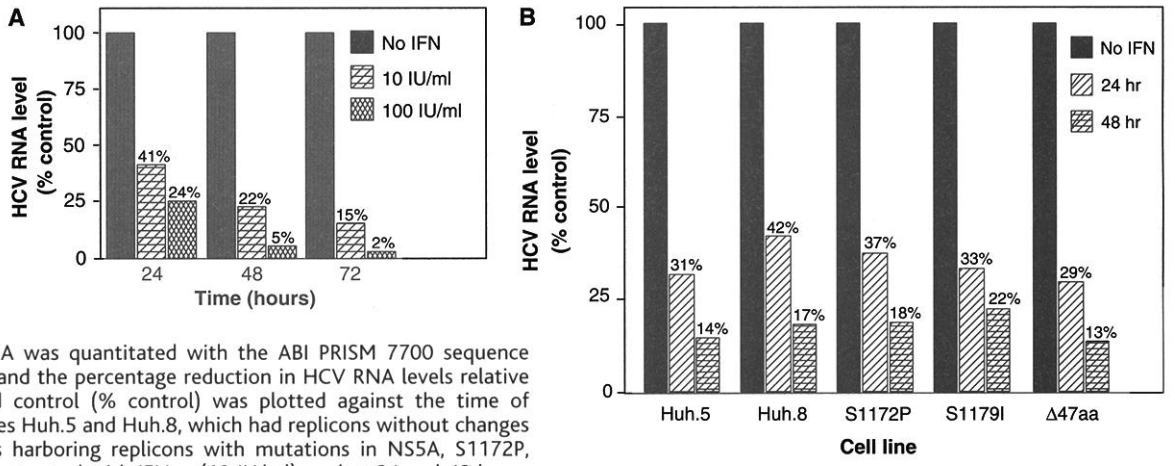

Fig. 3. Effect of IFN-α on HCV replication. (**A**) Huh.5 cells (4×10^5), containing a replicon with a wild-type NS5A sequence, were incubated in the absence of IFN-α or with IFN-α at 10 and 100 IU/ml. RNA was extracted from duplicate wells with Trizol reagent at 24, 48, and 72 hours after treatment. HCV-specific RNA was quantitated with the ABI PRISM 7700 sequence detection system (*12*), and the percentage reduction in HCV RNA levels relative to the No IFN–treated control (% control) was plotted against the time of treatment. (**B**) Cell clones Huh.5 and Huh.8, which had replicons without changes in NS5A, and cell lines harboring replicons with mutations in NS5A, S1172P, S1179I, and Δ47aa were treated with IFN-α (10 IU/ml), and at 24 and 48 hours after treatment, RNA was harvested from duplicate wells and HCV RNA was measured. The percentage decrease of HCV RNA relative to the No IFN–treated control (% control) was plotted for each replicon-harboring cell line.

or the amino acid changes in NS5A altered the ability of IFN to repress HCV replication, we treated the G418-resistant cells, harboring the engineered adapted replicons, with IFN-α. All cell lines were sensitive to IFN (Fig. 3B), demonstrating that irrespective of the NS5A sequence, IFN inhibited HCV RNA replication.

The striking convergence of mutations within a defined region of NS5A suggests that NS5A is important for the establishment of HCV replication in vitro. One possibility is that this region of NS5A mediates an interaction with a host cell protein that inhibits HCV replication. The NS5A mutations identified here may disrupt this interaction, thereby allowing HCV replication to proceed.

In conclusion, our findings highlight an important constellation of adaptive mutations in NS5A, identify an ISDR deletion mutant that may represent a starting point for the design of live, attenuated vaccines, and importantly, establish a robust system for future genetic and functional analyses of HCV replication in vitro.

