Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus

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The JFH1 strain of hepatitis C virus (HCV) is unique among HCV isolates, in that the wild-type virus can traverse the entire replication cycle in cultured cells. However, without adaptive mutations, only low levels of infectious virus are produced. In the present study, the effects of five mutations that were selected during serial passage in Huh-7.5 cells were studied. Recombinant genomes containing all five mutations produced 3–4 logs more infectious virions than did wild type. Neither a coding mutation in NS5A nor a silent mutation in E2 was adaptive, whereas coding mutations in E2, p7, and NS2 all increased virus production. A single-cycle replication assay in CD81-deficient cells was developed to study more precisely the effect of the adaptive mutations. The E2 mutation had minimal effect on the amount of infectious virus released but probably enhanced entry into cells. In contrast, both the p7 and NS2 mutations independently increased the amount of virus released.

single-cycle growth | CD81-dependent

Hepatitis C virus (HCV), a small enveloped RNA virus of the *Hepacivirus* genus of the *Flaviviridae* family, is an important cause of acute and chronic liver disease worldwide (1, 2). HCV research was severely hampered by the lack of a robust *in vitro* cell culture system until isolation of a unique HCV genotype 2a sequence (JFH1) provided the essential key for development of a useful cell culture system (3). Wakita *et al.* (4) demonstrated that Huh-7 cells transfected with full-length JFH1 genomes secreted infectious virus particles (HCVcc), albeit with low efficiency [10^2 to 10^3 focus-forming units (ffu) per milliliter]. Increased virion production was achieved by propagating virus in especially permissive subclones of Huh-7 cells (5), by serially passaging JFH1 to select adaptive mutations (6), or by creating a chimeric virus (7) between JFH1 and another 2a strain, J6 (8). Such manipulations resulted in infectious virus titers of 10^4-10^5 ffu/ml.

A number of recent studies have identified adaptive or compensatory mutations that enhance infectious virus production from either wild-type JFH1 (6, 9, 10) or intergenotypic chimeras (11–13). To date, cell culture-selected mutations have been found in nearly all of the structural and nonstructural proteins, but the majority of these have mapped to the core-NS2 coding region, with a noticeable preference for the p7 and NS2 proteins (11, 12). The functions of p7 and NS2 are not well defined, but accumulating evidence suggests that these proteins may be involved in assembly and/or release of infectious virus particles (14–16), and that adaptive mutations in p7 and NS2 improve these putative functions in virus assembly and/or release (12). Significant enhancements in virus expansion have also been attributed to adaptive mutations in the E2 glycoprotein (6, 9). However, the majority of these adaptive mutations have been selected and studied in highly permissive cells in which multiple rounds of infection and release make it difficult to determine when in the viral life cycle these mutations act.

In this study, JFH1-transfected Huh-7.5 cells were cultured until virus with enhanced replication capacity was selected. Adaptive mutations were identified and analyzed by transfecting mutant RNA genomes into a virtually noninfectable CD81-deficient subclone of Huh-7.5 cells to limit the replication cycle to one round: this system ensured that the vast majority of genomes contained the sequence being studied and made it possible to ask whether a specific mutation increased virus entry or enhanced virus production.

Results

Selection of Mutations During Serial Passage. Huh-7.5 cells were transfected with *in vitro* transcribed genomes of JFH1, and cells, and later media, were passaged sequentially. Consensus sequencing of the entire coding region at various time points demonstrated the acquisition and fixation of five mutations between day 20 posttransfection and round 2 of virus passage. The mutations included one synonymous mutation at nucleotide 1681 of E2 and one nonsynonymous mutation in each of E2, p7, NS2, and NS5A (Table 1). Because all but the p7 mutation were unique to this virus, the questions were asked, which, if any, increased the efficiency of virus production and at which step?

