

Identification of Human Kinases Involved in Hepatitis C Virus Replication by Small Interference RNA Library Screening^{*S}

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The propagation of the hepatitis C virus (HCV) is a complex process that requires both host and viral proteins. To facilitate identification of host cell factors that are required for HCV replication, we screened a panel of small interference RNAs that preferentially target human protein kinases using an HCV replicon expressing the firefly luciferase gene as a genetic reporter. Small interference RNAs specific for three human kinases, Csk, Jak1, and Vrk1, were identified that reproducibly reduce viral RNA and viral protein levels in HCV replicon-bearing cells. Treatment of replicon cells with a small molecule inhibitor of Csk also resulted in a significant reduction in HCV RNA and proteins, further supporting a role for Csk in HCV replication. The effects of siRNAs targeting eight kinases known to be negatively regulated by Csk were then examined; knock down of one of these kinases, Fyn, resulted in up-regulation of the HCV replicon, suggesting that Csk mediates its effect on HCV replication through Fyn. This conclusion was further corroborated by demonstration that replicon cells treated with Csk inhibitor contained lower levels of the phosphorylated form of Fyn than control cells.

Hepatitis C virus (HCV)² has emerged as a major cause of human liver disease, with ~3% of the world population persistently infected with the virus and more than 1 million new cases of infection reported annually (1). In ~70% of the cases, HCV escapes the immune system and establishes a chronic infection. In the long term, these chronic carriers are at risk of developing life-threatening liver disease, including hepato-cellular carcinoma (1). HCV is an enveloped virus that belongs to the *Hepacivirus* genus in the Flaviviridae family. Its genome consists of RNA of positive polarity ~9.6 kb in length that contains a large open reading frame. Translated polyprotein is processed by cel-

lular and viral proteases into at least 10 individual structural and nonstructural (NS) proteins. NS proteins are sufficient to support viral RNA replication and include the metalloprotease NS2, serine protease/helicase NS3, NS3 protease cofactor NS4A, RNA-dependent polymerase NS5B, and two other proteins with poorly characterized function, NS4B and NS5A. According to the current model, virus replication occurs within a complex that comprises viral RNA and NS proteins and is associated with the host endoplasmic reticulum (2, 3).

Current therapy for hepatitis C involves treatment with a combination of interferon- α and ribavirin. However, this regimen is effective only in half of patients, often poorly tolerated, and unsuitable for certain patient populations (4). Thus, there is an intense effort to develop new, better treatments, mostly by targeting viral enzymes (5). A complementary approach is to inhibit nonessential host cell proteins that are required for the viral life cycle. Several such host cell factors have been identified to date and represent potential candidates for this strategy. For example, inhibition of geranylgeranylation by lovastatin, a small molecule inhibitor of geranylgeranyl transferase 1, blocks HCV RNA replication and assembly of viral replication complex by preventing the geranylgeranylation of FBL2, a host cell factor interacting with NS5A (6). Another host protein, cyclophilin B, was found to stimulate binding of NS5B to RNA; its inhibition by various cyclosporin derivatives prevents propagation of HCV replicons in cells (7). And lastly, the inhibition of peroxisome proliferator-activated receptor α by 2-chloro-5-nitro-*N*-(pyridyl)benzamide results in down-regulation of HCV replication (8, 9), likely through effects on lipid metabolism.

The sequencing of the human genome and availability of highly specific methods for gene inactivation now allow a systematic examination of the roles of individual human genes in HCV replication and the identification of new potential drug targets. To this end, we have individually knocked down expression of 510 human genes, including 380 Tyr and Ser/Thr kinases, and examined the effect on HCV replicon propagation. Three kinases, Csk, Jak1, and Vrk1, were identified that when targeted with siRNAs caused a substantial reduction in cellular viral RNA and protein expression. The role of Csk in the HCV replicon propagation was confirmed with a small molecule inhibitor of Csk. Furthermore, we show that Csk exerts its effect on HCV replication through Fyn, the downstream effector of Csk and a member of the Src family of kinases.

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1.

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² The abbreviations used are: HCV, hepatitis C virus; siRNA, small interference RNA; NS, nonstructural; EMCV, encephalomyocarditis virus; Csk, carboxyl-terminal Src kinase; Jak, Janus kinase; Vrk, Vaccinia-related kinase; SFK, Src family kinase; STAT, signal transducer and activator of transcription; IRES, internal ribosome entry site.

EXPERIMENTAL PROCEDURES

Cell Culture—Huh7 human hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin (all from Invitrogen), and 10% heat-inactivated fetal bovine serum (Gemini Bio-Products). Cell lines harboring replicons were maintained in the same medium, which was further supplemented with 500 μ g/ml G418 (Invitrogen). Cells were grown at 37 °C and 5% CO₂ and were passaged twice per week.

Oligonucleotides—All oligonucleotides were purchased from Sigma Genosys, and their sequences and modifications were as follows: Neoluc1, 5'-CGATACGTAGG-3'; Neoluc2, 5'-CGC-GCCTACGTATCG-3'; HCVFor, 5'-GTCTGCGGAACCGG-TGAGTA-3'; HCVRev, 5'-GCCCAAATCTCCAGGCATT-3'; HCVDet, 6FAM-5'-ACGACCGGGTCCTTTCTTGGAT-CAA-3'-TAMRA.

Restriction Endonucleases—All restriction endonucleases were purchased from New England Biolabs.

