Generation of recombinant adenoviruses

rAdV, AxCANCre, expressing Cre recombinase tagged with nuclear localization signal under CAG promoter was prepared as described previously (Baba et al., 2005). The target rAdV AxCALNLH-CNS2 expressing HCV core-NS2 polyprotein with adaptive mutations in E2, p7 and NS2 was generated as follows. Cosmid pAxCALNLwit2 is identical to pAxCALNLw (Sato et al., 1998), except that both the terminal sequences of the rAdV genome are derived from pAxCAwit2 (Fukuda et al., 2006). The core-NS2 fragment obtained from pCAGC-NS2/JFH1am by StuI-EcoRI digestion and subsequent Klenow treatment was inserted into the SwaI site of pAxCALNLwit2. The resultant cosmid pAxCALNLH-CN2it2 was digested with PacI and transfected into 293 cells to generate rAdV AxCALNLH-CNS2.

Preparation of packaging cells for HCVtcp

Huh7.5.1 cells were coinfected with AxCANCre at an MOI of 1 and AxCALNLH-CNS2 at an MOI of 3 for expression of JFH-1 core-NS2 polyprotein containing the adaptive mutations in E2, p7 and NS2.

RNA preparation, RT-PCR and sequencing

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and subjected to reverse transcription with random hexamer and Superscript III reverse transcriptase (Invitrogen). Three fragments of HCV cDNAs that cover the entire HCV subgenomic replicon genome, were amplified by nested PCR with TaKaRa Ex Taq polymerase (Takara, Shiga, Japan). Amplified products were separated by agarose gel electrophoresis, and were used for direct DNA sequencing.

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A Cell-Based, Microplate Colorimetric Screen Identifies 7,8-Benzoflavone and Green Tea Gallate Catechins as Inhibitors of the Hepatitis C Virus

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We describe a cell-based, microplate colorimetric screen for anti-hepatitis C virus (HCV) drugs that exploits the HCV-JFH1 viral culture system. Antiviral activity was assessed by measuring protection against the HCV-JFH1-induced cytopathic effect (CPE) in Huh7.5.1 cells using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay. The use of serum-free medium substantially sensitized Huh7.5.1 cells to HCV-induced CPE, causing sufficient cell death to perform colorimetric assays for anti-HCV activity in 96-well plates. As a proof of concept, we carried out a pilot screen of an inhibitor library and identified cyclosporin A and tamoxifen, two compounds with reported anti-HCV activity. Using the assay, we discovered the anti-HCV properties of the plant flavonoids epigallocatechin gallate (EGCG) and 7,8-benzoflavone (α-naphthoflavone). Other gallate-type catechins and flavones also displayed anti-HCV activity, but 5,6-benzoflavone (β-naphthoflavone), flavanone, and non-gallate catechins were inactive. EGCG apparently acted mainly on HCV entry, although it may also block other steps. In contrast, 7,8-benzoflavone was presumed to inhibit later stages of the HCV life cycle. This assay is simple, reliable and cost-effective; does not require any specially engineered cell lines or viruses; and should be useful in the identification of compounds with anti-HCV activity.

Key words hepatitis C virus; 7,8-benzoflavone (α-naphthoflavone); epigallocatechin gallate

More than 170 million people worldwide are chronically infected with the hepatitis C virus (HCV) and are at risk for developing liver diseases such as cirrhosis and hepatocellular carcinoma. Vaccines against HCV are not currently available; furthermore, the standard interferon/ribavirin combination therapy is not effective in approximately half of HCV-infected patients, and it has considerable side effects.[1,2] Thus, there is an obvious and urgent need for new agents that can enhance or replace current HCV therapies.

Screening programs using HCV replicon-based systems have successfully identified compounds that act on HCV RNA replication. However, replicon systems do not reproduce the entire HCV life cycle, and they cannot isolate inhibitors of many important steps such as viral entry, assembly, and egress. HCV cell culture infection models that recapitulate the entire viral life cycle *in vitro* have greatly enhanced the opportunity for HCV drug discovery. Several reports, including ours, have demonstrated that this model can overcome the limitations of the HCV replicon system and enable the discovery of compounds that target various stages of the HCV life cycle.[3–8]

Using the HCV cell culture system, we previously developed a tube-capture-reverse transcription-polymerase chain reaction (RT-PCR) assay for screening HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication.[4] Here, we describe another screening method for the detection of anti-HCV activity. This assay measures the inhibition of the HCV-induced cytopathic effect (CPE) in Huh 7.5.1 cells. We show that the use of serum-free medium improves the sensitivity of the assay and permits the identification of drug candidates that may be overlooked in other assays due to their serum-binding capacities. The validated assay was used to evaluate a compound library, in which it successfully identified the anti-HCV activities of cyclosporin A and tamoxifen.

Many biological properties, including anti-oxidative, anti-inflammatory, anti-tumorigenic, anti-bacterial, and anti-viral activities, have been documented for plant flavonoids, particularly the green tea catechin epigallocatechin gallate (EGCG).[9] We tested various flavonoids in the assay and found that EGCG and 7,8-benzoflavone (α-naphthoflavone) inhibit HCV infection. Other gallate-type catechins were also active, but non-gallate catechins and 5,6-benzoflavone (β-naphthoflavone) did not exhibit prominent anti-HCV activity. EGCG has recently been shown to block HCV entry.[10] Our results using the JFH1 viral culture system also suggested that EGCG mainly targets virus entry but implied that it may also act on other stages of the HCV life cycle. In contrast, 7,8-benzoflavone appeared to inhibit post-entry phases.

The assay described here is simple, reliable and cost-effective, does not require any specially engineered viruses or cell lines, and should allow the high-throughput screening of HCV drug candidates.

MATERIALS AND METHODS

Materials Epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, cyclosporin A and tamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Catechin gallate and gallocatechin gallate were purchased from Nagara Science Co., Ltd. (Nagase, Japan). Flavone, flavanone, 5,6-benzoflavone, and 7,8-benzoflavone were obtained from Wako Pure Chemicals (Osaka, Japan). The SCADS Inhibitor Kit I was provided by the Screening Committee of

The authors declare no conflict of interest.

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Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on Innovative Areas in the Scientific Support Programs for Cancer Research, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The insulin-transferrin-selenium-X (ITS-X) supplement was purchased from Invitrogen (Grand Island, NY, U.S.A.). The anti-NS5 monoclonal antibody was obtained from Austral Biologicals (San Ramon, CA, U.S.A.), and antibodies against extracellular-regulated kinase 1 (ERK1) and ERK2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-core monoclonal antibody (2H9) has been described previously.[11] Huh 7.5.1 cells were kindly provided by Francis V. Chisari.[12] The pJFH1 plasmid, containing a full-length cDNA corresponding to the JFH1 isolate, was used to produce HCV in culture.[11] To prepare virus stock for screening, naïve Huh 7.5.1 cells were infected with the passaged supernatant virus, and the medium was collected 7 d post-infection and stored at −80°C until use.[4] The cells harboring the genotype 1a full-genomic replicon (RCYM1)[13] and the genotype 2a subgenomic replicon (clone 4-1)[14] have been described previously.

HCV CPE Inhibition Assay Huh 7.5.1 cells were suspended in 1 : 1 Dulbecco's modified Eagle's medium : Nutrient Mixture F-12 (DMEM/F12) supplemented with 0.5% ITS-X and seeded in wells of collagen-coated 96-well plates at a density of 5000 cells per well in a volume of 125 μL. The cells were allowed to attach to the plates, and 15 μL of test compounds in DMEM/F12 were added to the wells. The cells were then infected with HCV-JFH1 virus stock at a multiplicity of infection (MOI) of 1. A duplicate plate without the virus was prepared in parallel to assess the cytotoxicity of the compounds. After 4 d, cell growth was monitored using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (15 μL at 5 mg/mL in phosphate buffered saline) was added and incubated for 4 h. The resulting formazan was extracted by adding 100 μL of 20% sodium dodecyl sulfate (SDS) in 10 mM HCl, and the absorbance was monitored after 24 h at 570 nm with a reference wavelength of 690 nm.

Immunoblotting Analysis Huh 7.5.1 cells were seeded in 24-well collagen-coated plates at a density of 1.5×10^5 cells per well and infected with HCV-JFH1 at an MOI of 0.5. Drug treatment was initiated at various timepoints before and after infection. At 48 h after infection, cells were fixed with 10% trichloroacetic acid and lysed with 9 M urea, 2% Triton X-100 and 2% lithium dodecyl sulfate. The lysates were neutralized with 2 M Tris and analyzed by immunoblotting. Replicon cells were seeded in 24-well collagen-coated plates at a density of 5.0×10^4 cells per well, treated with the compounds for 72 h, and processed for immunoblotting as described above.

RESULTS

Assay Development As reported by Sekine-Osajima,[15] infection with HCV-JFH1 induces CPE in Huh 7.5.1 cells. We postulated that compounds with anti-HCV activity would protect cells from CPE and improve cell viability. Thus, by measuring the recovery of cell growth from HCV-induced CPE in microtiter well plates, it should be possible to screen for inhibitors of HCV.

To increase the sensitivity of the primary screen and identify as many compounds with anti-HCV activity as possible, we reduced the concentration of serum in the assay medium; many compounds are known to bind to serum proteins and thus may not show activity in media containing high concentrations of serum. We found that Huh 7.5.1 cells can proliferate on a collagen-coated surface in DMEM/F12 supplemented only with insulin and transferrin at a rate fully comparable to the growth in serum-containing medium.

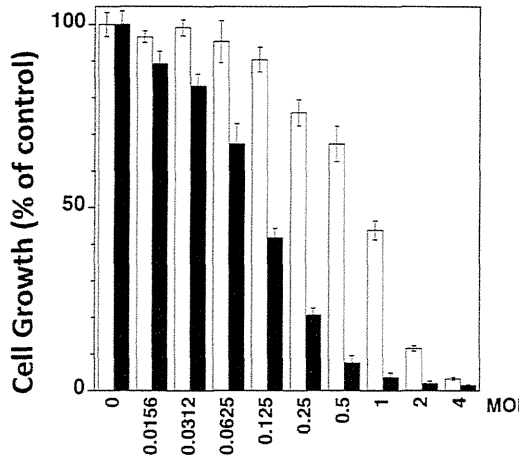

Fig. 1. HCV-Induced CPE in Serum-Free (Closed Bars) and Serum-Supplemented (Open Bars) Medium

Huh 7.5.1 cells were suspended in serum-free or serum-supplemented medium and seeded in 96-well plates at a density of 5000 cells per well. Cells were infected with HCV-JFH1 at various MOIs and cultured for 4 d. Cell growth was monitored by MTT assay and is presented as percentage of control (uninfected) cells. The values presented are the means±S.D. of at least triplicate wells.