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6. Purified plasmid DNAs were linearized, extracted once with phenol-chloroform, and precipitated with ethanol. DNA pellets were washed in 80% ethanol and resuspended in 10 mM tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0). RNA transcripts were synthesized at 37°C for 90 min in a 100-μl reaction mixture containing 40 mM tris-HCl (pH 7.9), 10 mM NaCl, 12 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 3 mM of each nucleoside triphosphate, 0.025 U of inorganic pyrophosphatase, 100 U of RNasin, 100 U of T7 RNA polymerase, and 2 μg of linearized DNA. RNA was extracted with phenol-chloroform, ethanol precipitated, and the RNA pellet washed in ethanol before resuspension in H_2O. DNA template was removed by three serial digestions for 20 min at 37°C with 10 U of DNase I.
7. RNA (0.1 to 10 μg) was electroporated into 5×10^6 hepatoma cells (*8*) and plated into 150-mm dishes. Between 24 and 48 hours, 1 mg of G418 per milliliter was added to the media, and thereafter the media was changed every 3 to 5 days.
8. Human hepatoma cell lines included HepG2, PH5CH (*27*), and two different Huh7 lines: our laboratory stock and another (*5*).
9. cDNAs spanning 600 to 750 bases in length were assembled in a stepwise PCR assay with 10 to 12 gel-purified oligonucleotides [60 to 80 nucleotides (nt)] with unique complementary overlaps of 16 nt. PCR products were purified, digested with appropriate restriction enzymes, and ligated into the similarly cleaved pGEM3Zf(+) plasmid vector. Multiple recombinant clones were sequenced, correct clones were identified, and the overlapping cDNA fragments were assembled into the contiguous replicon sequence: 5′ NTR-Neo-EMCV IRES-NS3-4A-4B-5A-5B-3′ NTR. Two derivatives were assembled that either lacked two U nucleotides in the poly (U/UC) tract (HCVrep1bBartMan/Δ2U′s) or carried an Ava II restriction enzyme site in the variable region of the 3′ NTR (HCVrep1bBartMan/AvaII). As negative controls, an RNA-dependent RNA polymerase–defective replicon (pol^-) was constructed where the Gly-Asp-Asp (GDD) motif was mutated to Ala-Ala-Gly (AAG) (*12*).
10. G418-resistant colonies were evident after 2 to 3 weeks, and 22 independent colonies were isolated. Authentic HCV replication was confirmed by (i) detection of ^{32}P-labeled, actinomycin D–resistant RNA of the expected size; (ii) amplification of sequences within the 5′ and 3′ NTRs in a quantitative RT-PCR assay revealing copy numbers of 50 to 5000 HCV RNA molecules per cell; (iii) immunoprecipitation of the HCV proteins NS3, NS4B, NS5A, and NS5B from ^{35}S-labeled cell lysates; and (iv) cytoplasmic localization of NS3 by immunostaining of cell monolayers.
11. Electroporation of replicon RNA (*7*) into our laboratory stock of Huh7 cells resulted in a higher yield of G418-resistant foci (~0.0005%) than electroporation into Huh7 cells obtained from R. Bartenschlager (~0.0001%) (*5*). Both replicon derivatives, HCVrep1bBartMan/Δ2U′s and HCVrep1bBartMan/AvaII (*9*), had similar G418 transduction efficiencies.
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13. Total cellular RNA was isolated from G418-resistant clones (*10*) with Trizol reagent (Gibco-BRL) and precipitated with isopropanol. The RNA pellet was washed in ethanol and resuspended in H_20. HCV RNA (~10^4 molecules) was mixed with the HCV-specific primer, and the primer extended at 43.5°C for 1 hour, with Superscript II reverse transcriptase. cDNAs were then amplified with KlenTaqLA DNA polymerase with 35 cycles at 95°C for 30 s, 55° to 60°C for 30 s, and 68°C for 4 min. PCR products were recovered from preparative low–melting point agarose electrophoresis by phenol extraction, and ~40 ng of purified PCR product was directly sequenced. For two clones, complete terminal sequences were determined; no change in the 5′ or 3′ NTRs was observed.
14. One replicon contained Q87R (NS3) and S1179I (NS5A), and the other contained K584E (NS3), S925G (NS4B), and R1164G (NS5A).
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23. The NS5A expression levels observed correlated with HCV RNA replication rate. The levels of NS3 and NS4B were comparable to that of NS5A in the different cell lines.
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25. The ISDR amino acid sequences of replicon I_{377}/NS3-3′ (*5*), HCV-J (*28*, *29*), and IFN-sensitive strains (*28*, *29*) were aligned. On the basis of this analysis, the I_{377}/NS3-3′ ISDR sequence corresponds to that of isolates from IFN nonresponders.
26. The NS5A coding region in clone Huh.5 did not contain any mutations. However, the remainder of the genome was not sequenced, so we cannot exclude the possibility that mutations elsewhere influence the IFN response.
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30. To determine the G418 transduction efficiency of transcripts with potential adaptive mutations, we transfected Huh7 cells with 1 μg of replicon RNA and plated them at multiple densities (10^3 to 2×10^5 cells), together with Huh7 cells transfected with pol^--defective RNA transcripts (*12*) such that the total cell number was maintained at 1×10^5 to 2×10^5 cells per 100-mm dish. Three weeks after G418 selection, the resultant foci were counted after staining with crystal violet. The relative transduction efficiency was calculated on the basis of the number of Huh7 cells electroporated.

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