Plasmids—Plasmid pLS1 was created by inserting a SnaBI restriction site adapter oligonucleotide (obtained by annealing Neoluc1 and Neoluc2) between the NruI and AscI sites of pFK-I389luc/NS3-3'/5.1 (10). This step removed ~120 bp from the 3'-end of the HCV IRES located just before the firefly luciferase open reading frame. Plasmid pLS2 was derived from pLS1 by inserting the BstUI-SmaI fragment from pIRES plasmid (Clontech), which harbors encephalomyocarditis (EMCV) IRES, into the SnaBI site of pLS1. To construct pFK-I389neo/luc/NS3-3'/5.1, a NruI-SpeI fragment from pLS2 was used to replace the PmeI-SpeI fragment of pFK-I389neo/NS3-3'/5.1. The resulting plasmid (pFK-I389neo/luc/NS3-3'/5.1) harbors three cistrons, neo^r, firefly luciferase, and the HCV NS open reading frame, which are translated from HCV IRES, EMCV IRES, and EMCV IRES, respectively. All plasmid constructs were verified by sequencing.

Generating Stable Cell Lines Harboring Replicons—pFK-I389neo/NS3-3'/5.1 and pFK-I389neo/luc/NS3-3'/5.1 plasmid DNAs were linearized with ScaI restriction endonuclease, extracted first with a phenol:chloroform mixture (1:1) and then with chloroform, and finally precipitated with ethanol. The resulting DNAs were used for the production of the synthetic replicon RNA with a T7-Megascript *in vitro* transcription kit (Ambion) according to the manufacturer's instructions. The DNA template was removed by digestion with 0.2 units/ μ l DNase I, and the *in vitro* transcribed RNA was purified with the RNeasy kit (Qiagen). 10 μ g of purified RNA were mixed with 400 μ l of Huh7 cell suspension in Cytomix containing 2 mM ATP and 5 mM glutathione. Electroporation conditions were 960 μ F and 270 V with a Gene Pulser system and a cuvette width of 0.4 cm (Bio-Rad). After electroporation, the cells were diluted with complete Dulbecco's modified Eagle's medium and seeded at a density ~10⁵ cells/cm². The medium was replaced 24 h later with a fresh one containing 500 μ g/ml of G418. Colonies harboring stably replicating HCV replicon appeared ~3 weeks post-transfection.

siRNAs—Predesigned siRNAs (3/gene) targeting Blk, c-Src, Fyn, Hck, Fgr, Lck, Lyn, and Yes kinases were purchased from

Ambion. Library and custom-designed siRNAs were purchased from Dharmacon as single-stranded RNA oligonucleotides.

siRNA Library Preparation and High-throughput Transfection—The sequences of the library siRNAs have been described previously (11). The single-stranded RNA oligonucleotides were annealed in 96-well plates according to the manufacturer's instructions. The whole collection was normalized to a final concentration of 100 ng/ μ l in 100 mM KOAc, 30 mM HEPES-KOH, and 2 mM MgOAc (pH 7.4). One microliter of each siRNA stock was transferred to 96-well plates, and the plates were stored at -80 °C until use. For transfection experiments, Lipofectamine 2000 (Invitrogen) was diluted 50-fold with Opti-MEM (Invitrogen), and 25 μ l of this mixture were added per well to 96-well plates containing the aliquoted siRNA library. The suspension of Huh7 cells harboring the tri-cistronic replicon (100 μ l, 4000 cells) was then added to the plates using a TiterTech 96/384 microplate liquid dispenser. After 48 h of cultivation, cell viability and activity of firefly luciferase in each well were determined.

Treatment with Small Molecule Inhibitor—To examine the inhibition of HCV replication by the small molecule inhibitor, Huh7 cells harboring the HCV replicon were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in the presence of 500 μ g/ml of G418. After reaching ~70% confluency, the inhibitor was added at the indicated concentrations in Me₂SO (1% final Me₂SO concentration). Control cells were treated in an identical manner except Me₂SO was added in place of the inhibitor solution. After 72 h of cultivation, cells were analyzed for NS5A, γ -tubulin, firefly luciferase expression levels, viability, and phosphorylation of Fyn kinase.

Determination of Cell Viability, Luciferase Activity, and Data Analysis—Cell viability was determined by incubating cells in 96-well plates with 10% Alamar Blue solution (Trek Diagnostic Systems) for 4 h, after which fluorescence was measured. To quantify firefly luciferase activity, the Bright-Glo luciferase assay system (Promega) was used according to the manufacturer's instructions. Luminescence was measured with an Analyst AD reader (Molecular Devices); data analysis was performed as described previously (9).

Immunoblot Analysis—To determine protein expression levels, cells were washed with phosphate-buffered saline and then resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 75 mM NaCl, 75 mM KCl, 20% glycerol (v/v), 4 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, 0.5% Triton X-100 (v/v), protease inhibitor mixture (Sigma)). Aliquots of extracts were subjected to either 7.5 or 10% SDS/PAGE and then transferred to a nitrocellulose membrane (Invitrogen). The blots were probed with monoclonal antibodies against NS5A, Fyn (Santa Cruz Biotechnology), or γ -tubulin (Sigma/Aldrich/Fluka) and then with anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad). Immunoreactive proteins were detected with the ECL Plus Western blotting detection system (Amersham Biosciences) and Kodak Biomax films (Eastman Kodak Co.).

