Template preparation (Steps 1–24): 6–7 h
RNA transcription and purification (Steps 25–31): 5 h
Transfection (Steps 32–39): 2 h
Collection (Steps 40–43): 3 d
Titration (Steps 44–59): 6 d

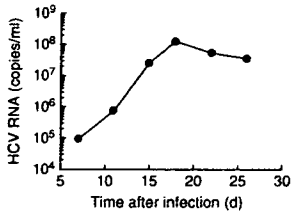

Figure 4 | HCV RNA titers in culture supernatants of inoculated Huh7.5.1 cells.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Cause	Solution
Step 4	Incomplete digestion	Inappropriate quality and quantity of plasmid DNA	Check quality and quantity of plasmid DNA
		Ruined enzyme	Use new enzyme
		Incubation time too short	Prolong incubation time
Step 24	Insufficient template DNA	Sample lost during procedures	Repeat digestion and purification steps more carefully
		Incorrect plasmid DNA concentration	Check plasmid DNA concentration
Step 31	Low yield of synthesized RNA	Contamination of RNase, SDS or EDTA	Repeat proteinase K treatment and purification step
		Insufficient template	Increase amount of template
		Incubation time too short	Prolong incubation time
	Synthesized RNA shows smear band	Contamination of RNase	Repeat proteinase K treatment and purification step
Steps 44 and 59	Low yield of generated HCV	Low transfection efficiency	Check transfection efficiency by indirect immunofluorescence

ANTICIPATED RESULTS

Huh7 cells transfected with synthesized full-length JFH-1 RNA were passaged at 2, 6, 9 and 12 d after transfection. At each time point, culture media from three independent transfected cells were collected and HCV RNA in culture media was measured in culture media by quantitative RT-PCR. HCV RNA titers in culture medium continuously increased up to 9 d after transfection (**Fig. 2**). At 72 h after transfection, HCV had spread to 60–80% of cells (**Fig. 3**). With this protocol, approximately 1×10^5 copies per ml of HCV can be obtained 72 h after transfection of 10 μg JFH-1 RNA into Huh7 cells, and 6 d after transfection, up to 1×10^7 copies per ml can be obtained. However, virus production is not always consistent and should be confirmed by HCV RNA titration or by counting infected foci in each experiment.

Huh7.5.1 cells (8×10^4 cells per well) were infected with concentrated culture medium from RNA-transfected cells containing 1×10^8 copies of HCV RNA. Inoculated cells were serially passaged and culture supernatants were collected at 7, 11, 15, 18, 22 and 26 d after infection. Amounts of HCV RNA in culture medium were determined by quantitative RT-PCR. By inoculation of cell culture-generated HCV into naive Huh 7.5.1 cells, infection and production of HCV could be monitored and maintained for 26 d or more after inoculation (**Fig. 4**).

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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Replication of a hepatitis C virus replicon clone in mouse cells

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Abstract

Background: Hepatitis C Virus (HCV) is a significant public health burden and small animal models are needed to study the pathology and immunobiology of the virus. In effort to develop experimental HCV mouse models, we screened a panel of HCV replicons to identify clones capable of replicating in mouse hepatocytes.

Results: We report the establishment of stable HCV replication in mouse hepatocyte and fibroblast cell lines using replicons derived from the JFH-1 genotype 2a consensus sequence. Viral RNA replication efficiency in mouse cells was comparable to that observed in human Huh-7 replicon cells, with negative-strand HCV RNA and the viral NS5A protein being readily detected by Northern and Western Blot analysis, respectively. Although HCV replication was established in the absence of adaptive mutations that might otherwise compromise the *in vitro* infectivity of the JFH-1 clone, no infectious virus was detected when the culture medium from full length HCV RNA replicating mouse cells was titrated on Huh-7 cells, suggesting that the mouse cells were unable to support production of infectious progeny viral particles. Consistent with an additional block in viral entry, infectious JFH-1 particles produced in Huh-7 cells were not able to establish detectable HCV RNA replication in naïve mouse cells.

Conclusion: Thus, this report expands the repertoire of HCV replication systems and possibly represents a step toward developing mouse models of HCV replication, but it also highlights that other species restrictions might continue to make the development of a purely murine HCV infectious model challenging.

Background

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus that causes acute and chronic hepatitis [1]. Between 70–90% of those who become infected fail to clear the virus and remain chronically infected with the risk of developing liver cirrhosis and hepatocellular carcinoma [2]. Unfortunately, there is no vaccine available to prevent this infection, and the only approved treatment has toxic side effects and is only effective in a subset of patients [3,4].

Even though recent work has led to the development of *in vitro* HCV infection systems, which allow for molecular analysis of the entire viral life cycle [5-7], the study of the immunobiology and pathogenesis of HCV still requires the development of genetically defined small animal

models. One obstacle to the development of HCV mouse models has been the limited host range of the virus. The restrictions that block HCV infection in mice are not well defined, but appear to involve multiple steps such as viral entry and genome replication.

Notably however, HCV replicons based on engineered viral genomes into which the antibiotic resistant marker neomycin phosphotransferase (neo) has been inserted [8](Fig. 1A) provide a means of experimentally by-passing viral entry and actively selecting for HCV replication after transfection of RNA into cells. The ability to select for cells replicating the neo-expressing replicon RNA led to the discovery that efficient replication of most HCV replicons in cell culture requires adaptive mutations in the viral genome [9-13]. Although HCV replication initially could only be achieved in the human hepatoma cell line, Huh-7, the ability to select for replication enhancing mutations eventually led to the establishment of HCV replication in other hepatic (HepG2 and IMY-N9 [14]) and nonhepatic (HeLa [15,16] and HEK293 [15,17]) human cell lines.

Unlike other published studies that focus exclusively on HCV replication in human and/or primate cell lines, Zhu et al (2003) further demonstrated that replication of the HCV-N genotype 1b subgenomic replicon could be initiated in one of the several mouse cell lines tested. However, this replication could only be established in a single mouse cell line after transfection of total RNA extracted from HeLa cells that were already replicating the adapted replicon (i.e. total human cellular RNA presumably containing a quasispecies of HCV replicons)[16]. In contrast, HCV replication could not be initiated in these mouse cells by transfection of *in vitro* transcribed replicon RNA generated from either the parental replicon construct or from any of the "adapted" replicon clones isolated from their original mouse replicon cells. Hence, no "mouse-permissive" HCV replicon clone was identified.

Because the development of HCV mouse models would be greatly facilitated by the identification of defined HCV clone(s) capable of establishing and maintaining replication in mice, we assembled a panel of HCV replicons derived from different HCV genotypes and assessed their ability to replicate in mouse cells. We show that JFH-1 genotype 2a subgenomic and full length replicons are able to stably replicate in multiple mouse hepatocyte and fibroblast cell lines following transfection of the replicon RNA. Although the HCV replication achieved in mouse hepatocytes was not dependent on adaptive mutations that might compromise the infectivity of the viral clone, no infectious HCV was detected in the media of mouse cells replicating full length HCV RNAs, nor were infectious JFH-1 virus particles produced in Huh-7 cells able to infect naïve mouse cells. Thus, these mouse cells are permissive for JFH-1 HCV replication, but exhibit blocks in both viral entry and production of infectious particles.

Results

Establishment of G418-resistant mouse hepatocyte colonies after transfection of subgenomic replicons encoding the neomycin selection gene

Because different HCV replicon clones exhibit a range of replication efficiencies in Huh-7 cells, we hypothesized that different replicons might also display differences in their ability to replicate in mouse cells. Thus, we screened a panel of subgenomic HCV replicons derived from genotypes 1b, 1a, and 2a for their ability to confer G418 resistance to mouse hepatocytes *in vitro*. We initially screened for HCV replication in immortalized Met Mouse Hepatocytes (MMH) cells; however, because HCV replication efficiency within different Huh-7 cell lines can vary, we tested

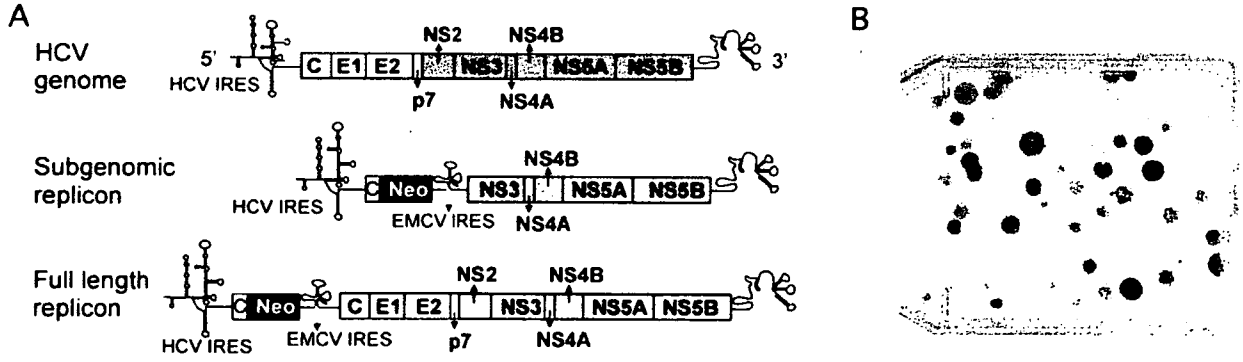

Figure 1

(A) Schematic diagram of HCV genomic and replicon RNA. (B) Representative crystal violet staining of G418-resistant colony formation in MMHD3 mouse hepatocytes after transfection with sgJFH-1 HCV RNA. Notably, although MMH cells exhibit relatively low transfection efficiency, numerous G418-resistant colonies form following transfection of HCV replicon RNA.

HCV replication in two independently derived MMH cell lines, MMHD3 [18] and MMH1-1 [19].

Replicon RNA from the clones listed in Table 1 was transcribed *in vitro* and electroporated into MMH cells. Transfected cells were then treated with 500 μg/ml G418, which was the minimal concentration required to effectively kill untransfected control cell cultures. Despite the fact that G418-resistent Huh-7 cell colonies survived the selection process after transfection with all of the replicon RNAs tested, RNA transcribed from the HCV genotype 1b clones (Con1 and HCV-N) and the HCV genotype 1a H77 clone did not generate G418-resistent mouse cell colonies (data not shown). In contrast, transfection of genotype 2a subgenomic (sg) JFH-1 RNA produce G418-resistant mouse hepatocyte colonies in both MMH cell lines in multiple independent transfection experiments (Fig. 1B).

Expression and replication of sgJFH-1 HCV replicons in immortalized mouse hepatocytes

To confirm the expression and replication of HCV in MMHD3 and MMH1-1 cells, G418-resistant cell colonies were expanded for further analysis. Western Blot analysis of total cell lysate verified that the viral NS5A protein was present in each of the MMHD3 and MMH1-1 replicon cell clones (Fig. 2A, lanes 5, and 6–11, respectively). Yet, constitutive HCV protein expression was not due the integration of an HCV transgene, as PCR analysis on cellular genomic DNA revealed no evidence of JFH-1 sequences (data not shown).

Clones were also examined for the presence of HCV RNA by strand-specific Northern Blot. Positive-strand (Fig. 2B, top panel) and negative-strand (Fig. 2B, middle panel) HCV RNA was detected not only in Huh-7 control cell clones (Fig. 2B, lanes 11–15), but also in the mouse clones derived from MMHD3 and MMH1-1 cells (Fig. 2B, lanes 2 and 4–9, respectively). Although the signal intensity observed with these independently labeled and hybridized riboprobes can not be compared to determine the ratio of positive-strand to negative-strand HCV RNA, both probes demonstrated stringent strand-specific hybridization when incubated with membranes containing serial dilutions (10^9, 10^8, and 10^7 copies) of *in vitro* transcribed positive-strand (Fig. 2B, lanes 16–18) and negative-strand (Fig. 2B, lanes 19–21) sgJFH-1 RNA controls, clearly demonstrating that negative-strand HCV RNA replication intermediates were being produced in the clones.

