Iron Inactivates the RNA Polymerase NS5B and Suppresses Subgenomic Replication of Hepatitis C Virus*

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Clinical data suggest that iron is a negative factor in chronic hepatitis C; however, the molecular mechanisms by which iron modulates the infectious cycle of hepatitis C virus (HCV) remain elusive. To explore this, we utilized cells expressing a HCV replicon as a well-established model for viral replication. We demonstrate that iron administration dramatically inhibits the expression of viral proteins and RNA, without significantly affecting its translation or stability. Experiments with purified recombinant HCV RNA polymerase (NS5B) revealed that iron binds specifically and with high affinity (apparent $K_d \approx 6$ and $60 \mu M$ for $Fe^{2+}$ and $Fe^{3+}$, respectively) to the protein’s $Mg^{2+}$-binding pocket, thereby inhibiting its enzymatic activity. We propose that iron impairs HCV replication by inactivating NS5B and that its negative effects in chronic hepatitis C may be primarily due to attenuation of antiviral immune responses. Our data provide a direct molecular link between iron and HCV replication.

Infection with hepatitis C virus (HCV) poses a serious health care problem worldwide and is the leading cause of blood-transmitted chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1). HCV is a positive-polarity, single-stranded RNA virus, a member of the Hepacivirus genus of the Flaviviridae family (2). There are at least six major HCV genotypes and a large number of subtypes (3). The viral genome comprises ~9600 nucleotides and contains a single, large open reading frame, which encodes a precursor polypeptide of ~3010 amino acids (4). This is proteolytically cleaved to yield the functional proteins of the virus by combined actions of host-derived signal peptidase and viral-encoded protease activities.

The course of infection is affected by various factors, including the body iron status (5). Chronic hepatitis C is often associated with mild to moderate iron accumulation in the liver, primarily in sinusoidal/Kupffer cells. Clinical studies show a positive correlation between elevated iron indices, such as hepatic iron content, serum ferritin levels, or transferrin saturation, and liver damage in HCV infection (6–8). Increased iron indices have also been associated with poor response to treatment with interferon-α (9–12). In other studies, however, hepatic iron concentration did not influence the response to interferon-α treatment (13, 14), and phlebotomy did not substantially improve the efficacy of antiviral therapies (15, 16).

Iron overload is, by itself, a risk factor for liver fibrosis, cirrhosis, and hepatocellular carcinoma (17), and it appears to aggravate the clinical picture of chronic hepatitis C. A pathogenic synergism is evident in the combination of HCV infection and the common disease of iron overload hereditary hemochromatosis, which is associated with accelerated liver damage (18, 19). The most prevalent form of hereditary hemochromatosis is linked to mutations in the HFE gene, encoding an atypical major histocompatibility complex class I type molecule that appears to control dietary iron absorption and body iron reutilization (20). Clinical studies suggest that HFE mutations exacerbate hepatic fibrogenesis in chronic hepatitis C (5), mostly at early stages (8). A failure to find such a correlation in some reports may be related to the lack of control for confounding variables (5).

Whereas clinical data suggest that iron metabolism is tightly linked to HCV pathology, it is unknown whether and how iron interferes with viral replication and the expression of viral proteins. The molecular mechanisms by which HCV affects iron metabolism are also poorly understood. The development of subgenomic replicon systems has provided a powerful tool not only for basic studies on the biology of HCV but also for the design and evaluation of pharmacological interventions (21, 22). Here, we utilized HCV replicon cells to search for molecular links between iron metabolism and HCV replication.
Modulation of HCV Subgenomic Replicon by Iron

EXPERIMENTAL PROCEDURES

Materials—Hemin was purchased from Sigma, and desferroxamine (DFO) was purchased from Novartis (Dorval, Quebec, Canada). Fe-SIH was prepared by mixing SIH with ferric citrate in 2:1 ratio (23).