Determination of Relative Cellular Levels of Replicon RNA by Real-time Quantitative Reverse Transcription PCR—Replicon cells grown in 96-well plate were washed once with 0.2 ml of ice-cold phosphate-buffered saline. Sequential cell lysis, cellu-

lar RNase inactivation, cellular DNA degradation, and total cellular cDNA synthesis were performed using the Cells-to-cDNA II kit (Ambion) according to the manufacturer's instructions. The reaction for the multiplex real-time quantitative PCR was set up in a total volume of 20 μ l and included 80 nM HCVFor, 120 nM HCVRev, and 100 nM HCVDet primers, as well as 10 μ l of AmpliTaq Gold PCR Master Mix and 1 μ l of Human RPLP0 (large ribosomal protein) Endogenous Control (VIC/MGB Probe, Primer Limited); both reagents were purchased from Applied Biosystems. Real-time quantitative RT-PCR was performed using 7900HT Sequence Detection System instrument (Applied Biosystems). The reaction mixture was first incubated for 2 min at 50 $^{\circ}$ C and then for 10 min at 95 $^{\circ}$ C and finally cycled 40 times between 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. C_t values for the replicon and large ribosomal protein cDNAs were calculated for each sample using the instrument software. To calculate the relative cellular abundance of the HCV replicon, $2^{-\Delta\Delta C_t}$ method was used (N -fold difference = $2^{-\Delta C_t}$ treated - ΔC_t control), where ΔC_t is the difference between the replicon and large ribosomal protein cDNA C_t values.

Detection of the Cellular Phosphorylated Form of Fyn—Huh7 cells harboring the bi-cistronic replicon were cultured in 6-well plates as described above. When the cells were 80% confluent, compound (dissolved in Me_2SO at 1 mM concentration) was added to cells at 5 μM final concentration. Cells were then incubated in the presence of the compound or Me_2SO for 24 h. The cells were washed three times with phosphate-free Dulbecco's modified Eagle's medium, incubated for 4 h in the same medium containing 400 $\mu\text{Ci/ml}$ of [^{32}P]orthophosphate (MP Biomedicals), and washed three times with ice-cold phosphate-buffered saline. Cells were lysed in 300 μl of radioimmune precipitation buffer (20 mM Tris-HCl, pH 8.0, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) supplemented with protease and phosphatase inhibitor cocktails (both from Sigma/Aldrich/Fluka). 20 μl of cell lysates were mixed with the Laemmli sample buffer and boiled for 5 min. The remaining lysates were precleared by incubation with 30 μl of protein A/G-agarose bead slurry (50%; Santa Cruz Biotechnology) for 1 h at 4 $^{\circ}$ C. After brief centrifugation, the supernatants were incubated with 10 μl of monoclonal anti-Fyn antibody (Upstate) for 2 h at 4 $^{\circ}$ C, followed by the addition of 100 μl of protein A/G-agarose beads (50% slurry, prewashed with radioimmune precipitation buffer), and subsequent incubation overnight at 4 $^{\circ}$ C. Immunoprecipitates were washed twice with phosphate-buffered saline and radioimmune precipitation buffer, resuspended in the Laemmli sample buffer, and boiled for 5 min. The samples were separated on a 4–20% SDS-PAGE gel and then dried. ^{32}P -labeled proteins were visualized by a PhosphorImager scanner (GE Healthcare).

Determination of Inhibitor Specificity and IC_{50} —JK239 specificity was assessed based on its inhibition profile in biochemical enzymatic assays against the panel of purified human kinase enzymes at 1 μM inhibitor concentration (performed at Invitrogen). The IC_{50} of JK239 for Csk kinase was determined with HTScanTM Csk Kinase Assay kit (Cell Signaling Technology) according to the manufacturer's instructions in the presence of various inhibitor concentrations and 2% Me_2SO .

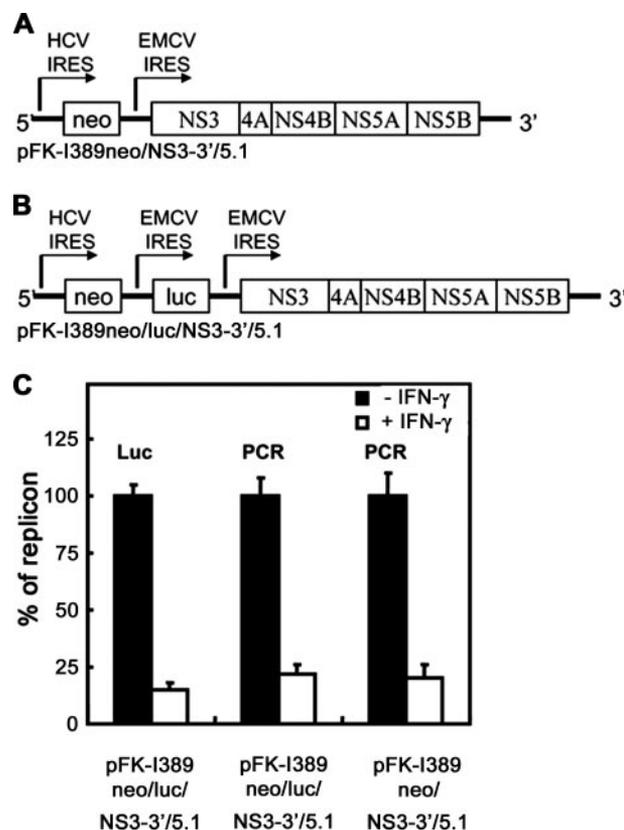


FIGURE 1. Examination of the effect of interferon- γ on the different subgenomic HCV constructs. A, three different replicon variants used in this study. All three replicons encode a 3'-part of the HCV polyprotein, starting with NS3, which is translated from the EMCV IRES. Two bi-cistronic variants, pFK-I389neo and pFK-I389luc, encode 5'-terminal neomycin resistance and firefly luciferase genes, respectively, translated from the HCV IRES and fused to the amino terminus to the 16th amino acid of HCV core protein. B, the tri-cistronic variant pFK-I389neo/luc was derived from pFK-I389neo by inserting the firefly luciferase gene translated from the EMCV IRES between two cistrons originally present. C, Huh7 cells harboring the indicated replicon variant were treated with interferon- γ at 10 units/ml, and the decrease in the amount of replicon RNA in cells was determined either by firefly luciferase measurement (*Luc*) or by quantitative RT-PCR (*PCR*). Averaged values with S.D. are shown.

