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Hepatitis C Virus Subgenomic Replicons in the Human Embryonic Kidney 293 Cell Line

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Hepatitis C virus (HCV) infects liver cells and its replication in other cells is incompletely defined. Human hepatoma Huh-7 cells harboring subgenomic HCV replicons were used in somatic cell fusion experiments with human embryonic kidney 293 cells as a means of examining the permissiveness of 293 cells for HCV subgenomic RNA replication. 293 cells were generally not permissive for replication of Huh-7 cell-adapted replicons. However, upon coculturing of the two cell lines, we selected rare replicon-containing cells, termed 293Rep cells, that resembled parental 293 cells. Direct metabolic labeling of cells with ^{33}P in the presence of actinomycin D and Northern blotting to detect the negative strand of the replicon demonstrated functional RNA replicons in 293Rep cells. Furthermore, Western blots revealed that 293Rep cells expressed the HCV nonstructural proteins as well as markers of the naïve 293 cells but not Huh-7 cells. Propidium iodide staining and fluorescence-activated cell sorting analysis of 293Rep cells revealed that clone 293Rep17 closely resembled naïve 293 cells. Transfection of total RNA from 293Rep17 into naïve 293 cells produced replicon-containing 293 cell lines with characteristics distinct from those of Huh-7-derived replicon cell lines. Relative to Huh-7 replicons, the 293 cell replicons were less sensitive to inhibition by alpha interferon and substantially more sensitive to inhibition by poly(I)-poly(C) double-stranded RNA. This study established HCV subgenomic replicons in nonhepatic 293 cells and demonstrated their utility in expanding the study of cellular HCV RNA replication.

Infection with hepatitis C virus (HCV), the causative agent for non-A, non-B hepatitis (8), is a serious worldwide health problem. The number of infected individuals is estimated to exceed 170 million (38), and >80% of contracting patients become chronically infected (2, 34). The disease is characterized by symptoms of varying severity and may progress to cirrhosis of the liver and hepatocellular carcinoma (17). The etiologic agent is an enveloped positive-sense RNA virus belonging to the *Flaviviridae* family (33). Translation of the 9.6-kb genome produces a polyprotein that is co- and posttranslationally cleaved into individual viral proteins, after which structural proteins are processed by host signal peptidases and nonstructural proteins are cleaved by virally encoded proteases (reviewed in reference 32).

The lack of robust cellular systems for virus replication has been overcome, in part, by the development of selectable, dicistronic, subgenomic replicons derived from the HCV genotype 1 strains (3, 4, 14, 23, 31). These RNA replicons harbor the viral 5′ and 3′ untranslated regions and use the HCV internal ribosome entry site (IRES) to express the neomycin phosphotransferase gene in place of the structural proteins in the first cistron; the second cistron translates the nonstructural region from the encephalomyocarditis virus IRES. RNA replicons have been established in the human hepatoma Huh-7 cell line and display sustained replication in the cytoplasm of these cells (1, 13, 31). Adaptive mutations in the nonstructural region improve the frequency with which replicons can be established (22), yet full-length viral genomes that encode these adaptive mutations fail to infect chimpanzees (7).

The Huh-7 cell line is permissive for adapted HCV replicons in vitro, although variability in the permissiveness for replicons has been observed for these cells and, recently, for two other cell lines (5, 21, 31, 39). One manner by which the permissiveness of Huh-7 cells and the nonpermissiveness of other cell types can be explored is through somatic cell fusion experiments. Heterokaryons generated by somatic cell fusion can combine the characteristics of two distinct cell types. Such heterokaryons may be used to differentiate between permissive and nonpermissive phenotypes of cells and to illustrate either the presence of essential or absence of inhibitory cellular factors that account for the phenotype of cells that host viral genome replication (16, 24, 35, 37).

For this study, we examined fusions between replicon-containing Huh-7 cells and human embryonic kidney 293 cells and demonstrated that 293 cells are less permissive than Huh-7 cells for HCV RNA replication. In rare instances, we isolated colonies of cells harboring subgenomic replicons that displayed all of the characteristics of naïve 293 cells. Moreover, the replicon RNA extracted from these cells was passaged successfully into naïve 293 cells. The replicon-containing 293 cells displayed responses to treatment with alpha interferon (IFN-α), as well as to poly(I)-poly(C), that were distinct from those of Huh-7 cells. The 293 cell replicons represent a novel system for investigating the biology of HCV RNA replication and may overcome some of the limitations inherent to the Huh-7 cell line.

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MATERIALS AND METHODS

Cell culture and generation of neomycin- and zeocin-resistant stable cell lines. Cell lines used for this study were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Sigma or HyClone). Huh-7 cell lines resistant to neomycin (Huh-7^{Neo}) or zeocin (HuH-7^{Zeo}) were generated by electroporation of naïve Huh-7 cells with plasmid DNA encoding the specified resistance gene followed by selection with neomycin or zeocin, respectively, for 3 weeks. Resistant cell lines were continuously grown in the presence of neomycin (500 μg/ml) (Geneticin; Invitrogen) or zeocin (100 μg/ml) (Invitrogen) in the growth medium. S22.3 cells are a Huh-7-derived cell line and contain a neomycin resistance gene encoded by an adapted HCV subgenomic replicon that was made with a construct resembling I_{377}/NS2-3′ (23). The cell line was continuously grown in DMEM–10% FBS containing neomycin (500 μg/ml). Zeocin-resistant Flp-In 293 cells (293^{Zeo}; Invitrogen) were maintained in DMEM–10% FBS containing zeocin (100 μg/ml).

PEG fusion. Zeocin-resistant cells (0.5×10^6 to 1×10^6) were cocultured with an equivalent number of neomycin-resistant cells in 6-well plates. After 20 h of incubation, cells were incubated with 0.5 ml of DMEM, without FBS, containing 50% polyethylene glycol (PEG) 3000-3700 (Sigma) solution for 5 min (at 37°C, 5% CO_2), rinsed three times with phosphate-buffered saline, and incubated with 3 ml of DMEM–10% FBS overnight. The following day, cells were transferred to 100-mm-diameter plates and medium containing neomycin (500 μg/ml) and zeocin (100 μg/ml) was added. Medium was changed twice a week for 3 weeks. Plates were used to isolate colonies for expansion into cell lines or were fixed with 1% glutaraldehyde and stained with crystal violet.

^{33}P labeling of replicons in the presence of actinomycin D. Cells were grown to 50 to 80% confluency in DMEM–10% FBS in 6-well plates. The cells were rinsed with phosphate-free DMEM–2% FBS (Wisent Inc.) supplemented with 2 mM L-glutamine and incubated in 1 ml of medium containing 1 μg of actinomycin D (actinomycin D-mannitol; Sigma) per ml for 3 h. Following incubation, 1 ml of labeling medium containing 5 μg of actinomycin D per ml and 80 μCi of ^{33}P radionuclide (NEN) was added and cells were incubated for 16 h. Total cellular RNA was extracted by use of the Qiagen RNeasy kit, denatured for 5 min at 75°C in buffer (1× morpholinepropanesulfonic acid [MOPS], 2.2 M formaldehyde, 50% formamide, 40 μg of ethidium bromide per ml), and then loaded onto a 0.6% denaturing agarose gel. The gel was dried, exposed to a storage phosphor screen, and scanned with a Storm 860 phosphorimager scanner (Molecular Dynamics). In experiments in which the HCV protease inhibitor (36) was used, the cells were first cultured in the presence of the inhibitor for 3 days prior to ^{33}P metabolic labeling.