Cell Culture—Replicon and parent human Huh7 hepatoma cells (24, 25) and human embryonic kidney cells (26) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. The replicon cells were maintained in media containing 500 μg/ml G418 (Genetecin; Invitrogen) in addition to the above-mentioned supplements. For a typical experiment, 1 × 10⁶ Huh7 or 5 × 10⁵ 293 cells were seeded into 10-cm plates and subjected to iron manipulations on the next day.

Generation of Additional Replicon Huh7 Clones—Total RNA from the replicon Huh7 clone described in Ref. 25 was transfected into parent Huh7 cells by the Lipofectamine reagent (Invitrogen), and stable clones were selected in the presence of 500 μg/ml G418 (27).

Western Blotting—Cytoplasmic lysates were resolved by SDS-PAGE on 10% gels and transferred onto Immobilon™ polyvinylidene difluoride membranes (Millipore Corp.), as described in Ref. 25. The blots were saturated with 10% non-fat milk in phosphate-buffered saline and probed with 1:1000-diluted antibodies against NS5A (Biogenesis), NPT-II (Cortex Biochem), phosphorylated (at Ser³⁵) ElF-2α (25), transferrin receptor 1 (Zymed Laboratories Inc.), or β-actin (Sigma). Dilutions were in phosphate-buffered saline containing 0.5% Tween 20 (PBS). After a wash with PBS, theblots with monoclonal antibodies were incubated with peroxidase-coupled rabbit anti-mouse IgG (1:4000 dilution), and the blots with all other polyclonal antibodies were incubated with peroxidase-coupled goat anti-rabbit IgG (1:5000 dilution). Detection of peroxidase-coupled antibodies was performed with the ECL method (Amersham Biosciences).

Northern Blotting—Cells were lysed with TRIzol reagent (Invitrogen), and RNA was prepared according to the manufacturer’s recommendations. Total cellular RNA (10 μg) was electrophoretically resolved on denaturing agarose gels, transferred onto nylon membranes, and hybridized to radiolabeled cDNA probes against replicon RNA (25), human Mt-2, or rat GAPDH. Autoradiograms were quantified by phosphorimaging.

Reporter Gene Assays—Huh7 cells expressing the HCV IRES/3′-UTR (25) or the EMCV IRES (29) bicistronic construct (29) were subjected to iron manipulations, and cell extracts were prepared for reporter gene assays. The expression of HCV IRES/3′-UTR or EMCV IRES was analyzed by CAT/luciferase (25) or dual luciferase (Promega) assays, respectively. The luciferase/CAT and firefly/Renilla luciferase ratios were used to estimate the activities of the HCV and EMCV IRESes, respectively.

Assessment of Replicon RNA Stability—Replicon and parent Huh7 cells were pretreated for 2 h with 4 μg/ml actinomycin D and metabolically labeled (in the presence of actinomycin D) for 16 h in phosphate-free media with 200 μCi of [α-³²P]UTP (ICN), as described in Ref. 29. Subsequently, the cells were washed and chased in cold media. Total RNA (0.5 μg) was extracted from each plate and resolved on a 1% formaldehyde-agarose gel. The gel was dried, and RNA was visualized by autoradiography and quantified by phosphorimaging.

Expression and Purification of NS5B21 to NS5B21, a truncated form of HCV NS5B protein lacking the 21 C-terminal amino acids, was expressed and purified as described previously (30).

Fluorescence Measurements—Fluorescence was measured using a Hitachi F-2500 fluorescence spectrophotometer. Background emission was eliminated by subtracting the signal from either buffer alone or buffer containing the appropriate sample of substrate. The extent to which ligands bind to the NS5B protein was determined by monitoring the fluorescence emission of a fixed concentration of proteins and titrating with a given ligand in binding buffer (50 mM Tris-Cl, pH 7.5, 50 mM KOAc). In experiments with ferrous iron (provided by FeSO₄) as a ligand, the redox state of Fe²⁺ was maintained by the addition of 1 mM ascorbate to the binding buffer. The binding can be described by Eq. 1:

$$ K_j \frac{[\text{NS5B}][\text{ligand}]}{[\text{NS5B-ligand}]} = \text{Eq. 1} $$

where $K_j$ is the apparent dissociation constant, [NS5B] is the concentration of the protein, [NS5B-ligand] is the concentration of complexed protein, and [ligand] is the concentration of unbound ligand.