RESULTS

Construction and Validation of a Selectable Tri-cistronic Subgenomic HCV Replicon Expressing the Firefly Luciferase Reporter—Several HCV replicon variants have been described previously that allow measurement of cellular replicon RNA levels through the activity of a reporter enzyme and, at the same time, also harbor an antibiotic resistance gene that enables the selection of transfected cells with stably replicating HCV replicon RNA (12–14). In parallel with these reports, we developed another HCV replicon, pFK-I389neo/luc/NS3-3'/5.1, which harbors the firefly luciferase gene and the Geneticin resistance gene *neo^r*. This new tri-cistronic construct was derived from the previously reported genotype 1b (con1) bi-cistronic replicon pFK-I389neo/NS3-3'/5.1 (Fig. 1A). pFK-I389neo/NS3-3'/5.1 includes the 5'-nontranslated end of the HCV genome together with the first five amino acids of core (C) protein that are fused to the *neo^r* gene (10). The second part of the replicon harbors the NS3-NS5B segment of the HCV polyprotein followed by the complete non-translated 3'-end of the HCV genome. The expression of NS3 through NS5B proteins, which are essential for the replication phase of the HCV life cycle, is

Human Kinases Involved in HCV Replication-siRNA Screen

driven by the heterologous EMCV IRES. In addition to these genetic elements, this tri-cistronic replicon (pFK-I389neo/luc/NS3-3'/5.1) harbors the third open reading frame encoding firefly luciferase translated from the second EMCV IRES (Fig. 1B). This modification allows a simple evaluation of the cellular levels of replicon RNA through the measurement of firefly luciferase activity and is fully compatible with high-throughput screening technologies. When tested for sensitivity to interferon- γ treatment, the cell lines harboring the above two replicon variants responded in a very similar manner, a decrease in the HCV RNA cellular contents by 78 and 80%, respectively (Fig. 1C). Changes in tri-cistronic RNA levels were also accurately reflected by the decrease in the activity of firefly luciferase (Fig. 1C). Furthermore, the replication of both replicon variants was equally affected by treatment with interferon- α (as measured by quantitative RT-PCR), and in the case of pFK-I389neo/luc/NS3-3'/5.1, this effect could be assayed through the activity of the reporter enzyme (data not shown). Thus, pFK-I389neo/luc/NS3-3'/5.1 is suitable for a rapid and accurate evaluation of changes in cellular replicon levels.

A Kinase siRNA Library Screen for Genes Involved in HCV Replicon Propagation—To identify cellular protein kinases involved in HCV replicon propagation, we employed an siRNA library described previously that predominantly targets human protein kinases (380 of 510 library members target protein kinases) (11). Expression of approximately two-thirds of the targeted RNAs is reduced at least 70% in HeLa cells. Two parallel transfection experiments, each executed in duplicate, were performed with this library in Huh7 cells harboring the tri-cistronic replicon pFK-I389neo/luc/NS3-3'/5.1. Control siRNAs against firefly luciferase (siGL2 and siGL3) (15) and HCV NS3 genes were included in each library plate. The effects of individual siRNAs on cell viability and replicon levels (measured by firefly luciferase activity) were determined. The use of a transfection protocol compatible with high-throughput screening resulted in a lower (~40%) transfection efficiency than observed (~70%) under optimal transfection conditions. Because of the low transfection efficiency observed during the screen, we initially selected all of the hits from the primary screen that caused at least 25% replicon down-regulation and did not significantly affect cell viability. These siRNAs were then tested using optimal transfection conditions. The activity of only three siRNAs was re-confirmed (Fig. 2A), Csk (carboxyl-terminal Src kinase), Jak1 (Janus kinase 1), and Vrk1 (Vaccinia-related kinase 1).

Csk, Jak1, and Vrk1 Play an Important Role in HCV Replicon Propagation—To strengthen the association between the knock down of these three protein kinases and the down-regulation of HCV replicon cellular levels, transfections were repeated with another set of siRNAs against these three kinases (see Table 1 for siRNA sequences). Transfection with any of six siRNAs resulted in roughly a 60% decrease of the targeted mRNA as determined by quantitative RT-PCR. For all three kinases transfection with either set of siRNAs resulted in a significant decrease in luciferase activity (~40% Csk, ~50% Vrk1, and ~50% Jak1 on average, Fig. 2A). At the same time, an Alamar Blue assay showed no effect of any tested siRNA on cell proliferation, excluding the possibility of decreased reporter activity due to siRNA cytotoxicity (Fig. 2B). We also eliminated

