Response of Hepatitis C Virus to Long-Term Passage in the Presence of Alpha Interferon: Multiple Mutations and a Common Phenotype

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Cell culture-produced hepatitis C virus (HCV) has been subjected to up to 100 serial passages in human hepatoma cells in the absence or presence of different doses of alpha interferon (IFN-\(\alpha\)). Virus survival, genetic changes, fitness levels, and phenotypic traits have been examined. While high initial IFN-\(\alpha\) doses (increasing from 1 to 4 IU/ml) did not allow HCV survival beyond passage 40, a gradual exposure (from 0.25 to 10 IU/ml) allowed the virus to survive for at least 100 passages. The virus passed in the presence of IFN-\(\alpha\) acquired IFN-\(\alpha\)-resistance as evidenced by enhanced progeny production and viral protein expression in an IFN-\(\alpha\) environment. A partial IFN-\(\alpha\)-resistance was also noted in populations passed in the absence of IFN-\(\alpha\). All lineages acquired adaptive mutations, and multiple, nonsynonymous mutations scattered throughout the genome were present in IFN-\(\alpha\)-selected populations.

Highly variable viruses exploit a number of strategies to counter selective constraints intended to prevent their replication (reviewed in reference 7). Mutations that confer resistance to DAAs generally map in the target protein or in a protein that interacts with the target (28–30). However, the complexity of the cellular IFN response pathway is expected to require greater diversity of viral resistance mutations. Current evidence suggests that the response of HCV to IFN-\(\alpha\)-based therapy is influenced by several host (i.e., interleukin-28B [IL-28B] gene polymorphisms) and viral genetic (i.e., viral genotype and population complexity) factors (31–37).

The advent of cell culture systems for sustained replication of HCV offers new prospects to approach the evolutionary dynamics of HCV and the host cell-virus relationship, including the anti-HCV activity of IFN-\(\alpha\). Using these systems, Garaigorta and Chisari documented that HCV-induced protein kinase R (PKR) phosphorylation inhibited translation of IFN-stimulated genes (ISGs) in infected hepatocytes (38). Here we describe the adaptation of the HCV replication system of genotype 2a (39–41) to perform serial passages of HCV in the Huh-7.5 hepatocyte cell line with sustained, efficient viral replication for at least 100 serial passages. This cell culture system allows for investigations on population dynamics similar to those carried out with different RNA
viruses (7). We have subjected HCV to 100 serial passages in the absence or presence of increasing doses of IFN-α. The IFN-α-selected populations displayed increased fitness in the presence of IFN-α. Consensus genomic nucleotide sequences of the viral populations after 30, 45, and 100 passages revealed multiple mutations throughout the HCV genome associated with IFN-α resistance in addition to several cell-culture adaptive mutations. Virus passed in the absence or presence of IFN-α displayed partial resistance to IFN-α plus ribavirin and a gradual increase in shutoff host cell protein synthesis. This trait was accentuated in the IFN-α-resistant populations replicating in the presence of IFN-α.

**MATERIALS AND METHODS**

**Cells, viruses, and drugs.** The origin of Huh-7.5, Huh-7 Lunet, and Huh-7.5 reporter cell lines and procedures for cell growth in Dulbecco’s modification of Eagle’s medium (DMEM) have been previously described (42–45). Infected and uninfected cultures were cultured at 37°C and 5% CO2.

The HCV cell culture (HCVcc) derivatives used in the experiments, ψC1FLAG2/p7-nsGlu2CA and its polymerase-defective negative control (GNN), have been previously described (46). To control for the absence of contamination, the supernatants of mock-infected cells maintained in parallel with the infected cultures were titrated; no infectivity in the mock-infected cultures was detected in any of the experiments.

A stock solution of alpha interferon 2b (IFN-α) (GenScript) was prepared in DMEM at a concentration of 104 IU/ml and stored at −70°C. Prior to use, the IFN-α was analyzed electrochemically and tested in a vesicular stomatitis virus infectivity reduction assay. The 50% inhibitory concentration (IC50) of IFN-α that results in cytotoxicity for 50% of the cells was calculated from four different determinations as described previously (49), and it was higher than 105 IU/ml for Huh-7.5 reporter cells.

Production of master infectious virus stock HCV p0 and specific mutants. Huh-7 Lunet cells were electroporated with 10 μg of parental HCVcc or polymerase-defective negative-control (GNN) RNA transcripts (Gene Pulser Xcell electroporation system; Bio-Rad) (260 V, 950 μF) and then subcultured every 3 to 4 days before cells reached confluence for up to 30 days postelectroporation (47). A viral stock was prepared by pooling cell culture supernatants from day 17 to day 24 postelectroporation. The viral stock was then concentrated 20-fold using 10,000 molecular weight cutoff (MWCO) spin columns (Millipore) as indicated by the manufacturer and stored in aliquots at −70°C. To increase virus infectivity, Huh-7.5 reporter cells were infected with concentrated virus stocks (multiplicity of infection [MOI]) of approximately 0.5 tissue culture infectious doses [TCID50]/cell, and 3 days later the cells were subcultured and maintained a further 3 days. After a second infection and two subsequent cell passages, aliquots of the cell culture supernatants were stored and used in all future experiments as the master HCV viral stock, HCV p0.