Table 1: HCV Replicons Screened

Genotype	Clone
1b	sgCon1 WT
1b	sgCon1 S1179I
1b	sgHCV-N
1a	sgH77
2a	sgJFH-1

sg = subgenomic

RT-QPCR analysis of the same RNA samples indicated that HCV RNA levels among the mouse sgJFH-1 replicon clones ranged from $2.9 \times 10^6 - 7.0 \times 10^7$ copies per microgram (copies/μg) of total cellular RNA. This was similar to the $7.4 \times 10^6 - 8.5 \times 10^7$ copies/μg observed in the human sgJFH-1 replicon clones. Thus, the average 2.0×10^7 HCV RNA copies/μg calculated in the mouse cells was comparable to the average 5.0×10^7 HCV RNA copies/μg detected in the Huh-7 clones indicating that while the level of the HCV RNA does vary between individual cells clones, no statistical difference in RNA level was detectable between mouse and human cells.

Adaptive mutations are not required for replication of sgJFH-1 replicons in mouse hepatocytes

Because adaptive mutations are required for efficient replication of many HCV replicons in cell culture [9-11,20-22], we sequenced replicon clones obtained from our mouse cell lines to determine whether the population of sgJFH-1 replicons present in the MMH cells had acquired any adaptive mutations. RNA was extracted from 3 of the MMH replicon cell lines (MMHD3-sgJFH#1 and MMH1-1sgJFH#4 and #9). After reverse transcription, 4 PCR primer sets were used to amplify overlapping segments spanning the sgJFH-1 cDNA (Fig. 3A). These PCR products were ligated into the pGEMT-Easy vector and 12 individual clones of each fragment were sequenced. Although random mutations were observed throughout the cloned genomes, none of the changes were detected in more than 1 of the 12 clones sequenced (Fig. 3B), supporting the conclusion that adaptive mutations had not become established in the mouse replicon population. Also consistent with the absence of replicon adaptive mutations, total RNA isolated from both MMH and Huh-7 replicon cells containing comparable copies of sgJFH RNA (as determined by RT-QPCR) formed equivalent numbers of G418-resistant colonies/μg of viral RNA when re-transfected into naïve MMH cells (data not shown).

To determine if the initial establishment of the mouse replicon clones had selected for adapted MMH cells that were more permissive for HCV replication, we also "cured" the sgJFH-1 replicon from our MMH replicon cells with a 3 week treatment of IFNα (1000 U/ml) and IFNγ (500 U/ml), but subsequent sgJFH-1 RNA transfections into these "cured" (e.g. G418-sensitive, HCV-negative) mouse cells did not produce higher numbers of G418-resistent colonies/μg of input RNA compared to parallel transfections into the parental MMH cell lines indicating that we had

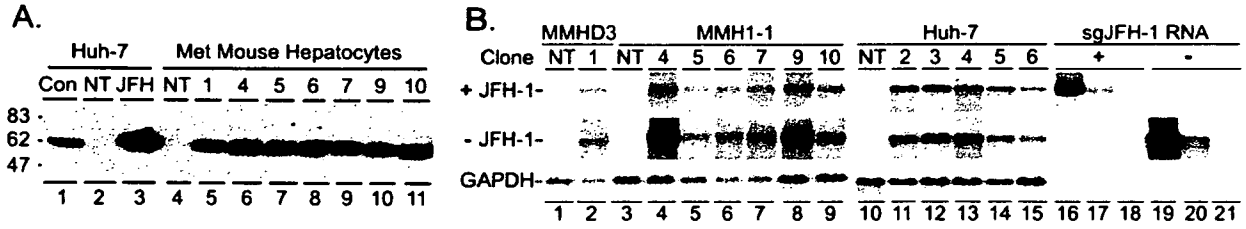

Figure 2

HCV protein and RNA detection in immortalized mouse hepatocytes. (A) Western Blot detection of HCV NS5A. Cell lysate was harvested from individual cell clones and resolved by SDS-PAGE. Samples include cell lysate from Huh-7 sgCon1 (lane 1); non-transfeted (NT) Huh-7 (lane 2); Huh-7 sgJFH-#2 (lane 3); NT MMHD3 cells (lane 4); MMHD3 sgJFH#1 (lane 5); and MMH1-1sgJFH#4, 5, 6, 7, 9, and 10 (lanes 6–11). (B) Northern Blot detection of sgJFH-1 RNA. Total RNA was isolated from non-transfected (NT) and individual MMHD3 (lanes 2), MMH1-1 (lanes 4–9), and Huh-7 (lanes 11–15) sgJFH-1 replicon clones. Positive-strand (top panel) and negative-strand (middle panel) HCV RNA was detected with ^{32}P-labeled strand-specific riboprobes. Cellular GAPDH was detected with a ^{32}P-labeled cDNA probe (bottom panel). Serial dilutions of *in vitro* transcribed positive-strand and negative-strand sgJFH-1 RNA (10^9, 10^8, 10^7) are shown in lanes 16–18 and lanes 19–21, respectively.

not selected MMH cells that more efficiently allow for establishment of HCV replication (data not shown).

Replication of sgJFH-1 HCV in other mouse cell lines

Because the sgJFH-1 replicon was able to replicate in MMH cells without any apparent viral or cellular adaptive mutations, we proceeded to determine if this ability was restricted to MMH cells or if the JFH-1 clone could replicate in other mouse cell lines. Hence, we transfected *in vitro* transcribed sgJFH-1 RNA into mouse AML12 hepatocytes as well as mouse NIH3T3 embryonic fibroblasts and selected for cells that were able to support replication of the neo-expressing replicon. Figure 4A shows strand-specific HCV Northern Blot analysis that verifies the presence of both positive-strand (Fig. 4A, top panel) and negative-strand (Fig. 4A, middle panel) HCV RNA in all the AML12 mouse hepatocyte cell clones. Similarly, negative strand-specific HCV Northern Blot analysis of total RNA from 9 NIH3T3 replicon cell clones also confirmed the presence of negative-strand HCV RNA in these non-hepatic mouse cells demonstrating that the sgJFH-1 HCV clone can replicate in a variety of mouse cell lines (Fig. 4B).

Replication of full length JFH-1 HCV replicons in mouse cells

To determine if full length HCV replication also could be established in mouse cells, *in vitro* transcribed full length (fl) JFH-1 replicon RNA was electroporated into MMH1-1 and Huh-7 cells, and cells supporting replicon replication were selected with 500 μg/ml G418. Northern Blot analysis of total RNA isolated from the resulting G418-resistant clones confirmed that negative-strand flJFH-1 RNA was present in both MMH1-1 (Fig. 5, lanes 1–4) and Huh-7 (Fig. 5, lanes 5–10) transfected cultures. However, unlike sgJFH replicon clones in which HCV RNA levels were equivalent between human and mouse cells clones, RT-QPCR analysis indicated that HCV RNA levels were approximately 3-fold lower in flJFH-1 MMH1-1 cell clones ($9.5 \times 10^4 - 7 \times 10^5$ copies/μg total RNA) compared to flJFH-1 Huh7 cell clones ($3.1 \times 10^5 - 1.9 \times 10^6$ copies/μg total RNA).

Lack of infectious HCV particle entry into and secretion from mouse hepatocytes

Because the flJFH-1 is capable of producing infectious HCV particles, we proceeded to determine whether the MMH1-1 mouse cells were able to secrete infectious HCV particles and/or were permissive for flJFH-1 entry. To determine if the flJFH-1 replicon clones were secreting infectious HCV particles that could transmit G418-resistance, the culture media from individual clones was collected and used to inoculate naive Huh-7 and MMH1-1 cells. Although G418-resistant colonies formed when Huh-7 cells were inoculated with the supernatant from Huh-7 flJFH-1 replicon cells, the culture supernatant from MMH1-1 flJFH-1 replicon cells did not confer G418-resistance to Huh-7 cells suggesting that the mouse cells were not able to secrete infectious HCV particles. Titration of the same media on Huh-7 cells further confirmed that all the Huh-7 flJFH-1 cell clones were secreting infectious HCV particles (expressed as foci forming units per ml), while no infectious HCV was detected in the media from MMH1-1 flJFH-1 cells, even after 50-fold concentration by centrifugal filtration (Fig. 5B).

Notably, inoculation of naïve MMH1-1 cells with media from either Huh-7 flJFH-1 or MMH1-1 flJFH-1 cell cultures did not confer G418-resistence to the mouse cells confirming that HCV particles are unable to enter mouse cells. Importantly, inoculation with high titer authentic

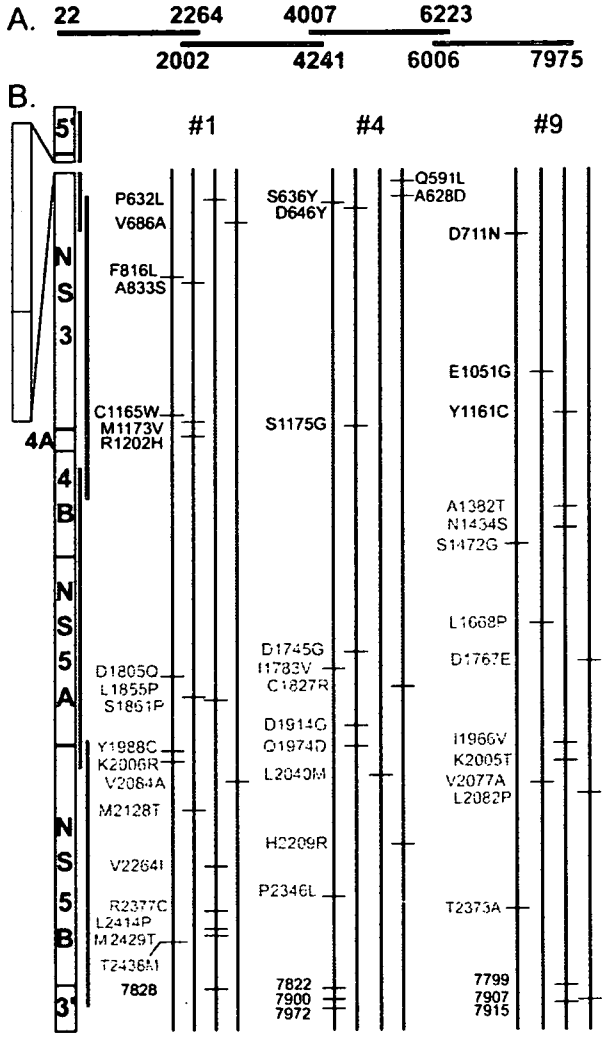

Figure 3

Sequence analysis of sgJFH-1 replicons in mouse hepatocytes. (A) PCR primer sets used to amplify overlapping HCV DNA segments. (B) Twelve clones of each fragment were sequenced with an ABI automatic DNA sequencer. Mutations within viral proteins are indicated by the amino acid position of the mutation within the context of the full length HCV open reading frame. Mutations in the 3' UTR are designated by their nucleotide position in the context of the full length HCV genome.

HCV virus produced in Huh-7 cells also failed to establish JFH-1 replication in MMH1-1, AML12, or NIH3T3 mouse cells (data not shown).