The infection of Huh 7.5.1 cells cultured in serum-free medium with JFH1 caused marked CPE. HCV-mediated CPE was also observed in serum-supplemented medium, but cells were less susceptible and required several-fold higher viral titers to achieve equivalent growth inhibition (Fig. 2). We chose an MOI of 1 in serum-free medium as the condition for screening. Although there is some serum carry over from the virus stock, at this MOI, the final serum concentration is generally reduced more than 100-fold compared to serum-supplemented medium. Cell growth usually decreased to less than 10% of the control, as measured by MTT assay.

To test the ability of the assay to identify HCV inhibitors, we performed a test screen using an inhibitor kit obtained from the Screening Committee of Anticancer Drugs (SCADS inhibitor kit I). This kit consists of 92 compounds with various biological activities. Cyclosporin A and tamoxifen, two compounds with known anti-HCV activity, were identified as HCV inhibitors in the assay, providing a proof-of-concept for anti-HCV screening. As shown in Fig. 3, infection with JFH1 at an MOI of 1 reduced the growth of Huh 7.5.1 cells to less than 7% of the control in this experiment. In the presence of 2 μM cyclosporin A and tamoxifen, cell growth recovered to 73% and 71% of the control, respectively.

Identification of Plant Flavonoids as Inhibitors of HCV We then used the assay to evaluate the potential anti-HCV activities of various compounds. As shown in Figs. 3 and 4, various plant flavonoids, including the green tea gallate catechin EGCG and 7,8-benzoflavone, reversed the CPE of HCV. The growth of Huh 7.5.1 cells recovered from less than 7% to 90% and 84% in the presence of 10 μM EGCG or 2.5 μM 7,8-benzoflavone, respectively. The morphology of cells infected in the

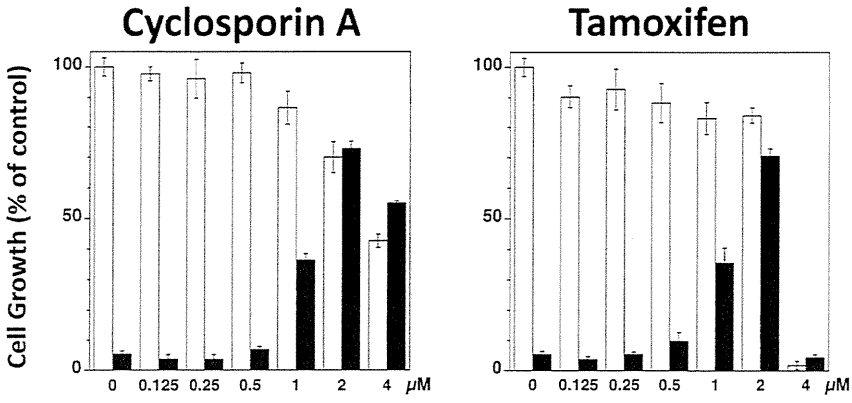

Fig. 2. Cyclosporin A and Tamoxifen Protect Huh 7.5.1 Cells from HCV-Induced CPE

Huh 7.5.1 cells were seeded in 96-well plates and treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 1. Cell growth is expressed as percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of quadruplicate wells.

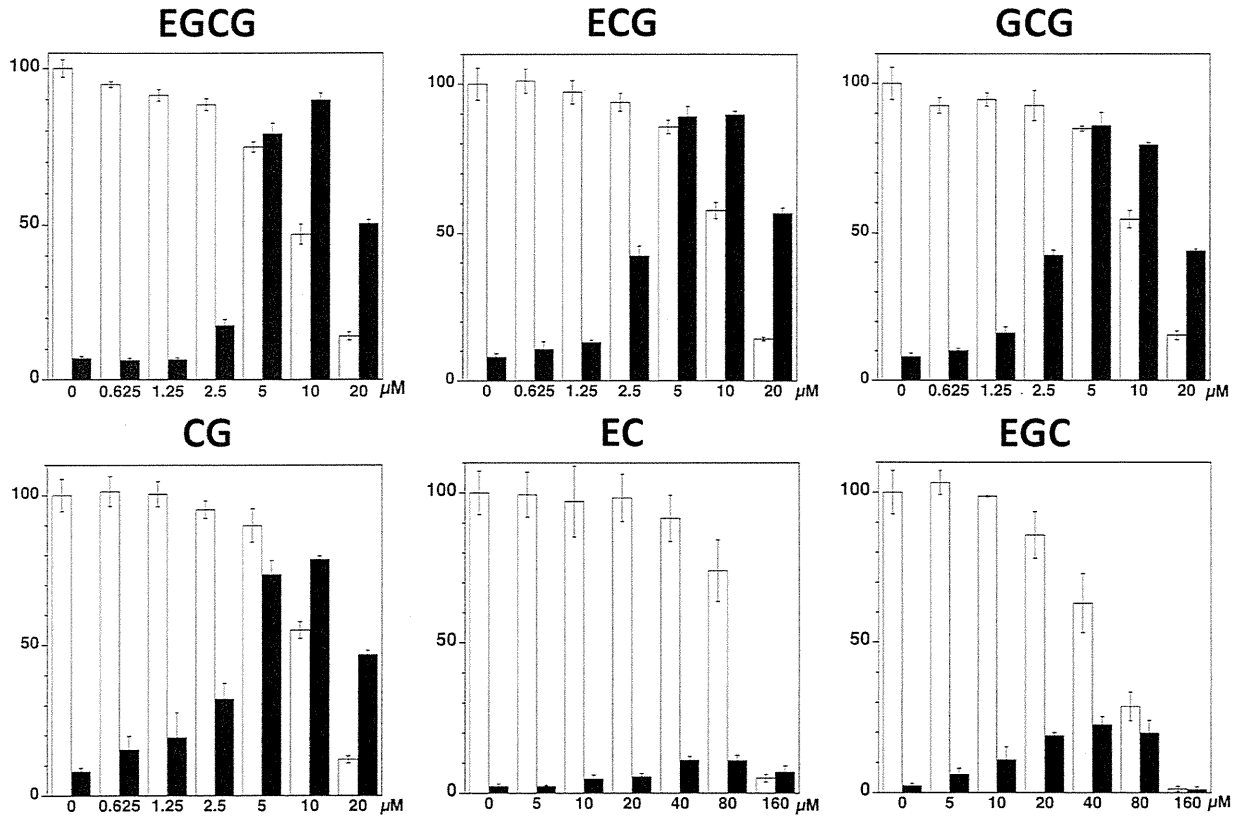

Fig. 3. The Effect of Green Tea Catechins on HCV-Induced CPE

Huh 7.5.1 cells were treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 1. Cell growth is presented as a percentage of mock-infected cells without inhibitors. EGCG, epigallocatechin gallate; ECG, epicatechin gallate; GCG, gallocatechin gallate; CG, catechin gallate; EC, epicatechin; EGC, epigallocatechin. The values presented are the means±S.D. of triplicate wells.

presence of EGCG or 7,8-benzoflavone was indistinguishable from that of control cells without virus (not shown). Other gallate-type catechins (catechin gallate, gallocatechin gallate, and epicatechin gallate) were approximately as active as EGCG (Fig. 3). Flavone also displayed a protective effect (Fig. 4), although only at concentrations approximately 10-fold higher than that of 7,8-benzoflavone, but 5,6-benzoflavone, flavanone (not shown), and the non-gallate-type catechins epicatechin and epigallocatechin did not show significant anti-HCV activity (Fig. 3).

Effects of Serum on Anti-HCV Activities of Various Compounds We next performed the assay in a medium containing 5% FBS to assess the effects of serum on the anti-HCV activities of the identified compounds. To obtain CPE comparable with that observed in serum-supplemented medium (Fig. 3), cells were infected at an MOI of 4 in serum-supplemented medium.

In the absence of HCV infection, cyclosporin A was

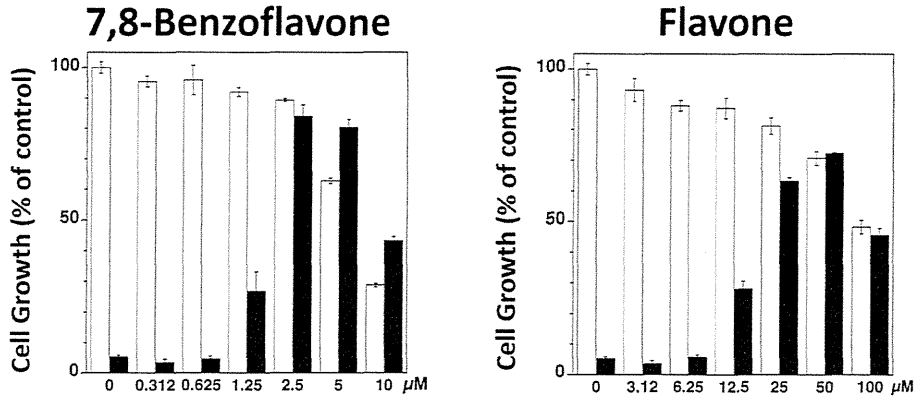

Fig. 4. The Effects of Flavonoids on CPE Induced by HCV

Huh 7.5.1 cells were treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 1. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of triplicate wells.