The cDNA that expressed HCV p0 was also used as the genomic backbone for the construction of three mutant infectious clones, T17A (in E1), S18G (in E2), and T28P (in NS2), by overlap extension PCR. Two overlapping PCR fragments were assembled each for E1-T17A (primers EcoRI-F plus BCH-O-7 and primers Jc1-E1-R1 plus BCH-O-8), for E2-S18G (primers Jc1-E1-F1 plus BCH-O-9 and primers NotI-R plus BCH-O-10), and for NS2-T28P (primers Jc1-LUC-F1 plus BCH-O-13 and primers Jc1-NS3-R2 plus BCH-O-14) (see Table S1 at http://www.cbmb.ums.es:8080/cv-303/SupplMatPerales.pdf). The fusion PCR products were then TA cloned into pT2ZfRT/v vector (Fermentas) and completely sequenced to confirm that no unwanted mutations had been introduced. Correct TA clones for the three mutants were digested using EcoRI and BsiWI, BsiWI and BglII, and BglII and SpeI, respectively, and mutant genome fragments were subsequently ligated with the similarly digested parental HCV CDNA clone to construct a full-length infectious HCV CDNA clone of each mutant. Infectious RNA transcripts were produced and used to electroporate Huh-7 Lunet cells as described for production of HCV p0.

**Virus titration.** Virus titers were determined by calculating the TCID50/ml (48). Briefly, cell culture supernatants were serially diluted and used to infect Huh-7.5 cells that had been seeded in 96-well plates at 6,400 cells/well 16 h earlier. Three days postinfection, cells were washed with PBS, fixed with ice-cold methanol, and stained for the presence of N55A using an anti-N55A monoclonal antibody, 9E10, as described previously (39).

**IFN-α toxicity and inhibitory activity.** The toxicity of IFN-α was measured in Huh-7.5 reporter cells by seeding 96-well plates at 70% confluence and exposing the cells to up to 104 IU/ml IFN-α for 72 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well at a final concentration of 500 μg/ml; the cells were incubated 4 h, 100 μl of dimethyl sulfoxide (DMSO) was added, and the optical density was measured at a wavelength of 550 nm. The IC50 (the concentration of IFN-α that results in cytotoxicity for 50% of the cells) was calculated from four different determinations as described previously (49), and it was higher than 105 IU/ml for Huh-7.5 reporter cells.

The 50% inhibitory concentration (IC50) of IFN-α with respect to HCV infectivity was determined by seeding Huh-7.5 reporter cells in 24-well plates at 70% confluence and exposing the cells to serial dilutions of IFN-α (0.1 to 100 IU/ml) for 72 h. IC50 was measured using the virus titration as described above and calculated relative to the untreated control levels (defined as 100%).

**Cell culture system for long-term serial passage of HCV in the absence or presence of IFN-α.** To initiate serial passages of HCV, 4 × 105 Huh-7.5 reporter cells per well of a 6-well tissue culture plate were infected with HCV p0 (MOI = −0.5 TCID50/cell) for 5 h at 37°C followed by removal of inoculum and addition of 2 ml of culture medium. Appropriate quantities of IFN-α were added to the infected cells that were further incubated at 37°C for 72 to 96 h. For each subsequent passage, 4 × 103 Huh-7.5 reporter cells were infected as described above using 0.5 ml of cell culture supernatant from the previous passage. The MOI for each infection was calculated from the infectivity values given for each experiment described in Results, and it ranged between 0.1 and 0.5 TCID50/cell. Passage number is here abbreviated with p followed by the number (e.g., p10 represents passage 10; viral populations are referred to with a passage number followed by “IFN” and the number of IU/ml in the cell culture supernatant in the case of populations passages in the presence of IFN-α). Mock-infected cells and cells infected with HCV p0 without IFN-α treatment were maintained for 100 passages in parallel. No evidence of contamination of mock-infected cells with virus was observed at any time.

Due to increasing cytotoxicity associated with elevated levels of infectious virus in cultures not treated with IFN-α, the infection volume was decreased from 500 μl to 20 μl after passage 59, as indicated in Results.

**RNA extraction, cDNA synthesis, PCR amplification, and nucleotide sequencing.** Intracellular viral RNA was extracted from infected cells after each passage using a Qiagen RNaseasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription was performed using AMV reverse transcriptase (Promega), and PCR amplification of the entire HCV genome was carried out using AccuScript (Agilent Technologies) with specific oligonucleotides (Table S1 at [http://www.cbmb.ums.es:8080/cv-303/SupplMatPerales.pdf]). Amplification products were analyzed by agarose gel electrophoresis using the fragments produced by digestion of Φ-29 DNA with HindIII as molecular mass standards. Negative controls without template RNA were included in parallel to confirm the absence of cross-contamination by template nucleic acids. Nucleotide sequences of genomic HCV RNA were determined on the two strands of an amplified cDNA copy as previously described (50). Numbering of HCV genomic residues is according to JFH-1 accession number AB047639.

**Quantification of HCV RNA using qRT-PCR.** Quantitative real-time PCR (qRT-PCR) of HCV RNA was carried out in triplicate using a Light Cycler RNA Master SYBR green I kit (Roche), as described previously.
RESULTS

Active HCV replication in cell culture and response to IFN-α.