Mutated Virus Produces More Progeny. Because mutations in E2, p7, and NS2 often have been associated with adaptation in other studies (6, 9–12), the four mutations in these genes were cloned into the wild-type JFH1 genome in the absence (JFH-AM1) or presence (JFH-AM2) of the NS5A mutation, and the replication capacity of these recombinant viruses was compared with that of wild type. After transfection, at day 5, both viruses had produced 100- to 1,000-fold more infectious virus than had wild-type (data not shown). Kinetics of virus production were determined by inoculating equal amounts of the infectious virus produced by transfection onto naïve cells at a multiplicity of infection (m.o.i.) of 0.0001 and monitoring viral RNA and infectious virus accumulation in the medium. Target cell cultures grew at similar rates until just before cytopathic effects (CPE) similar to those reported (6) were observed at peak virus titer, i.e., slower cell

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Table 1. Mutations selected during JFH1 culture

	Position changed				
Virus*	E2 417	(E2)† (1681)	p7 765	NS2 1012	NS5A 2175
Wild type	N	(T)	N	Q	L
D12, D16 PT	N	(T)	N	Q	L
D20 PT, D4 PI	N/S	(T/C)	N/D	Q/R	L/V
Rnd2,3,4 VP	S	(C)	D	R	V

*D, day; PT, posttransfection; PI, postinfection; Rnd VP, round of virus passage.
†The synonymous change in E2 is shown in parentheses.

growth and the appearance of dead cells floating in the culture fluids. At m.o.i.s ranging from 0.001 to 1.00, the only differences noted were that CPE and peak titers occurred at earlier time points. In general, RNA levels and infectious titer decreased immediately after severe CPE was observed. Culture fluids from cells transfected with a JFH1 subgenomic replicon (SGR-JFH1), which does not make infectious virus particles, were used as a mock infection control. The RNA patterns (Fig. 1*A*) and production of infectious virions (Fig. 1*B*) were virtually identical for JFH-AM1 and -AM2, and both released >3 logs more virus than wild type at the time of peak production. Therefore, adaptive

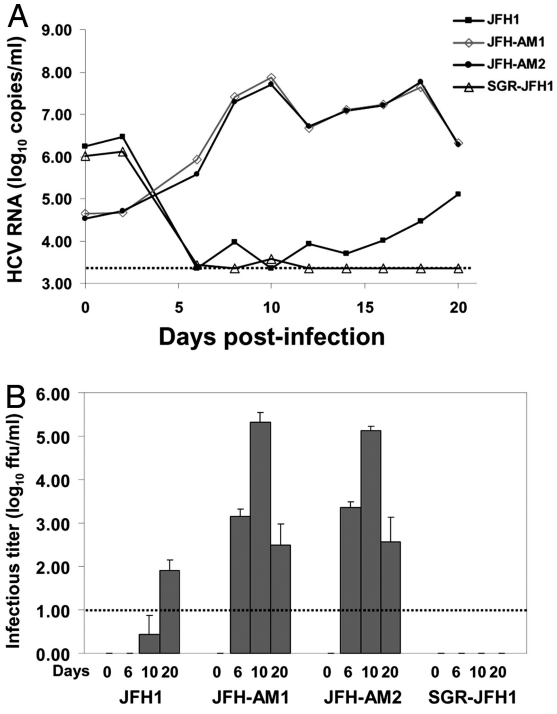

Fig. 1. Mutations permit 3- to 4-log higher levels of virus production. One million Huh-7.5 cells were inoculated with day 5 transfection supernatants containing 100 ffu (m.o.i. = 0.0001) of indicated HCVcc. (*A*) HCV RNA levels in culture fluids at indicated time points postinfection were measured by TaqMan real-time RT-PCR. At day 5 posttransfection, all culture fluids still contained large amounts of transfection-related RNA. Therefore, dilution of infectious virus to produce the desired m.o.i. resulted in a corresponding dilution of residual transfection RNA with the result that at day 0 RNA levels differed even though the amount of each infecting virus was the same. Culture supernatant from an SGR-JFH1 transfection was used as a mock infection control. Data points represent the mean value obtained from duplicate TaqMan amplifications of the same sample. (*B*) Infectious viral titers were measured at indicated time points postinfection by a limiting dilution assay for ffu. Results are representative of at least two independent transfection/infection experiments. Assays of ffu were performed in triplicate, and the means plus standard error are plotted. The dotted line represents the cutoff of the assay, which was 10 ffu/ml.