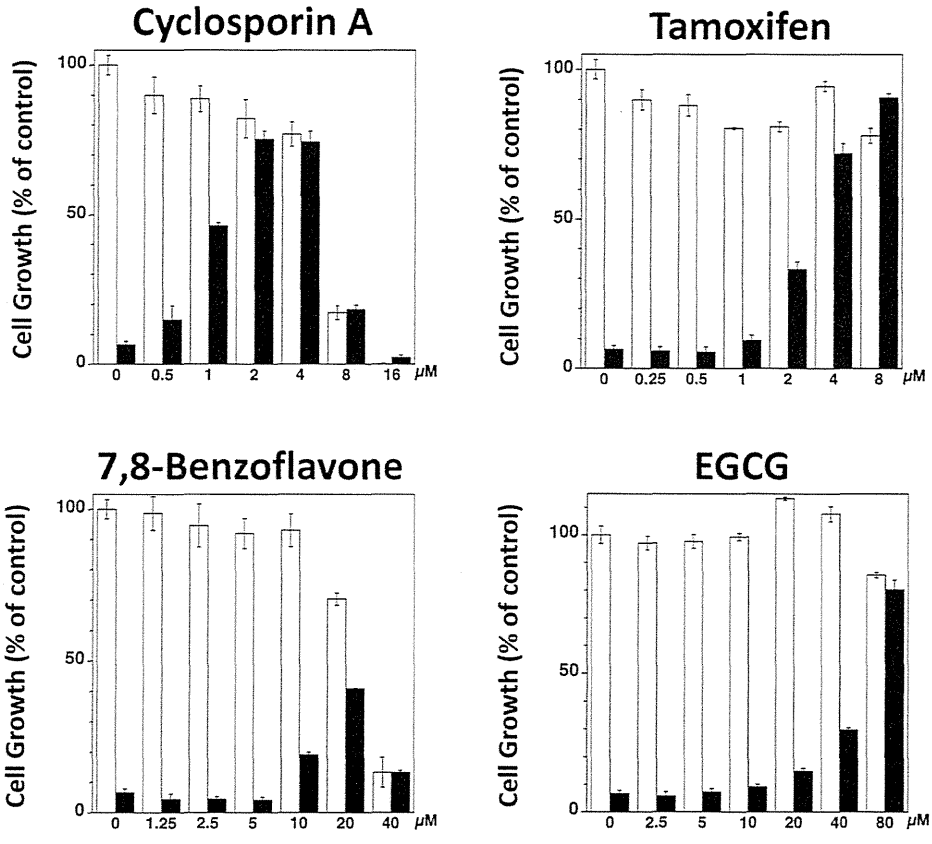

Fig. 5. The Effects of Serum on the Anti-HCV Activity and Cytotoxicity of Cyclosporin A, Tamoxifen, EGCG and 7,8-Benzoflavone

Huh 7.5.1 cells were suspended in serum-supplemented medium and seeded in 96-well plates. Cells were then treated with compounds in the absence (open bars) or presence (closed bars) of HCV-infection at an MOI of 4. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of triplicate wells.

somewhat more toxic to Huh 7.5.1 cells in serum-free medium than in serum-supplemented medium. However, growth recovery from HCV-induced CPE did not appear to be noticeably influenced by the increase in MOI (not shown) or by the addition of serum (Fig. 5). Other compounds did show anti-HCV activity and cytotoxicity in the presence of serum but required higher concentrations to achieve equivalent activities.

In serum-free medium, tamoxifen showed the highest HCV-inhibitory activity at 2 μM (Fig. 2). The increase in MOI from 1 to 4 slightly reduced the anti-HCV activity of tamoxifen (not shown). In 5% serum, the most effective dose of tamoxifen shifted to highest tested concentration of 8 μM, although the maximum observed growth improvement (6.6% to 91%) was better than that in serum-free medium. The cytotoxic concentrations also changed. In serum-free medium, tamoxifen was highly toxic to Huh 7.5.1 cells at concentrations

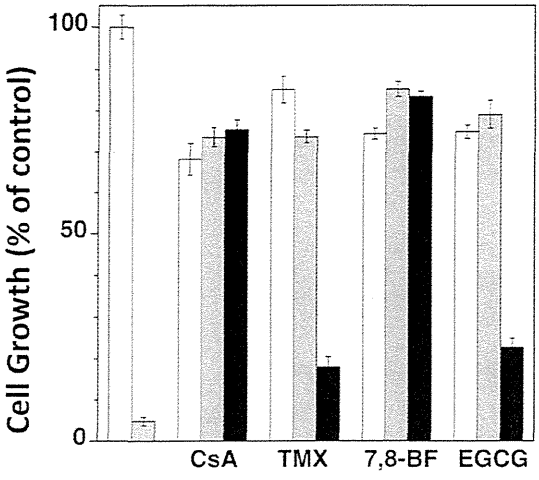

Fig. 6. The Effects of Time of Addition on the Anti-HCV Activity of Cyclosporin A (CsA), Tamoxifen (TMX), 7,8-Benzoflavone (7,8-BF) and EGCG

Compounds were added immediately before (shaded bars) or 2 h after (closed bars) HCV infection, and cell growth was monitored after 4 d. Open bars represent cell growth in the absence of HCV-infection. Cell growth is presented as a percentage of mock-infected cells without inhibitors. The values presented are the means±S.D. of quadruplicate wells. The concentrations used were: cyclosporin A, 2 μM; tamoxifen, 5 μM; 7,8-benzoflavone, 2.5 μM; EGCG, 5 μM.

greater than 4 μM (Fig. 2), but in the presence of serum, the cells still showed 78% growth at 8 μM (Fig. 5).

At 2.5 μM, 7,8-benzoflavone clearly protected cells from HCV-induced CPE in serum-free medium, but was completely inactive in the presence of 5% serum. Even at 20 μM, cell growth only recovered from 6.6% to 41% in serum-supplemented medium. The cytotoxic concentrations also changed; the 50% growth inhibitory concentrations in the absence of HCV infection without and with serum were 4.2 μM and 26 μM, respectively.

Among the compounds tested, EGCG was most influenced by serum addition and increased MOI. For example, 5 μM EGCG stimulated recovery from 7% to 80% at an MOI of 1 (Fig. 3), but no significant improvement could be observed at an MOI of 4 (not shown). The highest anti-HCV activity of EGCG at an MOI of 4 was observed at 20 μM (recovery from 3.1% to 78% of control), and its activities were further attenuated by the addition of serum. In 5% serum-supplemented medium, growth only recovered from 6.1% to 15% upon the addition of 20 μM EGCG, and the optimum concentration was 80 μM or higher (Fig. 5).

The results indicate that serum proteins may mask the anti-viral activities of some compounds and that the use of serum-free medium can improve the sensitivity of a cell-based screen.

EGCG and 7,8-Benzoflavone Target Different Steps of the HCV Life Cycle To determine which step of the HCV life cycle is blocked by flavones, we examined the effect of the time of addition. Compounds were added either immediately before HCV infection or at 2 h after infection, when the entry process had presumably been completed. The activity of entry inhibitors would be expected to decrease when added after the completion of entry, whereas compounds that interfere with post-entry steps should still be effective.

The protective effects of tamoxifen and EGCG decreased when added at 2 h after infection, suggesting that these compounds mainly target the early phases of infection (Fig. 6). By contrast, the anti-HCV activities of cyclosporin A and 7,8-benzoflavone were not affected by time of addition.

Immunoblot analysis of the HCV core protein supported the results of the MTT assay. Compounds were added at various time points before and after infection, and the levels of the HCV core protein were analyzed at 48 h after infection. As shown in Fig. 7, tamoxifen and EGCG completely blocked core expression if added at or before the time of infection, but addition later than 1 h post-infection resulted in markedly decreased efficacy. On the contrary, 7,8-benzoflavone and cyclosporin A were still effective even when added 4 h after infection. Our data suggested that EGCG and tamoxifen mainly inhibited the early steps of the HCV life cycle, such as attachment and entry, whereas 7,8-benzoflavone and cyclosporin A blocked later stages.

Effects of Inhibitors on Replicon Cells We next compared the effects of cyclosporin A, tamoxifen, EGCG and 7,8-benzoflavone on cells that harbor a genotype 2a subgenomic or a genotype 1b full-genomic replicon. Cells were

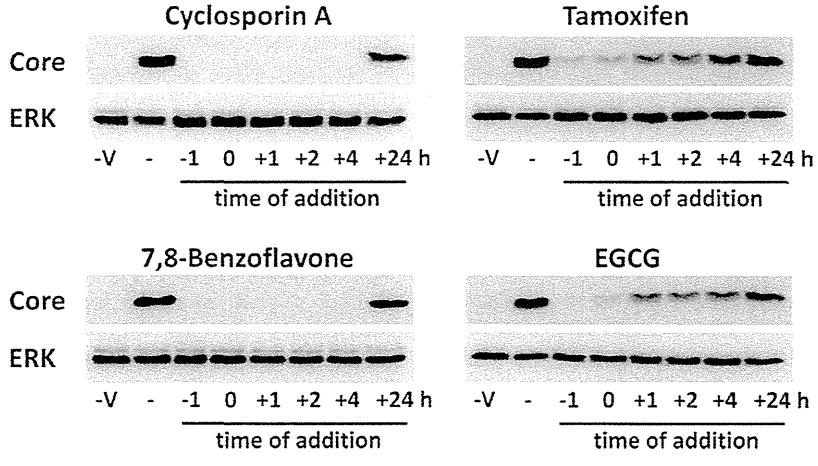

Fig. 7. The Effect of Time of Compound Addition on the Expression of Core Protein

Huh 7.5.1 cells were seeded in 24-well plates. Compounds were added at the indicated times before and after infection. Cells were fixed 48 h after infection and analyzed by immunoblotting with an anti-core antibody; anti-ERK antibody was used to confirm equal loading. −V indicates control without infection.