Quasispecies dynamics dictates that large population sizes and active viral replication ensure a dynamic mutant repertoire that may favor the presence of viral mutants with increased fitness in the presence of a selective agent (7). To achieve a robust HCV replication, an initial viral stock (p0) was prepared from the viral progeny obtained between 17 and 24 days postelectroporation of Huh-7 Lunet cells with HCVcc RNA; viral production was shown to steadily increase about 10-fold from day 2 through day 30 post-electroporation (Fig. S1A [http://www.cbm.ual.es:8080/cv-303/SupplMatPerales.pdf]). During each passage of the electroporated cells, the supernatant was collected and fresh medium added to the cell monolayer. Active HCV replication was confirmed by staining of cells with a NS5A-specific monoclonal antibody (Fig. S1B [see URL mentioned above]) and by reactivity of core- and NS5A-specific monoclonal antibodies in Western blot assays using an extract of infected cells (Fig. S1C [see URL mentioned above]). Pooled, concentrated cell supernatants yielded viral preparations p0 and GNN-p0 (negative control). The IC50 of IFN-α under our infection conditions for p0 was 0.43 ± 0.33 IU/ml. Its CC50 was >105 IU/ml. Thus, the selective (or therapeutic) index (CC50/IC50) was >2 × 104. To further measure the sensitivity of p0 to IFN-α treatment, the virus was serially passed in the presence of 1, 2, 5, and 10 IU/ml of IFN-α (Fig. S2A and B [see URL mentioned above]). HCV replication was sustained for at least 10 passages in the absence of IFN-α, with a dose-dependent decrease in the presence of IFN-α. Infectivity and viral RNA progeny production decreased modestly in the presence of 1 IU/ml of IFN-α, while a sharp decrease occurred in the presence of 2, 5, and 10 IU/ml IFN-α, rendering infectivity and HCV RNA undetectable by passage 4 to 6 (Fig. S2C [see URL mentioned above]). Thus, the system is adequate to provide a large viral load to study HCV adaptive mechanisms and the response to IFN-α.

Selection of IFN-α-resistant HCV populations during serial passages in the presence of increasing concentrations of IFN-α.

To obtain HCV populations resistant to IFN-α, HCV p0 viral progeny was passaged in either the absence or the presence of different concentrations of IFN-α. Because drug-resistant viral mutants are often more easily obtained by replicating a virus in the presence of gradually increasing drug concentrations (53, 54), we chose to establish three parallel HCV lineages, each involving serial infections under conditions of increasing IFN-α concentrations. For simplicity, we distinguish the three lineages with the terms “low” (initial IFN-α concentration of 0.25 IU/ml in the culture medium), “medium” (initially 0.5 IU/ml), and “high” (initially 1 IU/ml), with doubling of their corresponding prior IFN-α concentrations at passages 10 and 30 (Fig. 1). The “low” lineage was further passaged in the presence of IFN-α, with the concentration increased by 1 IU/ml after passages 45, 55, 60, 65, 70, 80, 85, 90, and 95, reaching a final IFN-α concentration of 10 IU/ml (Fig. 1A). In addition, two control lineages were estab-
lished: HCV p0 passaged in the absence of IFN-α and GNN supernatant (replication-defective control) passaged in parallel as a negative control. Virus infectivity and viral RNA were quantified for each passage, with striking differences seen between the parallel lineages (Fig. 1B and C). During the first 10 passages, the three viral lineages behaved similarly and, despite some minor fluctuations in infectious titer, each lineage approached the progeny production of HCV p10 (first vertical discontinuous line at p10 in Fig. 1B and C). After the IFN-α concentration was doubled at p11, a decline in progeny production followed in a dose-dependent manner. Interestingly, the “low” and “medium” lineages that were increased to 0.5 and 1 IU/ml IFN-α, respectively, at p11 showed a decrease in viral production that was more accentuated than when p0 was subjected to those same IFN-α concentrations (compare p1 to p10 with p11 to p20 in Fig. 1B and C). Thus, a history of HCV replication in the presence of IFN-α enhanced the sensitivity of the virus to IFN-α.

The “medium” and “high” lineages showed decreased viral production between p18 and p26, with some values below the detection limit, prior to a sharp recovery of virus production in both lineages by p30 (Fig. 1B and C). Thus, a severe population bottleneck occurred in the “medium” and “high” lineages but not in the “low” lineage. As a consequence of raising the IFN-α concentration to 4 IU/ml beginning at p31, the “high” lineage had infectivity and viral RNA levels below the limit of detection by p40 (Fig. 1B and C). HCV acquired resistance to IFN-α, as suggested by the high level of progeny production attained by the “low” lineage in the presence of 10 IU/ml IFN-α at p95 to p100. This progeny level was comparable to that of HCV p100 in the absence of IFN-α (Fig. 1B and C). In contrast, progeny production of the
“high” lineage became undetectable after only a few passages in the presence of 4 IU/ml of IFN-α (Fig. 1B and C). These results suggest a difference in fitness cost of short-term (few passages) versus long-term (many passages) adaptation of HCV to IFN-α.