Northern blot analysis. Total cellular RNA was extracted (RNeasy; Qiagen) and quantified by measuring optical density at 260 nm. Twenty micrograms of total RNA from each cell line was loaded in a 0.8% glyoxal-dimethyl sulfoxide gel (Northern Max-Gly; Ambion). Following electrophoresis, RNA was transferred onto a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech) and then cross-linked to the membrane by UV irradiation. The hybridization probe was generated by using the T7 promoter-based Riboprobe in vitro transcription system (Promega) on a 1,620-nucleotide region, spanning the NS5A and NS5B segments of an HCV 1b clone, in the presence of 50 μCi of [α^{32}P]GTP. The blot was hybridized by use of ULTRAhyb solution (Ambion) and then was exposed to a phosphor screen. Detection was performed with a Storm 860 phosphorimager scanner (Molecular Dynamics). As a control, the full-length negative strand of the HCV genome, cloned into the pCR3 vector (Invitrogen), was transcribed with the Riboprobe in vitro transcription system (Promega) and loaded on the gel.

PI staining and FACS analysis. Cells were digested with trypsin, washed with phosphate-buffered saline, and resuspended at a concentration of 10^6 cells/ml in propidium iodide (PI) staining buffer (0.1% sodium citrate, 0.3% NP-40, 0.05 mg of PI per ml, 0.02 mg of RNase A per ml). After a 10-min incubation on ice, 2×10^6 cells were centrifuged, resuspended in 2 ml of fresh PI staining buffer, and kept on ice. The DNA content of single cells was then determined by fluorescence-activated cell sorting (FACS) analysis using a Beckman Coulter cytometer.

Western blot analysis. Whole-cell extracts obtained by lysing cells in Laemmli buffer were resolved by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and then blocked in 5% nonfat milk in Tris-buffered saline–Tween buffer. Blots were incubated with either E1A monoclonal antibody (NeoMarkers), α-fetoprotein (AFP) polyclonal antibody (NeoMarkers), albumin polyclonal antibody (Sigma), NS3 polyclonal antibody (27), or NS5A monoclonal antibody (Maine Biotech Services). Membranes were then incubated with the appropriate mouse- or rabbit-specific antibodies conjugated with horseradish peroxidase, developed by use of LumiLight Western blotting solution (Roche), and exposed to film (Hyperfilm; Amersham Pharmacia Biotech).

Immunofluorescence. Approximately 10^5 cells were grown on LabTek 4-well chamber slides (Nalge Nunc International) that were precoated with 150 μg of poly-D-lysine per ml. At 1 day postseeding, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then incubated with either a polyclonal antibody against NS3 protein (27) or a monoclonal antibody against NS5A (Maine Biotech Services), and bound antibodies were detected with AlexaFluor 488 goat-anti-rabbit or AlexaFluor 488 goat anti-mouse antibody, respectively (Molecular Probes). Cells were visualized with a fluorescence detection microscope (Olympus).

Amplification and cloning of replicon RNA by RT-PCR and in vitro transcription of RNA. Total RNA extracted from cells (RNeasy; Qiagen) was used for reverse transcription (RT)-PCRs (One-Step RT-PCR; Qiagen) that amplified the *Kpn*I/*Sal*I, *Sal*I/*Sal*I, and *Sal*I/*Kpn*I fragments of the HCV nonstructural and 3′ untranslated region segments. The three fragments were first cloned into the pCRII vector (Invitrogen) for sequence verification and then excised by restriction digestion with the appropriate enzymes and assembled into a complete subgenomic replicon by subcloning into pET-9a vector (Novagen). Replicon RNA was synthesized by use of the T7 promoter-based RiboMax large scale RNA production system (Promega).

RNA transfection of cells. Total cellular RNA or in vitro synthesized RNA was transfected into cells by use of either Lipofectamine 2000 or DMRIE-C transfection reagents (Invitrogen). Lipofectamine 2000 transfection was performed by seeding either 2.2×10^6 293 cells or 1×10^6 Huh-7 cells in 60-mm-diameter plates overnight in DMEM–10% FBS (HyClone). On the day of transfection, 20 μg of RNA and 40 μl of transfection reagent (each premixed in 300 μl of OPTI-MEM I medium [Invitrogen] for 5 min) were combined and incubated for 20 min at room temperature, and then the 600-μl reaction was added to cells for 18 h. The cells were then detached with trypsin and seeded into two 150-mm-diameter plates in the presence of 0.25 mg (Huh-7 cells) or 0.4 mg (293 cells) of neomycin (Invitrogen) per ml. DMRIE-C transfection was performed by seeding 7.5×10^5 293 cells in 6-well plates overnight. Four micrograms of RNA was added to a mixture of 1 ml of OPTI-MEM I reduced serum medium and 12 μl of DMRIE-C reagent and was incubated with the cells for 6 h. Following incubation, the medium was changed to DMEM–10% FBS and the cells were incubated for 18 h. At that point, cells were split and plated in 100-mm-diameter plates in medium containing 0.35 mg of neomycin per ml. Selection was performed over a period of 3 weeks.

Replicon RNA inhibition assays and real-time RT-PCR. S22.3 cells (8×10^3) or 293rep17 cells (15×10^3) were seeded in 96-well plates in DMEM–10% FBS for 16 h, which was replaced with medium containing the indicated concentration (refer to particular experiments) of poly(I)-poly(C) (Sigma), IFN-α (Sigma), or an HCV NS3 protease inhibitor previously described as compound 4C ($C_{39}H_{46}N_4O_8$; molecular weight, 698.81) (36). After 72 h of incubation, total cellular RNA was extracted with the Qiagen RNeasy 96 kit, quantified by use of RiboGreen (Molecular Probes) on the Wallac Victor^2 *V* system (Perkin-Elmer), and used in quantitative *Taq*-Man real-time RT-PCRs (Applied Biosystems) performed with the ABI Prism 7700 sequence detection system (Applied Biosystems). Primers used for PCR were 5′-CCGGCTACCTGCCCATTC-3′ as forward primer and 5′-CCAGATCATCCTGATCGACAAG-3′ as reverse primer. The probe used was 5′-(FAM)-ACATCGCATCGAGCGAGCACG TAC-(TAMRA)-3′, which hybridizes within the neomycin phosphotransferase sequence of the HCV replicon RNA (9). The cycling conditions used for RT-PCR were as follows: 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C, followed by 20 s at 94°C and 1 min at 55°C for 40 cycles. The replicon copy number, given as copies per microgram of total RNA, was interpolated from predetermined (in vitro synthesized) HCV replicon RNA concentration standards.

RESULTS

Somatic cell fusion between S22.3 and 293 cells. HCV subgenomic replicons have been previously adapted for human hepatoma Huh-7 cells (3, 15, 22, 23, 31). These subgenomic RNAs replicate persistently and efficiently in Huh-7 cells. Our attempts to introduce in vitro-transcribed replicon RNA into nonhepatic cell types were unsuccessful. We tried to introduce Huh-7-adapted replicons into human embryonic kidney 293 cells through transfection of in vitro-synthesized RNA by electroporation or by using multiple cation-based transfection re-