To determine whether the lack of detectable infectivity in the culture medium of flJFH-1 MMH1-1 replicon clones was due to an inability to secrete HCV particles or due to the secretion of non-infectious HCV particles, the culture supernatant from Huh-flJFH#7 and MMH1-1-flJFH#2 cultures were subjected to sucrose density gradient centrifugation. Fractions were collected, total RNA extracted, and RT-QPCR analysis performed to determine the amount of viral RNA in each fraction (Fig. 5C). HCV RNA in the supernatant of the Huh-flJFH#7 cells was detected over a broad density range of 1–1.15 g/ml, with a predominant peak at 1.07 g/ml, supporting the conclusion that the viral RNA was associated with enveloped particles (Fig. 5C). This is similar to the HCV RNA density profile reported for *in vitro*-derived infectious JFH-1 particles from which RNA was observed in density fractions from 1–1.15 g/ml with a peak at 1.09–1.10 g/ml [5,6]. In contrast, there was no specific low density peak of HCV RNA in the media of MMH1-1flJFH#2 cells and total viral RNA levels in the supernatant were significantly lower than that seen the in Huh-7 replicon cell supernatant. Hence, these results support the conclusion that mouse hepatocytes do not efficiently secrete enveloped, flJFH-1 RNA-containing particles.

Discussion

In this study, we have shown that replicon clones derived from the HCV JFH-1 consensus cDNA can establish and maintain efficient replication in both mouse hepatocytes (e.g. MMHD3, MMH1-1, AML12) and mouse embryonic fibroblasts (e.g. NIH3T3). This discovery reveals a previous unrealized breadth in the host range of the JFH-1 HCV clone, and expands our ability to develop experimental HCV model systems. In addition to observing stable expression of viral proteins and replication of viral RNA (Fig. 2), we found that HCV replication in mouse hepatocytes is not dependent on adaptive mutations in the viral genome (Fig. 3), but is sensitive to inhibition by interferons (data not shown) and the HCV-specific small molecule inhibitor, BILN2061 (data not shown). Taken together, these results demonstrate replication of non-adapted HCV replicon clones in mouse cells, and they potentially provide the basis for the production of a mouse model of HCV replication.

While Zhu et al., (2003) previously provided evidence that HCV replication in mouse hepatocytes was possible, the data presented here differs from that report in three fundamental ways. First, Zhu et al., (2003) could only initiate HCV replication in mouse cells by transfection with a HCV RNA quasispecies obtained from previously established HeLa replicon cells, but they were not able to establish replication in mouse cells with any individual replicon clone. In contrast, we have been able to identify a defined HCV replicon clone capable of replicating in various mouse cell lines. The identification of a specific mouse-permissive HCV clone is advantageous because it facilitates mouse model development and allows for

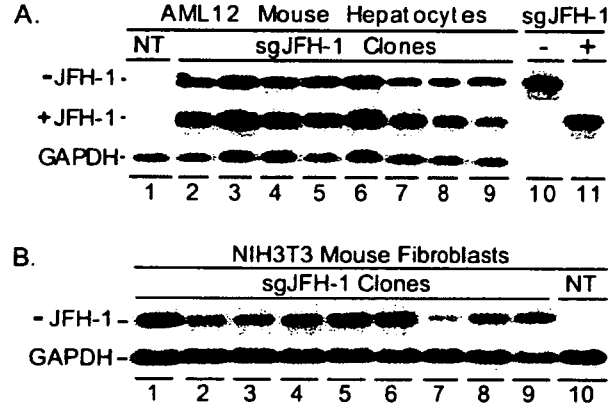

Figure 4
Northern Blot detection of sgJFH-1 RNA in AML12 mouse hepatocytes and NIH3T3 mouse fibroblasts. (A) Negative-strand HCV (top panel), positive-strand HCV (middle panel), and cellular GAPDH (bottom panel) was detected by Northern Blot analysis of total RNA isolated from non-transfected (NT) parental AML12 cells (lane 1) and 8 individual sgJFH-1 RNA transfected clones (lanes 2–9). *In vitro* transcribed positive-strand (lane 10) and negative-strand (lane 11) sgJFH-1 RNA was included as controls. (B) Northern Blot analysis for negative-strand HCV (top panel) and cellular GAPDH (bottom panel) was performed on total RNA isolated from non-transfected (NT) parental NIH3T3 cells (lane 10) and 9 randomly chosen sgJFH-1 RNA transfected clones (lanes 1–9).

reverse-genetics experimentation. Second, whereas replication of the subgenomic 1b HCV-N replicon reported by Zhu et al., (2003) was restricted to one specific mouse cell line, we have been able to establish JFH-1 HCV replication in multiple mouse cell lines significantly enhancing the likelihood that future *in vitro* and *in vivo* experimental HCV mouse models based on the JFH-1 clone might be possible. Finally, while the previous success was dependent on cell culture adaptive mutations that prevent HCV infectivity [23], we have demonstrated that replicons derived from the JFH-1 consensus sequence can replicate in mouse hepatocytes, in the absence of adaptive mutations that might otherwise alter the infectivity of the clone.

The ability of the JFH-1 clone to replicate efficiently in tissue culture in the absence of adaptive mutations has recently led to the development of *in vitro* HCV infection models in Huh-7 cells [5-7] and the demonstration that full length HCV replicons are capable of producing infectious viral particles (Fig. 5B–C). Likewise it provided us the opportunity to study the ability of mouse cells that support JFH-1 replication to uptake and secrete infectious HCV JFH-1 particles. Consistent with previous HCV pseudoparticles data, we observed no evidence that HCV particles were able to productively enter mouse cells, even when we used G418 treatment to select for cells that might have become HCV positive following inoculation. More unexpectedly, we also observed that mouse hepatocytes replicating infectious JFH-1 RNA were not able to secrete infectious HCV RNA containing particles. Though it is possible there were low levels of infectious particles present that were below the detection limits of our assays, sucrose density gradient analysis and RT-QPCR analysis of HCV RNA isolated from the mouse culture supernatants supports the conclusion that very few, if any, enveloped, HCV RNA-containing particles were being secreted from these mouse cells (Fig. 5C). This may reflect an inability to assemble infectious particles, secrete viral particles, or both. Hence, in addition to productive viral entry, infec-

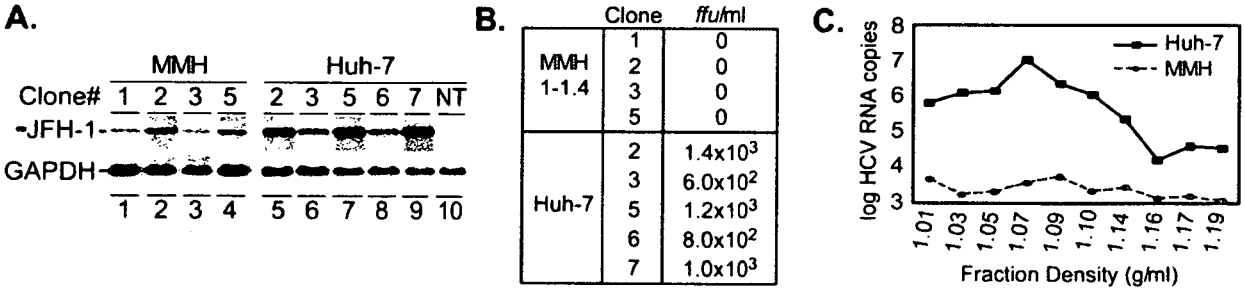

Figure 5
Intracellular and extracellular HCV RNA produced by fJFH-1 mouse replicon clones. (A) Northern Blot analysis for negative-strand HCV (top panel) and cellular GAPDH (bottom panel) was performed on total RNA isolated from MMH1-1 replicon clones (lanes 1–4), Huh-7 replicon clones (lanes 5–9), and non-transfected (NT) Huh-7 cells (lane 10). (B) HCV infectivity titer in the culture medium of the same MMH and Huh-7 clones was determined by incubating serial dilutions on naïve Huh-7 cells and performing immunofluorescence for HCV 3 days post-inoculation, as previously described [5]. The titer is expressed as NS5A-positive focus-forming units (*ffu*) per ml of medium. (C) RT-QPCR detection of extracellular HCV RNA following sucrose density gradient centrifugation.

tious particle production appears to be another aspect of the HCV life cycle that may not be readily recapitulated in mouse hepatocytes. Clearly, elucidating the basis of this phenomenon may help us understand the key steps required for infectious HCV particle secretion in human cells.

Conclusion

The data presented in this report demonstrate that JFH-1 derived HCV replicon clones can efficiently replicate in mouse cells. While this discovery certainly represents an advance in our ability to develop mouse models of HCV replication, further analysis of infectious JFH-1 HCV entry into and egress from mouse cells also identified at least 2 other aspects of the HCV life cycle that are not supported by the mouse hepatocytes tested. Such species restrictions will likely continue to make the development of a purely murine HCV infectious model challenging.

Methods

HCV constructs

Constructs containing the non-adapted wild type (pHCVrep1b BartMan_AvaII) and Huh-7 cell adapted [pHCVrep1bBB7 (S1179I)] HCV Con1 genotype 1b subgenomic replicons [9] and the genotype 1a H77 subgenomic replicon [H/SG-Neo(L+I)] [22] were provided by Dr. Charles Rice (Rockefeller University, NY). The pNNeo/3-5B(SI) construct encoding the genotype 1b HCV-N subgenomic replicon was provided by Dr. Stanley Lemon (University of Texas Medical Branch, Galveston)[13]. The constructs pSGR-JFH1, pFGR-JFH1, and pJFH-1 containing the sgJFH-1 HCV genotype 2a JFH-1 replicon, the full length (fl)JFH-1 replicon, and the genomic JFH-1 viral clone have been described [7,24,25]. In all constructs, the HCV cDNA is located at the 1+ position 5' of the T7 promoter. Plasmids were linearized at the 3' end of the HCV cDNA and used as a template for *in vitro* transcription by T7 RNA polymerase (MEGAscript; Ambion, Austin, TX). To generate strand-specific RNA probes, a 1 kb fragment of the JFH-1 NS5B coding region (HindIII-EcoRV) was cloned into the pBSKII+ vector to allow for T7 and SP6-driven transcription of JFH-1 negative and positive strand probes, respectively.

Cell culture

Huh-7 human hepatocytes and NIH3T3 mouse fibroblasts (CRL-1658; ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% fetal calf serum and Penicillin-Streptomycin-Glutamine (100×, liquid)(Gibco Invitrogen Corporation, Carlsbad, CA). AML12 mouse hepatocytes (ATCC; CRL-2254) were maintained in DMEM/F12 supplemented with 10% fetal calf serum, Penicillin-Streptomycin-Glutamine (100×, liquid), insulin-transferrin-selenium (100×, liquid)(Gibco Invitrogen), and 40 ng/ml dexamethasone (Sigma, St. Louis, MO). MMHD3 (Met Murine Hepatocyte) cells were obtained from Marco Tripodi (Università La Sapienza, Italy)[18]. This immortalized mouse hepatocyte cell line was derived from the liver of transgenic mice, which express the constitutively active cytoplasmic domain of the human hepatocyte growth factor receptor (cMet) in their livers. The MMH1-1 cells were independently derived from double transgenic cMet transgenic mice that also have a hepatitis B virus (HBV) transgene [19]. Although these cells contain a HBV transgene, expression and replication of HBV in these cells only occurs after the cells become confluent and are further differentiated in the presence of 2% DMSO for 8 days [19]. All of the experiments presented here were performed on subconfluent cell cultures in the absence of DMSO; therefore, no HBV replication was occurring in these cells (data not shown). Both MMHD3 and MMH1-1, were plated on collagen I Biocoat dishes (Becton Dickinson, Franklin Lakes, NJ) in RPMI 1640 (Gibco Invitrogen) supplemented with 10% fetal calf serum (Gibco Invitrogen), 55 ng/ml EGF (Becton Dickinson), 16 ng/ml IGF-II (Calbiochem, San Diego, CA), 10 μg/ml insulin (Sigma) and Penicillin-Streptomycin-Glutamine (100×, liquid)(Gibco Invitrogen)[19]. G418 was added to culture media as indicated (Invitrogen).