**IFN-α resistance of passaged HCV populations.** To obtain additional evidence that HCV populations passaged in the presence of IFN-α acquired an IFN-α-resistant phenotype, populations from p30, p45, and p100 were subjected to 10 further passages in the absence or presence of IFN-α and their behavior was compared with that of the initial virus p0. The amounts of infectivity and viral RNA indicate higher HCV progeny production in the presence of IFN-α of those populations that had a passage history in the presence of IFN-α compared with the population with a passage history in the absence of IFN-α (Fig. 2A). Differences in viral protein expression were confirmed by Western blot analysis of HCV-specific proteins in intracellular extracts from cells infected with viruses from passages 30, 45, and 100 (Fig. 2B).

Growth competition assays further confirmed IFN-α resistance phenotypes in the HCV passaged in the presence of IFN-α relative to the virus passaged in the absence of IFN-α (Fig. 2C and Table S2 [http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf]). A history of replication in the presence of 2 IU/ml of IFN-α did not confer higher resistance than a history of replication in the presence of 1 IU/ml (compare the p30 IFN1/p30 and p45 IFN2/p45 panels in Fig. 2C). Also, p30 IFN2 had a lower fitness relative to p30 in the absence or presence of 2 IU/ml of IFN-α (Fig. 2C and Table S2 [see URL mentioned above]). The fitness decrease might have contributed to loss of infectivity of the “high” lineage before p45 (compare with Fig. 1B and C). Unexpectedly, HCV p30, p45, and p100, passed in the absence of IFN-α, displayed a clear resistance to IFN-α compared with p0 (Fig. 2A). Several mechanisms may account for partial IFN-α resistance in HCV populations not exposed previously to exogenous IFN-α, and this point is under investigation (see Discussion). Thus, passage of HCV in Huh-7.5 cells in the presence of increasing concentrations of IFN-α results in selection of HCV populations that display an increased capacity to replicate in the presence of IFN-α.

**Mutations in HCV populations passaged in the absence or presence of IFN-α.** To evaluate the genetic diversification of the different HCV populations passaged in the absence or presence of IFN-α and to detect possible mutations associated with IFN-α resistance, the consensus nucleotide sequence of the entire genome of HCV populations after 30, 45, and 100 passages in the presence or absence of IFN-α was compared with that of the parental HCV p0 (Tables S3 to S9 [http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf]). A total of 115 different mutations were identified with a dominance of transitions (74%) versus transversions (26%) (55). The ratio of nonsynonymous to synonymous mutations, corrected per nonsynonymous and synonymous sites (dN/dS), gave a range of 0.2187 to 0.4444 (average, 0.3474) for comparisons between populations passaged in the absence of IFN-α and a range of 0.1929 and 1.2500 (average, 0.4489) for comparisons between populations passaged in the presence of IFN-α (dN/dS ratios for each comparison are given in Table S7 [see URL mentioned above]). The mutation frequencies (calculated relative to the genomic sequence of p0) were very similar for the populations passaged in the absence of IFN-α and for those passaged in its presence, with average values of 2.6 × 10⁻³ substitutions per nucleotide (s.n⁻¹) (range of 4.1 × 10⁻³ to 1.5 × 10⁻³ s.n⁻¹) and 2.3 × 10⁻³ s.n⁻¹ (range of 4.7 × 10⁻³ to 1.5 × 10⁻³ s.n⁻¹), respectively. Thus, the levels of diversification of HCV with regard to the consensus genomic sequence were comparable in the presence and absence of IFN-α. The HCV genotype 2a chimera used as the parent in our experiments has a Gauussia luciferase (Gluc) gene inserted between p7 and NS2 (46). By p30, the first 516 nucleotides (nt) of the 576-bp Gluc gene had been spontaneously deleted in all lineages and remained so in all viruses sequenced at p45 and p100.

The amino acid replacements found in the different populations (Fig. 3) could be divided into several classes: (i) replacements acquired by all populations examined, irrespective of IFN-α treatment (i.e., N34D in E2, N17D in p7, and Y618F in NS3); (ii) replacements that were associated with passage with IFN-α treatment and that tended to revert when the selective pressure of IFN-α was removed (i.e., T17A in E1 and S18G in E2 of “low” lineage p30 and p45 and K122Q in NS3 of “high” lineage p30); (iii) substitutions found only in populations passaged in the presence of IFN-α that did not revert when the virus was further passaged in the absence of IFN-α; and (iv) replacements that we could not associate with resistance to IFN-α. Each passage condition yielded a unique set of amino acid substitutions (Fig. 3).

To test whether individual amino acid substitutions could confer IFN-α resistance, we prepared HCVcc encoding amino acid replacements T17A (in E1), S18G (in E2), and T28P (in NS2) selected under conditions of “low,” “medium” and “medium-high” IFN-α concentrations, respectively (Fig. 1). Because these amino acid substitutions may impair fitness and T17A (in E1) and S18G (in E2) reverted upon virus passage in the absence of IFN-α, we prepared minimal virus stocks, ascertainment that they included the mutations, and tested infectious virus progeny production in a single infection at a low MOI. The results (Fig. 4) indicate that while HCVcc did not produce detectable infectious progeny in the presence of IFN-α, the three mutants yielded progeny in the presence of IFN-α. Thus, despite lacking fitness-enhancing genomic mutations, these mutant viruses displayed an IFN-α resistance phenotype.