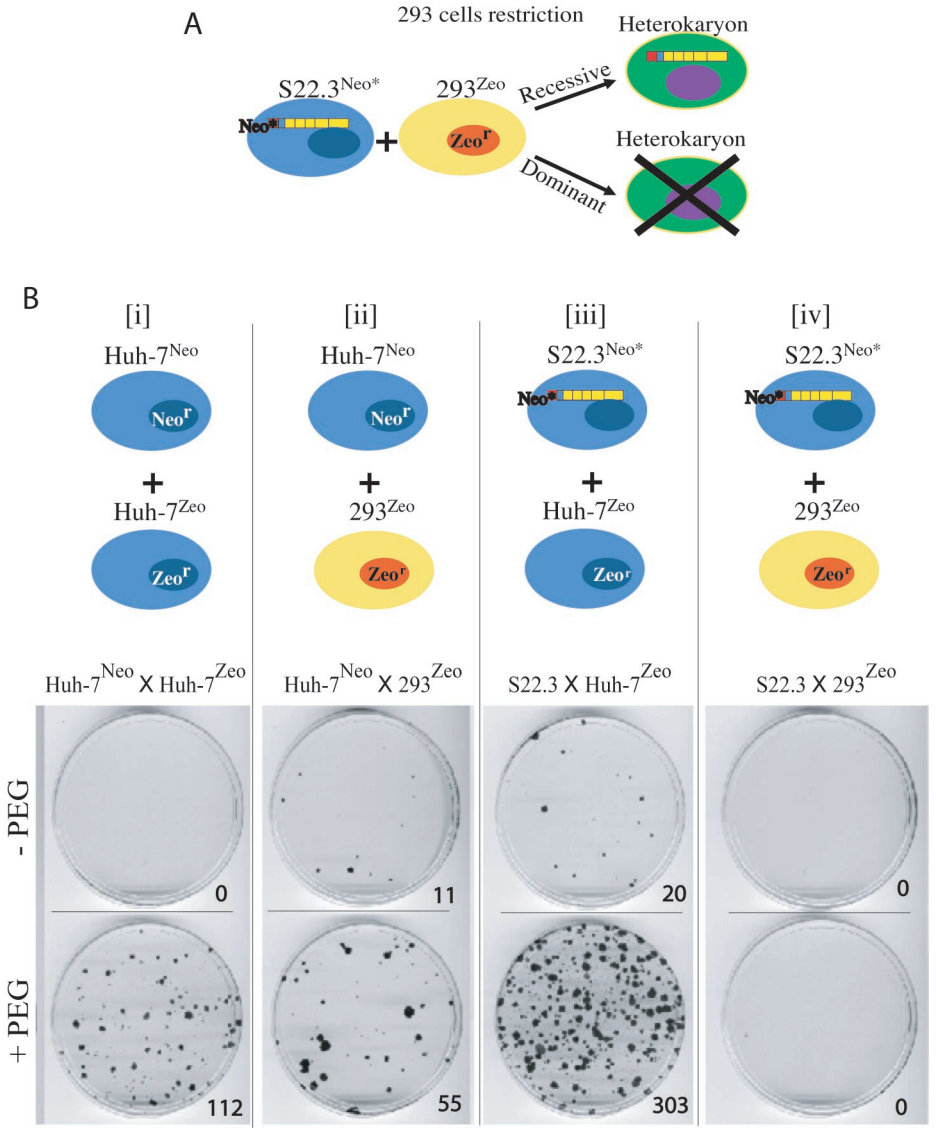

FIG. 1. Neomycin- and zeocin-resistant colony formation with Huh-7- and 293-derived cell lines. (A) Schematic of somatic cell fusion between replicon-containing Neo^r S22.3 cells and Zeo^r 293 cells. The generation of heterokaryons from S22.3 and 293 cells would indicate that 293 cells are recessive for an activity present in Huh-7 cells that is essential for RNA replication; the lack of clones would indicate that the restriction of the 293 cells is dominant over HCV RNA replication. (B) Cellular fusions performed with Huh-7^{Neo} and Huh-7^{Zeo}, two distinct Huh-7-derived cell lines with chromosomally integrated markers (i); Huh-7^{Neo} and 293^{Zeo} cells, each with the respective chromosomally integrated marker (ii); S22.3 and Huh-7^{Zeo}, two homologous cell lines with distinct RNA and DNA selectable markers (iii); and S22.3 and 293^{Zeo}, two heterologous cell lines with RNA and DNA selectable markers (iv). Cellular fusions were performed by coculturing the cells for 20 h and then either leaving cells untreated (−PEG) or treating them with a 50% PEG solution for 5 min (+PEG). Cells were then transferred to 100-mm-diameter plates and selection was done with neomycin and zeocin for 21 days. Colonies obtained were stained with crystal violet and counted (numbers in lower right corners).

agents; however, no neomycin-resistant 293 cell colonies with the replicon were obtained. A possible interpretation of this result is that the cells were nonpermissive for HCV replicons because either (i) the cells express a dominant activity or factor that prevents replication or (ii) the cells lack an essential activity that is necessary for replication (29).

To investigate the potential nonpermissiveness of non-Huh-7 cells, we examined heterokaryon formation between replicon-containing Huh-7 cells (clone S22.3) and human embryonic kidney 293 cells through cell fusion. Successful formation of heterokaryons between 293 and S22.3 cells would suggest that the nonpermissiveness of 293 cells is recessive and that they lack an activity provided by Huh-7 cells for HCV RNA replication (Fig. 1A). Alternatively, failure to generate heterokaryons would be consistent with the hypothesis that 293 cells express a dominant inhibitor of HCV RNA replication. We used 293 cells that have a stably integrated chromosomal copy of the zeocin resistance gene (293^{Zeo}). As controls, we also constructed a Huh-7^{Neo} cell line, which has the neomycin resistance gene stably integrated, and a Huh-7^{Zeo} cell line,

which has the zeocin resistance gene stably integrated. Cell-to-cell fusions were enhanced by the brief addition of the fusogen PEG, whereas in the controls (−PEG), the two cell types were simply cocultured without the addition of PEG (Fig. 1B, panel iv). After an overnight incubation, cells were replated in 10-cm-diameter plates with a selection medium containing 100 μg of zeocin per ml and 500 μg of neomycin per ml, and selection was maintained for 21 days. PEG-mediated cellular fusions between S22.3 and 293^{Zeo} cells (Fig. 1B, panel iv) produced no doubly resistant colonies in repeated attempts ($n = 4$). In parallel, control fusions were performed with combinations of control cell lines that have chromosomally integrated markers (Fig. 1B, panels i, ii, and iii). The control fusion of Huh-7^{Neo} with Huh-7^{Zeo} (homologous cell lines with different chromosomally integrated resistance markers) resulted in no colony formation without the addition of PEG and in 112 colonies for the PEG-treated sample (Fig. 1B, panel i). Coculturing of the heterologous Huh-7^{Neo} and 293^{Zeo} cells (both with DNA chromosomal markers) in the absence of PEG resulted in 11 colonies, and PEG treatment enhanced this result to 55 colonies (Fig. 1B, panel ii). Hence, Huh-7 cells and 293 cells, with appropriate markers, could be used to select for potential heterokaryons that harbor markers from both cells, and the addition of PEG increased the efficiency of colony formation. In order to examine the proficiency for such experiments with neomycin-resistant Huh-7 cells (S22.3) that encode the marker on an RNA replicon, we cocultured S22.3 cells with Huh-7^{Zeo} cells that have a chromosomal zeocin resistance marker and demonstrated relatively efficient colony formation (303 colonies [Fig. 1B, panel iii]). Therefore, PEG can also be used to enhance the selection of potential heterokaryons with RNA and DNA markers in replicon-permissive cell lines, although PEG treatment reduced the number of viable cells. Throughout these experiments, we obtained significant variations in colony formation (up to fivefold) between repeated experiment sets; however, the relative efficiency of colony formation among the samples was consistent with the experiment presented in Fig. 1B.

Invariably, PEG-enhanced fusion between S22.3 and 293^{Zeo} replicon cells repeatedly failed to generate neomycin- and zeocin-resistant colonies. However, coculturing of the two cell lines without PEG treatment, followed by neomycin and zeocin selection, produced rare resistant colonies. In the first trial, we obtained three clones (clones 5, 6, and 7). Upon repeating the coculturing experiment four times, we obtained four other clones (clones 15, 16, 17, and 18). The result presented (Fig. 1B, panel iv) is from a PEG-independent coculture experiment by which no resistant colonies were obtained. Therefore, the frequency of obtaining doubly antibiotic-resistant colonies was low and independent of PEG-enhanced fusion. The neomycin- and zeocin-resistant colonies, termed 293Rep cells, were expanded into cell lines.

293Rep cells contain the HCV subgenomic replicon. In order to assess whether 293Rep cells contain the HCV subgenomic replicon acquired through the coculturing process with S22.3, cellular RNA was metabolically labeled with ^{33}P in the presence of actinomycin D. Two representative cell lines resulting from coculturing experiments, 293Rep5 and 293Rep17, respectively, were chosen for the metabolic labeling experiment. In addition, parental Huh-7 and 293 cells and our original replicon-containing S22.3 cells were included as controls. Metabolically labeled RNA from clones 293Rep5 and 293Rep17 revealed an RNA band that was identical in size to the replicon band found for S22.3 cells (Fig. 2A), suggesting that the neomycin-resistant 293Rep5 and 293Rep17 cell lines contain a functional replicon. No RNA was detected in negative control naïve 293 cells or Huh-7 cells.

