The chemokine CXCL-8 (interleukin-8) is induced by many viruses, including hepatitis C virus (HCV). In the current study, we examined CXCL-8 levels in the context of acute and chronic HCV replication in vitro. Two different small interfering RNAs were used to silence CXCL-8 mRNA and protein expression in Huh7 and BB7 replicon cells. HCV RNA synthesis in BB7 cells was inhibited by CXCL-8 knockdown. Furthermore, antibody neutralization of endogenous CXCL-8 activity inhibited HCV replication, while addition of recombinant human CXCL-8 stimulated NS5A protein expression. Moreover, CXCL-8 protein levels correlated positively with HCV RNA levels in four independent subgenomic and genomic replicon lines (R = 0.41, P = 0.0013). However, CXCL-8 mRNA levels correlated inversely with CXCL-8 protein and HCV RNA levels in all replicon lines and in Huh7 cells. Transient replication assays with strongly permissive and weakly permissive Huh7 cells and three independent subgenomic replicons with various replicative capacities revealed that CXCL-8 protein levels were higher in weakly than in strongly permissive cells. The JFH-1 subgenomic replicon, which replicated to high levels in both strongly and weakly permissive Huh7 cells, induced CXCL-8 protein to high levels in both cell types. The data indicate that in the replicon system, CXCL-8 protein levels are positively associated with chronic HCV replication and that CXCL-8 removal inhibits HCV replication. During acute HCV replication, CXCL-8 production may be inhibitory to viruses with low replicative capacity. The data underscore the complex regulation of CXCL-8 mRNA and protein expression and further suggest that in addition to contributing to HCV pathiology via proinflammatory actions, CXCL-8 may have opposing antiviral and proviral effects depending on the level of HCV replication, the cellular context, and whether the infection is acute or chronic.
responses, cell growth, and immunity (13). For example, the HCV NS5A protein induces interleukin-8 (CXCL-8) mRNA and protein expression (5, 14, 37, 40), which inhibits the antiviral actions of interferon (IFN) (19). Thus, in the context of IFN antiviral pressure, these and other studies (20, 21) suggest that CXCL-8 is a proviral chemokine. Moreover, CXCL-8 is induced during the inflammatory response to many other virus infections (22, 31), further suggesting that modulation of CXCL-8 expression during HCV infection may affect viral replication and pathogenesis. Indeed, it has been demonstrated that serum levels of CXCL-8 are elevated in chronic HCV infection, and serum CXCL-8 is also higher in patients who fail to respond to antiviral therapy than in patients who respond (30, 38). The question arises as to the relationship between HCV replication and CXCL-8 production. However, since Huh7 and replicon cells produce CXCL-8 (15), strategies that inhibit CXCL-8 production were invoked.

RNA interference (RNAi) is an evolutionarily conserved system for negative regulation of gene expression, which occurs as a result of processing of double-stranded RNA into 21- to 23-nucleotide small interfering RNAs (siRNAs) (42, 50). The antisense strand of the siRNA directs the double-stranded RNA oligonucleotide to the correct mRNA, whereby message degradation occurs via the RNA-induced silencing complex. Through simple transfection of siRNA duplexes, it is now possible to determine the specific effects of any cellular gene. In this study, we used RNAi to silence CXCL-8 gene expression and assess the effects on HCV replication in a stable replicon cell line. We further examined the correlation between CXCL-8 protein and mRNA and HCV RNA synthesis in three independent subgenomic and one genomic-length stable replicon cell line. We also examined CXCL-8 production during acute HCV replication of replicons with various replicative capacities in strongly permissive and weakly permissive Huh7 cells.

MATERIALS AND METHODS

Cells. Human hepatoma Huh7 cells were grown in Dulbecco modified Eagle medium containing 9% fetal bovine serum; 1% penicillin, streptomycin, and amphotericin B (Fungizone); 10 mM l-glutamine; and 1% nonessential amino acids (all reagents were from Invitrogen, Carlsbad, CA). BB7 cells are Huh7 cells that support the replication of a subgenomic genotype 1b HCV replicon containing an S2204I adaptive mutation in the NS5A gene (3). FL-Neo cells are a stable Huh7-derived cell line containing a genome-length 1b HCV replicon with the S2204I mutation in NS5A (21). P1996G cells in NS5A, Huh7, BB7, and FL-Neo cells were obtained from Apath, LLC. Subgenomic replicon cell lines 9-13 and 5-15-9-2-3 (hereafter referred to as 5-15 cells) containing different adaptive mutations (9-13, R2884G mutation in NS5B; 5-15, S2197P mutation in NS5A) (23, 28, 29) were provided by Ralf Bartenschlager. Replicon cell lines were maintained in Huh7 medium containing 400 μg/ml of G418 (Calbiochem, San Diego, CA). For transient HCV replication assays, “strongly permissive” and “weakly permissive” Huh7 cells were used. “Strongly permissive” Huh7 cells refer to a cured replicon cell clone allowing strongly efficient HCV replication (12). “Weakly permissive” Huh7 cells are naïve Huh7 cells of a low passage number that support only low-level HCV replication in transient replication assays (27). Cells were maintained in humidified incubators at 37°C with 5% CO2.