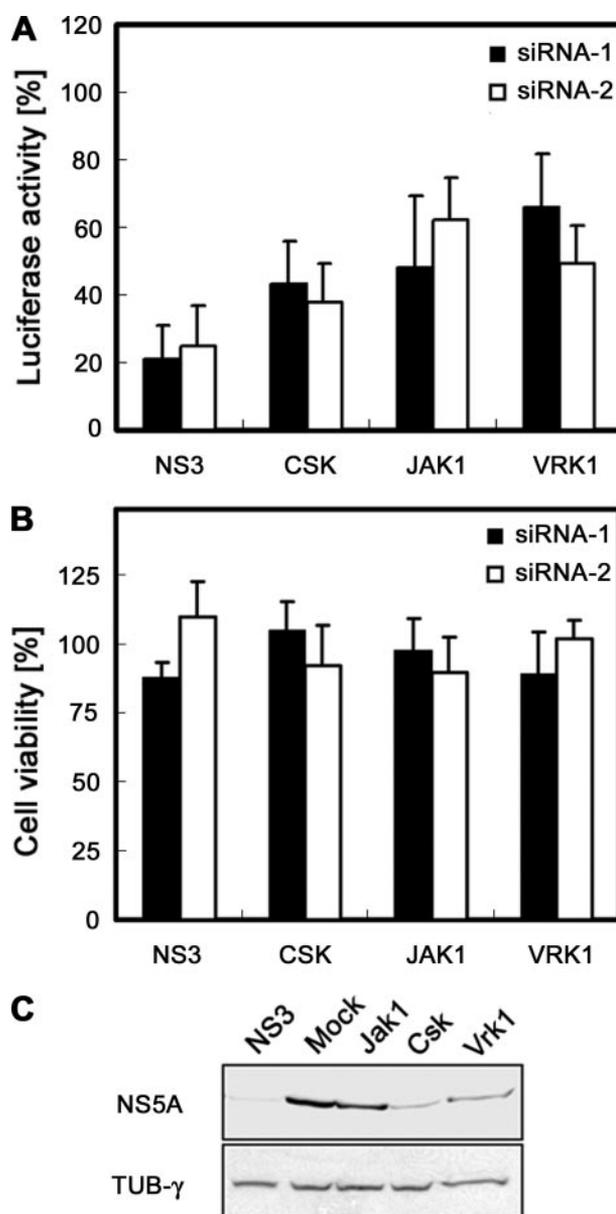


FIGURE 2. Effect of anti-NS3, -Csk, -JAK1, and -VRK1 siRNAs on replicon levels. Huh7 cells harboring pFK-I389neo/luc were transfected with the indicated siRNA and their effects on either replicon level (luciferase assay) (A) or cell viability (Alamar Blue) (B) were measured after 48 h. All shown values were normalized relative to transfection with non-coding siRNA, and averages with S.D. are shown. C, effect of interferon- γ at 10 units/ml and siRNAs (CSK1, JAK11, VRK12) at 100 nm final concentration on NS5A levels in Huh7 cells harboring the pFK-I389neo replicon. To normalize the amount of NS5A protein in cells, cell lysates were also examined for the amount of tubulin- γ (TUB- γ).

TABLE 1
siRNA kinase sequences

Gene	Sequence
NS3-1	GGGCCCAAUCACCCAAUUG
NS3-1	CCCAAUUGUACACCAAUUGU
CSK-1	CUACCGCAUCAUGUACCAU
CSK-2	CTGCGGCATAGCAACCTGG
JAK1-1	TGCTCAGGGACAGTATGAT
JAK1-2	GCTCTGGTATGCTCCAAAT
VRK1-1	GGCAAUUGGACCUCAGUG
VRK1-2	CCAGGUGAAUUGCCAAU
FYN-1	GGACUCACCUCUUUGGAG
FYN-2	GGUGUGAACUCUUCGUCUC
GL2	CGUACGGAAUACUUCGA
GL3	CUUACGCUGAGUACUUCGA

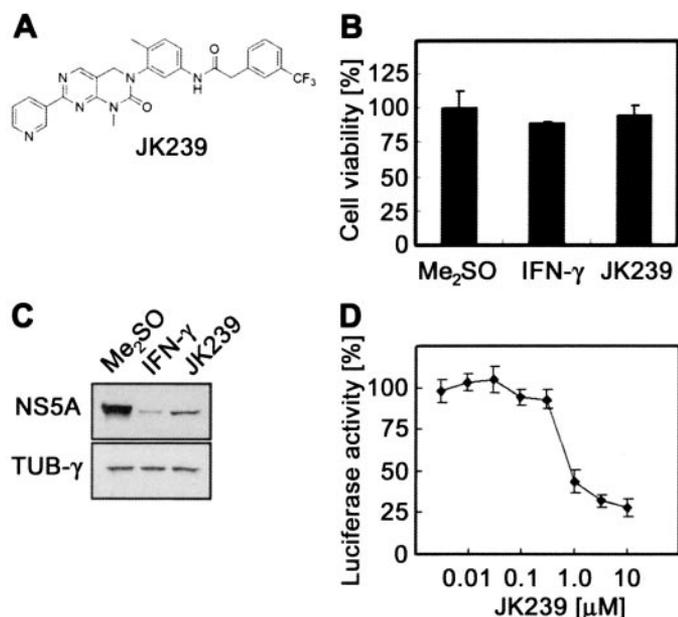


FIGURE 3. Determination of inhibitory activity for Csk inhibitor JK239. *A*, structure of JK239 inhibitor. *B*, cell viability was determined after 72 h of incubation by Alamar Blue. Concentration of interferon- γ was 10 units/ml, and concentration of JK239 was 10 μ M. *C*, intracellular levels of NS5A protein in Huh7-pFK-I389 neo cells after treatment with 10 μ M CSK inhibitor for 72 h. Western blot and antibody treatment were performed as described under "Experimental Procedures" in the absence of added compound Me₂SO (DMSO) or in the presence of 10 units/ml interferon- γ . *D*, luciferase activity in Huh7 cells harboring pFK-I389neo/luc after treatment with various concentrations of JK239 inhibitor for 72 h. Averages and the S.D. are shown.