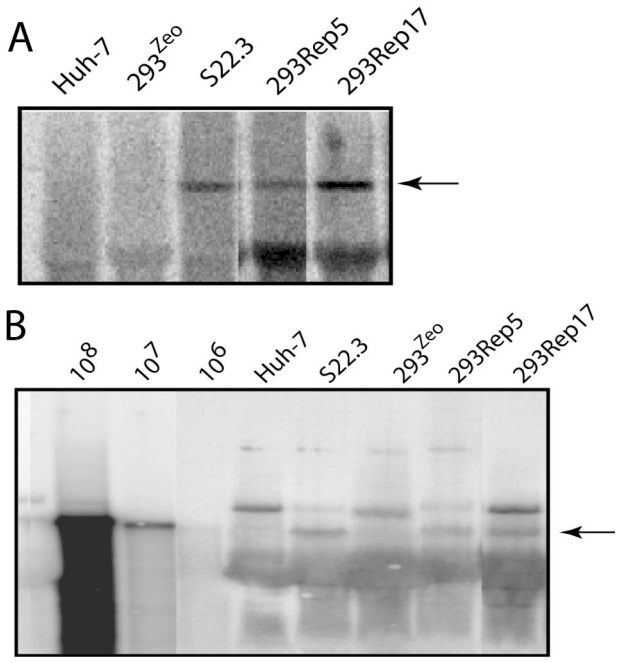

FIG. 2. 293Rep cells contain the HCV subgenomic replicon. (A) ^{33}P metabolic labeling of replicon RNA in the presence of actinomycin D. Total cellular RNA was extracted from cells treated with ^{33}P and actinomycin D for 16 h and was resolved in a 0.6% denaturing agarose gel. (B) Northern blots detecting the negative strand of HCV subgenomic replicons. Total cellular RNA was extracted and resolved in a glyoxal-dimethyl sulfoxide agarose gel. The RNAs were transferred and hybridized with an HCV negative-strand-specific ^{32}P-labeled RNA probe. The first three lanes are positive controls of in vitro-synthesized negative strand RNA, with 10^8, 10^7, and 10^6 copies used, as indicated.

Further confirmation that 293Rep cells harbor the subgenomic replicon was obtained from a Northern blot specifically probing for negative-strand RNA. Blots with RNA isolated from 293Rep5 and 293Rep17 revealed replicon RNA bands consistent with the S22.3 positive control (arrow in Fig. 2B). The replicon was not detected in the control samples from naïve 293 and Huh-7 cells. Hence, 293Rep cells contained the subgenomic negative-strand replicative intermediate of the replicon RNA. Based on the intensities of standards loaded in lanes 1 to 3 (Fig. 2B), the negative-strand replicon copy number is estimated to be in the range of 5×10^5 replicon copies per μg of total RNA for the 293Rep5 and 293Rep17 cell lines; this is approximately an order of magnitude lower than the quantity (as determined by *Taq*-Man real-time RT-PCR) of 1×10^7 plus-strand copies/μg of total RNA estimated for these cell lines.

293Rep cell protein markers. 293 cells and Huh-7 cells express distinct protein markers. 293 cells express the adenovirus transforming E1A protein (12), whereas Huh-7 cells express

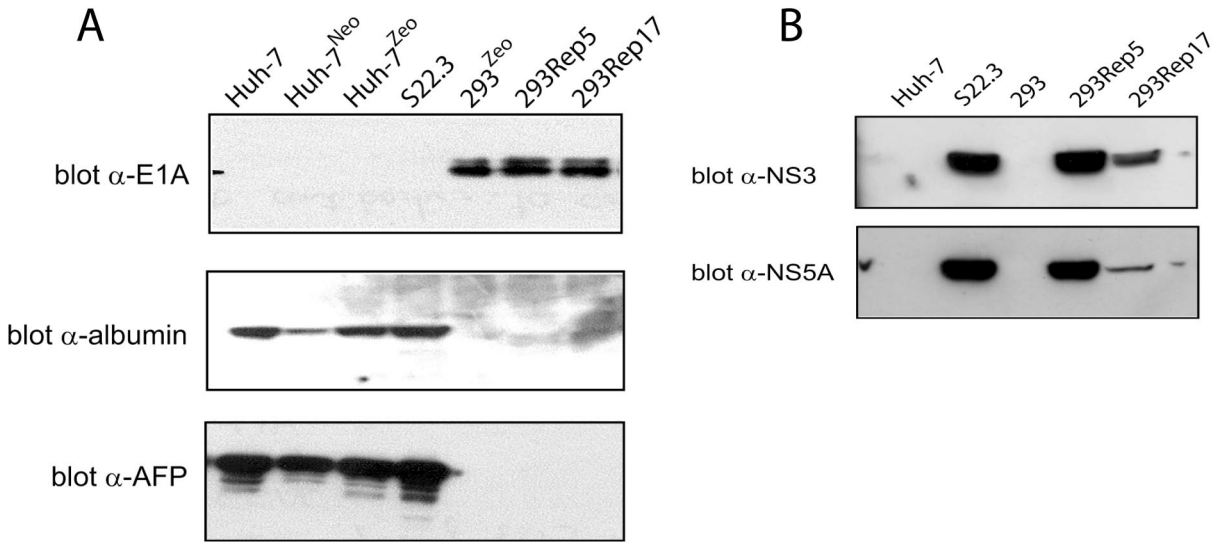

FIG. 3. 293Rep cells express 293- and HCV-specific proteins. (A) Western blot analysis of 293Rep cells. Equivalent concentrations of total cellular proteins, obtained from cell lysates and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were immunoblotted with either anti-E1A monoclonal antibody or anti-AFP polyclonal antibody. The anti-AFP blot was stripped and then reblotted with anti-albumin polyclonal antibody. (B) Western blot analysis of proteins from 293Rep cells. Total cellular proteins, prepared as for panel A, were immunoblotted with either anti-NS3 polyclonal antibody or anti-NS5A monoclonal antibody.

albumin and AFP markers (6). Western blot analysis was performed on total cellular lysates obtained from Huh-7, Huh-7^{Neo}, Huh-7^{Zeo}, S22.3, 293, 293Rep5, and 293Rep17 cells to examine their protein expression patterns (Fig. 3A). Polyclonal antibodies specific for albumin or AFP, or monoclonal antibodies against E1A, showed that Huh-7 and its derived cell lines (S22.3, Huh-7^{Neo}, and Huh-7^{Zeo}) all expressed the liver cell markers albumin and AFP but lacked expression of the 293 cell marker E1A. In contrast, 293 cells, as well as the 293Rep cells, expressed the E1A marker but lacked expression of Huh-7 cell protein markers albumin and AFP. Western blots examining HCV protein expression were performed with a polyclonal antibody against NS3 protein or a monoclonal antibody against NS5A protein. 293Rep5 and 293Rep17 cells expressed both NS3 and NS5A proteins, similar to S22.3 cells, albeit at lower levels, particularly for 293Rep17 cells.

The subcellular localization of NS3 and NS5A was also examined in 293Rep cells by immunofluorescence microscopy. Huh-7, S22.3, 293, 293Rep5, and 293Rep17 cells were fixed and then probed with either a polyclonal antibody against NS3 or a monoclonal antibody against NS5A (Fig. 4). 293Rep5 and 293Rep17 cells expressed both NS3 and NS5A proteins in the cytoplasm, similar to S22.3 cells. In contrast, naïve Huh-7 and 293 cells lacked this cytoplasmic staining. Notably, the gross morphology of 293Rep cells, characterized by size and shape, resembled that of naïve 293 cells. Moreover, 293 and 293Rep cells lacked the distinctive lipid droplets that are readily detectable in Huh-7 and S22.3 cells (arrows in Fig. 4).