**Response to a high IFN-α level and to IFN-α plus ribavirin.** To investigate the response of HCV populations passaged 45 times in the absence or presence of IFN-α to higher IFN-α concentrations, viral populations p45, p45 IFN1, and p45 IFN2 were subjected to 10 serial passages in the presence of IFN-α (12 IU/ml). A remarkable resistance of populations passaged 45 times in the presence of IFN-α (2 IU/ml) was observed relative to that of the parental virus p45 (Fig. 5A).

To investigate the response of HCV populations passaged in the absence or presence of IFN-α to a combination of IFN-α and ribavirin, viral populations of passages 45 and 100 were subjected to 10 serial passages in either the absence or the presence of IFN-α. The results show a modest increase of resistance to IFN-α plus ribavirin of p45 IFN1 and p45 IFN2 relative to the parental virus (Fig. 5B) and a remarkable resistance of the population passaged 100 times in the presence of elevated IFN-α (12 IU/ml) relative to p100 (Fig. 5C). Thus, resistance to IFN-α is reflected in a diminished response to the combination of IFN-α plus ribavirin.

**Phenotypic effects of IFN-α selection.** To investigate whether IFN-α resistance could be associated with some common phenotype regarding intracellular protein expression, populations p0, p45, p45 IFN1, p45 IFN2, p100, and p100 IFN10 were used to infect Huh-7.5 reporter cells at an MOI of about 0.03 TCID₅₀/cell,

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and 56 h later cells were treated with 2 IU/ml (for p0 and p45 populations) and 12 IU/ml (for p100 populations) of IFN-α for 16 h. Under these conditions, at the time of labeling at least 90% of the cells are infected. (Preliminary experiments showed that under our experimental conditions, maximal inhibition of host cell protein synthesis was achieved when IFN-α was added at the peak of the infection). The infected and mock-infected cultures were pulse-labeled with [35S]Met-Cys, and proteins were extracted and analyzed electrophoretically and by Western blotting with specific antibodies (Fig. 6). Populations passed in the absence of IFN-α produced a shutoff host cell protein synthesis that increased with the virus passage number. Shutoff was more evident in populations with a history of passage in the presence of IFN-α (Fig. 6A, row − IFN). Shutoff was further enhanced when the infection was carried out in the presence of IFN-α (Fig. 6A, row + IFN). Infection in the presence of IFN-α increased the level of shutoff, and the effect was more prominent with IFN-α-resistant populations at p45. This enhancement was lost with IFN-α-resistant populations at p100, presumably because they had acquired a high shutoff activity without IFN-α (Fig. 6A, row ± IFN). Thus, the IFN-α-selected populations accentuated a phenotypic trait that was partially expressed as a result of passage of the virus in Huh-7.5 cells.

Since host cell protein synthesis can be shut off by phosphorylation of translation initiation factor eIF2α and a candidate to exert such a control is PKR, we tested by Western blotting the phosphorylation state of PKR and its substrate eIF2α under our infection conditions (Fig. 6B). As expected, infections in the presence of IFN-α resulted in enhanced PKR expression (Fig. 6B). An increase of the ratio of the phosphorylated to nonphosphorylated forms of PKR and eIF2α proteins was observed with all viruses in the presence of IFN-α as compared with the same infection in the absence of IFN-α. The increase was slightly higher for the p100 virus with a history of passages in the presence of IFN-α, but it was similar for p45 viruses, despite a pronounced decrease in host cell protein synthesis in the infections with p45 IFN1 and p45 IFN2 viruses (Fig. 6B). Viral protein expression and progeny production were not significantly affected, as expected from the fact that HCV translation is internal ribosome entry site (IRES) dependent and may be less affected by eIF2α phosphorylation (56, 57) (Fig. 6C). As a control, under the same conditions, telaprevir did not increase shutoff host cell protein synthesis in any of the viral populations (Fig. S4 [http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf]), further supporting the idea that the IFN-α environment may be directly involved in the shutoff trait. Under these treatment conditions (leading to a less than 4-fold decrease in viral yield), IFN-α and telaprevir (an inhibitor of HCV NS3) did not cause a significant reduction of infectious virus production. Passage of the virus in the presence of IFN-α may select for viral populations with mutations at several viral genes that display an enhanced capacity to suppress host protein synthesis in the presence of IFN-α.

**DISCUSSION**

Replication of RNA viruses in cell culture has provided an extremely useful tool to study the response of viruses to selective constraints and passage regimens under controlled conditions. No fundamental discrepancies have been observed between the basic adaptive mechanisms displayed by viruses in cell culture and in vivo, a fact which has greatly improved the understanding of viral quasispecies dynamics (reviewed in reference 7). The possibility of completing infectious cycles of HCV in human hepatoma cells (39–41) renders HCV amenable to studies of virus evolution and virus-host interactions in cell culture (58). Here we have adapted the HCVcc–Huh-7.5 virus-cell system to show that a robust, sustained viral replication can be maintained for at least 100 passages. We have explored the response of HCV to IFN-α treatment, which is a major component of the current standard of care treatment for HCV infections. Previous studies that investigated whether HCV mutations were associated with unsuccessful IFN-α therapy have not yielded conclusive results (34, 59–65). In contrast to what has been generally observed with standard antiviral agents that target a defined viral function (53, 54), a history of passage of HCV in the presence of low concentrations of IFN-α did not prepare the virus to overcome higher IFN-α doses, at least not immediately. However, we have observed long-term fitness gains (measured in an IFN-α environment) upon virus replication in the presence of IFN-α (compare Fig. 1 and 2). A difference between IFN-α and DAAs with regard to low drug concentrations in preparing the virus to attain drug resistance may relate to the triggering by IFN-α of multiple pathways leading to an antiviral state in the cells. In the case of Huh-7.5 cells, this antiviral state is likely to involve the JAK/STAT system since Huh-7.5 cells—a clonal derivative of Huh-7 cells that supported effective HCV replication (42)—include a substitution in the first CARD domain of