the possibility that these siRNAs affect the luciferase reporter rather than HCV replication. The cell line harboring the original pFK-I389neo/luc/NS3-3'/5.1 replicon was transfected with the control, CSK, JAK1, and VRK1 siRNAs, and their effects on the bi-cistronic replicon were determined by measuring cellular levels of the HCV NS5A protein. As was the case with the tri-cistronic replicon, each transfected siRNA resulted in a decrease in the level of NS5A protein present in the cells (Fig. 2C) that was comparable with that observed with the tri-cistronic replicon-encoded luciferase. In summary, the above data suggest that the three host kinases, Csk, Jak1, and Vrk1, are involved in HCV replicon propagation.

A Small Molecule Inhibitor of Csk Blocks Phosphorylation of Fyn and Inhibits Replicon Propagation—Because even under optimal transfection conditions we could observe only ~50–60% replicon inhibition after transfection with various siRNAs (likely a consequence of low transfection efficiency), we sought to strengthen the above conclusions by examining the effects of small molecule inhibitors of these kinases on replicon-containing cells. One such inhibitor, JK239 (Fig. 3A) was identified for Csk based on data derived from previous profiles of kinase inhibitors in biochemical assays against a panel of various human protein kinases (data not shown). Of 90 kinases tested, JK239 inhibited only two, Csk and MAPK11, by >50% at 1 μ M inhibitor concentration. The IC₅₀ of this inhibitor in biochemical assay of Csk activity was found to be 0.43 μ M. To assess the effect of JK239 on the viability of bi- or tri-cistronic replicon-containing cells, these cell lines were grown in the presence of 10 μ M JK239 for up to 3 days. This treatment did not result in any observable cytotoxicity (Fig. 3B). To confirm that JK239

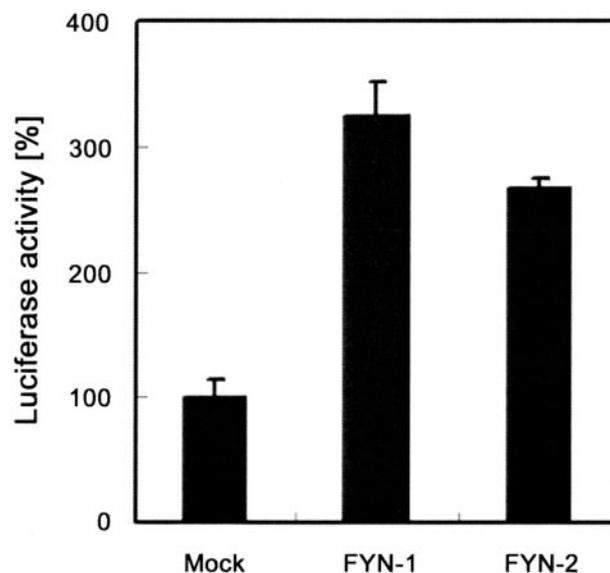


FIGURE 4. Effect of anti-Fyn siRNAs on the level of HCV replication. Huh7 cells harboring the HCV replicon were treated with siRNAs Fyn-1 and Fyn-2 at 100 nM concentration for 48 h. An untreated control was set up in parallel. The levels of HCV subgenomic replicon RNA are expressed as percentage of luciferase activity and were calculated from at least two experiments done in triplicates.

treatment leads to a decrease in HCV replicon copy number, the amount of NS5A protein in Huh7 cells harboring bi-cistronic replicon was determined after compound treatment. In agreement with the previous experiment, addition of JK239 (10 μ M) resulted in elimination of >80% NS5A protein from cells (Fig. 3C). Additionally, the compound did not inhibit the expression of firefly luciferase translated from EMCV IRES and transcribed from the cytomegalovirus promoter in the control experiment. Further characterization of this inhibitor revealed that it inhibited replicon propagation in tri-cistronic replicon cells (measured through luciferase activity) with an EC₅₀ = 0.63 μ M (Fig. 3D). These data, together with the results of siRNA experiments, strongly indicate that inhibition of Csk results in inhibition of HCV replication in the cell-based, HCV replicon model of HCV infection.

Csk Facilitates HCV Replicon Propagation through Fyn—Csk negatively regulates Src family kinases by tyrosine phosphorylation. The Src family includes at least eight genes in humans, five genes with expression restricted to cells of hematopoietic origin (Blk, Hck, Fgr, Lck, and Lyn) and three genes expressed ubiquitously (Fyn, c-Src, and Yes) (16). One possible explanation for inhibition of HCV replication by the reduction in Csk protein expression could be an increased activity of one or more members of the Src family kinase. To explore this possibility, we tested the effects of knock downs of these eight kinases on the tri-cistronic HCV replicon. Although siRNAs targeting seven of these eight kinases did not show any effect, the siRNA targeting Fyn elevated replicon levels (measured by luciferase reporter) ~3-fold upon transfection. This observation was also confirmed with a second siRNA independently targeting Fyn, which also increased the cellular replicon level ~3-fold upon transfection (Fig. 4). The up-regulation of replicon after Fyn depletion suggests that Fyn plays an important role in HCV replicon propagation under standard culture conditions.