Genetic content of 293Rep17 cells. The gross karyotypic content of 293Rep cells relative to the parental 293 and Huh-7 cells was examined by FACS analysis of PI-labeled samples. Huh-7, S22.3, 293, 293Rep5, 293Rep17, and Huh-7^{Neo}/293^{Zeo} (a control hybrid cell line resulting from the fusion of Huh-7 and 293 cells) cells were analyzed. PI staining of parental 293 and Huh-7 cells (Fig. 5A), as well as of S22.3 cells (Fig. 5B), revealed the two peaks characteristic of diploid cells in the G_1 stage and the G_2/M phase of the cell cycle. The PI staining pattern of the Huh-7^{Neo}/293^{Zeo} fusion cells (Fig. 5C) reflected the polyploid characteristic of the hybrid cell line; cells in G_1 were sorted in a fashion consistent with tetraploidy (note that the G_1 peak for this hybrid cell line coincides with the G_2/M peak for parental cells). PI staining of 293Rep5 cells (Fig. 5E) suggested that these cells were not equivalent in DNA content to 293 cells, containing additional genetic material that may have been acquired during the heterologous cell fusion. In contrast, PI staining of 293Rep17 cells (Fig. 5D) resembled the pattern observed for naïve 293 cells; the pair of peaks (representing cells in G_1 and G_2/M) for 293Rep17 cells were coincident with those of the 293 cell sample. 293Rep17 appeared to bear a greater similarity to parental 293 cells.

Transfection of RNA from 293Rep17 cells into naïve 293 cells. In order to determine whether the 293Rep cell replicons were uniquely adapted for replication in 293 cells, we isolated total cellular RNA from 293Rep5, 293Rep17, and S22.3 cells and attempted to reintroduce the RNA by cationic lipid transfection into naïve 293^{Zeo} cells. Transfection of total RNA from S22.3 cells resulted in no colony formation. Similarly, transfection of total RNA from 293Rep5 cells also failed to establish colonies. In contrast, transfection of total RNA isolated from 293Rep17 cells into naïve 293 cells produced 76 neomycin-resistant colonies.

We sequenced the RT-PCR-amplified products from the replicon RNA that was isolated from 293Rep5 and 293Rep17 cells. The sequences were compared to those of the replicons of S22.3 cells, at passage 98 or 79, that represent different lineages (diverging at passage 20) which were used for coculturing with 293 cells in the fusion experiments described above. The sequences of all of the replicons were compared in turn to

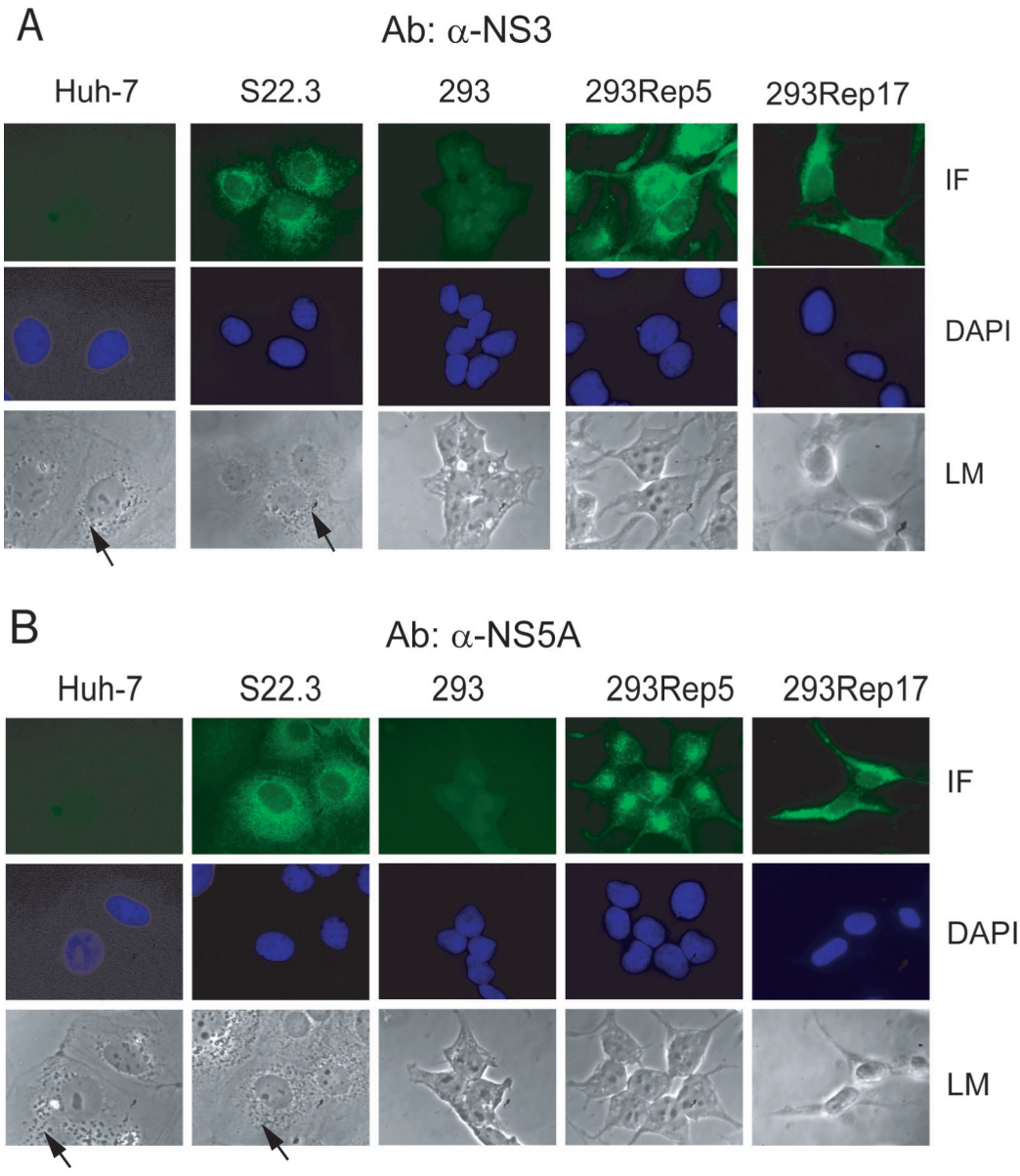

FIG. 4. Detection of HCV proteins in 293Rep cells by immunofluorescence. Fixed and permeabilized cells were incubated with either a polyclonal antibody against the NS3 protein (A) or a monoclonal antibody against the NS5A protein (B) and appropriate secondary antibodies. IF, immunofluorescence with the indicated primary antibody; DAPI, 4′,6′-diamidino-2-phenylindole DNA staining; LM, phase-contrast light microscopy of the same cells. Arrows define the characteristic cytoplasmic lipid droplets of Huh-7 cells.

the sequence of the previously published (AJ238799) HCV Con1 replicon (23). Table 1 lists the nucleotide mutations as well as amino acid substitutions in the replicons. 293Rep5 and 293Rep17 cells each contain unique mutations that are dispersed throughout the NS2-NS5B region. Most of the mutations resulting in amino acid substitutions were within the NS5A region. We also sequenced four replicon clones derived from 293 cells that were transfected with early-passage 293Rep17 total RNA and only identified minor deviations (i.e., <6 nucleotide transitions in the nonstructural region) from the 293Rep17 sequence. These predominantly silent mutations (encoding no more than 2 amino acid substitutions) were unique to each clone and were not found in other 293 replicons.