RNA interference. Two synthetic double-stranded RNA oligonucleotides referred to as CXCL-8-1 (5’-AAGTGCCAGTGGCCTTACGAAAG and CXCL-8-2 (5’-AAGTCTGACTGGTGGTGGTGG) were synthesized in a ready-to-use format (Pharmona, Inc., Biberach, Germany). CXCL-1 and CXCL-2 span nucleotides 62 to 82 and 151 to 169 of CXCL-8 mRNA, respectively (GenBank accession number Y00787). Control siRNAs included two commercially available siRNAs: lamin A/C and fluorescein (Pharmona). The day prior to transfection, 10,000 cells were plated in complete medium lacking G418 in 12-well plates. Cells were then transfected with or without 0 to 100 nM of duplexed RNA using Oligofectamine (Invitrogen) in complete medium without G418.

Western blotting. ELISA, antibody neutralization, and real-time RT-PCR. Protein lysates were quantitated (bicinchoninic acid protein assay; Pierce, Rockford, IL), and equal amounts (typically, 10 μg) of total protein were separated on 4 to 20% sodium dodecyl sulfate-polyacrylamide gels. NS5A and NS3 proteins were detected using random, deidentified HCV-infected patient serum, as described previously (36). Infected serum was inactivated by adding Triton X-100 to 1% prior to use. NS5A was also detected with a monoclonal antibody (Bio-source, Camarillo, CA). Lamin A/C was detected using polyclonal rabbit antisera (Santa Cruz Biotechnology, Santa Cruz, CA). Differences in NS5A protein content were determined by calculating pixel intensity using Image J, a version of NIH Image for Macintosh OSX. NS5A pixel intensities were determined by calculating pixel intensity using Image J, a version of NIH Image for Macintosh OSX. NS5A pixel intensities were quantitated by real-time RT-PCR, using recently described procedures (8, 15, 35).

HCV replicons and transient HCV replication assay. Replicons ET, S2197P, and GND used in this study are derived from the Con1 genotype 1b isolate; contains the HCV 5’ nontranslated region (nucleotides 1 to 341), the gene encoding firefly luciferase under translational control of the poliovirus IRES, the encephalomyocarditis virus IRES, the HCV nonstructural proteins NS3 to NS5B, and the HCV 3’ nontranslated region; and have been described elsewhere (repiPLu luc [27]). Replicon ET harbors adaptive mutations in NS3 and NS4B (E1202G, T1280I, and K1846T; numbers refer to the amino acid position of the polyprotein from the Con1 isolate, EMBL database accession number AJ242654), replicon S2197P has the indicated adaptive mutation in NS5A, and the GND replicon contains an inactivating mutation in the active center of the polymerase NS5B (D2737N). The replicon designated JFH has the same architecture, but all HCV sequences are derived from plasmid pSGR-JFH1, which contains a selectable replicon of the JFH-1 isolate, which is grouped into genotype 2a and exhibits very high replication efficiency in cell culture (18). Construction of this replicon has been recently described (46). In vitro transcription, electroporation, and transient replication assays including luciferase measurement in cell lysates have been described in detail elsewhere (27). Briefly, 4 × 10^6 strongly permissive or weakly permissive Huh7 cells were electroporated with 5 μg of in vitro-transcribed RNA derived from the indicated replicons or in the absence of RNA (mock), suspended in 12 ml complete Dulbecco modified Eagle medium, and seeded in 2 ml aliquots in six-well plates. Cell lysates and supernatants were harvested at 4, 24, 48, and 72 h posttransfection. CXCL-8 protein was measured on culture supernatants, and luciferase activity was measured on cell lysates.

Statistics. Linear mixed models were applied to determine whether a significant correlation existed between CXCL-8 protein and HCV RNA. Measures were transformed to the logscale.