HCV RNA transfection and G418-resistant colony formation

In vitro transcribed HCV RNA or total cell RNA was transfected into cells using a modified electroporation protocol [10]. Trypsinized cells were washed twice with serum-free medium and resuspended to a final concentration of 1×10^7 cells/ml. One to ten micrograms of HCV RNA was then mixed with 0.4 ml of the cells in a 4 mm cuvette. A Gene Pulser system (BioRad Laboratories, Hercules, CA) was used to deliver the following single pulses: Huh-7 (0.27 kV, 100 OHMS, 960 μF); Met-based and AML12 cells (0.45 kV, 100 OHMS, 960 μF); NIH3T3 (0.27 kV, 100 OHMS, 960 μF). For the generation of replicon clones, transfected cultures were maintained in the presence of G418 at a concentration of 500 μg/ml until all cells died or distinct G418-resistant cell colonies formed. To visualize colony formation, cells were fixed and stained with crystal violet.

RNA analysis

Total cell RNA was isolated by the guanidine thiocyanate method using standard protocols [26]. RNA was resolved in 1% agarose, 2.2 M formaldehyde gels and transferred to nylon membrane (Schleicher & Schuell, Keene, NH). Membranes were cut across the 28S ribosomal band so that the top of the blot could be hybridized with ^{32}P-labeled strand-specific riboprobes (MAXIscript; Ambion), while the bottom was hybridized with ^{32}P-labeled cellular GAPDH cDNA probe (Random Prime Synthesis; Invitro-

gen, Carlsbad, CA). Hybridized probe was visualized using a storage phosphor system (Cyclone; Packard Instrument Co.). Alternatively, 1 μg of RNA was DNAse treated (DNA-*free* reagent; Ambion) for reverse transcription quantitative polymerase chain reaction (RT-QPCR). RNA was used for cDNA synthesis using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA), followed by real-time PCR quantification using a BioRad iCycler (BioRad Laboratories). HCV and GAPDH transcript levels were determined relative to a standard curve comprised of serial dilutions of plasmid containing the HCV cDNA or the mouse GAPDH gene. The PCR primers used to detect JFH-1 (GenBank AB047639) were 5'-TCTGCGGAACCGGTGAGTA-3' (sense) and 5'-TCAGGCAGTACCACAAGGC-3' (antisense). The PCR primers used to detect H77C (GenBank AF011751) were 5'-GTCTGCGGAACCGGTGAG-3' (sense) and 5'-GGCATTGAGCGGGTTTATC-3' (antisense). The PCR primers used to detect both genotype 1b HCV clones (Genbank AI242652 and AF139594) were 5'-ATGGCGTTAGTATGAGTGTC-3' (sense) and 5'-GGCATTGAGCGGGTTGATC-3' (antisense). The PCR primers used to detect mouse GAPDH (GenBank M32599) were 5'-TCTGGAAAGCTGTGGCGTG-3' (sense) and 5'-CCAGTGAGCTTCCCGTTCAG-3' (antisense).

Western Blot analysis

Cells were harvested in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with a protease inhibitor cocktail (Roche Applied Science, Indianapolis). Fifty micrograms of protein was resolved by SDS-PAGE and transferred to Hybond nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ). Membranes were sequentially blocked with 5% Nonfat Milk, incubated with a 1:500 dilution of the polyclonal rabbit NS5A antibody, M15 (provided by Dr. Michael Houghton, Chiron, Emeryville, CA), washed 3 times with PBS/0.05% Tween20, incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce, Rockford, Illinois), and washed again. Bound antibody complexes were detected with SuperSignal chemiluminescent substrate (Pierce).

Sequencing HCV replicons

Total cell RNA was extracted from 3 mouse replicon cell lines (MMHD3sgJFH#1, MMH1-1sgJFH#4, and MMH1-1sgJFH#9). Reverse transcription was carried out with random hexamers using 1 μg of RNA (Transcriptor Reverse Transcriptase; RocheApplied Sciences, Indianapolis, IN). HCV DNA was subsequently amplified with high fidelity Tgo polymerase (Roche). Four PCR primer sets were used to amplify overlapping segments spanning positions 22 to 2264, 2002 to 4241, 4007 to 6223, and 6006 to 7975 of the sgJFH-1 replicon (Fig. 3A). The PCR products were cloned into the pGEM-T Easy plasmid (Promega, Madison WI), and 12 clones of each fragment (4 from each cells line) were sequenced with an ABI automatic DNA sequencer using the M13 forward and reverse primers in the vector as well as 20–21 bp sequencing primers located within the HCV subgenomic replicon at nucleotide positions: 1252, 1790, 2053, 2290, 2571, 2820, 3269, 3836, 4256, 4634, 4813, 5215, 5425, 6148, 6555, and 7416.

Titration of infectious HCV

Cell supernatants were serially diluted in complete medium and used to infect naïve MMH and Huh-7 cells in 96-well plates. The level of HCV infection was determined 3 days post-infection by immunofluorescence staining for HCV NS5A as previously described [5]. The viral titer is expressed as focus-forming units per milliliter of supernatant (*ffu*/ml), determined by the number of NS5A-positive foci detected at the highest HCV-positive dilution.

Sucrose density gradient analysis

Sucrose density-gradient ultracentrifugation was performed as described [5]. Supernatant from flJFH-1 replicon cultures was collected, centrifuged at 4,000 rpm for 5 min to remove cellular debris, and concentrated by centrifugal filteration when indicated (Amicon Ultra; Millipore, Billerica, MA). Samples were loaded onto TNE buffer (50 mM Tris·HCl, pH 8;100 mM NaCl;1 mM EDTA)-based 20–60% sucrose gradient and centrifuged at 120,000 × *g* for 16 h at 4°C using a SW60 rotor in a Beckman Coulter L8-80 Ultracentrifuge (Fullerton, CA). Fractions were collected from the bottom of the gradient, and analyzed for HCV RNA by RT-QPCR.

Note

While this manuscript was being prepared, Chang et al., (JVirol. 2006 Aug;80:7364-74) published a report consistent with these data showing that JFH-1 replicons can replicate in mouse embryonic fibroblasts (MEFs).

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SLU conceived and designed the study, obtained funding, and then performed the experiments. JC did the sequence analysis of sgJFH-1 from mouse cells and cloned the JFH-1 RNA probe template plasmid. FVC participated in data analysis, revising of the manuscript, and provided funding for JC. TW provided the critical unpublished reagents, participated in data analysis, and revising of the manuscript. All authors read and approved the manuscript.

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Regulation of CXCL-8 (Interleukin-8) Induction by Double-Stranded RNA Signaling Pathways during Hepatitis C Virus Infection[▽]

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Hepatitis C virus (HCV) infection induces the α-chemokine interleukin-8 (CXCL-8), which is regulated at the levels of transcription and mRNA stability. In the current study, CXCL-8 regulation by double-stranded (ds)RNA pathways was analyzed in the context of HCV infection. A constitutively active mutant of the retinoic acid-inducible gene I (RIG-I), RIG-N, activated CXCL-8 transcription. Promoter mutagenesis experiments indicated that NF-κB and interferon (IFN)-stimulated response element (ISRE) binding sites were required for the RIG-N induction of CXCL-8 transcription. IFN-β promoter stimulator 1 (IPS-1) expression also activated CXCL-8 transcription, and mutations of the ISRE and NF-κB binding sites reduced and abrogated CXCL-8 transcription, respectively. In the presence of wild-type RIG-I, transfection of JFH-1 RNA or JFH-1 virus infection of Huh7.5.1 cells activated the CXCL-8 promoter. Expression of IFN regulatory factor 3 (IRF-3) stimulated transcription from both full-length and ISRE-driven CXCL-8 promoters. Chromatin immunoprecipitation assays demonstrated that IRF-3 and NF-κB bound directly to the CXCL-8 promoter in response to virus infection and dsRNA transfection. RIG-N stabilized CXCL-8 mRNA via the AU-rich element in the 3′ untranslated region of CXCL-8 mRNA, leading to an increase in its half-life following tumor necrosis factor alpha induction. The data indicate that HCV infection triggers dsRNA signaling pathways that induce CXCL-8 via transcriptional activation and mRNA stabilization and define a regulatory link between innate antiviral and inflammatory cellular responses to virus infection.

Hepatitis C virus (HCV) infects an estimated 3% or 170 million of the world's population and causes an estimated 476,000 deaths per year due to complications of end-stage liver disease (56, 62). In the United States, about 1.8% of the general population or 4 million persons are infected. Of those acutely infected with HCV, approximately 85% develop chronic infection, and about 70% of these patients develop histological evidence of chronic liver disease. Moreover, viremia is not cleared in about 50% of infected patients treated with pegylated interferon (IFN)-ribavirin therapy, the current standard of care. Compounding this issue are the predictions that in the next 20 years, HCV-related complications, including hepatic decompensation, hepatocellular carcinoma, and liver-related deaths, will increase by 106%, 81%, and 180%, respectively (12). Thus, chronic hepatitis C is a serious global medical problem necessitating effective treatment. Given the propensity of HCV for chronic infection, association with severe liver disease, and difficulty of treatment, many studies are focused on HCV-host interactions that contribute to HCV persistence and pathogenesis.

The inflammatory response to virus infection involves the regulated induction of cytokines and chemokines. The response is initiated within the infected cell by pathogen-associated molecular pattern (PAMP) recognition but soon thereafter affects neighboring cells and tissues due to the paracrine effects of cytokine and chemokine release. Cytokine and chemokine release occurs rapidly in response to virus infection, and its chief objectives are to recruit inflammatory leukocytes, limit virus replication and spread, and induce adaptive immunity. However, prolonged expression of chemokines in the context of chronic viral infections may be detrimental to the host. For example, patients with chronic hepatitis C have elevations in serum levels of α-chemokine interleukin-8 (CXCL-8), and patients who are nonresponsive to IFN therapy have high pretreatment levels of CXCL-8 (39, 51). When expressed in cell culture, the HCV NS5A protein induces CXCL-8, which is associated with the inhibition of the antiviral effects of IFN (4, 16, 50).

CXCL-8 is a 71-amino-acid chemokine belonging to the CXC family and is produced by many cell types, including monocytes, epithelial cells, fibroblasts, and hepatocytes (42). CXCL-8 elicits many effects, including neutrophil, T-lymphocyte and basophil chemotaxis and degranulation, oxidative burst, and lysosomal-enzyme release (42). CXCL-8 has been shown to be an important mediator of the inflammatory responses to many viruses and bacteria. For example, CXCL-8 is induced in response to the expression of the HCV NS5A protein (3, 4, 16, 50) and HCV replication (19). Moreover,

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CXCL-8 inhibits the antiviral actions of IFN-α (29), and recent studies indicate that CXCL-8 protein levels are associated with HCV replication (30).

CXCL-8 is induced by a variety of stimuli, including those from CXCL-1, tumor necrosis factor alpha (TNF-α), phorbol esters, lipopolysaccharide, and virus infection (42). The induction of CXCL-8 involves both the transcriptional activation of the CXCL-8 promoter and the stabilization of CXCL-8 mRNA (61). CXCL-8 mRNA stabilization involves the binding of regulatory proteins to AU-rich elements (AREs) in the 3′ untranslated region (3′UTR) of the mRNA (1, 6, 9, 53). The CXCL-8 promoter contains binding sites for NF-κB, NF-interleukin 6 (IL-6), AP-1 protein, and an IFN-stimulated response element (ISRE)-like element, which has been shown to bind proteins belonging to the IFN regulatory factor (IRF) family (8, 65). The mechanisms of CXCL-8 gene induction have been investigated using different stimuli and cell types, and deletion and mutational analyses of the promoter indicate that CXCL-8 is activated in a cell-type- and stimulus-specific manner (43, 45). It has previously been shown that HCV NS5A-induced transcriptional activation of the CXCL-8 promoter requires intact NF-κB and AP-1 binding sites (4, 50). However, the role of upstream regulatory elements, such as the ISRE site, is not known.