**FIG 2** Interferon resistance of HCV p0 passaged in the absence or presence of IFN-α, as determined by infectious progeny production in the cell culture supernatant. (A) The upper box indicates the viral populations subjected to analysis. The blue symbols for p30, p45, and p100 represent the populations that resulted from passaging HCV p0 30, 45, and 100 times, respectively, in the absence of IFN-α. Yellow, red, and green symbols represent populations that resulted from passaging HCV p0 30, 45, and 100 times in the presence of the indicated IFN-α concentrations. The number following IFN corresponds to the IFN-α concentration at the indicated passage. Each of the populations was subjected to 10 passages in the absence (left panels) or presence (concentration given in the filled inset in each panel) of IFN-α. No infectious virus was detected for the “high” lineage after passage 40 (see Fig. 1B); thus, only p30 was tested for this lineage. The corresponding intracellular RNA levels are given in Fig. S3 (http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf). (B) At 72 h postinfection (h.p.i.) (one passage), cellular extracts were subjected to SDS-PAGE and analyzed by Western blotting using monoclonal antibodies specific for the NS5A, core, and actin proteins. Procedures are described in Materials and Methods. (C) Relative fitness values of interferon-resistant HCV mutants, in the absence or presence of IFN-α. The mutant populations from “low,” “medium,” and “high” lineages were mixed with the HCV from p30, p45, and p100 (without IFN-α). Growth competition experiments were performed by infecting Huh-7.5 cells with each mixture (MOI = 0.03 TCID₅₀/cell) followed by four serial passages in the absence or presence of IFN-α (2 IU/ml for p30 and p45 virus mixtures; 12 IU/ml for p100 viral mixture). The asterisk indicates that for the competition depicted by the yellow bar, the relative fitness value was calculated based on changes in population ratios determined during a single passage (at 24, 48, or 72 h.p.i.) rather than 4 passages. Viral RNA present in the initial mixtures and at each passage was sequenced, and the proportion of the two competing populations was estimated by comparison of the areas of the relevant nucleotide peaks. Mean fitness values of each IFN-α-resistant population relative to HCV p30, p45, or p100 in the presence or absence of IFN-α are plotted, with error bars indicating the standard deviations. The nucleotide residues that differ between the two competing viruses and whose peak areas were used to calculate the proportion of the two viral populations and the individual fitness values used to calculate the average and standard deviations are given in Table S2 (http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf). Relative fitness values of greater than or less than 1 indicate mutant populations that have increased or decreased fitness, respectively, relative to HCV p30, p45, or p100. Procedures are detailed in Materials and Methods.
RIG-I that prevents the association of RIG-I with mitochondrial anti-viral signaling (MAVS), rendering the RIG-I pathway deficient (66, 67). The IFN-α/H9251-mediated antiviral state may provide an unfavorable environment for the virus that initially cannot find mutations to maintain or increase its fitness. Overt IFN-α resistance was achieved by extensive passage of HCV under conditions of gradually increasing IFN-α concentrations.

Three amino acid substitutions (N34D in E2, N17D in p7, and B31K in E2) were selected for further analysis. These substitutions were found to be present in both the low and medium passage lines, indicating that they may play a role in the development of IFN-α resistance. The specific amino acid substitutions in the consensus genome sequences of p0 after 30, 45, and 100 passages in the absence or presence of IFN-α are shown in Figure 3A.

FIG 3 Amino acid substitutions (relative to the initial infectious transcript HCVcc) in the consensus genome sequences of p0 after 30, 45, and 100 passages in the absence or presence of IFN-α. (A) Proteins core, E1, E2, NS2, NS3, NS4A, and NS4B. (B) Proteins NS5A and NS5B. HCV proteins containing nonsynonymous mutations are indicated on the top row, with the numerical position and single-letter code of the original amino acid listed directly below. Amino acids are numbered individually for each protein. Colored panels indicate numbers of passages in the absence of IFN-α (blue, top) or in the presence of IFN-α in the “low” (yellow), “medium” (pink), or “high” (green) lineages (as described for Fig. 1). Boxes on the left indicate passage history, including passage number (p) and the IFN-α concentration at the time of collection, sometimes followed by a number indicating further passages in the absence (p10 IFN 0) or presence (p10 IFN 2 or p10 IFN 12) of IFN-α; the number following IFN indicates the number of IU/ml of IFN-α included in the culture medium.