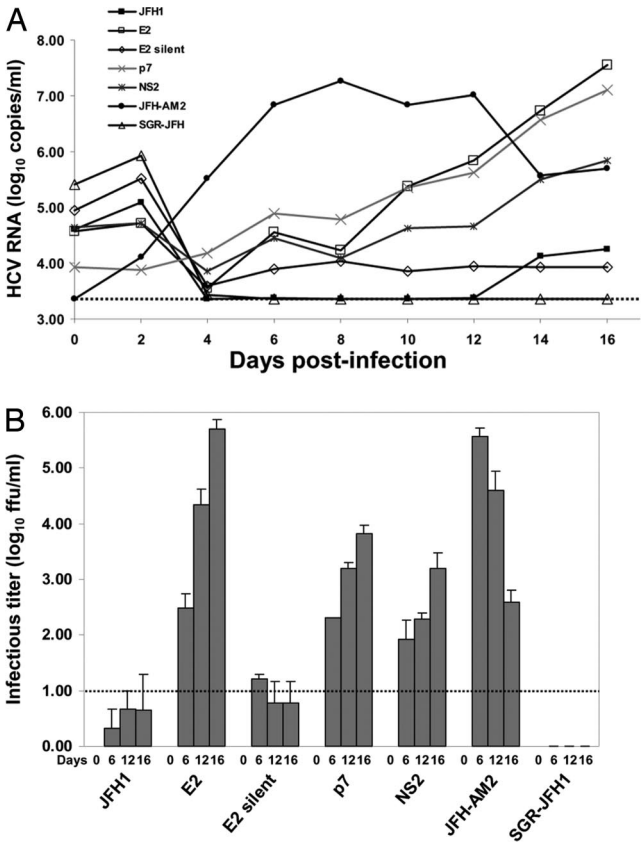

Fig. 2. Effects of individual mutations on virus replication capacity. One million Huh-7.5 cells were inoculated with RNase-treated transfection supernatants containing 500 ffu (m.o.i. = 0.0005) of indicated HCVcc. (*A*) HCV RNA levels in culture fluids at indicated time points postinfection were measured by TaqMan Real Time RT-PCR. (*B*) Infectious viral titers were measured at indicated time points postinfection by a limiting dilution assay for ffu. See Fig. 1 legend for details.

mutations had been selected. Because these data suggested that the NS5A mutation was not important for increased virus yield, it was omitted from subsequent experiments.

Effect of Individual Mutations During Prolonged Culture. Recombinant genomes containing only one of the four selected mutations were constructed to test individual effects. At day 5 posttransfection, virus productions by wild type and the E2 mutant with the synonymous mutation were approximately equivalent, whereas that of each of the other three mutants was at least 1 log higher (data not shown). For precise comparison, equal amounts of infectious virus harvested at day 4 posttransfection were inoculated onto naïve Huh-7.5 cells (m.o.i. = 0.0005), and virus release was monitored. RNA analyses of culture fluids indicated that input RNA (SGR negative control) was no longer detected by day 4 (Fig. 2*A*). Wild-type JFH1 RNA levels remained constant until day 14, then increased. In contrast, JFH-AM2 RNA levels immediately and rapidly increased until peaking at $\approx 10^7$ copies/ml by day 8. Although all of the viruses with a single coding mutation lagged behind JFH-AM2, they all produced RNA levels significantly higher than that of JFH1, suggesting that each coding mutation could increase virus production: both the E2 and p7 mutants produced the same peak titer of RNA as the JFH-AM2 mutant, but it took much longer.

The wild-type and E2 synonymous mutant again produced only low levels of infectious progeny (Fig. 2*B*). As with the RNA levels, the E2 nonsynonymous mutant produced the same high

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levels of infectious virus as JFH-AM2 but again with delayed kinetics: however, the p7 mutant was less effective in producing infectious virus, although it and the NS2 mutant each produced >2 logs more infectious virus than JFH1, confirming that these two mutations were also adaptive. Similar patterns of virus replication kinetics were consistently observed in at least two independent experiments. These results seemed to demonstrate that the E2 mutation was the most critical for efficient virus production, and that the NS2 and p7 mutations were approximately equivalent. However, the relative differences in level of virus production changed with time, suggesting that virus spread and/or newly acquired mutations were a confounding factor. Indeed, we found that the virus at day 16 in the culture fluids of the E2 and p7 mutants retained the original mutations but also had acquired additional consensus sequence amino acid changes (P251L in E1 and T2438S in NS5A for the E2 mutant; V1132A in NS3 and Y2158H in NS5A for the p7 mutant). It is possible one or more of these affected the replication kinetics with the degree of impact coupled to the time of acquisition and, therefore, unpredictable (Fig. 2*A*). Nevertheless, these results indicated that a mutant virus with improved but suboptimal replication capacity may continue to adapt in culture, and very quickly, making it difficult to ascertain the contribution of a specific adaptive mutation if multiple infectious cycles are permitted.