RESULTS

CXCL-8 knockdown by RNAi in human liver cell cultures. Two CXCL-8-specific siRNAs (CXCL-8-1 and CXCL-8-2) were designed according to established procedures (Pharmona), and the efficiency of CXCL-8 knockdown was assessed by transfecting different concentrations of the siRNAs into Huh7 and BB7 cells. Control siRNAs targeted introns to knockdown the cellular gene, lamin A/C, and fluorescein as an additional control for nonspecific effects since this gene is not carried in the human genome. Figures 1A and B demonstrate dose-dependent reduction of CXCL-8 protein expression by CXCL-8-1 and CXCL-8-2 siRNAs relative to the fluorescein siRNA, in Huh7 and BB7 cells, respectively. CXCL-8-1 inhibited CXCL-8 production by 4- to 14-fold in Huh7 cells and 2- to 13-fold in...
BB7 cells. CXCL8-2 inhibited CXCL-8 production by three- to sixfold in both Huh7 and BB7 cells. Similar effects were observed when the reduction (n-fold) in CXCL-8 protein was calculated by comparing CXCL-8 siRNA with lamin siRNA results (data not shown). CXCL-8 mRNA was similarly inhibited by the CXCL-8 siRNAs (see Fig. S1 in the supplemental material).

To determine the kinetics of CXCL-8 silencing, Huh7 and BB7 cells were transfected with 25 nM of lamin, fluorescein, CXCL8-1, and CXCL8-2 siRNAs, and CXCL-8 protein in culture supernatants was quantitated by ELISA. Data are expressed as the reduction (n-fold) in CXCL-8 protein levels by the CXCL-8 siRNA compared to fluorescein. (C and D) Kinetics of CXCL-8 silencing. Huh7 cells and BB7 cells were transfected with 25 nM of fluorescein (Fluor), lamin, or CXCL8-1 and CXCL8-2 siRNAs, and CXCL-8 protein levels in culture supernatants were quantitated at days 1, 3, and 6 posttransfection. (E) IL-1β, IL-6, and TNF-α protein levels in BB7 cells transfected with CXCL8-1 siRNA or mock transfected. (F) Western blot analysis of lamin protein expression in Huh7 cells that were transfected with lamin siRNA or mock transfected. Lysates were from days 4 and 5 posttransfection. Experiments were repeated three times for each cell line and produced similar results.

FIG. 1. RNAi-mediated silencing of CXCL-8 expression. (A and B) Huh7 and BB7 cells were transfected with 10, 25, and 50 nM of CXCL8-1, CXCL8-2, or fluorescein (fluor) siRNAs, and at day 3 posttransfection, CXCL-8 protein in culture supernatants was quantitated by ELISA. Data are expressed as the reduction (n-fold) in CXCL-8 protein levels by the CXCL-8 siRNA compared to fluorescein. (C and D) Kinetics of CXCL-8 silencing. Huh7 cells and BB7 cells were transfected with 25 nM of fluorescein (Fluor), lamin, or CXCL8-1 and CXCL8-2 siRNAs, and CXCL-8 protein levels in culture supernatants were quantitated at days 1, 3, and 6 posttransfection. (E) IL-1β, IL-6, and TNF-α protein levels in BB7 cells transfected with CXCL8-1 siRNA or mock transfected. (F) Western blot analysis of lamin protein expression in Huh7 cells that were transfected with lamin siRNA or mock transfected. Lysates were from days 4 and 5 posttransfection. Experiments were repeated three times for each cell line and produced similar results.
Culture supernatants was measured by ELISA at days 1, 3, and 6 posttransfection. Figures 1C and D demonstrate that the CXCL8-1 and CXCL8-2 siRNAs inhibited CXCL-8 protein expression as early as 1 day posttransfection compared to lamin and fluorescein siRNAs. Effective silencing of CXCL-8 was maintained at days 3 and 6 posttransfection, with three- to eightfold suppression of CXCL-8. Note also that BB7 replicon cells produced two to four times more CXCL-8 than Huh7 cells did, indicating that HCV replication in this cell line is associated with elevated CXCL-8 expression (15). The data demonstrate that single transfections of CXCL-8 siRNAs inhibit CXCL-8 protein production for up to 1 week in BB7 and Huh7 cells.

To demonstrate the specificity of CXCL-8 silencing, culture supernatants from BB7 cells that were mock transfected or transfected with CXCL8-1 siRNA were tested for the cytokines IL-1β, IL-6, and TNF-α by fluorescent multiplex ELISA (Fig. 1E). The levels of these cytokines were not affected by CXCL-8 knockdown. To verify that lamin protein expression was inhibited by the lamin siRNA, Western blot assays were performed.
Figure 1E demonstrates that the lamin siRNA effectively inhibited lamin protein expression in Huh7 cells. The data demonstrate specific inhibition of CXCL-8 mRNA and protein production using RNAi.