IRF proteins play pivotal roles in innate antiviral responses and bind to DNA sequences containing ISREs (2, 36). IRFs are activated by virus infection when viral PAMPs, such as double-stranded (ds)RNA, are sensed by Toll-like receptor 3 (TLR3) or by cytoplasmic dsRNA sensors such as the retinoic acid-inducible gene I (RIG-I) (66). IPS-1 (also known as the MAVS, Cardif, or VISA protein) (21) is an adaptor protein for RIG-I signaling (34). RNA binding to RIG-I and interaction with IPS-1 or TLR3 activate IRF-3 and NF-κB. IRF-3 and NF-κB translocate to the nucleus, interact with other transcription factors, and bind to their cognate promoter elements to induce gene expression, which classically includes IFN-α and IFN-β genes.

IRFs and NF-κB also regulate transcription of chemokine genes. For instance, IRF-1 is involved in the respiratory syncytial virus (RSV) and *Helicobacter pylori* induction of CXCL-8 by binding to one of several ISRE-like elements in the CXCL-8 promoter (8, 65). Similarly, IRF-3 induces the chemokine RANTES by binding to ISREs in the RANTES promoter (32). Moreover, NF-κB is the principal transcription factor responsible for CXCL-8 induction (42). Thus, the regulation of chemokines by NF-κB and especially by IRFs may represent a functional link between innate antiviral and inflammatory responses. In the current report, we examined the induction of CXCL-8 by dsRNA-triggered innate antiviral pathways in the context of HCV infection in vitro.

MATERIALS AND METHODS

Cells and viruses. Human hepatoma Huh7 cells and HEK293 cells were grown in Huh7 medium which contained Dulbecco's minimum essential medium, 10% fetal bovine serum, 1× penicillin, streptomycin, amphotericin B (Fungizone) (except for HEK cells), 10 mM L-glutamine, and 1× nonessential amino acids (all reagents were from Invitrogen, Carlsbad, CA). Huh7 cells were obtained from Apath, LLC. Huh7.5.1 cells were obtained from Francis Chisari (68) and cultured in Huh7 medium. All cell lines were checked for mycoplasma by using a MycoAlert assay (Cambrex Bio Science, Rockland, ME) and found to be mycoplasma-free.

JFH-1 viral stock preparation, cell infection, and titration were performed exactly as described previously (59, 68).

Plasmids. BB7 replicon plasmid DNA was obtained from Apath, LLC. Generation of JFH-1 RNA and transfection of cells were performed as described previously (59). Various luciferase reporter genes under the control of different forms of CXCL-8 were also used and were obtained from Naofumi Mukaida (44) or generated as described previously (8) (see Fig. 1). Plasmid pQ150 expresses green fluorescent protein (GFP) under the control of the constitutive EF-1α promoter and was provided by Jeffery Vieira. Cytomegalovirus IRF-3 (CMV-IRF-3) (33) was obtained from John Hiscott. Wild-type RIG (RIG-WT), constitutively active (RIG-N), and dominant negative (RIG-C) plasmids were generated as previously described (14, 66).

Transfection. The day prior to transfection, 3×10^4 cells were plated in black, clear-bottomed, 96-well tissue culture plates. Endotoxin-free plasmid DNA was purified (Endofree kit; QIAGEN, Valencia, CA) and introduced into cells with Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen). For reporter gene studies, unless otherwise indicated, 100 ng of the luciferase gene under the control of the promoter construct of interest was transfected into cells in triplicate or quadruplicate. When expression plasmids were included in the transfection, 50 ng of each was added. Eighteen hours later, stimuli such as recombinant human TNF-α (rhTNF-α) (15 ng/ml; Pierce Biotechnology, Rockford, IL) or virus infection (Sendai virus at a multiplicity of infection [MOI] of 100 hemagglutinin units, or JFH-1 virus at an MOI of 0.01 focus-forming unit) were added to cells. In the case of poly(I) · poly(C) [poly(I:C)] (0.2 μg/well) or JFH-1 RNA (0.25 μg/well), cells were retransfected using Lipofectamine 2000 or transmessenger RNA (QIAGEN) reagents. Six h [for poly(I:C)] or 24 h later (for JFH-1 RNA), luciferase activity was measured on cell lysates using a Britelite assay system (Perkin Elmer, Boston, MA). Before protein harvest, cells were visualized under a fluorescent microscope, and the transfection efficiency was determined by comparing the proportion of GFP-positive cells to the total cell number, as described previously (30, 40, 47). The results, in relative light units, shown in Fig. 2, 3, and 4 are corrected for transfection efficiency.

Western blot analysis. Protein lysates were quantitated (BCA Protein Assay; Pierce), and equal amounts of total protein (10 to 20 μg) were separated on 4 to 20% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels. IRF-3 and Stat2 were detected using polyclonal antiserum (Santa Cruz Biotechnology). HCV proteins were detected using random, deidentified HCV-infected patient serum, as described previously (49). Prior to use, infected serum was inactivated by adding Triton X-100 to a final concentration of 1%. NS5A protein was also detected using a polyclonal antibody to NS5A (Chiron, Emeryville, CA). RIG proteins were detected using a polyclonal antibody (25).

RNA quantitation. CXCL-8 mRNA was quantitated by real-time reverse transcriptase (RT)-PCR, as recently described (19). Dilutions of precisely quantitated CXCL-8 cDNA in the PCMGSNEO plasmid (64) (kindly provided by Naofumi Mukaida), ranging from 0 to 10^7 copies per tube, were run in triplicate to generate a standard curve, which served as a reference to calculate the CXCL-8 copy number based on the cycle threshold. RNA copy numbers were normalized to 10 ng of total cellular RNA.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's specifications (Upstate USA, Charlottesville, VA). Briefly, 2×10^6 cells were plated on 10-cm dishes and then treated with various inducers, including TNF-α, poly(I:C) or encephalomyocarditis virus infection, for various times. Transcription factors were cross-linked to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubated for 10 min at 37°C. Cells were washed and scraped into phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml pepstatin A. Cells were pelleted and resuspended in SDS lysis buffer (Upstate), and DNA was sheared into lengths of 200 to 1,000 base pairs by sonication at 50 V and 30% amplitude for a total of eight times for 5 seconds each on ice. Lysates were cleared by centrifugation for 10 min at 13,000 rpm at 4°C. Supernatants were diluted ninefold in ChIP dilution buffer (Upstate) and precleared with protein A agarose-salmon sperm DNA (50% slurry; Upstate) for 30 min at 4°C with agitation. One to two microliters of IRF-3 antibody (polyclonal, from Michael David, or monoclonal, from Pharmingen) was added to the precleared supernatants and incubated overnight with rotation at 4°C, followed by the addition of protein A agarose-salmon sperm DNA. ChIP samples were washed, DNA protein complexes eluted, cross-links reversed, and DNA extracted. CXCL-8 and IFN-B promoter-specific PCRs were performed using primer sets CXCL-8F (AAGAA AACTTTCGTCATACTCCG), CXCL-8R (TGGCTTTTTATATCATCACCC TAC), IFN-BF (CCTCACAGTTTGTAAATCTTTTTCCC), and IFN-BR (AC

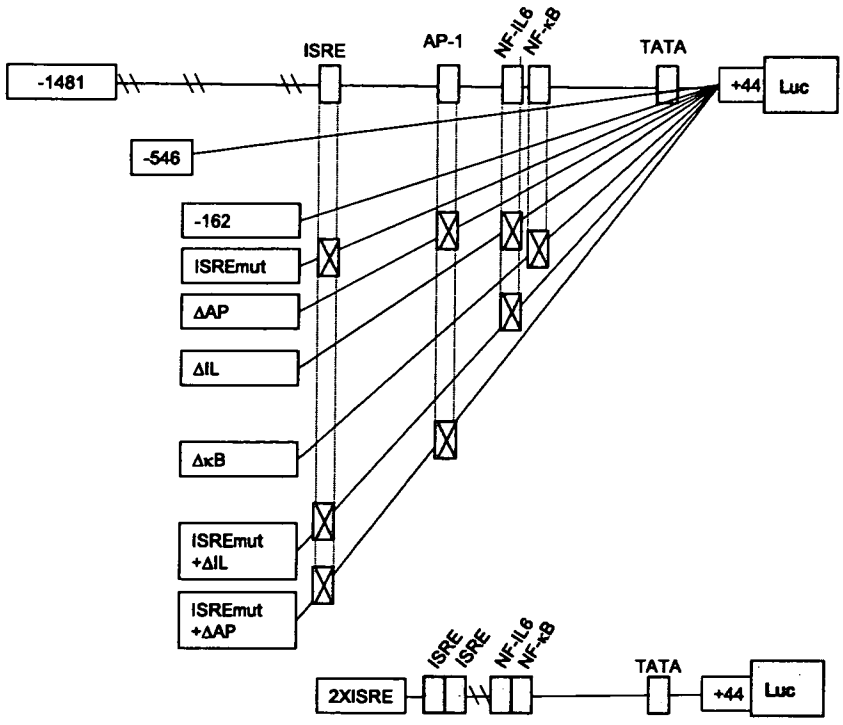

FIG. 1. Schematic representation of the CXCL-8 promoter. Locations of ISRE, AP-1, NF-IL-6, NF-κB sites are indicated. The names of the reporter constructs are found in the boxes to the left of each schematic. Luc, luciferase.

GAACAGTGTCGCCTACTACCTG), where F and R designate forward and reverse primers.

These primer sets hybridize to regions 1302 to 1473 of the genomic clone of CXCL-8 (NCBI accession number M28130) and to regions 97 to 348 of the IFN-β (NCBI accession number V00534.1) promoter and generate PCR products of 171 (CXCL-8) and 251 (IFN-β) base pairs. Forty-five cycles of PCR were performed as follows: 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

Cloning and reporter studies with the CXCL-8 3′UTR. A 237-base pair region (nucleotides 972 to 1209 of accession number NM000584) from the 1,250-base pair CXCL-8 3′ untranslated region (3′UTR) was amplified by RT-PCR. Briefly, total RNA was extracted by Tri Reagent (Molecular Research Center, Cincinnati, OH) from a THP-1 monocytic cell line (ATCC, Manassas, VA) that was previously stimulated with 10 μg/ml lipopolysaccharide in the presence of cycloheximide (5 μg/ml). cDNA was synthesized and amplified by PCR with the forward primer with a BamHI site (underlined), 5′ GCACCGGATCCGATGT TGTGAGGACATGTG 3′, and the reverse primer with an XbaI site (underlined), 5′ GCCAGTCTAGAACCCTGATTGAAATTTAT 3′. The additional 5′ sequences provided thermal stability to the oligonucleotides and facilitated the restriction digest. The PCR products were purified by phenol-chloroform extraction, followed by ethanol precipitation. The PCR products were cut by BamHI and XbaI sequentially, followed by phenol extraction and ethanol precipitation. The digested PCR products were ligated into an expression vector derived from a gWIZ plasmid (Gene Therapy Systems, Inc., San Diego, CA) that contains an enhanced GFP (EGFP) coding region under the constitutive expression of the CMV/intron A promoter and has a bovine growth hormone 3′UTR. Recombinant colonies were verified by PCR using a forward vector-specific primer and a CXCL-8 3′UTR reverse primer.

Cells (3×10^4 cells per well) in black clear-bottomed 96-well plates were transfected with the GFP plasmids. Transfections were performed as mentioned above. All transfections were performed in quadruplicate. The variance in GFP fluorescence among replicate microwells was <7%. Transfection efficiency in HEK293 was always 70% ± 5%. Data are presented as the mean values ± standard errors of the fluorescence intensity determined by using a ZENYTH 3100 model instrument with the following parameters: excitation filter, 485 nm; emission filter, 535 nm; integration time, 1s; and bottom fluorescence read setting.