Development of a Single Cycle Assay. To determine whether a mutation promoted virion entry or release, it was useful to separate these steps. Therefore, a cell line was sought that was resistant to infection but released infectious HCV after transfection. A panel of Huh-7 subclones that had been randomly generated by limiting dilution cloning for use in other studies (S.U.E., unpublished data) was screened for these characteristics, and the S29 cells were found to meet these criteria. These cells were at least 1,000-fold less susceptible than Huh-7.5 cells to infection by exogenous JFH-AM2 virus (data not shown). Only rarely was an infected cell detected. Because CD81 is the major receptor protein for HCV (17–19), we determined whether it was present on S29 cells. Immunofluorescent (IF) staining for CD81 did not detect CD81 on S29 cells, whereas it was easily detected on Huh-7.5 cells (data not shown).

The resistance of the majority of S29 cells to infection was confirmed by cocultivation of JFH-AM2-infected Huh-7.5 cells with either uninfected Huh-7.5 or uninfected S29 cells at a ratio of 1 infected per 100 uninfected cells. IF staining for HCV core antigen demonstrated that, although virus rapidly spread through the entire culture of Huh-7.5 cells, it was unable to infect the S29 cells [supporting information (SI) Fig. 6]. In a repeat experiment, cocultured cells were dually stained for HCV antigens and CD81; after 3 days of coculture, the largest focus in the S29 target cell population contained only four HCV-positive cells, which probably reflected division of a single preinfected Huh-7.5 cell, because all also were CD81-positive. In contrast, the Huh-7.5 target cell population displayed multiple foci containing as many as 50 infected cells each. In both cultures, virus was found only in cells that stained positive for CD81 (data not shown). This result suggested that infectability of S29 cells might be restored if CD81 were provided. Therefore, S29 cells transfected with a CD81 expression vector or an irrelevant vector were inoculated 1 day posttransfection with JFH-AM2 virus at an m.o.i. of 2.5 and doubly stained 2 days later for CD81 and HCV core protein. CD81 staining of control S29 cells was not observed. However, in the culture transfected with the CD81 vector, every cell that was successfully transfected with CD81 was also stained for HCV antigen (Fig. 3). Therefore, the CD81 deficiency alone was responsible for the resistance to infection, and all other factors, including receptor cofactors, required for progression through the entire HCV replication cycle were present. Additionally, these data suggested that HCV could not pass directly from one cell to another by a receptor-independent mechanism.

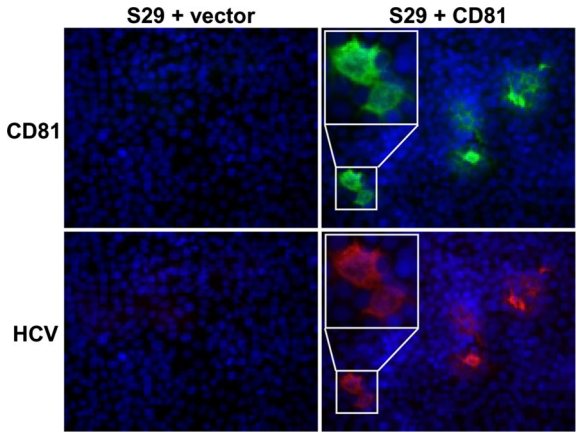

Fig. 3. Characterization of a cell line that can support virus replication but cannot be infected. Two hundred thousand S29 cells were transfected with either irrelevant vector (*Left*) or human CD81 (*Right*) in two-chamber culture slides and inoculated with 500,000 ffu (m.o.i. = 2.5) of JFH-AM2 24 h later. Two days postinoculation, cells were costained by direct IF for CD81 by using a FITC-conjugated murine monoclonal Ab against CD81, and indirect IF for HCV proteins using serum from an HCV-infected chimpanzee in combination with anti-human AlexaFluor 568. Green fluorescence represents CD81, red fluorescence represents HCV-infected cells, and DAPI-stained nuclei are shown in blue as observed using the ×40 objective. White boxes outline two cells that have been enlarged to better display the dual staining. Results are representative of two independent experiments.