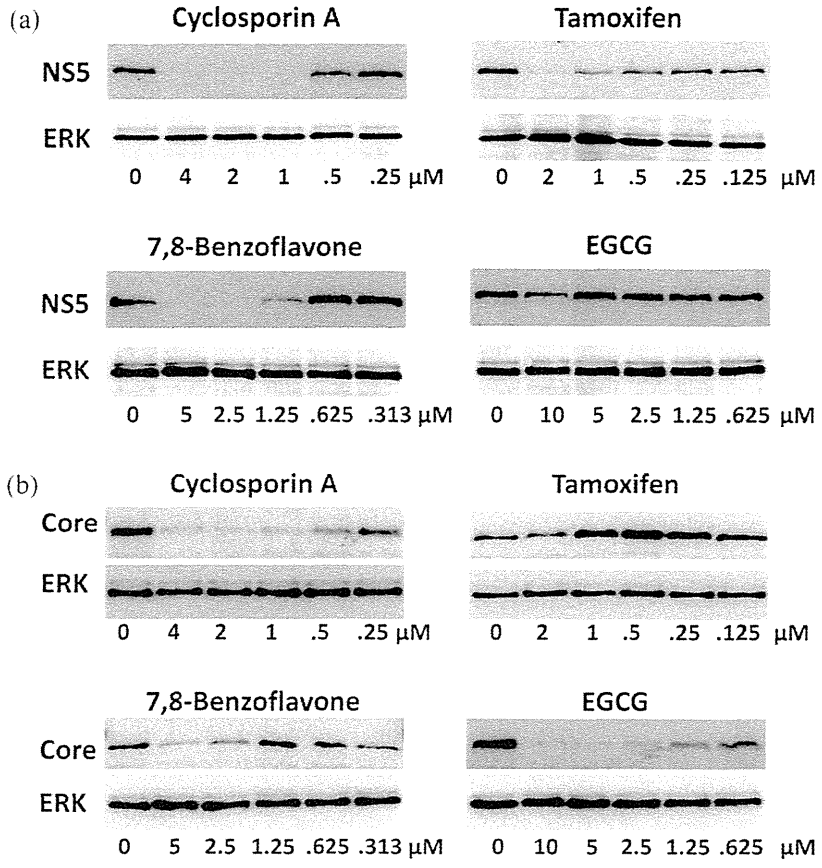

Fig. 8. The Effects of Cyclosporin A, Tamoxifen, EGCG and 7,8-Benzoflavone on the Expression of Viral Proteins in Replicon Cells

Replicon cells were treated for 72 h with the indicated concentrations of compounds and processed for immunoblotting. (a) NS5 in genotype 2a subgenomic replicon cells. (b) Core in genotype 1b full genomic replicon cells. Filters were probed with anti-ERK antibody to confirm equal loading.

treated with the inhibitory compounds for 72 h, and the levels of NS5 or core proteins were analyzed by immunoblotting. Replicon replication is completed within cells, so compounds that target steps other than RNA replication, such as attachment or entry, should not show activity. We hypothesized that cyclosporin A and 7,8-benzoflavone would be effective against replicon cells and that tamoxifen and EGCG would not be effective.

As expected, treatment with cyclosporin A and 7,8-benzoflavone noticeably reduced the level of NS5 in 2a subgenomic replicon cells (Fig. 8a). EGCG showed little effect, but tamoxifen appeared to show a moderate level of activity. In the genotype 1b full-genomic replicon cells, cyclosporin A and 7,8-benzoflavone also reduced the amount of core protein, although 7,8-benzoflavone appeared to be somewhat less effective (Fig. 8b). However, tamoxifen was completely inactive and even appeared to enhance core expression at some concentrations. Unexpectedly, EGCG displayed considerable activity, implying that it may inhibit stages of the HCV life cycle other than entry. The results indicate that the efficacy and even the mechanism of action of inhibitors may differ with viral genotype.

DISCUSSION

We established a microtiter plate method for anti-HCV drug discovery that measures the increased viability of infected cells. This method enables the screening of inhibitors that target all stages of the HCV life cycle, including steps that cannot be recognized by replicon systems, such as viral attachment, entry, and egress. An assay based on a similar principle using an engineered cell line has recently been described.[7,16] In that study, an HCV NS3-4A protease-cleavable derivative of Bid, which renders cells highly susceptible to HCV infection, was expressed in Huh 7 cells to increase the sensitivity of the assay. Here, we showed that the use of serum-free medium substantially enhanced HCV-induced CPE, and obviated the need for specially engineered cells. The mechanism of increased CPE in serum-free medium is not clear, but it is likely that serum proteins and/or lipids confer resistance to HCV-infection.

Many compounds are known to bind to serum proteins and may not show activity in a cell-based assay in the presence of high concentrations of serum. For example, EGCG has been reported to form water-soluble complexes with bovine serum albumin.[17] We showed that the anti-HCV activity of EGCG is diminished in the presence of serum. Thus, serum-free medium offers various benefits to screening programs. It increases the sensitivity of the assay by allowing the use of lower MOIs and by enabling the detection of compounds with high serum protein-binding capacity. Compounds that bind to serum proteins with high affinity may have undesirable

pharmacodynamic properties, but the object of primary screening is to identify as many potentially active compounds as possible, and pharmacodynamic properties can be improved by chemical modifications. Although serum-free medium does have certain negative aspects such as enhanced toxicity of some compounds, we anticipate that its use will expand the range of identified compounds and increase the possibility of discovering anti-HCV drug candidates.

In our trial screening, we identified active compounds with several different modes of action, including the plant flavonoids EGCG and 7,8-benzoflavone. The green tea catechin EGCG is most likely the best-studied plant flavonoid, and it is known to exert multiple biological effects. It has been reported to inhibit various viruses through a number of mechanisms. The documented anti-viral activities of EGCG include the inhibition of the influenza virus,[18] human immunodeficiency virus type 1 (HIV-1),[19,20] Epstein–Barr virus,[21,22] herpes simplex virus,[23] and hepatitis B virus.[24] *In vitro*, EGCG inhibited various viral enzymes, such as adenovirus protease adenain,[25] influenza A RNA polymerase,[26] and HIV-1 integrase.[27] EGCG bound to CD4 on T cells and prevented its interaction with HIV gp120.[28] EGCG has been shown to possess activity against the HCV NS3 serine protease[29] and NS5B,[30] although the actual impact of these effects on HCV replication have not been confirmed.

EGCG has recently been reported to inhibit the entry of HCV.[10] EGCG inhibited the entry of all genotypes tested (1a, 1b and 2a), but it was much less active against vesicular stomatitis virus (VSV) entry. The present study also suggested that the attenuation of the replication of genotype 2a HCV by EGCG is mainly due to entry inhibition. We tested the effect of EGCG on the entry of other genotypes using pseudoparticles and, as reported, observed that it inhibited the entry of all HCV genotypes (not shown). In our hands, however, the entry of VSV was blocked at comparable concentrations, demonstrating that EGCG was not specific to HCV. It has also been reported that EGC, EC, and ECG had no obvious inhibitory activity on HCV.[10] Although EGC and EC were not effective in our assay, ECG and the other gallate-type catechins GCG and CG showed anti-HCV activities similar to that of EGCG. Time-of-addition experiments suggested that these gallate-type catechins all mainly target viral entry, at least for genotype 2a HCV (not shown). It is likely that the gallate moiety is essential for the anti-HCV activity of catechins. The influence of the third hydroxyl group in the B-ring appeared to be marginal, at least in the present study.

EGCG had little effect on the expression of HCV proteins in genotype 2a replicon cells, a context that does not involve viral entry, further supporting the theory that EGCG is an entry inhibitor. However, EGCG substantially reduced the level of HCV proteins in genotype 1b replicon cells, implying that it may also target post-entry phases. Given that green tea catechins display diverse biological effects, it is conceivable that the mechanism of anti-HCV action is multimodal and that the main target may differ with genotypes.

In contrast to EGCG, 7,8-benzoflavone inhibited HCV replication even when added after the completion of the entry phase, and it effectively reduced the amount of NS5 in genotype 2a replicon cells, suggesting that it acts on post-entry phases. Furthermore, 7,8-benzoflavone displayed genotype selectivity and was not as active against the genotype 1b replicon. 7,8-Benzoflavone has been reported to inhibit aromatase[31] and breast cancer resistant protein (BCRP)[32] and modulate aryl hydrocarbon receptor (AhR) signaling.[33,34] Whether any of these biological activities are associated with the anti-HCV activity of 7,8-benzoflavone remains to be elucidated.

Other plant flavonoids with reported anti-HCV activity include naringenin[35] and quercetin.[36] Although the mechanisms of action of these compounds are not completely defined, naringenin acted as a PPAR α agonist to block virus assembly.[37] The inhibition of HCV particle production by quercetin was attributed at least partially to the reduction of HSP40 and HSP70 that is potentially involved in IRES translation.[36] More recently, quercetin was found to inhibit NS3 activity.[38] In our assay, 7,8-benzoflavone was more active than naringenin or quercetin in inhibiting HCV (not shown). We are currently testing various plant flavonoids to gain insight into the structure/activity relationship.

In conclusion, using the JFH1-HCV viral culture system, we developed a simple and cost-effective microplate colorimetric assay that will allow the high-throughput screening for HCV inhibitors that target various phases of the viral life cycle. The use of serum-free medium sensitized Huh 7.5.1 cells to HCV-induced CPE and eliminated the need for specially engineered cells or viruses. We anticipate that this assay will facilitate the discovery of compounds with anti-HCV activity and assist drug development. We have extended the screening to large chemical libraries and revealed anti-HCV activity in a wide variety of compounds.

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Replication of Hepatitis C Virus Genotype 3a in Cultured Cells

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See Covering the Cover synopsis on page 1; see editorial on page 13.

Hepatitis C virus (HCV) genotype 3a is widespread worldwide, but no replication system exists for its study. We describe a subgenomic replicon system for HCV genotype 3a. We determined the consensus sequence of an HCV genome isolated from a patient, and constructed a subgenomic replicon using this clone. The replicon was transfected into HuH-7 cells and RNA replication was confirmed. We identified cell culture-adaptive mutations that increased colony formation multiple-fold. We have therefore established a genotype 3a replicon system that can be used to study this HCV genotype.

Keywords: Virology; Experimental Model; HCVGT3; In Vitro Culture System.

Hepatitis C virus (HCV) infection leads to chronic infection and advanced liver diseases in most infected adults.[1] Of the 6 major HCV genotypes, genotypes 1 and 2 are the most prevalent in North America, Europe, and Japan,[2,3] and are the most highly studied. However, other genotypes display specific characteristics. For example, genotype 3a infection can result in hepatic steatosis[4] and telaprevir and boceprevir are less effective against genotype 3a.[5] Therefore, the pathogenesis and inhibitor sensitivity of all HCV genotypes should be studied. Although HCV subgenomic replicons are useful for understanding viral/host factors involved in HCV replication and inhibitor sensitivity, only HCV replicons for genotypes 1a, 1b, and 2a have been established.[6–9] Here, we report on the robust genotype 3a replication system.