Statistics. Differences between means of triplicate or quadruplicate samples of luciferase or fluorescence readings were compared using Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Figure 1 shows a schematic representation of the CXCL-8 promoter constructs used in this study. The figure shows the full-length (−1481) and truncated (−546 and −162) promoters used in this study. The −162 construct was also altered so that the ISRE, AP-1, NF-IL-6, and NF-κB binding sites were mutated to prevent transcription factor binding (8). The 2XISRE construct contains two copies of the ISRE site fused upstream to a minimal CXCL-8 promoter containing the NF-IL-6 and NF-κB binding sites, which was shown previously to be induced by RSV infection (8).

dsRNA sensor proteins activate CXCL-8 transcription. RIG-I and related CARD-containing cellular proteins have been shown to lead to NF-κB and IRF-3 activation following viral infection or dsRNA stimulation (14, 38, 55, 66). We first demonstrated the functionality of the RIG-WT, RIG-N (constitutively active), and RIG-C (dominant negative) proteins in our experimental system. Wild-type and mutant RIG-expressing plasmids were transfected along with the IFN-β luciferase reporter gene and transfected with poly(I:C), a synthetic source of dsRNA. RIG-WT transfection led to IFN-β transcription only following dsRNA transfection, while RIG-N induced IFN-β transcription independently of dsRNA. In contrast, RIG-C inhibited IFN-β transcription under all conditions (data not shown). We also verified the expression of the RIG-WT and RIG-N proteins (Fig. 2A). We then investigated

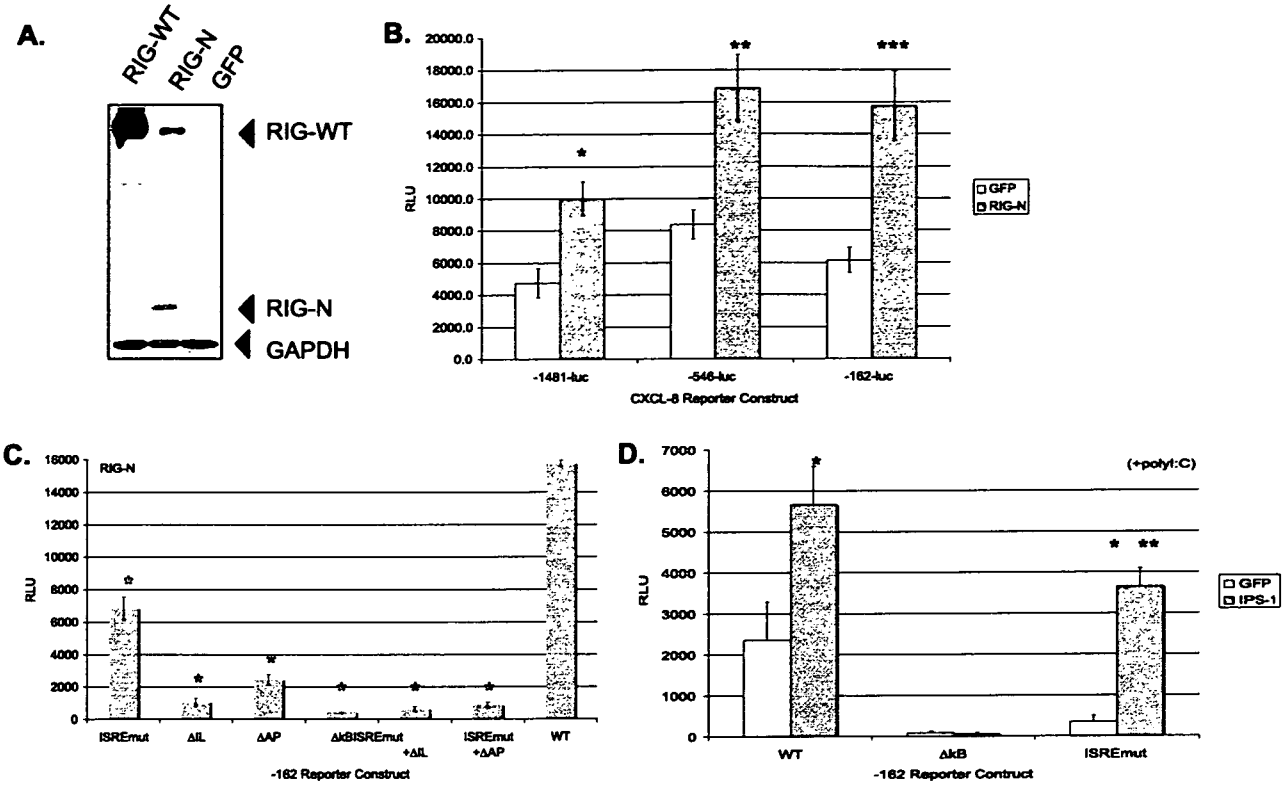

FIG. 2. Double-stranded RNA signaling pathways activate CXCL-8 transcription. (A) Expression of RIG-WT and constitutively active RIG-N proteins. Huh7 cells were transfected with plasmids expressing RIG-WT, RIG-N, or GFP, and whole-cell protein extracts were harvested 48 h later and analyzed by Western blotting with a polyclonal antiserum to RIG-I. Positions of the proteins are indicated with arrowheads. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cellular protein expression verified equal loading of proteins among the different conditions. (B) RIG-N activates CXCL-8 transcription. Huh7 cells were transfected with RIG-N- or GFP-expressing plasmids along with CXCL-8 reporter genes −1481-luc, −546-luc, or −162-luc, and luciferase readings were measured 24 h posttransfection. RLU, relative light units. Asterisks indicate significance of RIG-N luciferase values versus GFP values (*, $P = 0.002$; **, $P = 0.017$; ***, $P = 0.003$). (C) CXCL-8 promoter mutagenesis. Huh7 cells were transfected with RIG-N or GFP plasmids and the indicated CXCL-8 promoter constructs in the −162 backbone containing various mutations in transcription factor binding sites. Luciferase readings were measured 24 h posttransfection. Asterisks indicate that the luciferase values of mutant promoters compared to that of the wild-type −162 construct were significantly different (P, <0.01). (D) IPS-1 activates CXCL-8 transcription. Huh7 cells were transfected with IPS-1- or GFP-expressing plasmids along with wild-type −162 or mutant −162 promoters containing mutations in the NF-κB and ISRE binding sites, and 20 h posttransfection, cells were transfected with poly(I:C). Luciferase readings were measured 6 h later. The single asterisk indicates that IPS-1 luciferase readings were significantly different from the GFP readings (*, P <0.01). The double asterisk indicates that IPS-1-induced luciferase values from the ISRE mutant promoter were significantly lower than IPS-1 induced luciferase values from the wild-type promoter (**, $P = 0.008$).

whether RIG-I modulated CXCL-8 transcription. Huh7 cells were transfected with various CXCL-8 reporter gene constructs in the presence of constitutively active RIG-N or GFP as a negative control. As shown in Fig. 2B, RIG-N transactivated full-length (−1481) and truncated (−546 and −162) CXCL-8 promoter constructs by 2.1-, 2.0-, and 2.6-fold, respectively, relative to that of GFP (all P values were <0.02).

Because dsRNA signaling activates both IRFs and NF-κB and these transcription factors are involved in CXCL-8 induction, we examined the RIG-N activation of CXCL-8 transcription from the −162 constructs containing various mutations in their transcription factor binding sites. As shown in Fig. 2C, RIG-N activated the −162 construct, while the mutation of the ISRE caused a 2.3-fold reduction in luciferase activity (P, <0.01). Mutations of the AP-1, the NF-IL-6, and the NF-κB sites led to 6.5-, 14.8-, and 37.2-fold reductions, respectively, in transcription, while mutations of both the ISRE/NF-IL-6 and the ISRE/AP-1 sites caused 24.3- and 17.6-fold reductions, respectively, in transcription. The data indicate a requirement for NF-κB, NF-IL-6, and AP-1 in RIG-N-mediated induction of CXCL-8 transcription, with a subtle yet consistent regulation by transcription factors binding to the ISRE of the CXCL-8 promoter.

Since IPS-1 is an essential adaptor protein in RIG-I signaling (27, 38, 52, 63), we determined whether it regulates CXCL-8 transcription. Cells were transfected with reporter and expression plasmids and then transfected with poly(I:C) to provide dsRNA. As shown in Fig. 2D, IPS-1 expression activated CXCL-8 transcription from the wild-type −162 construct 2.4-fold compared to that of GFP (P, <0.01), and a mutation of the NF-κB binding site abrogated the response. A mutation of the ISRE binding site in the CXCL-8 promoter revealed that while IPS-1 still stimulated transcription 10.2-fold relative to that of GFP ($P < 0.01$), the relative levels of transcription

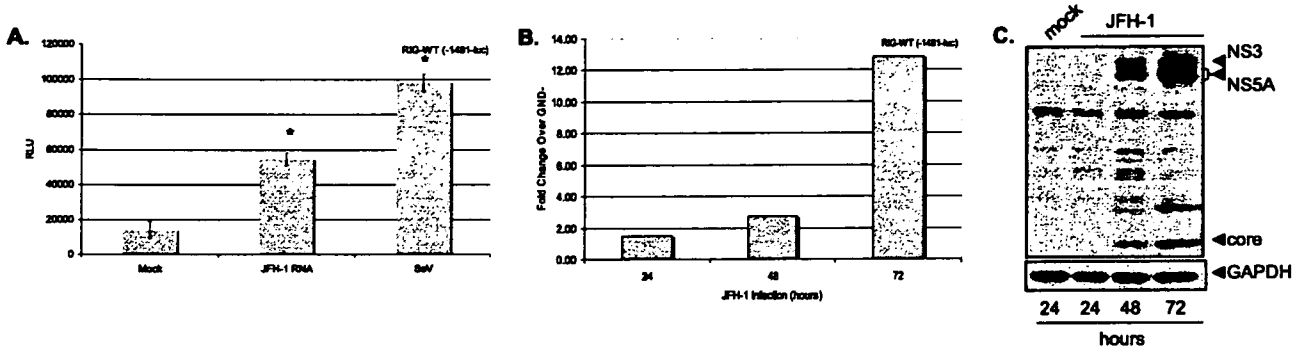

FIG. 3. HCV RNA and HCV infection activate CXCL-8 transcription. (A) Huh7.5.1 cells were transfected with RIG-WT and the full-length −1481 CXCL-8 luciferase construct, and 24 h later, cells were mock transfected, transfected with JFH-1 RNA, or infected with Sendai virus (SeV) at 100 hemagglutinin inhibitor units. Luciferase readings were measured 24 h later. Asterisks indicate that JFH-1- or Sendai virus-induced CXCL-8 transcription was significantly higher than mock-transfected cells (*, $P < 0.01$). RLU, relative light units. (B) Huh7.5.1 cells were transfected with RIG-WT and the full-length −1481 CXCL-8 luciferase construct, and 24 h later, cells were infected with JFH-1 virus stocks at an MOI of 0.01. Control cells were incubated with an equivalent volume of supernatants from cells transfected with replication-incompetent JFH-1 RNA containing the GND mutation in the viral polymerase. Luciferase readings were taken at the indicated times. Data are expressed as the increase (*n*-fold) in luciferase values over that of the GND control. (C) Western blot detection of HCV NS3, NS5A, and core proteins in Huh7.5.1 cells infected with JFH-1 at an MOI of 0.01. Blots were also probed with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) to verify equal protein loading.

were 1.6-fold lower than that of the wild-type CXCL-8 promoter ($P = 0.008$). In the absence of dsRNA, IPS-1 still activated transcription from the −162 wild-type construct (data not shown). The data indicate that IPS-1 and RIG-I signal to induce the CXCL-8 promoter and that in addition to the well-known role for NF-κB, ISRE-binding proteins also regulate CXCL-8 transcription.