The proportion of ligand-bound protein as described by Eq. 1 is related to measured fluorescence emission intensity by Eq. 2:

$$ \Delta F / \Delta F_{\text{max}} = \frac{[\text{NS5B-ligand}][\text{ligand}]}{[\text{NS5B}] \text{tot}} \text{Eq. 2} $$

where $\Delta F$ is the magnitude of the difference between the observed fluorescence intensity at a given concentration of ligand and the fluorescence intensity in the absence of ligand, $\Delta F_{\text{max}}$ is the difference at infinite [ligand], and $[\text{NS5B}]_{\text{tot}}$ is the total protein concentration.

If the total ligand concentration, [ligand]$_{\text{tot}}$, is in large molar excess relative to [NS5B]$_{\text{tot}}$, then it can be assumed that [ligand] is approximately equal to [ligand]$_{\text{tot}}$. Eqs. 1 and 2 can then be combined to give Eq. 3:

$$ \frac{\Delta F}{\Delta F_{\text{max}}} = \frac{[\text{ligand}]_{\text{tot}}(K_j + [\text{ligand}]_{\text{tot}})}{[\text{NS5B}] \text{tot}} \text{Eq. 3} $$

The $K_j$ values were determined from a nonlinear least square regression analysis of titration data by using Eq. 3.

Analysis of Competitive Metal Ion Binding—Analysis of the effect of a second ion on the concentration of one ion ligand (ligand) was performed in a manner analogous to that reported previously for analyzing the kinetics of a system in which two alternative substrates compete for the same enzyme binding site (31). The change in fluorescence ($\Delta F$) observed upon titration of NS5B with ion$_a$, in the presence of a fixed concentration of competing substrate (ion$_b$), can be described by Eq. 4:

$$ \frac{\Delta F}{\Delta F_{\text{max}}} = \frac{[\text{ion}_a][\text{NS5B}][\text{ligand}]}{[\text{ion}_b][\text{NS5B}] \text{tot} + [\text{ion}_a][\text{ion}_b]} \text{Eq. 4} $$

Results

Loading of Replicon Huh7 Cells with Iron Decreases the Expression of NS5A and NPT-II—The prototype replicon consists of a subgenomic HCV RNA, which is sufficient for replication in Huh7 hepatoma cells (24). In this system, the HCV structural region has been replaced by the NPT-II gene that is translated under the control of the HCV IRES. The translation of the viral proteins NS3 to NS5 is directed by the EMCV IRES (Fig. 1A). To explore the effects of iron on HCV replication, replicon Huh7 cells were exposed for 24 h to increasing concentrations of hemin, an iron donor, or DFO, an iron chelator, and...
the expression of the viral protein NS5A and the marker NPT-II was assessed by Western blotting (Fig. 1B). Quantitative conditions for the analysis of these proteins were established previously (25).

The treatment with hemin inhibited the expression of both NS5A and NPT-II (Fig. 1B, first and second panels, respectively, lanes 5–7), whereas DFO appeared to elicit opposite responses (lanes 1–4), and levels of control β-actin remained unchanged (bottom panel). The inhibitory effect of hemin was consistently much stronger on NS5A compared with NPT-II, possibly reflecting differences in the stability of these proteins (25). Similar results were also obtained following iron perturbations for 12 h, but the range of regulation was lower (data not shown). As a control for the hemin treatment, we evaluated the expression of replicon proteins (for example, in Fig. 1B, lanes 6–10) in the absence (lane 6) or presence of the indicated concentrations of SIH (lanes 2, 3, 7, and 8) or DFO (lanes 4, 5, 9, and 10), and the expression of NS5A and β-actin was analyzed by Western blotting.