Single-letter amino acid codes are used to indicate substitutions discovered in each sequenced viral population; two letters separated by a slash indicate a mixture of the indicated amino acids in the population. An asterisk means that the mutant amino acid is present at a proportion of 25% or lower. No infectious virus was recovered from the “high” lineage after p40 (see Fig. 1B); thus, only p30 IFN 2 and p30 IFN 2 [p10-IFN 0] were included in the analysis. Sequences correspond to the populations described for Fig. 1. To facilitate comparisons, the amino acid substitutions using the nomenclature of HCV strain H77 (129) are given in Table S10 (http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf). Procedures for RT-PCR amplification of the entire genome and nucleotide sequencing are described in Materials and Methods and in the data at http://www.cbm.uam.es:8080/cv-303/SupplMatPerales.pdf.
Y618F in NS3) were detected in all passaged HCV populations in both the absence and the presence of IFN-α (Fig. 3). Previous studies have described adaptive mutations throughout the genome when passaging JFH-1-derived viruses (68–79). Specifically, N34D in E2 results in the loss of a well-characterized N-glycosylation site (E2N1) that has been linked to modulation of entry functions of HCV envelope proteins without interfering with viral morphogenesis (78). Another report investigating N34S described enhanced entry, leading to a rapid amplification via efficient spreading (70). Amino acid 17 in p7 is an essential structural determinant for the p7 ion channel and may play a role as a pH sensor. Replacement of N17 by D may favor a more optimal function of the p7 (79) and could also increase viral production due to enhancement of assembly/morphogenesis steps (76). Y618F is localized in the helicase domain of NS3, though its significance with respect to HCV adaptation to Huh-7.5 cells is unknown. In most lineages, amino acid C298 was exchanged for an arginine in the absence of IFN-α/H9251 or a tyrosine in the presence of IFN-α/H9251. C298R has been also described as increasing infectious virus production and may play a role in regulating the release of infectious particles into the cell culture (68). The number following “IFN” corresponds to the IFN-α concentration at the indicated passage. (A) p45 viruses passaged in the presence of a high IFN-α concentration. Each of the populations was subjected to 10 passages in the presence of 12 IU/ml IFN-α, and infectious progeny production in the cell culture supernatant (left panel) and intracellular progeny RNA levels (right panel) were determined. (B) p45 viruses passaged in the presence of IFN-α+R. Each of the populations was subjected to 10 passages in the presence of 2 IU/ml IFN-α+R (50 μM), and infectious progeny production in the cell culture supernatant (left panel) and intracellular progeny RNA levels (right panel) were determined. (C) p100 viruses passaged in the presence of IFN-α+R. Each of the populations was subjected to 10 passages in the presence of 2 IU/ml plus ribavirin (50 μM), and infectious progeny production in the cell culture supernatant (left panel) and intracellular progeny RNA levels (right panel) were determined. Procedures are described in Materials and Methods.
FIG 6 The effect of infection by passaged HCV populations on host cell protein synthesis in the absence and presence of IFN-α. (A) Huh-7.5 cells were either mock infected or infected with the indicated HCV populations for 56 h. Subsequently, cells were treated with IFN-α (IU/ml indicated at the top) for 16 h before
a significant general fitness advantage, allowing for increased viral production in the absence or presence of IFN-α.

This study has identified amino acid substitutions in every HCV protein except NS4A (Fig. 3 and Tables S3 to S9 [http://www.cbm.uam.es/8080/cv-303/SupplMatPerales.pdf]) that became dominant (composed the majority of genomes in the mutant spectra) in the populations passed in the presence of IFN-α. The comparisons suggest that different substitutions at structural (not expressed in replicon systems) and nonstructural proteins can play a role in IFN-α resistance, though we have not distinguished substitutions that provide bona fide IFN-α resistance from substitutions that confer a fitness gain once key IFN-α resistance mutations have occurred. Also, we cannot exclude the possibility that some synonymous mutations may affect viral fitness (80). Several HCV proteins are known to abrogate or decrease the effects of IFN-α. The core protein can counteract IFN-stimulated gene (ISG) expression by interfering with JAK/STAT signaling (81–83), and substitutions at positions 70 and 91 resulted in a significantly decreased cellular response to IFN-α treatment in vitro (84); substitution G90A—present in the “low” lineage as a mixture at p45 and dominant at p100 (Fig. 3)—by virtue of being the neighbor to position 91 might confer a similar phenotype. E2 targets the IFN-α-induced PKR, and it has been suggested that this interaction may prevent the antiviral effects of IFN-α (85). E2 substitution S18G (present in the “low” lineage by p30), located within hyper-variable region 1 (HVR1) of E2, has been previously linked to IFN resistance in human patients (65). Substitution E2 L283Q (present in the “low” lineage by p100) is within the region of sequence identity shared among the phosphorylation sites of PKR, eIF2α, and HCV E2 (85). The protease NS3-4A can cleave TRIF and IPS-1/Visa/MAVS/CARDIF, which are essential for IFN-β and -α induction (86–88).

The possible contribution of NS5A to IFN-α resistance has been the focus of many studies (89–100). Amino acid substitutions within a 40-amino-acid stretch in NS5A of HCV of genotype 1b correlated with a complete response to IFN-α therapy (89, 101); the corresponding region was termed the IFN sensitivity-determining region (ISDR). This correlation was confirmed by other groups (102–106) but was not supported for HCV from other cohorts infected with genotype 1b or with other genotypes (107–113). The ISDR is part of a domain of NS5A that interacts with PKR; the ensuing decrease of PKR activity may permit HCV to avoid the antiviral effects of IFN-α (91, 92, 114, 115). In our study, substitutions N248H and E269K found in the “medium” lineage at p45 and S272P in the “low” lineage (mixed at p45 and dominant by p100) are located within the ISDR. Studies on the mechanisms by which NS5A counteracts IFN-α activity have not given conclusive results (91, 93–95, 112).