Single-Cycle Virus Production. S29 cells were transfected with the panel of HCV genomes, and the yield of infectious virus was determined by assays of ffu in Huh-7.5 cells. Transfection efficiencies were comparable, because real-time RT-PCR assays on 0.6 ng of total cellular RNA indicated that intracellular viral RNA levels at 6 h posttransfection differed no more than 3-fold (data not shown), and transfected cultures contained similar numbers of core-positive cells at day 6 (Fig. 4*A*). The results were strikingly different from those obtained by transfecting Huh-7.5 cells with the same plasmid preparations. Measurement on day 2 of total intracellular and extracellular infectious virus (Fig. 4*B*) indicated that the E2 coding mutant no longer produced the greatest amounts of virus but rather was similar to the wild-type and E2 synonymous viruses. In agreement with the Huh-7.5 results, both the p7 and NS2 mutants were virtually identical and produced ≈1 log more infectious virus than wild type in each compartment (Fig. 4*C*). The transfection was repeated, and this time, virus at day 3 was quantified; again, both p7 and NS2 mutants released more infectious virus into the medium than did the E2 mutants (data not shown).

Because the E2 adaptive mutation did not increase virus production from S29 cells, the effect of this mutation on virus entry was tested in a pseudoparticle system (HCVpp) that incorporated JFH1 E1E2. Unexpectedly, the JFH1 HCVpp containing the E2 adaptive mutation were ≈90% less infectious than HCVpp containing wild-type E1E2 (data not shown). This result could reflect differences between the structure and/or conformation of the glycoproteins found on 293T-derived HCVpp vs. Huh-7.5-derived HCVcc, but that remains to be determined.

Finally, S29 cells were transfected with the various viruses, and the levels of infectious virus produced per day were monitored (Fig. 5). One day posttransfection, wild-type, the E2 silent mutant, and the E2 mutant each either produced no or very little