**Effect of CXCL-8 inhibition on HCV replication in stable replicon cells.** HCV RNA levels were quantitated in BB7 cells that were transfected with the different siRNAs. Figure 2A demonstrates that the CXCL8-2 siRNA inhibited CXCL-8 protein production by 7.9-fold relative to the fluorescein siRNA and 7.2-fold relative to the lamin siRNA. This level of CXCL-8 knockdown was associated with 3.8 (relative to fluorescein)- and 2.5 (relative to lamin)-fold suppression of HCV
RNA when analyzed 3 days posttransfection. A similar reduction in HCV RNA levels by CXCL-8 inhibition was observed 6 days posttransfection (see Fig. S2 in the supplemental material). To independently verify the association between CXCL-8 inhibition and suppression of HCV replication, BB7 cells were treated with a neutralizing monoclonal antibody to CXCL-8. Figure 2B demonstrates that neutralization of CXCL-8 activity in BB7 cells was associated with 3.6-fold inhibition of HCV replication. HCV replication was not affected in control cultures treated with isotype-matched IgG. Furthermore, treatment of BB7 cells with recombinant human CXCL-8 induced a modest, dose-dependent increase in HCV NS5A protein expression (Fig. 2C). The data indicate that CXCL-8 is positively associated with chronic HCV replication in stable cell lines.

To examine this issue further, the relationships between basal HCV RNA, CXCL-8 protein, and CXCL-8 mRNA were temporally analyzed in three independent subgenomic and one...
highest level of HCV replication among all replicon lines, and CXCL-8 protein correlates with increased HCV RNA production. mRNA declines during the same period. The increase in CXCL-8 protein correlates with increased HCV RNA production in all cell lines, except 9-13 replicon cells, which had the highest level of HCV replication among all replicon lines, and remained sustained during the culture period. A positive association was found between CXCL-8 protein and HCV RNA on the log$_{10}$ scale ($R = 0.41$, $P = 0.0013$; see Fig. S3 in the supplemental material). Moreover, Fig. 3 shows that CXCL-8 mRNA levels were negatively correlated with HCV replication. An independent analysis of different passages of Huh7 cells with various permissivities for HCV replication revealed a similar negative correlation between HCV replicative efficacy and CXCL-8 mRNA expression (see Table S1 in the supplemental material). The data indicate that CXCL-8 protein levels correlate positively with HCV RNA levels in independent subgenomic and genomic replicon lines. However, since replicons may acquire genetic or epigenetic mutations during continuous selection in G418 medium and exposure to high-level HCV replication (4, 41), we next examined the relationship between CXCL-8 protein levels and acute HCV replication.

**CXCL-8 levels during acute HCV replication.** HCV replication as measured in transient replication assays is strongly dependent on the permissiveness of the host cell and on the efficiency of the viral sequence (27). In order to look for correlations between HCV replication and CXCL-8 secretion, we wanted to achieve various levels of HCV replication in Huh7 cells. Therefore, we used two sets of Huh7 cells and three different subgenomic luciferase replicons to cover a broad range of intracellular levels of HCV replication: strongly permissive cells, referring to a cured Huh7 replicon cell clone; weakly permissive cells, representing naive Huh7 cells of a low passage number; the JFH replicon, based on a genotype 2a isolate (18) exhibiting very high replication efficiency; ET, the most efficient replicon of the Con1 isolate with adaptive mutations in NS3 and NS4B (27); and S2197P, a moderately adapted Con1-based replicon with a mutation in NS5A (27). We included an RNA incapable of replication due to an inactivating mutation in the GDD motif of the NS5B polymerase (GND) to serve as a negative control and cells electroporated in the absence of RNA (mock). Figure 4A shows the time course of luciferase expression after transfection. Luciferase activity 4 h after transfection reflects transfection efficiency, which was similar between the different replicons and cell types. Replication efficiency was judged by increased luciferase activity compared to the GND mutant at 24 h, 48 h, and 72 h after transfection. The replicons with high (JFH), intermediate (ET), and low (S2197P) replicative efficiencies behaved as expected in strongly permissive cells, with JFH replicating to the highest levels, followed by ET, followed by S2197P. The three replication-competent RNAs replicated about 10-fold (JFH) to 250-fold (ET) less efficiently in the weakly permissive than in the strongly permissive Huh7 cells. Furthermore, in the weakly permissive cells, the differences in the replicative capacities of the different replicons were even more apparent: replication of the S2197P replicon was barely detectable in nonpermissive cells and ET replicated moderately efficiently in these cells, whereas JFH reached replication levels in weakly permissive cells similar to those of the ET replicon in strongly permissive cells. The highest replication efficiency was obtained for the JFH replicon in permissive Huh7 cells, reaching the maximum at 24 h posttransfection and staying at this level for the remaining 2 days.