The NS2-NS5B regions of 293Rep5 and 293Rep17 replicons were reverse transcribed, amplified, cloned, and assembled individually within a standard I_{377}/NS2-3′ dicistronic replicon construct (23). In vitro-synthesized replicon RNA was transfected into naïve 293 and Huh-7 cells and the frequency of colony formation was evaluated after a course of neomycin selection. Transfection of in vitro-transcribed replicon RNA that encodes Huh-7 cell-adapted mutations into naïve Huh-7 cells produces colonies in the range of 2×10^6 to 3×10^6 CFU per μg of RNA (3, 15, 22). We observed that transfection of in

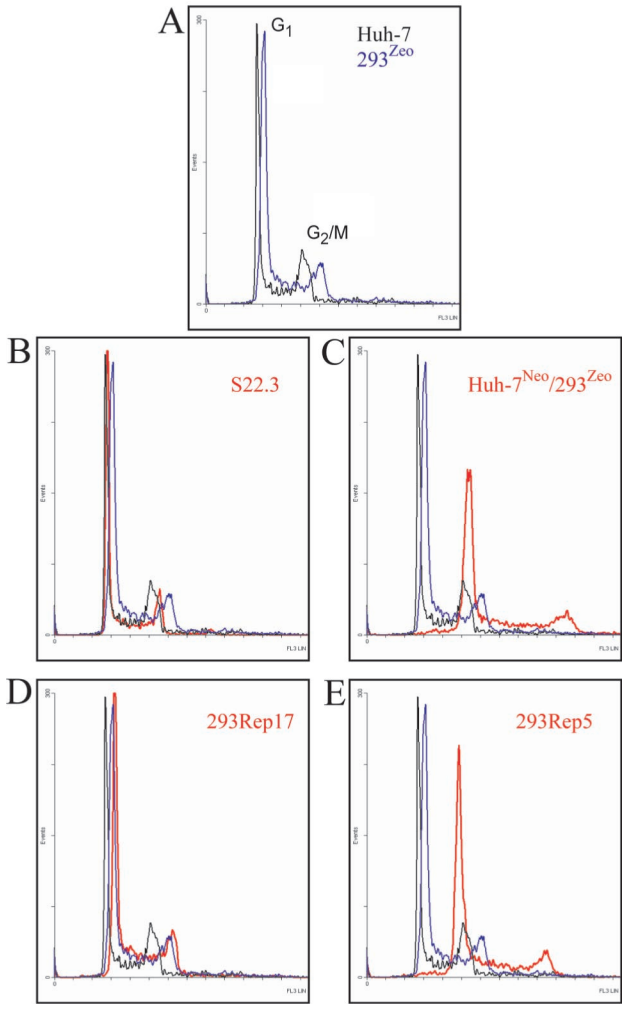

FIG. 5. PI staining and FACS analysis of cell lines. Huh-7, 293, S22.3, 293Rep5, and 293Rep17 cells were digested with trypsin and incubated in staining buffer containing PI for 10 min. DNA content was determined with a FACS analyzer. (A) Overlay of the FACS profiles of parental Huh-7 (black) and 293 (blue) cells depicting canonical G_1 and G_2/M phase peaks. (B) Overlay of FACS profile of S22.3 cells (red) onto the profile of panel A. (C) Overlay of FACS profile of Huh-7^{Neo}/293^{Zeo} hybrid cells (red) onto the profile of panel A, revealing different G_1 and G_2/M phase peaks. (D) Overlay of FACS profile of 293Rep17 (red) cells onto the profile of panel A. (E) Overlay of FACS profile of 293Rep5 (red) cells onto the profile of panel A.

vitro-transcribed 293Rep5 or 293Rep17 replicon RNA into naïve Huh-7 cells also displayed the characteristics of moderately adapted replicons by producing $>10^3$ CFU per μg of RNA (data not shown). However, transfection of either 293Rep5 or 293Rep17 in vitro-transcribed RNA into naïve 293 cells produced no colonies harboring a replicon. Transfections of naïve 293 cells with total cellular 293Rep17 RNA, performed in parallel, consistently ($n = 3$) produced 60 to 80 colonies and indicated that our transfection procedure for 293 cells functioned properly. Thus, the cloned and in vitro-transcribed 293Rep17 RNA, though active in Huh-7 cells, failed to establish 293 colonies and differed in functionality from the RNA directly isolated from 293Rep17 cells.

Transfection of total RNA isolated from 293Rep17 cells into naïve 293^{Zeo} cells produced clones that were expanded into cell lines. We characterized some of these cell lines to confirm the presence of replicon RNA and the expression of viral proteins. Metabolic labeling with ^{33}P in the presence of actinomycin D was performed with Huh-7, 293, S22.3, 293Rep 5, 293Rep17 cells and the LR1, DM1, DM3, DM4, DM6, DM8, LIP3, LIP5, and LIP7 second-generation replicon cell lines (Fig. 6A). Labeled replicon RNA was detected in 293Rep cells as well as in all of the cell lines obtained from the transfection of 293Rep17 total RNA into naïve 293 cells (LR1, DM1, DM3, DM4, DM6, DM8, LIP3, LIP5, and LIP7).

TABLE 1. Nucleotide and amino acid mutations found in 293Rep replicons relative to the Con1 replicon sequence

Protein	Mutation		Presence of mutation in replicon[a]			
	nt	aa	S22.3p98	293Rep5	S22.3p79	293Rep17
NS2	G2784A	A815T	X	X		
	T3004C	I888T	X	X	X	X
	T3134G		X			
NS3	C3459T	L1040F		X		
	C3535T	A1065V				X
	T3650C					X
	T3881C			X		
	C4028T			X		
	C4043T		X	X	X	X
	A4143G	M1268V		X		
	A4154G			X		
	C4172T					X
	T4394G			X		
	T4592C			X		
	C4685T				X	X
	A5019G	S1560G	X	X	X	X
NS4A	C5442T	L1701F				X
NS4B	T5489C					X
	A5646G	I1769V	X	X		
	T5681C			X		
	A5730G	I1797V			X	X
	C5774T					X
NS5A	A6334G	K1998R		X		
	G6465C	G2042R	X	X	X	X
	G6618A	V2093I	X	X		
	T6805C	L2155P		X		
	G6956A					X
	A7075G	E2245G				X
	T7106C				X	X
	C7314T	P2325S			X	X
	T7347C	S2336P	X	X		
	A7349G		X	X	X	X
	G7368A	A2343T		X		
	T7513C	L2391P		X		
	T7554A	W2405R				X
	T7557C	S2406P		X		
	A7565G					X
NS5B	A7653C	T2438P				X
	A7733G			X		
	C7767T			X		
	A8348G					X
	G8441A				X	X
	T8447C					X
	C8603T			X		
	C8860T	A2840V			X	X
	T8999C		X	X	X	X
	A9131G		X	X		
	T9225G	S2962V		X		
	C9226T	S2962V				
	C9368Y					X

[a] X, mutation is present.