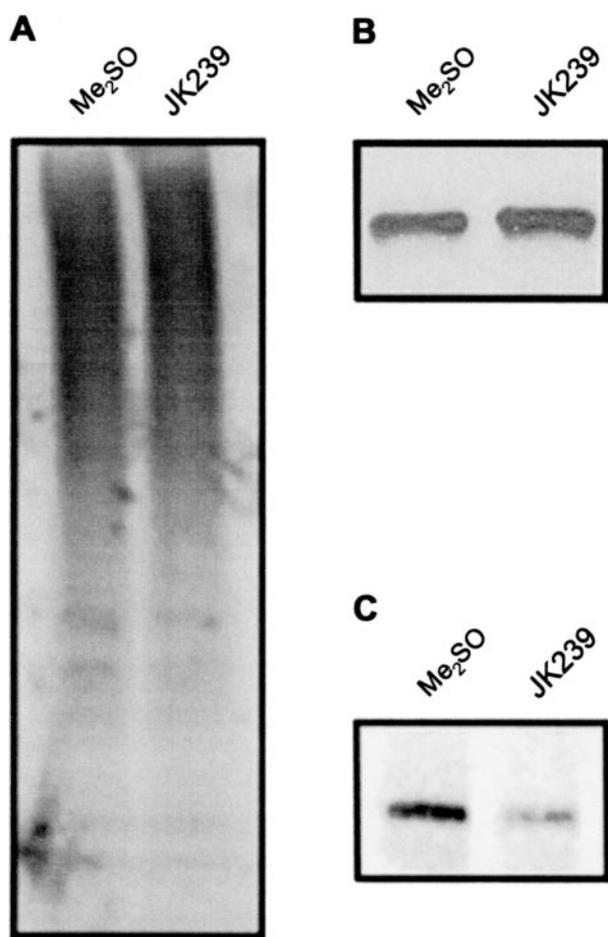


FIGURE 5. Inhibition of Fyn phosphorylation by Csk inhibitor JK239. *A*, comparison of Huh7 replicon cell phosphoprotein profiles after treatment with Me₂SO or JK239 for 24 h and subsequent labeling with [³²P]orthophosphate. *B*, detection of Fyn in cell lysates shown in *panel A* by Western blotting. *C*, immunoprecipitation of [³²P]orthophosphate-labeled replicon cell lysates with monoclonal anti-Fyn antibody. The lysates were processed as described under "Experimental Procedures."

Because Csk is the direct negative regulator of Fyn, it is likely that the inhibition of the HCV replicon propagation observed upon siRNA-dependent Csk knock down, or inhibition of Csk activity by JK239, results from increased Fyn activity (JK239 does not inhibit Fyn activity in biochemical assays). To further demonstrate that Csk mediates its effects on HCV replication through Fyn, we examined the effect of JK239 on the phosphorylation status of Fyn by first radioactively labeling the cells with [³²P]phosphate and then immunoprecipitating and quantifying the amount of phosphorylated Fyn in the labeled cells. As expected, 24 h of treatment of cells with 5 μM inhibitor significantly reduced the amount of the phosphorylated form of Fyn present in cells (residual phosphorylated form = 20%, Fig. 5C) without affecting the amount of total Fyn (Fig. 5B). At the same time, treatment of cells with JK239 did not result in any significant global change in protein phosphorylation shown by the comparison of the phosphoprotein profiles of treated *versus* control cells (Fig. 5A). These results reinforce the notion that Csk affects HCV replication by modulating the phosphorylation status and activity of Fyn.

DISCUSSION

An siRNA library directed against ~500 human genes (including 380 protein kinases) was screened in a cell-based assay to identify cellular kinases that are required for the efficient propagation of an HCV subgenomic replicon in Huh7 hepatoma cells. To facilitate the rapid identification of siRNAs that affect the HCV replication cycle, a tri-cistronic replicon was generated that expresses the genetic reporter firefly luciferase. This tri-cistronic replicon combines genetic features that allow selection of transfectants with a stably replicating HCV replicon and the detection of replicon levels through the activity of a firefly luciferase reporter. The stably replicating tri-cistronic replicon behaved quite similarly to the original bi-cistronic replicon when cells were treated with interferon-α or -γ in validation experiments. Both replicon variants also behaved similarly in subsequent experiments with siRNAs and a small molecule inhibitor, demonstrating the utility of this replicon as a screening tool. Of ~500 genes screened, we identified three known protein kinases, Csk, Jak1, and Vrk1, that are involved in HCV replication as demonstrated by the fact that their down-regulation through RNA interference resulted in at least a 2-fold (3-fold in the case of Csk) reduction in cellular replicon levels. None of the siRNAs showed any cytotoxicity under the conditions tested, indicating that replicon down-regulation was not caused by nonspecific cellular effects.