Figure 4B depicts the corresponding CXCL-8 ELISA data. Weakly permissive cells secreted high levels of CXCL-8 protein into the culture medium over time independently of the replicon construct. For replicons with low (S2197P) to moderate (ET) levels of HCV replication, CXCL-8 protein levels were up to five times lower in strongly permissive Huh7 cells than in weakly permissive Huh7 cells. In contrast, the JFH replicon, which replicated to high levels in both highly permissive and weakly permissive Huh7 cells, induced CXCL-8 to high levels in both cell types. The data suggest that in the replicon system, acute HCV replication is associated with CXCL-8 induction, which may be inhibitory to viruses with low replicative capacity.

**DISCUSSION**

CXCL-8 inhibits the antiviral actions of IFN (19, 37). Assuming the level of HCV replication is inversely proportional to the vigor of the innate antiviral response, it can be hypothesized that CXCL-8 removal would inhibit HCV replication. Since Huh7 and replicon cells produce CXCL-8 (15), CXCL-8 addition experiments were precluded. It is for this reason that we inhibited CXCL-8 production using RNAi, and we found that CXCL-8 knockdown was associated with inhibition of HCV replication in BB7 cells. Moreover, there was a statistically significant correlation between CXCL-8 protein production and HCV replication in independent replicon cell lines. These data demonstrate for the first time that CXCL-8 protein levels are positively associated with HCV replication in vitro. The increase of CXCL-8 protein could be a consequence of HCV-induced cellular stress and activation of the inflammatory response. Indeed, it has been shown that CXCL-8 is induced in response to cellular stress, including virus infection (22). Alternatively, CXCL-8 may modulate HCV replication via inhibition of IFN antiviral responses (37, 38), the mechanisms of which are currently under investigation.

The regulation of CXCL-8 expression is a very complex process, with multiple regulatory mechanisms operative at the transcriptional and posttranscriptional levels. CXCL-8 induction involves transcriptional activation of the CXCL-8 promoter (31) and mRNA stabilization (16, 24, 47), via regulatory AU-rich elements (AREs) in the 3′ UTR of the cellular mRNAs (1). We have previously shown that HCV induction of CXCL-8 involves increases in both transcription and mRNA stabilization (15, 37). In the current report, we found that CXCL-8 protein accumulated in culture supernatants along with HCV RNA, yet CXCL-8 mRNA levels progressively declined as all cell lines, including Huh7 cells, grew in culture. CXCL-8 protein may have accumulated because of a longer half-life than its corresponding mRNA’s half-life of about 45 min (11, 15, 39). Thus, HCV may induce stabilization of CXCL-8 mRNA via modulation of protein interactions on the 3′ UTR ARE sequences, but the innate mechanism which regulates ARE-dependent mRNA decay likely contributes to...
the CXCL-8 mRNA decay as cells grow in culture. These issues will be resolved using transient HCV replication/infection using the JFH-1 infectious culture system. Nonetheless, the inverse correlation between CXCL-8 mRNA and protein indicates that future studies focusing on CXCL-8 should not solely rely on assessments of CXCL-8 mRNA, as this message, like most ARE-containing genes, turns over rapidly (15). This is a particularly important consideration for microarray-based studies of the host response to HCV infection.

An intriguing finding in the current study is the inverse correlation between permissiveness of a cell for acute HCV replication and CXCL-8 protein expression. The data from transient HCV replication assays suggest that the cellular proinflammatory status may be inhibitory to HCV replication in replicons or viruses with low to moderate replicative capacity. In this scenario, high CXCL-8 could render cells refractory to HCV replication. This is reminiscent of the proinflammatory molecule TNF-α, which exerts both protective and deleterious effects in vivo. In addition to being closely associated with the pathology of many chronic diseases including hepatitis (9), TNF-α displays antiviral activity against several different viruses (48, 49). The duality of function of TNF-α further suggests that proinflammatory chemokines such as CXCL-8 may display both antiviral and proviral effects. By analogy with TNF-α, the biological effects of CXCL-8 may depend on a complex set of interdependent factors such as tissue type; cellular context; receptor expression; and the dose, duration, and timing of the CXCL-8 response (43).

The current study also suggests that innate antiviral responses are linked to proinflammatory responses via complex signaling networks. Indeed, interferon regulatory factor 3 regulates the expression of the chemokine RANTES (25), and signaling networks. Indeed, interferon regulatory factor 3 regulates the expression of the chemokine RANTES (25), and

REFERENCES