We therefore examined whether the inhibition on NS5A and NPT-II expression shown in Fig. 1B is an iron-dependent or a hemin-specific phenomenon. Treatment with the lipophilic, cell-permeable iron donor Fe-SIH (23) recapitulated the inhibitory effects of hemin (Fig. 1C, lanes 4 and 5) and clearly suggested that inorganic iron negatively regulates the expression of NS5A and NPT-II. Further evidence that inorganic iron is the critical component was provided by the fact that two iron chelators, DFO and SIH, efficiently antagonized the hemin-mediated decrease in NS5A expression (Fig. 1D). We noticed that in some experiments, DFO and SIH slightly stimulated the expression of replicon proteins (for example, in Fig. 1D, lanes 1–5); however, this was not consistent (for example Fig. 1C, lanes 1–3).

To analyze whether the inhibitory effects of iron are also shared by other metals, the replicon cells were exposed for 24 h to either 100 μM hemin or 100 μM copper, manganese, zinc, or cobalt salts. Note that only hemin significantly inhibited (p < 0.01, Student’s t test) the expression of NS5A (Fig. 2A). As a control for the cellular response to the metal treatments, we analyzed Mt-2 mRNA levels by Northern blotting (Fig. 2B). As expected (35), copper and zinc strongly induced Mt-2 mRNA expression. Taken together, the above results establish a molecular link between iron metabolism and HCV gene expression by showing that pharmacological modulation of cellular iron levels affects the expression of proteins of the subgenomic HCV replicon.

Translation via the HCV and EMCV IRES Is Not Affected by Iron—The iron-dependent inhibition of NS5A and NPT-II expression could result from changes in viral RNA translation, possibly via the HCV or EMCV IRES. To address this scenario, we employed a bicistronic CAT/firefly luciferase indicator containing the HCV IRES and 3′-UTR sequences of viral RNA and a bicistronic Renilla/firefly luciferase indicator containing the EMCV IRES (Fig. 3A). These constructs were transfected into parent Huh7 cells. The cells were subjected to iron manipulations, and lysates were prepared for the analysis of luciferase and CAT activities. No significant iron-dependent variations were observed in the activity of the firefly luciferase indicator after normalization with the respective CAT or Renilla luciferase values (Fig. 3B), suggesting that altered translation via the HCV or EMCV IRES cannot explain the observed effects on HCV gene expression. As expected (20), treatment with the iron chelator DFO stimulates the expression of endogenous transferrin receptor 1 by ~2.5-fold, whereas the iron donors hemin and Fe-SIH decrease transferrin receptor 1 steady-state levels by ~90% (Fig. 3C).

Iron Inhibits the Expression of Replicon RNA without Affecting Its Stability—We next analyzed whether iron alters the expression of subgenomic HCV RNA. The Northern blotting experiment depicted in Fig. 4A demonstrates that treatment of replicon cells with hemin for 24 h decreased the replicon RNA levels in a dose-dependent manner (top panel, lanes 1 and 5–7), whereas DFO had a slight stimulatory effect (top panel, lanes 5–7).
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Fig. 2. Iron specifically inhibits the expression of NS5B. Replicon Huh7 cells were either left untreated or treated for 24 h with 100 μM hemin, CuSO₄, MnCl₂, ZnSO₄, or CoCl₂. A, the expression of virally encoded NS5A and endogenous β-actin was analyzed by Western blotting. The data from three independent experiments were quantified by densitometry; the percentages of NS5A band intensities (mean ± S.D.) are plotted, after normalization with the respective β-actin values (right panel). B, the expression of Mt-2 and GAPDH mRNAs was analyzed by Northern blotting. The data from three independent experiments were quantified by phosphorimaging; the percentages of Mt-2 band intensities (mean ± S.D.) are plotted, after normalization with the respective GAPDH values (right panel).

1–4). Probing with the cDNA of cellular GAPDH serves as control (Fig. 4A, bottom panel).