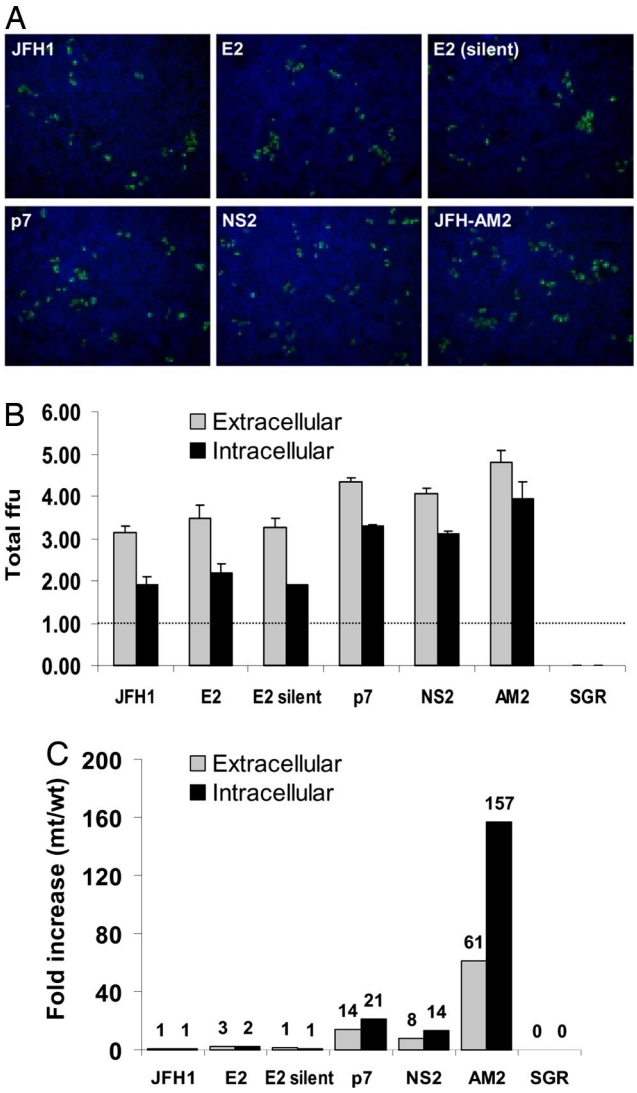

Fig. 4. Adaptive mutations enhance the accumulation of infectious virus. One million CD81-deficient S29 cells were transfected with 4 μl of a 20-μl DNase-treated T7 transcription reaction containing indicated wild-type JFH1, JFH1 plus culture-selected mutations, or SGR-JFH1 RNA. (*A*) Transfected HCV core-positive cells were visualized by indirect IF with murine monoclonal anti-core followed by anti-mouse AlexaFluor 488. Green fluorescence represents HCV core and blue represents DAPI-stained nuclei as observed using the 10X objective. (*B*) Total extracellular and intracellular infectious virus accumulated by day 2 was measured by assays for ffu in triplicate, and means plus standard errors are plotted. The dotted line represents the cutoff of the assay, which was 10 ffu/ml. (*C*) Levels of extracellular and intracellular infectious virus present in the cultures of JFH1 mutant viruses (mt) were compared with that of wild-type JFH1 (wt) and the ratios were plotted. Fold increases (mt/wt) are indicated above the respective bars. Results are representative of two independent transfection experiments.

detectable infectious virus. In contrast, the p7 and NS2 mutants both produced significant amounts during the first day (almost 3 logs). Interestingly, aside from JFH-AM2, all viruses reached a similar maximal level of daily virus production: JFH-AM2, containing all five mutations, plateaued at $\approx$1 log higher than all other mutants. Unfortunately, the large amount of residual transfection-related RNA precluded determinations of specific infectivities. However, in future studies, it should be possible to scale up the transfections and determine the specific infectivity of purified virions.

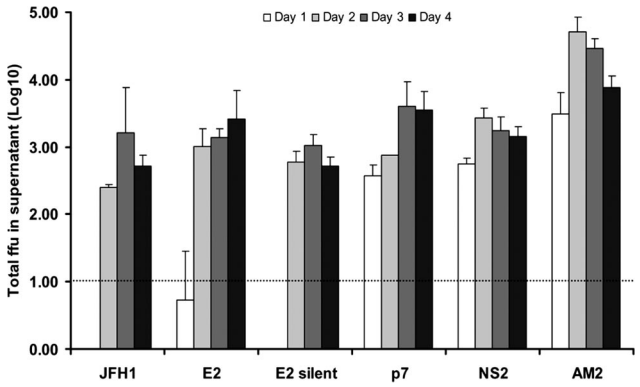

Fig. 5. Adaptive mutations increase the efficiency of infectious virus production. One million CD81-deficient S29 cells were transfected as described in Fig. 4, and total extracellular infectious virus produced each day was measured by assays for ffu in triplicate and means plus standard errors are plotted. The bar representing day 2 for the p7 mutant appears not to have an error bar, because the standard error of the three measurements taken for this sample was zero. The dotted line represents the cutoff of the assay, which was 10 ffu/ml. Results are representative of three independent experiments.