An almost complete HCV genome was recovered from the serum of a patient with post-transplantation recurrent HCV infection. This serum exhibited higher infectivity than other tested sera toward primary human hepatocytes (Supplementary Figure 1*A*). The isolate, named S310, contained the following structural elements: a 5′UTR (nt 1-339), an open reading frame encoding 3021 aa (nt 340-9402), and a 3′UTR (nt 9403-9654). Only the last 44 nt of the X-region (nt 9611-9654) could not be recovered. Two major virus populations were found; S310/A contained Ala, Thr, Thr, and Ile, and S310/B contained Thr, Ala, Ala, and Thr, at the 7th, 151st, 431st, and 472nd aa of the NS3 protein, respectively. S310 was clustered into genotype 3a by phylogenetic analysis (Supplementary Figure 1*B*). The complexity of the virus quasispecies in the serum was analyzed by sequencing the hypervariable region. Identical amino acid sequences in all 10 hypervariable region clones indicated a very low degree of diversity. The hypervariable region sequence of the JFH-1 strain also exhibited monoclonality,[10] which can be important for efficient replication in cultured cells.

Subgenomic replicons SGR-S310/A and SGR-S310/B were constructed and their replication efficiency was evaluated by G418-resistant colony-formation assay. After 3 weeks, a small number of colonies were visible for both replicons (Figure 1*A*). Because more colonies were observed in SGR-S310/A than in SGR-S310/B, we focused on SGR-S310/A (henceforth called SGR-S310). Ten cell colonies of SGR-S310 were isolated and analyzed for HCV replication. The mean RNA titer was $9.1 \times 10^7 \pm 4.6 \times 10^7$ copies/μg total RNA (Figure 1*B*). HCV RNA (approximately 8 kb) was detected by Northern blotting (Supplementary Figure 2*A*). Viral proteins in the replicon cells were detected by immunofluorescence and Western blotting (Supplementary Figure 2*B* and 2*C*). To determine whether the G418 resistance of the cells was transmissible by cellular RNA transfection, we electroporated total cellular RNA isolated from 4 replicon clones into naïve HuH-7 cells. Multiple G418-resistant colonies appeared after transfection of the RNA isolated from the replicon clones (Supplementary Figure 3*A*), but not from the naïve HuH-7 cells. These results indicate that the replicon RNA in the parental colonies could replicate in naïve cells. Thus, the G418-resistant colonies that were isolated from cells electroporated with SGR-S310 synthetic RNA contained replicating viral RNA.

Replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential. To examine whether SGR-S310 acquired mutations, the complete HCV sequences from 10 replicon clones were sequenced. At least one nonsynonymous mutation was detected in the NS3-NS5B region of each replicon clone (Figure 1B). The following mutations were identified: T1286I in the NS3 helicase (6 of 10

Abbreviation used in this paper: HCV, hepatitis C virus.

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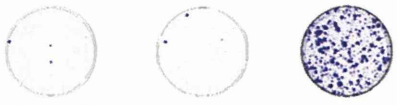
A



B

Replicon cell clone	Nucleotide mutation	Amino acid substitution	Affected protein	Replicon RNA amounts (copies/μg RNA)
1	5272 (6932) G→A	2198 R→H	NS5A	2.1×10^8
2	7362 (9022) A→G	2895 R→G	NS5B	9.9×10^7
3	2536 (4196) C→T	1286 T→I	NS3	9.5×10^7
4	2536 (4196) C→T	1286 T→I	NS3	9.9×10^7
5	2536 (4196) C→T	1286 T→I	NS3	5.6×10^7
6	2536 (4196) C→T	1286 T→I	NS3	4.0×10^7
7	2536 (4196) C→T	1286 T→I	NS3	8.3×10^7
8	2536 (4196) C→T	1286 T→I	NS3	8.7×10^7
9	6166 (7826) C→T 7363 (9023) G→A	2496 T→I 2895 R→K	NS5B NS5B	9.3×10^7
10	5241 (6901) A→G	2188 T→A	NS5A	5.1×10^7

C

Replicon constructs	Protein	Colony formation efficiency (cfu/μg RNA)	Transient replication efficiency (72h/4h†)
SGR-S310/A	None	0.4×10^0	4.4×10^{-3}
T1286I	NS3	2.9×10^3	6.5×10^{-3}
T2188A	NS5A	2.3×10^2	4.9×10^{-3}
R2198H	NS5A	3.7×10^3	6.0×10^{-2}
S2210I	NS5A	6.2×10^2	1.1×10^{-2}
T2496I	NS5B	0.2×10^0	5.0×10^{-3}
R2895K	NS5B	2.3×10^3	7.9×10^{-2}
R2895G	NS5B	9.8×10^2	2.6×10^{-2}
T2496I+R2895K	NS5B	4.1×10^3	2.6×10^{-1}
Con1-NK5.1	NS3+NS5A	9.1×10^2	6.3×10^0
N	None	NA††	7.1×10^{-2}
SGR-JFH1	None	5.3×10^3	1.5×10^2

Figure 1. S310 subgenomic replicon analysis. (*A*) Three million HuH-7 cells were electroporated with 10 μg RNA from SGR-S310/A or SGR-S310/B or 0.1 μg RNA from SGR-JFH1. G418-selected colonies were fixed and stained after 3 weeks. (*B*) Non-synonymous mutations identified in the replicon genomes and HCV RNA titers in the replicon clones. Nucleotide positions within the S310 subgenomic replicon and within the full-length S310 genome (in parentheses) are given. (*C*) Replication potential of the adaptive mutants as determined by the colony-formation assay using Neo-replicons and by the transient replication assay using Fluc-replicons. †72 h/4 h, transient replication efficiency was determined as a ratio of luciferase activity in the transfected cells between 72 h and 4 h post transfection. ††NA, not available.

clones); T2188A or R2198H in NS5A (2 clones); an R2895G substitution in NS5B (1 clone); and T2496I in NS5A plus R2895K in NS5B (1 clone). These mutations and the S2210I mutation (corresponding to S2204I in genotype 1 replicon)[7,8] were introduced, individually or in combination, into the parental SGR-S310 and the colony-formation efficiencies of the mutant replicons were tested. All mutations, except T2496I, increased the colony formation, indicating an adaptive phenotype (Figure 1*C*, Supplementary Figure 3*B*). Transient replication efficiency was also tested using firefly luciferase reporter replicons. SGR-S310/Luc did not replicate in Huh-7.5.1 cells, whereas the adaptive mutants displayed varying degrees of replication (Figure 1*C*, Supplementary Figure 3*C*). Adaptive mutations T2496I and R2895K, when combined together, most efficiently enhanced the colony formation as well as transient replication (Figure 1*C*). Interestingly, T1286I and R2895G found in our study correspond to the Con1 adaptive mutations T1280I and R2884G, respectively.[11,12] T2188A or R2198H in NS5A were identified in 2 replicon clones and are located close to S2210I. Indeed, S2210I also enhanced SGR-S310 replication, suggesting that this region might be important for HCV replication. S310 replicons with adaptive mutations were compared with genotype 1b (Con1 and N) and 2a (JFH-1) replicons. Colony-formation efficiencies of most S310 adaptive replicons were at levels comparable with Con1 and JFH-1 (Figure 1*C*, Supplementary Figure 3*B*). In contrast, S310 adaptive replicons replicated less efficiently than Con1-NK5.1 and JFH-1 replicons in transient replication assays. However, genotype 1b N replicon replicated at a level similar to some S310 adaptive replicons (Figure 1*C*, Supplementary Figure 3*C*). Future studies will dissect the detailed mechanisms that underlie the effects of these mutations.

Successful generation of a genotype 3a replicon provided a unique opportunity to compare the susceptibility of genotype 3a (SGR-S310), 1b (Con1[13]), and 2a (JFH-1/4-1[13]) replicons to HCV inhibitors. Interferon-alfa dose-dependently decreased the replication of all tested genotypes (Figure 2*A*), whereas a protease inhibitor, BILN-2061, was more effective against replicons from genotypes 1b and 2a than 3a (Figure 2*B*). The non-nucleoside polymerase inhibitor JTK-109 was more potent against genotype 1b and 3a (Figure 2*C*). However, the nucleoside polymerase inhibitor, PSI-6130, equally inhibited all genotypes (Figure 2*D*).

In conclusion, we established a subgenomic replicon for genotype 3a, which should be useful for understanding the specific characteristics of this genotype and for the screening of antiviral chemicals that are effective against this genotype. Construction of a full-length infectious S310 clone is in progress.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.09.017.