To elucidate whether the iron-dependent decrease in the steady-state levels of replicon RNA is a result of alterations in its stability, a pulse-chase experiment was performed (Fig. 4B). Parent and replicon Huh7 cells were incubated for 16 h with [α-³²P]UTP in the presence of 4 μg/ml actinomycin D to block cellular mRNA transcription by RNA polymerase II. Subsequently, the replicon cells were chased for different time intervals with cold media alone or with cold media containing 100 μM hemin or DFO, and RNA was prepared for analysis by agarose gel electrophoresis. Iron chelation seems to partially (~20%) stabilize the replicon RNA, which may contribute to the DFO-dependent increase shown in Fig. 4A. The treatment with hemin appeared to accelerate the decay of replicon RNA rather modestly (by ~20%) within 6 h and did not have any effects afterward. On the basis of this finding, we conclude that the profound iron-induced decrease in replicon RNA expression cannot be sufficiently explained by alterations in its half-life.

Iron Inhibits Subgenomic HCV Replication in Multiple Replicon Systems—The data presented thus far collectively suggest that iron may inhibit HCV replication. However, because these data were obtained with a single clone of replicon Huh7 cells (24), it was important to exclude that the inhibitory effects of iron may represent a clonal phenomenon. To address this, we established further replicon Huh7 clones and evaluated their response to iron. Three new clones were isolated, which express different amounts of replicon RNA. The cells were treated with hemin or DFO, and the expression of replicon RNA was analyzed by Northern blotting (Fig. 5A). Whereas no stimulatory effects of DFO can be observed, a profound iron-induced inhibition of replicon RNA expression is evident in all clones, confirming the previous data.

To further validate this interpretation, we utilized the recently described 293Rep replicon system, consisting of human embryonic kidney 293 cells fused with the S22.3 clone of replicon Huh7 cells into a heterokaryon (26). Parent 293 and replicon 293Rep cells were subjected to iron manipulations, and the expression of NS5A protein and replicon RNA was analyzed by Western and Northern blotting, respectively. Treatment of 293Rep cells with hemin dramatically decreased the steady-state levels of both NS5A and endogenous β-actin, as evident in all clones, confirming the previous data.

To further validate this interpretation, we utilized the recently described 293Rep replicon system, consisting of human embryonic kidney 293 cells fused with the S22.3 clone of replicon Huh7 cells into a heterokaryon (26). Parent 293 and replicon 293Rep cells were subjected to iron manipulations, and the expression of NS5A protein and replicon RNA was analyzed by Western and Northern blotting, respectively. Treatment of 293Rep cells with hemin dramatically decreased the steady-state levels of both NS5A and endogenous β-actin, as evident in all clones, confirming the previous data.

Fig. 3. Iron does not affect translation via the HCV or EMCV IRES. A, schematic representation of the HCV IRES/3’-UTR and EMCV IRES bicistronic constructs. B, Huh7 cells stably transfected with the HCV IRES/3’-UTR or transiently transfected with the EMCV IRES constructs were either left untreated (control) or treated for 24 h with 100 μM DFO, hemin, or Fe-SIH, respectively. Cell lysates were analyzed for firefly luciferase and either CAT or Renilla luciferase. The relative firefly luciferase activities corresponding to the HCV IRES/3’-UTR and EMCV IRES constructs were obtained after normalization with the respective values for CAT or Renilla luciferase. The values represent the mean ± S.D. from two independent experiments, each performed in quadruplicate. C, lysates from the stable transfectants with the HCV IRES/3’-UTR construct were analyzed by Western blotting for the expression of transferrin receptor 1 (TfR1) and β-actin.
Iron Binds Specifically to Purified HCV RNA Polymerase and Inhibits Its Activity—The results in Figs. 1–5 could be best explained if iron inhibited viral RNA transcription, which is mediated by the RNA-dependent RNA polymerase (RdRp) activity of the NS5B protein (36, 37). To investigate this directly, we utilized the soluble, catalytically active (38) NS5BΔ21 protein and tested whether iron affects its catalytic properties. The NS5BΔ21 protein was highly purified (Fig. 6A) and analyzed by endogenous tryptophan fluorescence emission spectroscopy (30) for the binding of ferrous (Fe²⁺) iron. Fluorescence spectra were quenched in the presence of increasing micromolar concentrations of Fe²⁺ without affecting the emission maximum and the spectra bandwidth (Fig. 6B), suggesting that iron directly binds to NS5BΔ21. Similar results were obtained for ferric (Fe³⁺) iron (data not shown).