Csk is a non-receptor protein tyrosine kinase that negatively regulates protein kinases belonging to the Src family (SFKs). These include c-Src, Yes, and Fyn, which are expressed ubiquitously, and Blk, Fgr, Hck, Lck, and Lyn, whose expression is restricted to cells of hematopoietic origin (16). SFKs have been implicated in numerous cellular processes including the innate immune response and signaling induced by integrins, cytokines, antigens, and growth factors. Csk-null mice showed hyperactivation of SFKs and striking embryonic phenotypes characterized by early lethality, neural tube defects, and reduced size (17, 18). Csk negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun NH₂-terminal kinase, and STAT (signal transducers and activators of transcription) pathways. Mice with a conditional knock out of Csk in granulocytes developed acute multifocal inflammation in skin and lung and were hypersensitive to lipopolysaccharide-induced shock. This hypersensitivity was associated with hyperadhesion, impaired migratory responses, and enhanced adhesiveness of granulocytes (19).

We were able to further confirm a role of Csk in the HCV replication by using a small molecule inhibitor of Csk (IC₅₀ = 0.43 μM) that inhibited replicon propagation with an EC₅₀ = 0.63 μM. The activity of JK239 is comparable with that of previously reported thiophene-2-carboxylic acid-derived inhibitor of HCV NS5B polymerase (EC₅₀ = 0.35 μM) (20). This result reinforces the conclusion derived from siRNA knockdown experiments regarding the importance of Csk in HCV replicon propagation. The dependence of viral propagation on the presence of active Csk is not exclusively associated with HCV; similar findings were recently reported for herpes simplex virus 1. In that case, the amount of virus produced by Csk null cells was 10-fold lower than that produced by wild-type cells (21).

The most straightforward explanation for the effects of siRNA-mediated Csk knock down on the HCV replicon is that it causes activation of one or more SFKs. It has been reported that some viruses manage to manipulate the activities of SFKs so as to benefit from cell signaling events regulated by SFKs. For example, hepadnaviruses activate Src kinases, leading to a 5- to 20-fold increase in the replication of hepatitis B virus and woodchuck hepatitis virus (22, 23). In another example, West Nile virus was shown to up-regulate expression of the c-Yes kinase, which when knocked down by siRNAs results in an accumulation of West Nile Virus virions in the endoplasmic reticulum and an inhibition of their transit through the secretory pathway (24). And lastly, the c-Src kinase was found to be essential for Dengue virus propagation through virion assembly (25). On the other hand, the up-regulation of SFKs by means of Csk inactivation results in inhibition of herpes simplex virus 1 replication (21), although the cellular mechanism is unknown at present.

To gain further insights into the interplay between SFKs and HCV replication, we individually knocked down eight SFKs and examined the effects on replicon activity. Knock down of one kinase, Fyn, led to a ~2.5- to 3-fold up-regulation in replicon levels, suggesting the effects of Csk on replicon activity are mediated through negative regulation of Fyn. To support this conclusion, we examined the phosphorylation status of Fyn in cells treated with the Csk-specific inhibitor JK239. Although the inhibitor did not significantly affect global protein phosphorylation or the total amount of Fyn in cells, it reduced the extent of Csk-dependent Fyn phosphorylation by 80%. Although the mechanism through which Fyn exerts its effect on the HCV replication is at present unclear, the previously reported physical interaction between Fyn and HCV NS5A (26, 27) suggests that Fyn might modulate NS5A biological activity, which is known to be essential for HCV replication (27). Finally, although inhibition of Csk is effective in modulating HCV replicon *in vitro*, it remains to be seen whether it can be used for the treatment of hepatitis C in humans. One concern with such therapy might be the induction of inflammatory associated side effects.

Another kinase identified in the screen, Jak1, is a ubiquitously expressed membrane-associated member of the JAK family of protein tyrosine kinases that plays an essential and non-redundant role in promoting biological responses induced by a select subset of cytokine receptors (28). Because it is absolutely required for mediating biologic responses to interferon- α and interferon- γ (29, 30), it appears counter-intuitive that it is required for the HCV replication. However, it has been shown that NS5A transfected into HepG2 cells interacts with and activates Jak1 kinase, which in turn phosphorylates STAT3. Phosphorylated STAT3 translocates into the nucleus and modulates the expression of Bcl-xL and p21, which may lead to HCV-mediated pathogenesis (31). Our data suggest that this complex may be necessary for HCV replication. Other transforming viruses have been shown to activate STAT3 in cooperation with other kinases. For example, Herpes virus saimiri Tip-484 activates STAT3 in cooperation with p56^{lck} (32), and human T-cell lymphotropic virus type-1-transformed cells exhibit constitutive activation of the Jak-STAT pathway (33).

Vrk1 is overexpressed in tumor cells as well as in highly proliferating cells, such as regenerating liver. It phosphorylates and

forms a complex with transcription factor ATF2, resulting in increased transcriptional activity. ATF2 contributes to the regulation of genes implicated in cell growth, differentiation, immune response, and response to stress (34). Vrk1 also phosphorylates p53, enhancing the protective role played by this tumor suppressor protein, a key element in the responses to different types of cellular stress (35–38). It was shown previously that NS5A associates and sequesters p53 into cytoplasm, resulting in p21 down-regulation. Based on that and other observations, it was proposed that p53-NS5A complex formation allows STAT3 to phosphorylate and translocate into the nucleus for promotion of cell growth. It may be that the interaction between p53 and NS5A requires phosphorylation of p53 by Vrk1.

In conclusion, this work demonstrates the involvement of the cellular Csk kinase in HCV replication, likely by modulating the activity of Fyn, which is known to directly interact with the NS5A protein of HCV, which is required for replication. This approach is currently being extended in two directions. First, siRNA libraries targeting a large fraction of human genes are available and can be screened. Second, the recent discovery of JFH1 HCV isolate that is capable of replicating in tissue culture may allow the identification of host genes necessary for other stages of HCV infection.

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