There are several possibilities to explain the disparity of results reported for HCV IFN-α resistance: several HCV genes may be involved, multiple IFN-α resistance pathways may be available to HCV, the selected pathway may depend on key population parameters (viral load, replicative capacity, quasispecies diversity, occurrence of genetic bottlenecks [7, 35]), and accompanying mutations may obscure the role of key mutations in IFN-α resistance, among other possibilities. In cell-HCV replicon systems that require passage of the cells, the mutations observed in HCV may be conditioned by mutations in the cells that coevolve with the HCV replicon (75, 96, 116, 117). Furthermore, selection of a cellular IFN-α resistance determinant may render unnecessary the selection of viral modifications to achieve an equivalent phenotype.

The HCVcc populations that were passaged 45 and 100 times in the absence of IFN-α acquired not only partial resistance to IFN-α (despite not having been exposed to IFN-α [Fig. 2 and 3]) but also the ability to suppress host cell protein synthesis (Fig. 6). It is unlikely that endogenous IFN produced by Huh-7.5 cells could provide a sufficiently selective constraint for the observed resistance, because Huh-7.5 cells have been reported not to express detectable IFN levels (66, 67). A possible alternative explanation is that in the exploration of sequence space inherent to unrestricted (large population) passages of HCV, subpopulations able to cope with the host cell response—generally manifested in the form of protein expression—gradually replace their ancestors. Such replacement is expected to be accelerated by the presence of IFN-α. An alternative possibility is that as the quasispecies expands, it becomes enriched in IFN-α resistance mutations. This would represent an extension of previous evidence of the presence of drug-resistant mutants never exposed to the corresponding drug (118–123; other examples are reviewed in reference 7). Evolution of HCV toward IFN-α resistance in the absence of IFN-α could be due to the chance occurrence of adaptive mutations that might also confer IFN-α resistance or a general fitness gain (due to repeated passages) that renders the virus more resistant to IFN-α (for example, C298R, C298Y in NS5A, N34D in e2, or N17D in p7; see Fig. 3). This point is under investigation. The fact that multiple, different mutations in the HCVcc lines can lead to decreased IFN-α sensitivity may explain why no unified picture on the molecular basis of HCV IFN-α resistance has been obtained in vivo, where additional environmental variables operate. Furthermore, different HCV isolates may not respond in similar manners to the selective pressure of IFN-α. In particular, the rescued HCVcc is rather unique in displaying high infectivity for Huh-7.5 cells, and it is not clear whether it shows a behavior representative of other HCV isolates (46, 58, 124).

A phenotypic trait associated with IFN-α resistance identified in the present study is an enhanced capacity of the virus to evoke a shutoff host cell protein synthesis in the presence of IFN-α. This

**Proteins were labeled with [35S]Met-Cys for 1 h (top box and headings; “No virus” means mock-infected cells). Cell extracts were subjected to SDS-PAGE and visualized by autoradiography as described in Materials and Methods. The numbers indicate the percentages of actin protein determined by densitometric scanning of the corresponding protein band and normalized to the value obtained in the absence of infection and IFN-α treatment (−IFN row) or to the value obtained in the absence of infection but presence of IFN-α treatment (+IFN row) or to the value obtained in the absence of IFN-α treatment for each virus (±IFN row). (B) Cellular extracts were subjected to SDS-PAGE and analyzed by Western blotting using antibodies specific for the phosphorylated and nonphosphorylated forms of PKR and eIF2α. The amount of cellular proteins was monitored by the amount of actin and visualized by Western blotting (actin panels). The bottom panels indicate the ratio of phosphorylated to nonphosphorylated forms of PKR and eIF2α calculated from the densitometry of the relevant bands. (C) Virus-specific proteins were analyzed by reactivity with specific monoclonal antibodies (NS5A and core) in Western blot assays. Determinations of virus titers in the supernatants of the infected cells at 72 h.p.i. in the absence and presence of IFN-α were carried out in triplicate using the titration assay described in Materials and Methods. The individual titer values are given in Table S11 (http://www.cbm.uam.es/8080/cv-303/SupplMatPerales.pdf).**
result is in agreement with the observation that HCV triggers the phosphorylation of PKR and eIF2α as a means to attenuate protein expression, including that by ISGs (38, 125, 126). However, the difference in shutoff among viral populations was not consistently reflected in the ratio of phosphorylated to nonphosphorylated forms of PKR and eIF2α (Fig. 6A and B). Thus, other host factors related to translation regulation and viral proteins are likely involved. The IFN-α-resistant populations obtained in the present study display partial resistance to IFN-α plus ribavirin, and they will allow probing the effects of viral fitness and the IFN-α resistance phenotype on the response to the new-generation DAAs. Thus, IFN-α-resistant HCV populations may provide a useful tool to penetrate some aspects of HCV biology that were previously beyond the reach of replicon-based systems. The use of IFN-α-resistant HCV populations and large HCV populations that may mimic a persistent chronic infection would also be a valuable tool in the design of new antivirals or the testing of new antiviral regimens based on lethal mutagenesis (127, 128).

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