Discussion

Cell culture-derived adaptive mutations can greatly improve the *in vitro* replication capacity of the JFH1 strain of HCV (6, 9–13). However, the mechanism of these adaptive mutations and the steps at which they exert their effects are difficult to ascertain with highly permissive cell lines such as Huh-7.5 cells that support multiple rounds of the complete viral life cycle. Not only can different rates of entry, replication, or release affect the kinetics of virus production in available *in vitro* systems, but multiple infection cycles or generation of a virus stock with sufficient titer to permit a high m.o.i. infection can select mutations in addition to those being studied. For example, the majority of the p7 and E2 mutant viruses shown in Fig. 2 had already acquired two new consensus mutations each after only 20 days (4 posttransfection and 16 postinfection days) in culture. Therefore, we used a cell line (S29) that produced infectious HCVcc in the absence of cell-to-cell spread because of an almost complete absence of CD81, thereby providing a single-cycle virus production assay. By comparing results obtained using this subclone of Huh-7 cells with those obtained with permissive subclones such as 7.5, it is possible to separate entry- from production-enhancing mutations. This system represents a specific test for distinguishing the effects of such mutations. In agreement with previous studies (6, 9–13), this report describes a set of amino acid changes within E2, p7, and NS2 that individually enhanced virus production from JFH1-transfected and -infected cells. In Huh-7.5 cells, the E2 adaptive mutation by itself increased infectious titers to a range of 10^5-10^6 ffu/ml. However, in the S29-based single-cycle virus production assay, adaptive mutations in p7 and NS2 significantly increased the rate of virion production, whereas the E2 mutation had a minimal effect. The most logical explanation for the different effects in the two cell lines is that the E2 mutation enhanced virus entry, thus leading to rapid amplification via efficient spreading in a fully permissive culture, whereas the p7 and NS2 mutations promoted assembly and/or egress, thus resulting in a more rapid accumulation of extracellular virus. That at similar RNA concentrations the E2 mutant produced more foci than the p7 mutant (Fig. 2) supports the contention the E2 mutant was able to infect cells more efficiently, and that the p7 and NS2 mutants produced more intracellular infectious virus (as well as extracellular) than did wild type (Fig. 4) is consistent with these mutations playing a role in steps leading to or involved in virion assembly. However, the "all or none" kinetics of virus release observed upon

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daily sampling (Fig. 5) were unexpected, and it is not possible to explain them at this time.

At present, we can only speculate on how the N to S E2 mutation might exert such a positive effect. The mutated Asn at position 417 is highly conserved, with 98% (198/202) of the E2 sequences listed in the Los Alamos HCV Sequence Database containing N, and none containing S. This Asn residue is a known glycosylation site, and mutation at this position in a genotype 1a pseudoparticle system resulted in >50% lower HCVpp infectivity (20, 21), which is consistent with the 90% decrease we observed in the HCVpp system. Until more is known about glycoprotein assembly in the context of authentic HCVcc, it will be difficult to explain exactly how these mutations affect virus production or entry, but two recent reports confirmed that mutation of N417 in the context of HCVpp caused impairment in entry (20, 22). In contrast, the observation with HCVcc that preventing addition of glycan at this position increased infectivity (Fig. 2) indicates there may be discrepancies with respect to the structure of the glycoproteins contained on pseudotyped viruses vs. authentic HCV particles. In this regard, the loss of a glycan causing a more favorable interaction with an HCV receptor has recently been proposed to explain the N543K JFH1 adaptive mutation in E2 (9).

The p7 protein contains only 63 amino acids, and mutations can have profound effects on the virus. It is essential for infectivity in chimpanzees: two basic residues in the cytoplasmic loop are necessary for infectivity *in vivo* (23). Basic residues in this loop are also critical for virus production *in vitro* (14); it is proposed that it oligomerizes to form ion channels (24, 25), but its exact function is unknown. The mutated Asn at residue 765 is unique to JFH1, and the Asp that it mutated to was found in only one other sequence (genotype 1b virus). All other genotype 2 sequences have a Ser at this position. Yet the Asn to Asp mutation clearly increased the levels of infectious virus in culture fluids of both Huh-7.5 and S29 cells (Figs. 2 and 4): this same mutation was recently isolated in another laboratory, suggesting that it plays a precise and critical role in adaptation (10).

Most studies have concluded that p7 functions at the stage of virion assembly: Yi *et al.* (12) propose that it protects the infectivity of newly assembled virions during release, whereas Jones *et al.* (14) concluded that p7 functions at an early stage of virion morphogenesis. A recent mutatgenesis study demonstrated that p7 is essential for assembly and release of HCVcc (16). Our data provide further support for the prevalent conclusion that p7 plays a central role in virus assembly and/or release. Based on the observation that the levels of intracellular, in addition to extracellular, infectious virus increased >10-fold in the presence of the p7 mutation, we propose that the mutation did not simply accelerate virus release, which would deplete the intracellular pool, but rather increased the absolute number of assembled virions within the cell.

The NS2 mutant behaved much like the p7 mutant, even though as a membrane-associated cysteine protease, it appears to be a very different protein. Its exact function is as unclear as that of p7 (26, 27). However, like p7, it is thought to play a role in production of infectious virus (12, 28) and to be important at an early stage of virion morphogenesis (14). Again, our data, especially those demonstrating the increased level of intracellular infectious virus, support these conclusions. At the next level, it should be very informative to test different combinations of mutations in the single-cycle system to determine whether there is a synergistic or additive effect or inhibition.