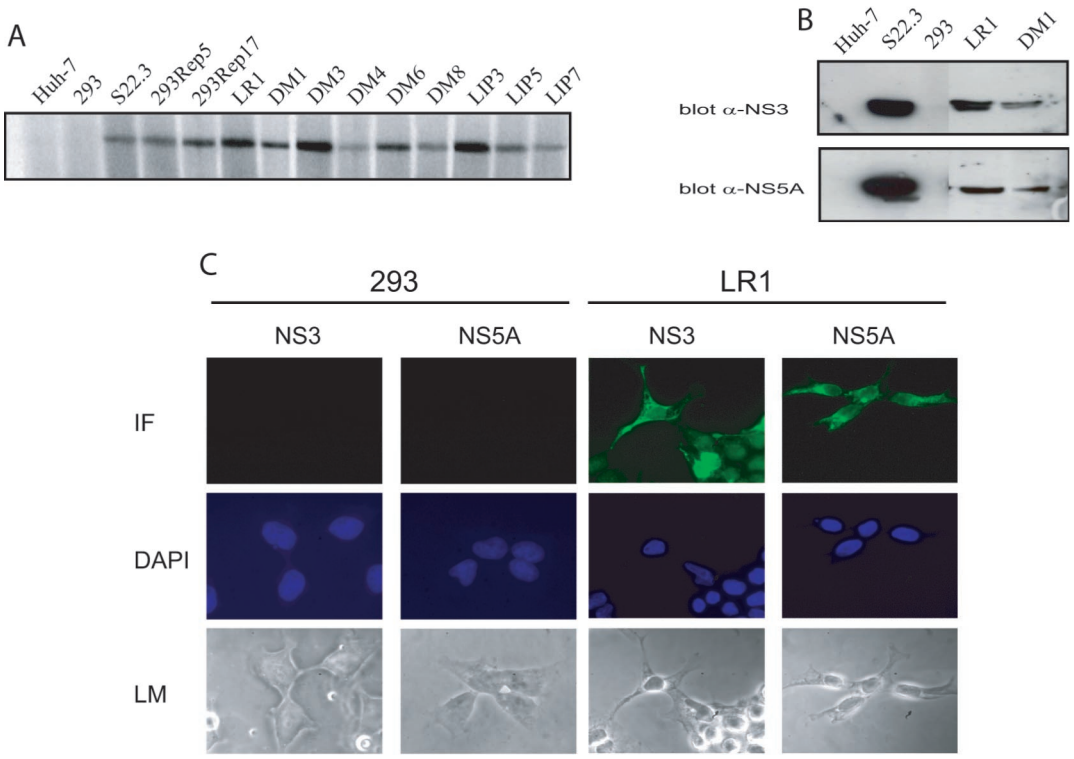

FIG. 6. Analysis of 293 cell lines generated by transfection of total RNA from 293Rep17 cells. (A) ^{33}P labeling of total RNA in the presence of actinomycin D. Selected 293 cell clones (LR1, DM1, DM3, DM4, DM6, DM8, LIP3, LIP5, and LIP7) were metabolically labeled with ^{33}P and actinomycin D for 16 h and visualized as described for Fig. 2. (B) Western blot with anti-NS3 or anti-NS5A antibody as described for Fig. 3. (C) Immunofluorescence detection of viral proteins in replicon-containing LR1 and naïve 293 cells was performed as described for Fig. 4.

Western blot analysis (performed as described for Fig. 3) of total cell lysates from two of the selected second-generation replicon-containing 293 cell lines, LR1 and DM1, revealed proteins detected by NS3 and NS5A antibodies, albeit at lower levels of expression than in S22.3 cells (Fig. 6B); negative control Huh-7 and 293 cells did not express these proteins. Furthermore, by immunofluorescence, both NS3 and NS5A were detected in the cytoplasm of LR1 cells (Fig. 6C), as previously observed for 293Rep17 and S22.3 cells (Fig. 4A and B).

Distinct biological characteristics of replicon-containing 293 cells. HCV subgenomic replicons in 293 cells present a distinct cellular system for the study of HCV RNA replication. In a preliminary effort, we examined the characteristics of these replicon-containing 293 cells in response to various inhibitors and compared them to Huh-7 replicon cells. The effect of a specific HCV NS3 protease inhibitor, 4C, previously demonstrated to inhibit HCV subgenomic replication in S22.3 cells (36) was examined with 293Rep17 cells (Fig. 7A). Cells were incubated for 3 days in the presence of protease inhibitor 4C concentrations ranging from 0.1 to 1 μM. Huh-7 and 293 cells were also included as controls. Metabolically labeled RNAs indicated that treatment of S22.3 cells as well as 293Rep17 cells with 0.1 μM protease inhibitor significantly reduced ^{33}P-labeled replicon levels, whereas 0.33 and 1 μM protease inhibitor concentrations eliminated the labeled replicon from both of these cell types. Moreover, the 50% inhibitory concentration (IC_{50}), as determined by quantitative *Taq*-Man RT-PCR measurement (27, 36) for 293Rep17 replicon RNA (IC_{50} = 0.05 ± 0.013 μM) (data not shown), was similar to the value reported for Huh-7 replicon cell lines (27, 36).

We next examined the effect of treating replicon-containing 293 cells with biological reagents that induce antiviral responses. S22.3, 293Rep17, and LR1 cells were treated with IFN-α at concentrations ranging between 0.023 and 80 U/ml (Fig. 7B). Treatment of S22.3 cells with IFN-α reduced replicon levels, and a 50% reduction relative to untreated controls (IC_{50}) was achieved at a concentration of 0.33 ± 0.12 U/ml (n = 9), consistent with previously reported values for IFN-α using this cell line (27). However, treatment of replicon-containing 293 cell lines 293Rep17 and LR1 required significantly higher (14-fold) concentrations of IFN-α to achieve 50% inhibition; the IC_{50} was 4.8 ± 1.2 U/ml (n = 8) for 293Rep17 cells and 7.2 ± 4.9 U/ml (n = 5) for the LR1 cell line (Fig. 7B). The 293Rep5 hybrid cell line displayed a similar sensitivity to IFN-α, with an IC_{50} of 4.5 ± 3.0 U/ml (n = 3) (data not shown). We also assessed the response of replicon-containing 293 cells to treatment with the double-stranded RNA mimic polyinosine-poly(C) [poly(I)-poly(C)] (28). This agent is proposed to stimulate the activation of cellular antiviral responses through the Toll-like receptor 3 (TLR-3) (18), although the detailed mechanism of activation and the upstream link to TLR-3 remain unknown. We treated the replicon-containing S22.3, 293Rep17, and 293LR1 cells with doses of poly(I)-poly(C) ranging from 0.9 to 2,000 μg/ml and also measured the decline in replicons by using *Taq*-Man real-time RT-PCR (Fig.

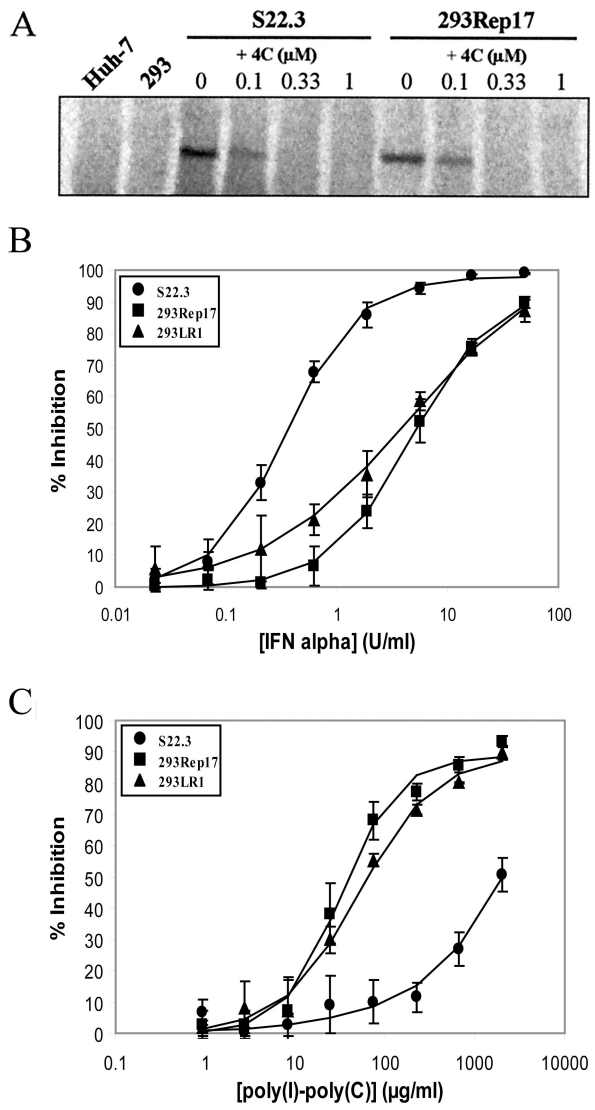

FIG. 7. Inhibition of subgenomic RNA replicon in 293 cells. (A) An HCV NS3 protease inhibitor (4C) decreases intracellular replicon levels. S22.3 and 293Rep17 cells were cultured with the protease inhibitor and labeled with ^{33}P and actinomycin D; total cellular RNA was resolved as described for Fig. 2. Protease inhibitor was tested at 0, 0.1, 0.3, and 1 μM concentrations. (B) Replicon cells were cultured in the presence of different doses of IFN-α, ranging from 0.023 to 50 U/ml, for 72 h. Total cellular RNA was extracted and the replicon copy number was quantified by *Taq*-Man real-time RT-PCR to obtain the percent inhibition of treated samples with respect to untreated control levels. Circles, S22.3 cells; squares, 293Rep17 cells; triangles, LR1 cells. (C) Inhibition of replicon in cells as described for panel B, but cultured in the presence of different doses (0.9 to 2,000 μg/ml) of poly(I)-poly(C). The plotted percentages of inhibition represent average values (with error bars) from at least three determinants.