Parameters of Fe²⁺ and Fe³⁺ binding to NS5BΔ21 are summarized in Table I. The results were calculated from binding experiments with Fe²⁺ (depicted in Fig. 7) and Fe³⁺ (data not shown; the patterns with Fe³⁺ and Fe²⁺ were identical). The binding of iron was saturable (Fig. 7A) and occurred within seconds (Fig. 7B). The apparent dissociation constants (Kᵩ) for Fe²⁺ and Fe³⁺ were 6 and 60 μM, respectively (Table I). The apparent association rates were 9.4 and 59.7 μM⁻¹ s⁻¹ for Fe²⁺ and Fe³⁺, respectively (Table I). The ionic strength had only minimal effects on the Kᵩ values (Fig. 7C), suggesting that electrostatic interactions only make minor contributions to the overall binding energy. Generation of Hill plots (Fig. 7D) yielded a Hill coefficient n of 0.99 for Fe²⁺ and 1.16 for Fe³⁺ (Table I), indicating a lack of cooperativity in iron binding.

Where does iron bind to NS5B? To address this question, a dual ligand titration assay was performed with NS5BΔ21 and increasing concentrations of Fe²⁺ in the presence of 20 or 50 mM Mg²⁺ (Fig. 8A). This experiment showed that Fe²⁺ competes with Mg²⁺ for binding to the protein. Because Mg²⁺ (or Mn²⁺, or a combination of both) are absolutely required for NS5B to function as an RdRp (30, 38, 39), we reasoned that the binding of iron may impair its enzymatic activity. This was directly assessed by a primer-independent RNA synthesis assay (Fig. 8B). In the absence of iron, the polymerization assay yields two major products of 227 and 246 nucleotides (Fig. 8B, lane 1), as described previously (32). Addition of 40 μM Fe²⁺ almost completely inhibited the generation of these products (Fig. 8B, lane 2), whereas 4 μM Fe³⁺ was already inhibitory, albeit to a lesser extent (Fig. 8B, lane 3). A dose titration curve (Fig. 8C) reveals that the IC₅₀ for Fe²⁺ was ~7.5 μM. We conclude that iron binds to the Mg²⁺-binding pocket of NS5B and inhibits its RdRp activity.

Iron-mediated Inhibition of RdRp Activity in a Cell-free System from Replicon Huh7 Cells—A cell-free system from replicon Huh7 cells (33, 40) was utilized to examine whether iron also inhibits the RdRp activity of NS5B in cellular replication complexes. Crude extracts prepared from parent and replicon Huh7 cells were incubated in a reaction mix containing [α-³²P]UTP to monitor the synthesis of radioactive replicon RNA, which reflects NS5B activity (Fig. 9). As expected, no RNA was synthesized in extracts from parent Huh7 cells (Fig. 9, lanes 1 and 2). In extracts from replicon Huh7 cells, the generation of replicon RNA completely depends upon the addition of Mg²⁺ (Fig. 9, lanes 3 and 4), as reported earlier (40) and consistent with the Mg²⁺ requirement for the RdRp activity of NS5B. The addition of Fe²⁺ profoundly inhibited the enzymatic activity in a dose-dependent manner (Fig. 9, lanes 4–7), with an
IC$_{50}$ value of $\sim$5 µM. These results corroborate the binding (Table I) and functional (Fig. 8, B and C) data obtained with purified NS5B$_{Δ21}$ and strongly suggest that the target of iron in replicon Huh7 cells is the viral RNA polymerase.

**DISCUSSION**

We utilized the subgenomic HCV replicon system to examine how iron metabolism affects HCV replication. We show that iron inhibits the expression of the selection marker NPT-II and the viral protein NS5A (Figs. 1 and 2), without affecting translation via the HCV or EMCV IRES (Fig. 3). The above responses are associated with iron-dependent alterations in replicon RNA content (Fig. 4). Whereas the initial results were obtained in a single replicon clone (24), the iron-mediated inhibition in replicon expression was recapitulated in newly established Huh7 replicon clones and in 293Rep cells (26) (Fig. 5). Collectively, these data show that iron inhibits HCV replicon activity in two independent systems.