That single-site mutants reached a similar plateau of virus production, and that the best combination was only 10-fold higher suggests there are real constraints on virus replication and assembly *in vitro*. From the practical standpoint, it will be interesting to see whether virus production *in vitro* can be pushed to levels high enough to make vaccine candidates feasible. From the biological standpoint, it will be very interesting to determine the factors that restrict virus multiplication *in vitro* since it can replicate so efficiently *in vivo*. Currently, we and others have identified a number of adaptive mutations in JFH1 and related intergenotypic chimeras (6, 9–13). Such viruses with the ability to replicate to high levels will be useful for both basic virological and immunological studies and can be used to test putative HCV antiviral agents. The establishment of the S29-based single cycle virus production assay provides a further advance toward determination of the degree and stage of the viral life cycle these mutations are affecting. Further elucidation of the mechanisms used by these mutations will undoubtedly provide a better understanding of the various complex interactions required for the HCV life cycle. For example, biologically relevant adaptive mutations have now been found in all of the HCV structural genes, and it will be interesting to determine how these modified proteins work together to assemble an infectious virus particle. In this regard, the S29-based single cycle virus production assay provides an additional tool for the study of HCV particle assembly and release in general and the involvement of cellular factors in these processes. For example, a paper was recently published suggesting HCV can spread from cell-to-cell by a receptor-independent mechanism (29): the results from experiments performed in the CD81-deficient S29 cells (Fig. 3 and SI Fig. 6) are in conflict with this conclusion, because they suggested that HCV absolutely requires CD81 for cell-to-cell spread. Additional experiments will be needed to resolve this question further.

Methods

For detailed methods, see *SI Text*.

Cells. HCVcc transfections and infections were performed in Huh-7.5 human hepatoma cells (30) (gift from C. Rice) or S29 cells, a subclone of Huh-7 cells (31) generated in-house by limiting dilution cloning (S.U.E., unpublished data).

Plasmid Constructs. The JFH1 and SGR-JFH1 plasmids were gifts from Takaji Wakita, and the human CD81 expression vector was a gift from T. Jake Liang. Custom plasmids incorporating cell culture-selected mutations were synthesized (GeneScript and Gene Oracle) and used to generate recombinant constructs containing selected mutations.

RNA Transfection. One microgram of XbaI-linearized JFH1 plasmids was transcribed *in vitro* with the T7-MEGAscript kit (Ambion) and transfected with either Lipofectamine 2000 (Invitrogen) or DMRIE-C (Invitrogen).

TaqMan Real-Time RT-PCR Quantitative Assay. Quantitative RT-PCR was performed as described (32).

Virus Sequencing. Viral RNA was extracted from 100 μl of filtered transfection and infection culture media with TRIzol (Invitrogen), and long RT-PCR was performed as described (8, 11).

Immunofluorescence Microscopy. HCV-positive cells were detected with mouse monoclonal antibody recognizing core protein (Anogen) and visualized with Alexa Fluor 488 anti-mouse secondary antibody (Invitrogen) or immune serum from an HCV-infected chimpanzee, followed by anti-human AlexaFluor 568 (Fig. 3). Human CD81 was visualized directly by using a FITC-conjugated mouse monoclonal Ab against human CD81 (BD PharMingen).

Virus Titration. Virus titers were determined by endpoint dilution assays of ffu, as described (5).

Infection by JFH1 Viruses. One million preadhered Huh-7.5 cells were inoculated with HCVcc-containing filtered supernatants diluted in complete medium to give desired m.o.i. For all mutant and wild-type virus cultures, cells were split at confluency every 3–4 days with all media replaced at each passage. Culture fluids were collected for virus quantification every 2–3 days on the same day for each culture in a given experiment.

Titration of Intracellular Virus. Levels of intracellular infectious virus were measured as described (33).

CD81 Transfection. S29 cells were transfected in two-chamber culture slides with 0.8 μg of either human CD81 expression vector (or an irrelevant vector) using Lipofectamine Plus reagents (Invitrogen).

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