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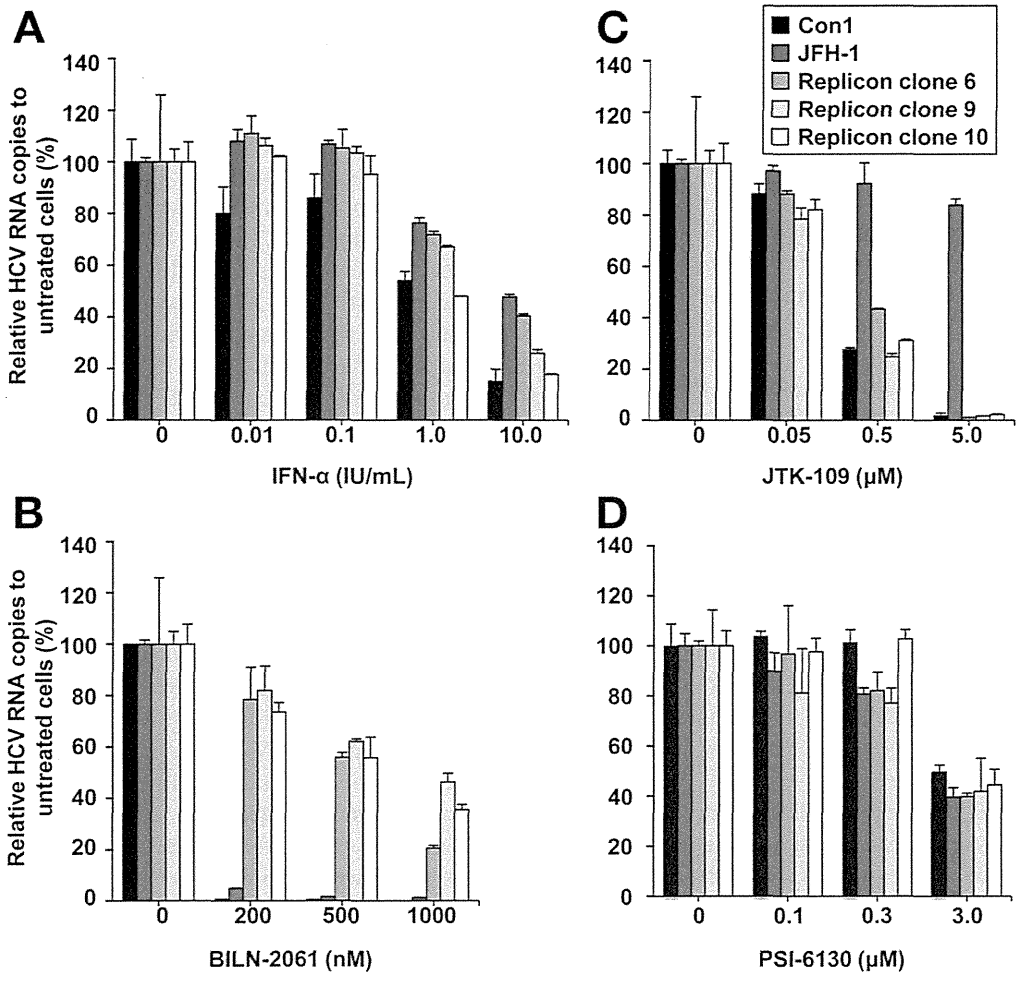

Figure 2. Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) interferon alpha, (B) HCV protease inhibitor BILN-2061, (C) the non-nucleoside polymerase inhibitor JTK-109, and (D) the nucleoside polymerase inhibitor PSI 6130 for 72 hours and replication levels were measured by quantifying intracellular HCV RNA. Results are means ± standard deviations of 3 replicates.

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Reprint requests

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DDBJ/EMBL/GenBank accession numbers: S310/A: AB691595, S310/B: AB691596, SGR-S310/A: AB691597, SGR-S310/B: AB691598, SGR-S310/Luc: AB691599.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Methods

Cell Culture

The human hepatoma cell line HuH-7[1] and its derivative cell line Huh-7.5.1[2] were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a 5% CO_2 incubator.

Primary human hepatocytes (PHH) were isolated from an encapsulated liver sample.[3] Isolated PHH were seeded in 12-well plates and cultured at 37°C in Lanford medium before infection.

PHH Infection With HCV-Positive Sera

Three days post seeding, PHH were inoculated with HCV-positive sera. After 16 h of inoculation, monolayers were washed with William's E medium and fresh Lanford medium was added. Cells were harvested at 72 h post infection. Total RNA was isolated using a guanidinium isothiocyanate solution (RNAble; Eurobio, Courtaboeuf, France) and intracellular levels of HCV RNA were quantified using the SuperScript III Platinium One-Step quantitative reverse transcription polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, CA) and a LightCycler480 real-time PCR system (Roche Diagnostics, Meylan, France).

HCV Genotype 3a Clone

Clone S310 was isolated from a 71-year-old female patient suffering from post liver transplantation HCV recurrence. She was diagnosed with HCV genotype 3a infection at the age of 59 years and underwent liver transplantation 4 years later due to liver cirrhosis. HCV-RNA titer was 2.8×10^6 copies/mL. Total RNA extracted from 100 μL serum using the acid-guanidinium isothiocyanate-phenol-chloroform method (Isogen-LS; Nippon Gene, Tokyo, Japan) was precipitated with isopropanol, washed with ethanol, and dissolved in 10 μL nuclease-free water. An aliquot of 4 μL was subjected to reverse transcription using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III; Invitrogen) at 42°C for 50 min and then at 50°C for 10 min.

Isolation of HCV

The sequences of 4 isolates of genotype 3a (accession numbers AF046866, D28917,[4] X76918, and D17763[5]) that were obtained from the HCV database (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html) were aligned and PCR primers were designed based on the conserved sequences. These primers were used to amplify the complementary DNA (cDNA) of S310 into 9 overlapping fragments by nested PCR (nt 1–370, nt 127–1284, nt 1117–1997, nt 1704–3352, nt 3152–5080, nt 4869–6842, nt 6601–8129, nt 7988–9145, and nt 9082–9576; nucleotide numbers refer to the positions on S310, with nt 1 being the first nucleotide of the 5′ UTR). The sequence of these primers is shown in Supplementary Table 1. Two microliters of cDNA was subjected to PCR using Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan) and the outer set of primers, and this first-round PCR product (2 μL) was further amplified by a second round of PCR using the inner set of primers. PCR conditions for the first and second rounds of PCR consisted of 35 cycles each of denaturation at 98°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min. A fragment encompassing the 5′ end of the viral genome (nt 1–370) was amplified by 5′RACE. Briefly, cDNA was synthesized with a 5′ UTR primer (antisense), tailed with a dCTP homopolymer by using terminal deoxynucleotidyl transferase, and amplified by PCR (5′ RACE System for Rapid Amplification of cDNA Ends; Invitrogen) using TaKaRa LA Taq polymerase (Takara Bio). The PCR products of all fragments were separated by agarose gel electrophoresis, cloned into the pGEM-T EASY vector (Promega, Madison, WI) and sequenced using the Big Dye Terminator Mix and an automated DNA sequencer. The consensus sequence of 5 to 9 isolated cDNA clones was adopted for each fragment. Two major populations of the virus were identified in the patient's serum that differed in 4 amino acids in the NS3 protein (aa 1039, 1183, 1463, and 1504), and these populations were designated as S310/A and S310/B (DDBJ/EMBL/GenBank accession number: AB691595 and AB691596, respectively). To assess the complexity of the HCV population in the patient's serum, the hypervariable region sequences of 10 clones were determined.

Computer Analysis

A phylogenetic tree was constructed using the neighbor-joining method to examine the relationship between the polyprotein region of S310 and that of other HCV genotype 3a isolates available in the database. In order to analyze the diversity in each subgenomic region, the genetic distance was calculated between all possible pairs of genotype 3a isolates and between S310/A and other isolates using MacVector software (MacVector, Inc., Cary, NC). The ratios of these 2 values (mean genetic distance between S310/A and other isolates/mean genetic distance among all genotype 3a isolates) were compared.

Construction of Replicons

Based on the consensus sequence of S310, we assembled pS310/A and pS310/B, which contained the full-length S310/A and S310/B cDNA, respectively, downstream of the T7 RNA polymerase promoter. Briefly the 9 amplicons described here were combined by overlapping PCR and ligated with pGEM-T EASY vectors to generate 6 plasmids (A through F) in such a way that each plasmid contained a unique restriction enzyme

cleavage site toward the 3′ end of the viral fragment, which overlapped with the 5′ end of the next fragment. For this purpose, we took advantage of the EcoRI restriction site that is present in the polycloning site of the plasmid toward the 5′ end of the viral fragment. Plasmid A contained the T7 promoter sequence followed by one G-nucleotide and nt 1–3352 of S310, while plasmids B, C, D, and E contained nt 1704–4307, nt 4044-6013, nt 5424–7755, and nt 7276–9425, respectively. Plasmid F contained the fragment constructed by combining the C-terminal end of NS5B (nt 9182–9402) and the variable and poly U/UC regions of the S310/A 3′UTR (nt 9403–9610) with the last 44 nucleotides of JFH-1. Restriction sites for EcoRI and XbaI were introduced upstream of the T7 promoter sequence and downstream of the conserved region, termed the *X-region*, of the 3′UTR, respectively, and the restriction sites of these enzymes that were present within the cDNA were removed by PCR-based mutagenesis. In the neomycin-based subgenomic replicons (SGR-S310/A and SGR-S310/B, accession number: AB691597 and AB691598, respectively), the cassette containing the neomycin phosphotransferase gene and the EMCV IRES replaced the region of S310 that encompasses amino acids 20–1032. Firefly luciferase–based subgenomic replicons (SGR-S310/Luc, accession number: AB691599) were generated from SGR-S310/A by replacing amino acids 20–1032 of S310/A with the cassette containing firefly luciferase and the EMCV IRES from pSGR-JFH1/Luc.[6]

RNA Synthesis

RNA was synthesized by in vitro transcription as described previously.[7] Briefly, the plasmids carrying the cDNA described here were linearized with the XbaI restriction enzyme and 5′ overhangs were removed by treating with mung bean nuclease. Reaction mixtures were further incubated at 50°C for 1 h with 2 μL 20 mg/mL proteinase K and 10 μL 10% sodium dodecyl sulfate to degrade nucleases, and templates were purified with 2 rounds of phenol-chloroform extraction and ethanol precipitation. Three micrograms of templates were subjected to in vitro transcription using a MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Synthesized RNA was treated with DNase I (Ambion) and then purified using ISOGEN-LS (Nippon Gene). The quality of the synthesized RNA was examined by agarose gel electrophoresis.

RNA Transfection

In vitro transcribed RNA or total cellular RNA isolated from replicon cells was introduced into cells by electroporation. Trypsinized cells were washed twice with serum-free Opti-MEM I (Invitrogen) and 3.0×10^6 cells were resuspended in 400 μL cytomix buffer.[8] RNA was delivered into cells by a single pulse of 260 V and 950 μF using the Bio-Rad Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately suspended in culture medium and transferred to the appropriate plates. For G418 selection of colonies, the transfected cells were seeded in 10-cm dishes, each containing 8 mL culture medium. G418 (500 μg/mL; Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 24 h after transfection. Culture medium supplemented with G418 was replaced every 3 days. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet or replicon colonies were picked and expanded.