7C). Treatment of the S22.3 cell line had little effect on replicon levels, even at the highest concentration used, consistent with previous reports (19). In contrast, the replicon-containing 293Rep17 and LR1 cell lines responded effectively even to relatively low doses of poly(I)-poly(C) treatment. The IC_{50} of poly(I)-poly(C) for the 293Rep17 cells was 36 ± 11 μg/ml (n = 8), and the IC_{50} for LR1 cells was 77 ± 29 μg/ml (n = 5).

DISCUSSION

In an attempt to examine and extend the tropism of HCV replicons, we conducted a series of somatic cell fusion experiments between replicon-containing human hepatoma Huh-7 cells (S22.3) and human embryonic kidney 293 cells. From the fusion experiments, we determined that homologous fusion of Huh-7 cells as well as heterologous fusion of Huh-7 and 293 cells could be achieved through selection for two antibiotic resistance markers. S22.3 cells were proficient in fusion experiments with Huh-7 cells harboring a DNA-integrated zeocin resistance marker. The selection of heterokaryons from S22.3 replicon cells and Huh-7^{Zeo} cells, which carry the resistance marker in the nucleus, occurred with a higher efficiency than heterokaryons from the homologous Huh-7^{Zeo} and Huh-7^{Neo} cells, which both have their respective resistance marker integrated in the chromosome. The higher efficiency may reflect a facilitated fusion process between a nuclear marker and a cytoplasmic RNA marker relative to the conventional cell fusion that involves two nuclear DNA markers. Alternatively, as subgenomic replicon RNA can be produced and released into the extracellular medium by Huh-7 cells in ill-defined RNase-resistant particles (30), the zeocin-resistant cells may have acquired the replicon independently of direct cell fusion.

In contrast to the successful fusion between Huh-7 cells and 293 cells, fusion of S22.3 with 293 cells did not produce viable colonies under the same conditions. One interpretation from these results is that 293 cells restrict replication of the Huh-7 cell-adapted replicons and that the restrictive phenotype of 293 cells is dominant over the permissive phenotype of Huh-7 cells. This phenotype of 293 cells may be due to the expression of an inhibitor that is absent (or differentially expressed) in Huh-7 cells. Due to the fact that we observed a difference between 293 and Huh-7 cells in responding to treatment with activators of antiviral responses (discussed below), we suspect that 293 cells may encode a replicon inhibitor that is less active in (or absent from) Huh-7 cells. Other groups have observed that Huh-7 cells are not uniformly permissive for HCV replicons, and variations in replication efficiency have been reported for different cell passages or for "replicon-cured" Huh-7 cells (4, 5, 21, 25). Whether a common inhibitory activity accounts for restriction in 293 and some Huh-7 cells is unknown, and the components of a potential repressive pathway(s) and their role in HCV subgenomic replication remain to be determined.

The nonpermissive phenotype of 293 cells for Huh-7 cell-adapted replicons was suggested from the repeated attempts that failed to produce cell fusions between 293^{Zeo} and S22.3 cells. When replicon-containing S22.3 cells were simply cocultured with 293 cells in the absence of PEG, we isolated rare neomycin- and zeocin-resistant colonies. We suspect that a PEG-independent fusion occurred between the two cell types or that 293^{Zeo} cells acquired an extracellular form of the RNA marker produced by S22.3 cells (30) (see above).

Analysis of the DNA content of the replicon-containing 293Rep cells, 293Rep5 and 293Rep17, by PI staining and FACS supported both proposals. The analysis revealed that 293Rep5 cells contain more than the normal diploid, but less than tetraploid, DNA content observed for either parental 293 or Huh-7 cells. This suggests that 293Rep5 cells originated from a fusion of the two cocultured parental cell types and then

lost chromosomes but maintained the replicon. A similar analysis on 293Rep17 cells, on the other hand, revealed that the DNA content of this cell line was indistinguishable from that of naïve 293 cells. Hence, either of the two proposed methods of replicon acquisition could apply for 293Rep17 cells. Both 293Rep5 and 293Rep17 cells expressed the E1A protein, a marker for the adenovirus Ad5 immortalized 293 cells (20), and lacked expression of the albumin and AFP protein markers of Huh-7 cells; the latter marker may not represent an ideal marker for Huh-7 cells, since its expression is commonly lost in somatic cell hybrids (26). Replicon RNA was detected in these cells by metabolic labeling with ^{33}P and by Northern blots that confirmed the presence of the negative-strand replicative intermediate. Both Western blots and immunofluorescence detected HCV nonstructural proteins in 293Rep cell lines. Our data supported the proposal that 293Rep17 has a 293 cell background and provided us with the means to isolate replicon HCV RNA that may have adapted to 293 cells.

Transfection of total cellular RNA isolated from the 293Rep17 cells into naïve 293 cells produced colonies. Notably, transfection of total RNA from 293Rep5 and 293Rep17 cells into naïve Huh-7 cells produced neomycin-resistant colonies, whereas only the total RNA from 293Rep17 cells produced replicon-containing colonies in naïve 293 cells. The consensus sequence of 293Rep17 replicon RNA differs from that of 293Rep5 replicon RNA by 41 nucleotides that encode five amino acid substitutions within the nonstructural region, some of which may represent adaptive mutations required for replication in 293 cells.

Though the in vitro-transcribed RNA that was generated from the 293Rep17 DNA clone encoded all of the nonstructural region mutations found in the total RNA isolated from 293Rep17 cells, only the latter efficiently established replicons in naïve 293 cells. We currently have not identified the cause of this difference, and a similar discrepancy was also noted by Zhu and colleagues (39) in replicons isolated and cloned from HeLa cells and a murine liver cell line. We can eliminate the possibility that the cloning of the replicon had inadvertently incorporated lethal mutations, as the in vitro-transcribed RNA derived from the 293Rep17 clone replicated and produced colonies when transfected into naïve Huh-7 cells. One possible explanation is that the nonstructural region adaptive mutations, though functional in Huh-7 cells, are insufficient to confer replication in 293 cells and additional mutations within or beyond this region are necessary; we have sequenced the 5′and 3′ untranslated regions of 293Rep17 but have not identified mutations that induce efficient 293 cell colony formation with in vitro-transcribed RNA. Alternatively, unidentified accessory RNA molecules, coisolated with the replicon from 293Rep17 cells, may play a role in RNA replication. A third possibility is that the replicon isolated directly from 293Rep17 cells has epigenetic modifications (lacking in vitro-transcribed RNA) that are essential. These possibilities are currently being explored.

Preliminary characterization of the replicon-containing 293 cells revealed differences relative to Huh-7 cells. For example, replicon sensitivity to IFN-α differed between the two cell lines; the IC_{50} was 14-fold higher for 293 cells than for S22.3 cells or our other Huh-7 adapted replicon cell lines. We also observed a significantly different response to the double-stranded RNA mimic poly(I)-poly(C). There was a substantial reduction of HCV replicon RNA levels in 293 cells following poly(I)-poly(C) treatment, whereas we observed only a limited effect in Huh-7 cells, as previously reported (19). This difference between the two cell types may be due to differences in the expression of a receptor for poly(I)-poly(C), such as TLR-3, or a downstream signaling component (10), such as IRF-3, which is proposed to regulate subgenomic RNA replication (11).

We have isolated 293 cells that replicate HCV subgenomic RNA. These cells can be used to investigate specific cellular inducers or effectors of HCV RNA replication beyond Huh-7 cells and to expand the available cell culture models to investigate the biology of HCV.

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