HCV RNA and JFH-1 infection activate CXCL-8 transcription. Huh7.5.1 cells are defective in dsRNA signaling due to a mutation in RIG-I (55). Therefore, to investigate the effect of transfection of HCV RNA on CXCL-8 transcription, cells were first cotransfected with RIG-WT and the full-length CXCL-8 promoter-luciferase construct and then transfected with JFH-1 viral RNA derived from in vitro transcription. As shown in Fig. 3A, JFH-1 RNA caused a fourfold increase in transcription from the full-length CXCL-8 promoter compared to that of the mock-transfected cells (P, <0.01). As a positive control, cells were infected with Sendai virus, which caused a sevenfold induction of CXCL-8 transcription (P, <0.01). Moreover, as shown in Fig. 3B, when Huh7.5.1 cells were transfected with RIG-WT, followed by JFH-1 virus infection, a progressive increase in CXCL-8 transcription was observed over time. Figure 3C shows the relative levels of HCV NS3, NS5A, and core proteins by Western blotting at 24, 48, and 72 h postinfection, respectively. Collectively, the data indicate that HCV infection activates CXCL-8 transcription via the RIG-I pathway. Note also that transfection of synthetic dsRNA in the form of poly(I:C) also induced CXCL-8 in a RIG-I dependent fashion (data not shown). Thus, the response is not necessarily specific to HCV RNA but may reflect a general cellular response to dsRNA exposure.

IRF-3 stimulates CXCL-8 transcription. IRF-3 has been shown to regulate the expression of other chemokines such as RANTES (32) and is central to the host's control of HCV infection (15). Since RIG-I signals to IRF-3 (66), we investigated the effects of IRF-3 expression on CXCL-8 transcription and HCV replication. Under basal conditions, IRF-3 induced a highly significant 8.5-fold increase (P, <0.01) in transcription from the full-length −1481 CXCL-8 promoter, while IRF-3 induced a 2.5-fold increase (P, <0.01) in CXCL-8 promoter activity in the presence of TNF-α (Fig. 4A). Shown below Fig. 4 A is a Western blot verifying the specific expression of IRF-3. Moreover, IRF-3 induced a statistically significant dose-dependent increase in activation of the 2XISRE CXCL-8 reporter construct (P, <0.01) (Fig. 4B). IRF-3-5D, a constitutively active mutant of IRF-3 (33), also activated the 2XISRE promoter construct (data not shown). The data indicate that IRF-3 transactivates the CXCL-8 promoter and are consistent with a recent report that during the early course of HCV infection, IRF-3 is activated (34).

IRF-3 binds directly to the CXCL-8 promoter in vivo. Since IPS-1 and RIG-I signaling to the CXCL-8 promoter requires the ISRE at positions −130 to −162 (Fig. 2C and 2D) and IRF-3 transactivates the CXCL-8 promoter (Fig. 4), we questioned whether IRF-3 binds directly to the CXCL-8 promoter. To investigate this issue, we established ChIP assays which measure binding of transcription factors to DNA in a cellular context. We first demonstrated that NF-κB, a central player in both CXCL-8 (42) and IFN-β (20) transcription, bound to the CXCL-8 and IFN-β promoters in response to double-stranded RNA transfection (Fig. 5A). The interaction was specific since CXCL-8- and IFN-β-specific amplification products were obtained only with extracted genomic DNA, input DNA, and DNA-protein complexes that were immunoprecipitated with anti-NF-κB antiserum. The data indicate that dsRNA treatment results in NF-κB activation and binding to the CXCL-8 and IFN-β promoters. Similarly, when Huh7 cells were transfected with IRF-3, the protein bound specifically to both the CXCL-8 and the IFN-β promoters (Fig. 5B). Cumulatively, the data indicate that dsRNA activates the RIG-I pathway to induce CXCL-8 transcription, which involves the binding of IRF-3 and NF-κB to the CXCL-8 promoter.

RIG-N stabilizes CXCL-8 mRNA in an ARE-dependent manner. In addition to transcriptional activation, modulation of the half-life of CXCL-8 mRNA plays a major role in CXCL-8 induction in response to various stimuli. For example,

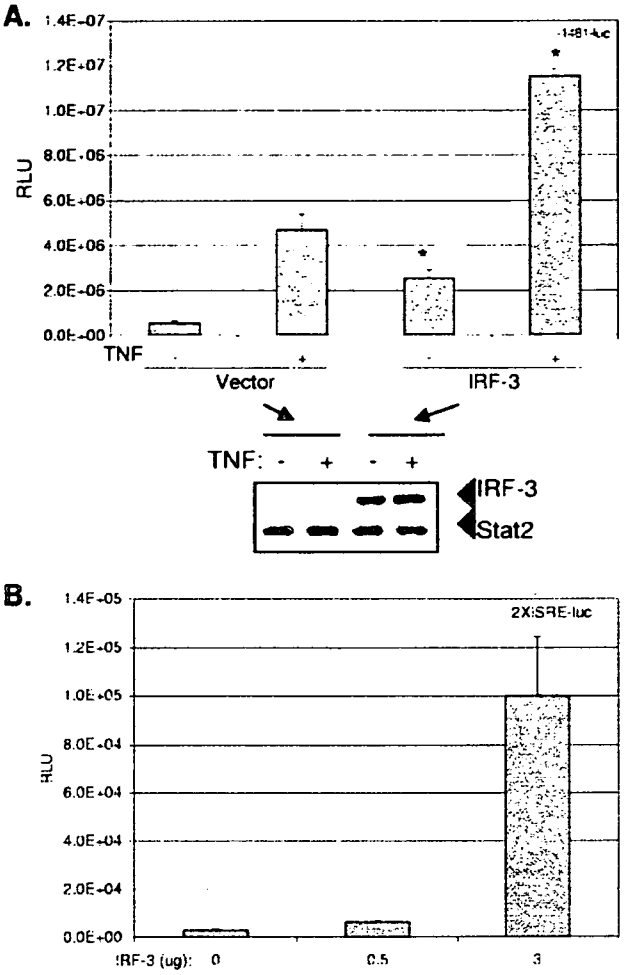

FIG. 4. IRF-3 transactivates the CXCL-8 promoter. (A) Huh7 cells were plated in 12-well plates and transfected with 0.5 μg of CMV-IRF-3 or vector plasmids along with 0.5 μg of the full-length −1481-luc CXCL-8 promoter-luciferase plasmid. Twenty-four hours later, cells were treated or not treated with 15 ng/ml of rhTNF-α for 6 h before luciferase activity was measured on cell lysates. The Western blot below the graphs depicts the expression of IRF-3 detected with polyclonal antiserum. Stat2 was also detected with polyclonal antiserum and served as a control for equal loading. Asterisks indicate significant stimulation of CXCL-8 transcription by IRF-3 compared to that of the vector control (*, $P < 0.01$). RLU, relative light units. (B) Increasing amounts of IRF-3 were cotransfected with 0.5 μg of the synthetic CXCL-8 promoter, 2XISRE-luc, into Huh7 cells, and cells and lysates were processed as described above.

TNF-α and virus induction of CXCL-8 involve mRNA stabilization (19, 23, 31, 61) via the binding of regulatory proteins to AREs in the 3'UTR of the mRNA (1, 6, 9, 53). We therefore determined whether RIG-I induction of CXCL-8 also involves stabilization of CXCL-8 mRNA. Huh7 cells were transfected with plasmids expressing RIG-N or GFP and treated with TNF-α to induce CXCL-8 mRNA. Actinomycin D was added to cultures to stop transcription, and CXCL-8 mRNA was quantified by real-time RT-PCR (19). As shown in Fig. 6A, expression of RIG-N led to a 7.6-fold enhancement of basal CXCL-8 mRNA (P, <0.01). Furthermore, RIG-N expression was associated with a 2.1-fold increase in the half-life of CXCL-8 mRNA following TNF-α stimulation. Statistical comparison of the decay rates by a one-phase exponential model (19) showed that this difference is significant ($P = 0.03$).

Since AREs are involved in the posttranscriptional regulation of CXCL-8 mRNA, we performed experiments with an EGFP reporter gene fused to the ARE region of CXCL-8 (Fig. 6B). The CXCL-8 construct contained 237 nucleotides from the CXCL-8 3'UTR that included the ARE (60). A control vector included a sequence of 200 nucleotides which lacked AREs. The CXCL-8 ARE caused a sixfold reduction in EGFP fluorescence compared to that of the wild-type vector (Fig. 6C, compare open bars). Fluorescence levels correlated with mRNA levels as detected by quantitative PCR (data not shown), indicating that the GFP reporter system reflects mRNA changes. Cotransfection experiments with RIG-N demonstrated that RIG-N was able to increase EGFP fluorescence by threefold in the presence of the CXCL-8 ARE compared to that of the wild-type vector that did not contain the ARE (Fig. 6C, solid bars). Overall, these results indicate that RIG-N stabilized CXCL-8 mRNA via ARE-dependent pathways.

DISCUSSION

The inflammatory response to virus infection, which is usually beneficial to the host, is often deregulated in the context of chronic viral infections like those caused by HCV. Indeed, inductions of inflammatory cytokines and chemokines such as CXCL-6, CXCL-8, and TNF-α have been reported in patients with chronic hepatitis C (11, 13, 18, 39, 51). In this case, the inflammatory response may do more harm than good because deregulation of inflammatory cytokines and chemokines creates an environment within the liver that is harsh and leads to hepatocyte turnover and regeneration (7, 17, 35, 37, 57). It has been suggested that chronic inflammation is mechanistically involved in the establishment of cancer (41), in particular hepatocellular carcinoma (5), so a deregulated inflammatory response may also have severe pathological sequelae.

In the current report, we demonstrate that innate antiviral signaling pathways that sense dsRNA during virus infection also trigger inflammatory chemokine expression. RIG-I and IPS-1 induced CXCL-8 transcription, and this involved, as expected, NF-κB. A role for the ISRE site in the CXCL-8 promoter was defined, and we demonstrated for the first time that IRF-3, which is downstream of RIG-I and IPS-1, also activated CXCL-8 transcription by directly binding to the CXCL-8 promoter. Moreover, RIG-N stabilized CXCL-8 mRNA. We propose that the regulation of CXCL-8 production by dsRNA signaling pathways represents a link between innate antiviral and inflammatory pathways. Based on previous studies (24, 50), HCV NS5A and core proteins could also play a role in the CXCL-8 induction observed in the present study. In this case, the mechanisms involved could be independent of dsRNA signaling responses and involve other transcription factors such as NF-κB and AP-1, reflecting the classical mode of CXCL-8 induction.