Analysis of G418-Resistant Cells

G418-resistant colonies were collected and used for further analysis. Colonies were independently isolated using cloning cylinders (Asahi Techno Glass Co., Tokyo, Japan) and were expanded until they were 80%–90% confluent in 10-cm dishes. Expanded cells were harvested for nucleic acid and protein analysis. Total RNA was isolated from the cells using the ISOGEN reagent (Nippon Gene). Another aliquot of the cell pellet was dissolved in RIPA buffer containing 0.1% sodium dodecyl sulfate for Western blot analysis. For immunofluorescence analysis of viral proteins, cells were seeded on 12-well slides.

Quantification of HCV RNA by Real-Time RT-PCR

Copy numbers of HCV RNA were determined by real-time detection RT-PCR, as described previously,[9] using the ABI Prism 7700 Sequence Detector System (Applied Biosystems Japan, Tokyo, Japan). The concentration of total RNA in the cells was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockford, IL).

Northern Blot Analysis

Isolated RNAs (3 μg) from replicon cells were separated on a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK Ltd., Buckinghamshire, UK) and immobilized using a FUNA-UV-LINKER (Funakoshi, Tokyo, Japan). Hybridization was carried out with a [α-^{32}P]dCTP-labeled DNA probe using Rapid-Hyb buffer (GE Healthcare UK Ltd.). The DNA probe was synthesized from a BsrGI-MfeI fragment of the S310 clone that contained NS3-5B genes using the Megaprime DNA labeling system (GE Healthcare UK Ltd.).

Indirect Immunofluorescence

Untransfected HuH-7 cells or S310 replicon-replicating cells were grown on a glass slide for 24 h and fixed in acetone-methanol (1:1 [vol/vol]) for 10 min at −20°C. Cells were then incubated in immunofluorescence buffer (phosphate-buffered saline, 1% bovine serum albumin, 2.5 mM EDTA). S310 patient serum was added at

a dilution of 1:200 in immunofluorescence buffer. After incubation for 1 h at room temperature, cells were washed and then incubated with an Alexa Fluor488–conjugated goat anti-human IgG antibody (Invitrogen) in immunofluorescence buffer. The glass slide was washed and a cover glass was mounted using PermaFluor mounting solution (Thermo Scientific, Cheshire, UK). Cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis of HCV Proteins

The protein samples were separated on 12.5% polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). Transferred proteins were incubated with 2% skim milk. Anti-NS3 mouse monoclonal antibody (clone 8G2, Abcam, Cambridge, UK) and peroxidase-labeled sheep anti-mouse IgG (Cell Signaling Technology, Danvers, MA) were used to detect HCV proteins. The signals were detected with a chemiluminescence system (ECL Prime; GE Healthcare UK Ltd.).

Identification of Mutations

cDNA was synthesized from total RNA that was extracted from replicon-expressing cells at 2 different times. These cDNAs were amplified into 5 overlapping fragments that spanned the 5′UTR and the NS3-NS5B region using LA Taq DNA polymerase (Takara Bio) and the primers described in Supplementary Table 1. The sequence of each amplified DNA was determined. The mutations identified were subsequently introduced into SGR-S310/A and SGR-S310/Luc by PCR-mediated mutagenesis.

Luciferase Assay

Five micrograms of RNA, prepared by in vitro transcription of S310/SG-FLuc constructs with or without adaptive mutations, were introduced into 3.0×10^6 Huh-7.5.1 cells by electroporation. Cells were harvested with Cell Culture Lysis Reagent (Promega) at 4, 24, 72, and 96 h post electroporation, and luciferase activity was determined by use of a Luciferase Assay System (Promega) and the Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany).

Inhibition of S310 Replicon Replication by Specific Inhibitors

S310 replicon cell clones 6, 9, and 10 and the genotype1b Con1 and 2a JFH-1 replicon cells,[10] were seeded into 24-well plates at a density of 5.0×10^4 cells/well. On the next day, the culture medium was replaced with medium containing 0.1% dimethyl sulfoxide with or without various concentrations of interferon alfa (Dainippon-Sumitomo, Osaka, Japan), the specific NS3 protease inhibitor, BILN-2061 (Boehringer Ingelheim Ltd., Québec, Canada), or the NS5B inhibitors, JTK-109 (Japan Tobacco, Inc., Osaka, Japan) and PSI-6130 (Pharmasset, Inc., Princeton, NJ). After 72-h incubation, cells were harvested and HCV RNA was quantified as described.

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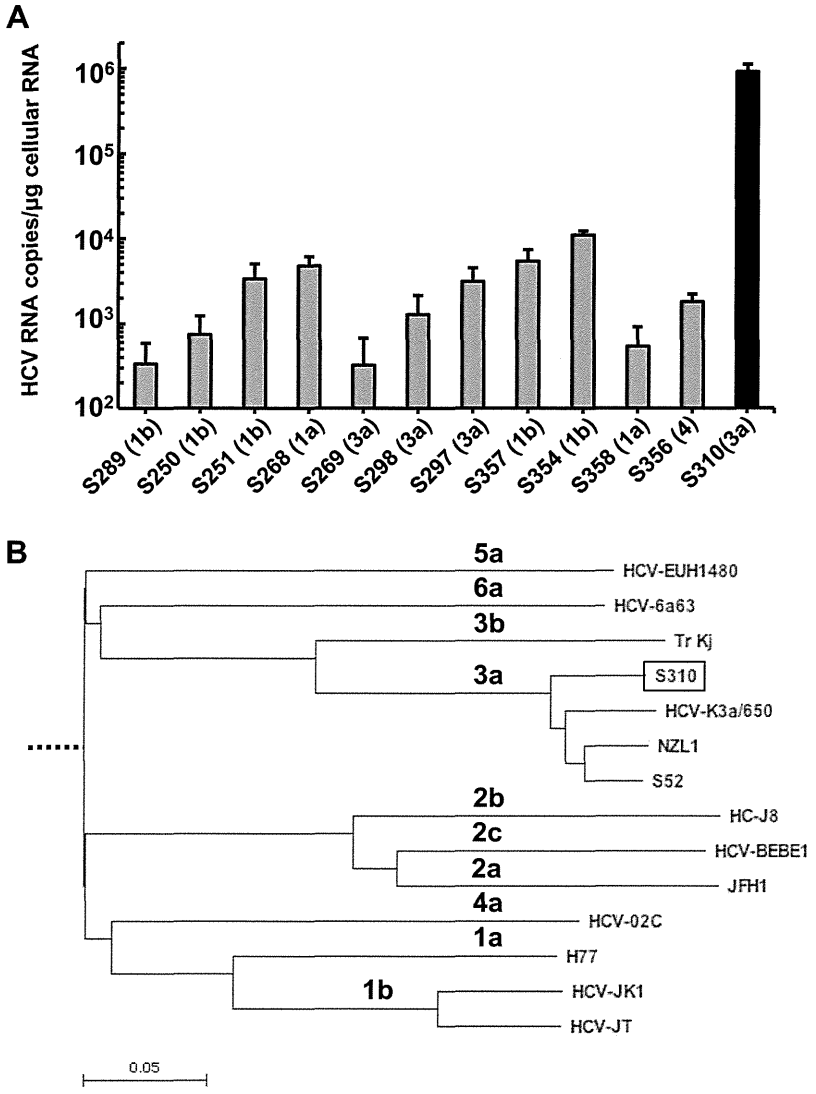
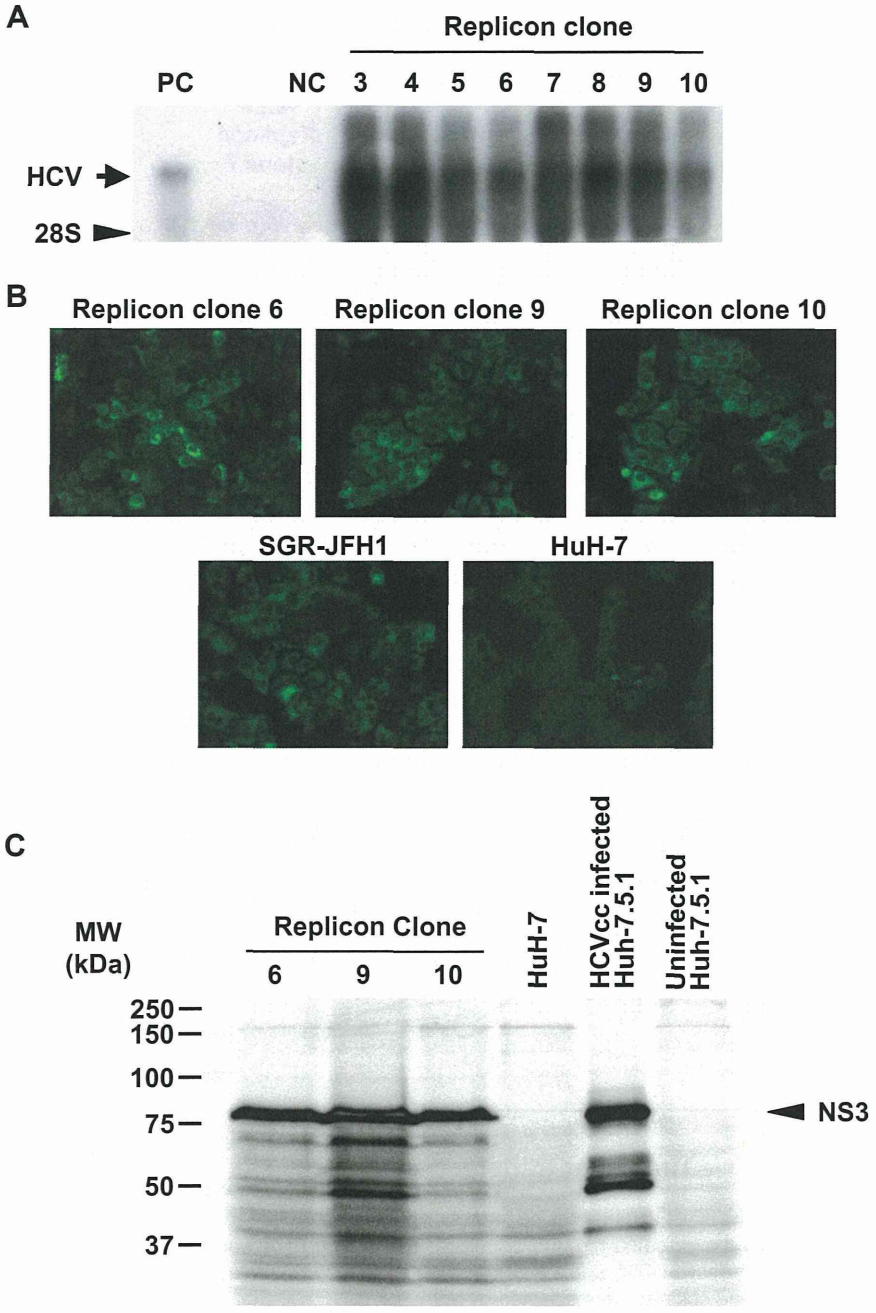
Supplementary Table 1. Primers for Amplification of the S310 HCV Strain

Fragment			Primer sequence (5′→3′)
1 (5′ RACE)[a]	Outer	Antisense	CTTGACGTCCTGTGGGCGA
	Inner	Antisense	TTTTTCTTTGGGGTTTAGG
2	Outer	Sense	GTCTTCACGCGGAAAGCGC
		Antisense	CACCCAAACCACCGACCAC
	Inner	Sense	CCGGGAGAGCCATAGTGGTC
		Antisense	TCCTGAAAGATGGCCTGGGTA
3	Outer	Sense	CTTGGCCCCTCTATGGTAA
		Antisense	GATGTTTCCTGAAGCAGTCG
	Inner	Sense	AGTCATGTGGACCTATTAGT
		Antisense	CACCCAAACCACCGACCAC
4	Outer	Sense	ATGGCTCGTGGCACATCAA
		Antisense	TAGTCATCAGCAGGTCCCAA
	Inner	Sense	GCTCAGCAGCTGCAAGCCCAT
		Antisense	CGCAAAGAATATCTCCGCAAG
5	Outer	Sense	ATTTTTGACATCACTAAGCTAC
		Antisense	AGTGTTGGCTTAAGCCGCA
	Inner	Sense	AATACTTCCAGATGATCATACT
		Antisense	GTGACAGAAAGTGGGCAT
6	Outer	Sense	GTTTCCCGCAGCCAACGT
		Antisense	GTCTCTCAACATCGAGGT
	Inner	Sense	CGGTGAAAGACCGTCTGGA
		Antisense	CAGGGGAGTTGAGATCCT
7	Outer	Sense	GGCCGCGTACATGTGCTAAC
		Antisense	CCGCAGACAAGAAAGTCCGGGT
	Inner	Sense	CTATGGCGCGTGGCTGCCA
		Antisense	ACCCCCAGGTCAGGGTACAC
8	Outer	Sense	CATAACCTAGTCTATTCAACG
		Antisense	TGGTCTTGGTGCGTACCG
	Inner	Sense	GCTCCGTCTGGGAGGACTTGC
		Antisense	CTCGTGCCCGATGTCTCCAA
9	Outer	Sense	TGCTCCTCCAACGTCTCCGT
		Antisense	GCGGCTCACGGACCTTTCAC
	Inner	Sense	GTCGCGGGGACACTCAGGAA
		Antisense	ACTAGGGCTAAGATGGAGCC

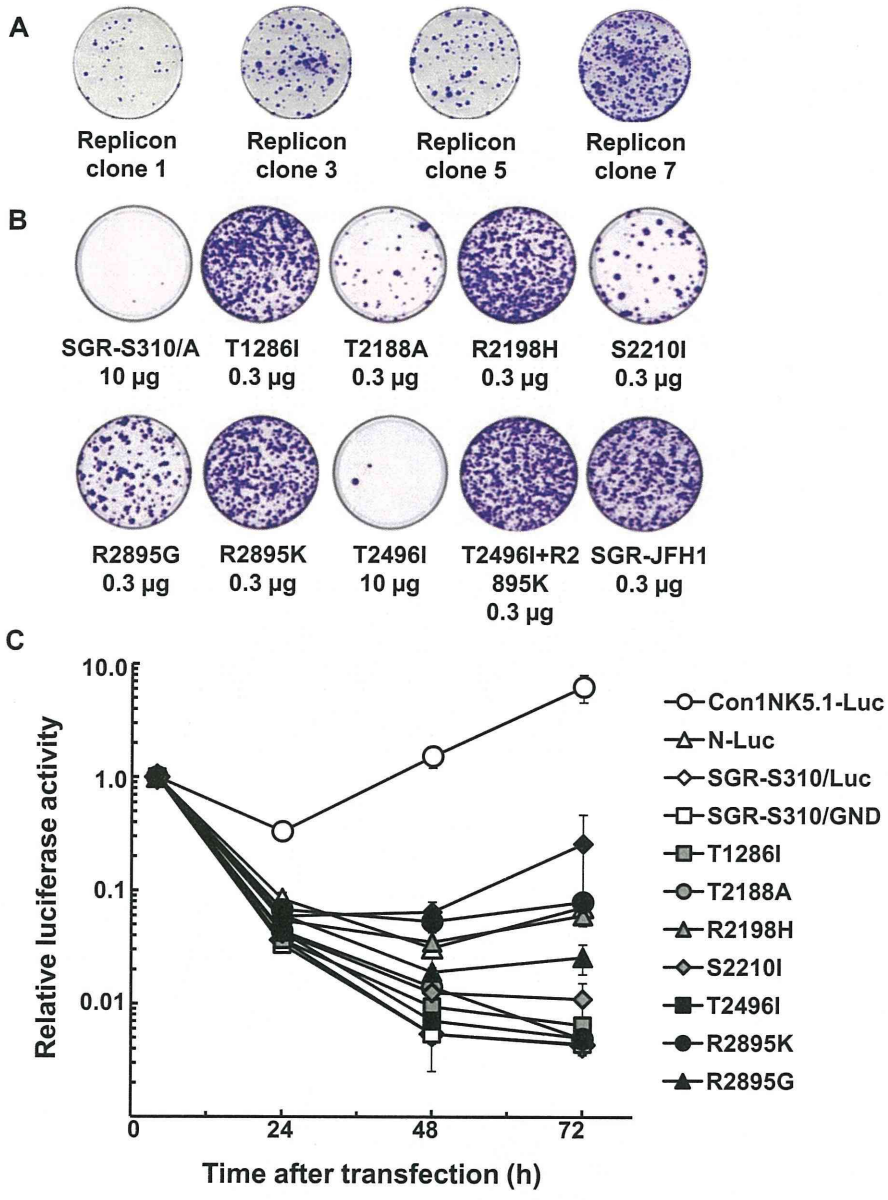
RACE, rapid amplification of complementary DNA ends.
[a]Forward primers used were those in the 5′RACE kit (Abridged Universal Amplification Primer (AUAP) for the first round of PCR and Universal Amplification Primer (UAP) for the second round of PCR).



Supplementary Figure 1. Infection of PHH with HCV patient sera and phylogenetic tree analysis of the S310 strain. (*A*) PHH were exposed to sera of patients infected with genotype 1b (S289, S250, S251, S357, S354), 1a (S268, S358), 3a (S269, S298, S297, S310), and 4 (S356) for 16 h (25 µL/well, except for S310, 10 µL). Intracellular HCV RNA was quantified 72 h post inoculation. Experiments were done in triplicate and data are presented as means ± standard deviation. (*B*) The phylogenetic tree was constructed using the polyprotein region of S310 and HCV strains of different genotypes. The HCV strains analyzed and their corresponding GenBank accession numbers are: K3a/650; D28917, NZL1; NC_009824, S52; GU814263, EUH1480, HCV-6a63; DQ480514, Tr Kj; D49374, HC-J8; D10988, BEBE1; D50409, JFH-1; AB047639, HCV-02C; DQ418784, H77; AF009606, HCV-JK1; X61596 and HCV-JT; D11168. The root of the tree was tentatively taken as the midpoint of the longest path. The length of the horizontal bar indicates the number of nucleotide substitutions per site.



Supplementary Figure 2. Detection and quantification of HCV RNA and proteins in replicon cells. (*A*) Total RNA (3 μg) from replicon cells was analyzed by Northern blot; 5.0×10^7 copies of in vitro–transcribed RNA were loaded in parallel as a positive control (PC), while total RNA from untransfected HuH-7 cells served as the negative control (NC). Replicon RNA was detected using a [α-^{32}P]dCTP-labeled DNA probe. Arrow and arrowhead indicate the positions of the replicon RNA and 28S ribosomal RNA, respectively. (*B*) Subcellular localization of viral proteins determined by immunofluorescence. S310 replicon cell clones, JFH-1 replicon cells, and untransfected HuH-7 cells were grown on glass slides for 24 h. After fixation, cells were incubated with patient serum. (*C*) Western blot analysis. Cell lysates were prepared from replicon clones 6, 9, and 10, untransfected HuH-7, and HCVcc (J6/JFH1)-infected Huh-7.5.1 cells and uninfected Huh-7.5.1 cells. Protein (10 μg) was resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and viral nonstructural protein NS3-specific bands were detected using an anti-NS3 mouse monoclonal antibody (clone 8G2). Arrow indicates the position of NS3.



Supplementary Figure 3. Analysis of the effect of mutations on the colony-forming efficiency and transient replication of the subgenomic replicon S310. (*A*) Total RNA was isolated from the indicated replicon cell clones and 10 μg RNA was introduced into 3 million naïve HuH-7 cells by electroporation. After 3 weeks of G418 selection (500 μg/mL), colonies were stained. (*B*) Three million HuH-7 cells were electroporated with the indicated amounts of transcribed RNA and colonies were selected by a 3-week G418 selection. The JFH-1 subgenomic RNA was included as a positive control. (*C*) Huh-7.5.1 cells were transfected with the transcribed RNA from pSGR-S310/Luc and pSGR-S310/Luc constructs with mutations (GND mutation in NS5B, T1286I, T2188A, R2198H, S2210I, T2496I, R2895K, R2895G, and T2496I+R2895K) and Con1-NK5.1/Luc and N/Luc replicon. Transfected cells were harvested at the indicated time points and at 4 h post transfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate and was normalized to the activity at 4 h post transfection. Assays were performed in triplicate, and data are presented as means ± standard deviation.