CXCL-8 induction requires transcriptional activation of the CXCL-8 promoter. Formation of the CXCL-8 "enhanceosome" involves the coordinated assembly of multiple transcrip-

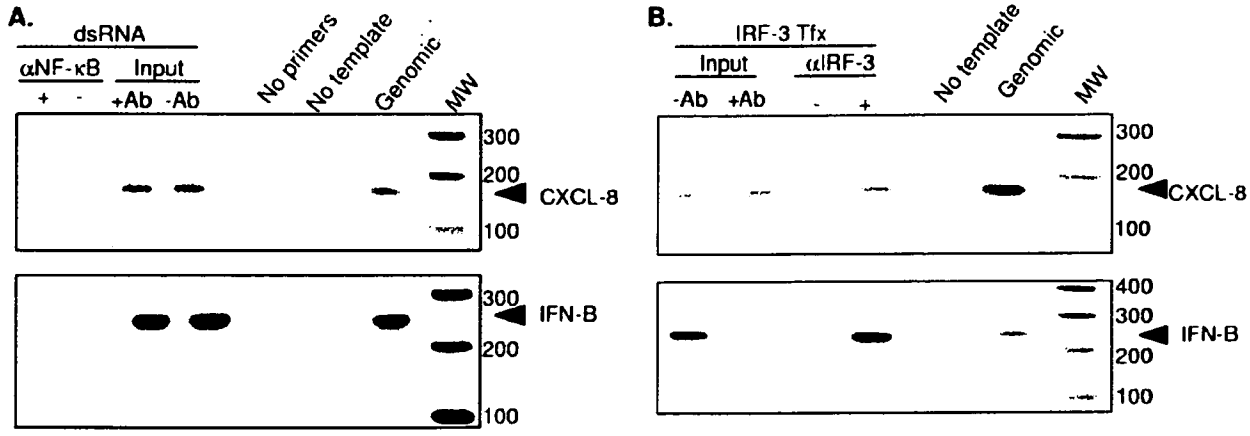

FIG. 5. IRF-3 binds directly to the CXCL-8 promoter in vivo. (A) A confluent 10-cm dish of Huh7 cells was transfected with 20 μg of poly(I:C) for 6 h. Nuclear DNA was isolated from fixed cells and sheared by sonication, and equal amounts were added to immunoprecipitations (IP) with (+) or without (−) antiserum to NF-κB. DNA was extracted from IP, and the CXCL-8 and IFN-β promoters were amplified by PCR. The PCR-positive control was genomic DNA, while PCR-negative controls contained either no primers or no template DNA. Positive controls for the IP included aliquots of the input samples that went into each IP (+antibody [Ab] or −Ab). (B) Huh7 cells were transfected with CMV-IRF3, and 24 h later, nuclear DNA was isolated from fixed cells as described above. Samples were handled as described above except polyclonal antiserum to IRF-3 was used in the immunoprecipitation. Arrows indicate the location of the CXCL-8- and IFN-β-specific PCR products.

tion factors (22). Under basal conditions, the CXCL-8 promoter is repressed by at least three mechanisms, including the binding of NF-κB repressing factor to a region that overlaps the NF-κB binding site, the deacetylation of histone proteins by histone deacetylase I (HDAC1), and the binding of octamer-1 (OCT-1) to a complementary site of the CAAT/enhancer-binding protein (C/EBP) binding sites (22). During transcriptional activation, NF-κB enters the nucleus and displaces NRF, OCT-1 is replaced by C/EBP, and CREB-binding protein (CBP)/p300 is recruited, which causes histone acetylation and remodeling of chromatin. In the current report, we demonstrate that NF-κB, AP-1, NF-IL-6, and IRF-3 participate in CXCL-8 transcriptional induction in response to HCV infection. Our data indicate that similar to RSV, HCV induces multiple transcription factors to bind to the CXCL-8 promoter. In other systems, NF-κB binding is essential to CXCL-8 transcriptional induction (42). AP-1, C/EBP, and NF-IL-6 are not required for transcriptional activation in some cells (22, 42), so it is thought that these transcription factors provide maximal gene induction. An important, unresolved issue is whether AP-1 and NF-IL-6 involvement in CXCL-8 induction requires the activation of AP-1 and NF-IL-6 signaling pathways or whether these transcription factors are noninducible and simply serve to amplify the effect of inducible IRF-3 and NF-κB. For example, it has been demonstrated that while the CXCL-8 AP-1 site is not TNF-α inducible, a mutation of the site significantly reduced TNF-α induction of the native CXCL-8 promoter (58).

RIG-I signaling is central for triggering the host response to HCV infection (34). It has been demonstrated that the HCV NS3 protease blocks RIG-I-dependent signaling of IRF-3 and NF-κB activation by its targeted proteolysis of IPS-1 (10, 34, 38). However, the host response may be transiently induced during early points of HCV infection prior to control by NS3/4A (34). Our data now indicate that the RIG-I pathway signaling through IPS-1 can also drive the expression of CXCL-8, thus connecting innate defense signaling to the inflammatory response to virus infection. We found that IRF-3 and NF-κB participate to induce CXCL-8 expression, suggesting that HCV might impose a similar blockade on CXCL-8 induction. We also note that among other transcription factors, NF-κB is essential for CXCL-8 induction and is responsive to many inflammatory stimuli other than dsRNA or virus infection (22). Thus, it is possible that viral triggering and control of CXCL-8 induction will depend on the cumulative and time-dependent cross-talk among signaling networks that is triggered and activated by NF-κB during HCV infection, with the subtle modulation imposed by the dsRNA activation of RIG-I signaling to IRF-3 serving to fine tune the response. Furthermore, while it has been suggested that IPS-1 mediates the bifurcation of the NF-κB and IRF-3 activation pathways, it remains possible that other adaptors of the RIG-I pathway are involved in this process (21).

Although transcriptional activation often plays a central role in gene expression, in certain situations, mRNA stabilization plays a more prominent role (1, 28). For instance, collagen expression in hepatic stellate cells is increased primarily through mRNA stabilization (54). Moreover, we have recently shown that Huh7 and replicon cells constitutively express CXCL-8 mRNA that is regulated posttranscriptionally (19). In the current study, we showed that RIG-N stabilized CXCL-8 mRNA, suggesting that a consequence of dsRNA signaling includes the activation of mRNA stabilizing responses. Note, however, that while ARE-mediated pathways primarily regulate mRNA turnover, they may also influence translation. However, it is likely that the RIG-N effect described in the current report acts at the mRNA level, since the half-life of CXCL-8 was prolonged as demonstrated by the actinomycin D chase experiment. The mRNA stabilization was dependent on AREs in the 3′UTR, since RIG-N increased reporter activity from a vector containing the 3′UTR region that harbors CXCL-8 AREs but not with a 3′UTR that lacked AREs. The

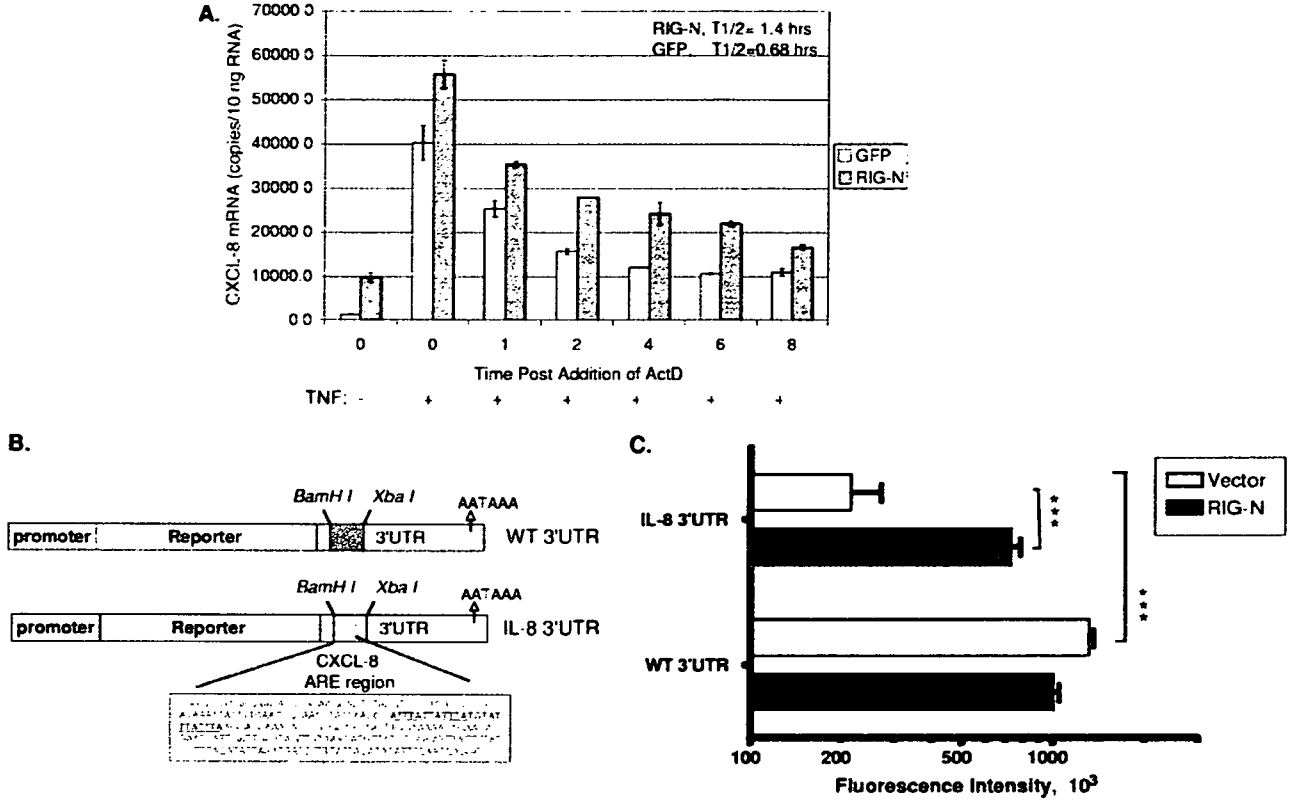

FIG. 6. RIG-N stabilizes CXCL-8 mRNA. (A) Huh7 cells were transfected with RIG-N- or GFP-expressing plasmids, and 24 h later, cells were treated with 10 ng/ml TNF-α for 2 h to induce CXCL-8 mRNA. Actinomycin D was then added to cell cultures to stop transcription, and total cellular RNA was isolated at the indicated times. CXCL-8 mRNA was quantitated by real-time RT-PCR, as described in Materials and Methods. (B) Schematic representation of the CXCL-8 ARE reporter genes. The EGFP protein was placed under the control of a constitutively active CMV promoter. The control of mRNA stability was mediated by sequences in the 3'UTR. The dashed box indicates a 200-nucleotide stuffer sequence which lacks AREs in the vector construct or 237 nucleotides from the CXCL-8 3'UTR that contains AREs. Underlined are the ARE pentamers (AUUUA). (C) Cells (3×10^4 cells per well) in black clear-bottomed 96-well plates were cotransfected with 25 ng of EGFP reporter vectors (CXCL-8 and WT) and 50 ng of modulator vectors (vector and RIG-N). After 48 h, the plates were read by bottom fluorescence. Data are presented as the means ± standard errors of four readings. ***, P values <0.001 using Student's t test.

CXCL-8 ARE was able to destabilize the reporter activity in the absence of any modulator but also was able to respond to RIG-N-induced stabilization. Thus, this region contains the necessary ARE regulatory and accessory domains for both destabilization and RIG-N-induced stabilization. This region has been shown to contain two functionally different domains in which four AUUUA-containing domains are sufficient for p38 mitogen-activated protein kinase-induced stabilization (60). Thus, it is possible that RIG-N induces the p38 pathway via a yet-unknown process. Since CXCL-8 mRNA stabilization involves the binding of regulatory proteins to the 3'UTR of the mRNA that enhance its mRNA stability (22), it is possible that RIG-N modulates the activity of these proteins. We are currently evaluating these issues. Furthermore, TLR5 and TLR9 signaling results in CXCL-8 induction by increasing the stability of CXCL-8 mRNA (46, 67). The degree to which dsRNA pathways, both TLR3 dependent and independent, regulate CXCL-8 mRNA stability during virus infection requires further study.

In summary, we have shown that dsRNA antiviral pathways cross-talk with inflammatory pathways. The combined action of these pathways is central to the control of acute HCV infection and replication. However, because HCV disrupts antiviral and inflammatory pathways (26, 48), these virus-host interactions likely contribute to HCV persistence and the pathogenesis of HCV-associated liver disease.

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