Although hemin was used as a convenient iron donor in many experiments, the results with the cell-permeable iron donor Fe-SIH (Fig. 1C) and, moreover, with the co-administration of hemin and the iron chelator (DFO or SIH) (Fig. 1D) suggest that inorganic chelatable iron is the species accountable for the inhibitory effects on subgenomic HCV replication. Depletion of intracellular iron with chelating drugs slightly stimulated the expression of the replicon in some experiments (Figs. 1B and 4A), but this was not consistent (Figs. 1C and 5),

**FIG. 5. Inhibitory effects of iron in additional replicon systems.** A, iron-mediated inhibition of replicon RNA expression in newly established replicon Huh7 clones. The cells were either left untreated (lanes 1, 4, and 7) or treated for 24 h with 100 µM DFO (lanes 2, 5, and 8) or hemin (lanes 3, 6, and 9). Replicon RNA and cellular GAPDH mRNA were analyzed by Northern blotting. B and C, iron inhibits the subgenomic HCV replicon in human embryonic kidney 293 cells. Parent 293 cells and replicon 293Rep cells were left untreated or treated for 24 h with 100 µM DFO or hemin. The expression of virally encoded NS5A and endogenous β-actin was analyzed by Western blotting (B). The expression of replicon RNA and cellular GAPDH mRNA was analyzed by Northern blotting (C).

**FIG. 6. Iron binds to HCV RNA polymerase.** A, an aliquot (2 µg) of purified recombinant NS5B$_{Δ21}$ was analyzed by electrophoresis on a 12.5% polyacrylamide gel containing 0.1% SDS and visualized by Coomassie Blue staining. The positions of molecular mass standards (in kDa) are indicated on the left. B, purified NS5B$_{Δ21}$ (300 nm) was incubated with the indicated amounts of ferrous sulfate, and the emission spectrum was scanned from 310 to 440 nm. Fluorescence spectra were recorded at an excitation wavelength of 290 nm.

**TABLE I**

| Ligand | $K_d$ (µM) | $\Delta F/F_0$ | $n$ | Association rate ($\mu M^{-1} s^{-1}$) |
|--------|-----------|---------------|-----|---------------------------------------|
| Fe$^{2+}$ | 6         | 0.90          | 0.99| 9.4                                   |
| Fe$^{3+}$ | 60        | 0.69          | 1.16| 59.7                                  |

The dissociation constants ($K_d$), maximal decrease of fluorescence ($\Delta F/F_0$), Hill coefficients ($n$), and association rates for the interaction of NS5B$_{Δ21}$ with Fe$^{2+}$ and Fe$^{3+}$.
possibly due to growth-related differences in intracellular iron status. Importantly, iron administration was always inhibitory. These findings are indicative of an inhibitory threshold for intracellular iron determining whether subgenomic HCV replication is permitted or impaired. Under permissive conditions, it appears that the efficiency of replicon expression largely remains unresponsive to any further reduction of intracellular iron with chelating drugs.

The iron-dependent inhibition of replicon RNA expression (Fig. 4A) is not associated with significant alterations in RNA stability (Fig. 4B). Considering that translation via the HCV or EMCV IRES is not affected by iron perturbations (Fig. 3), these results suggest that iron impairs the synthesis of replicon RNA. Furthermore, they raise the possibility that iron may directly target the RdRp activity of HCV, which is mediated by the viral NS5B protein (36, 37). An earlier study with HCV-infected PH5CH hepatocytes proposed that iron enhances HCV replication (41), but this conclusion was based solely on the outcome of a semiquantitative reverse transcription-PCR assay.

The hypothesis that iron may modulate the RdRp activity of HCV was directly investigated by analysis of purified recombinant NS5B. Solubilization of the otherwise insoluble protein was facilitated by deletion of a hydrophobic four-leucine motif (LLLL) within the C-terminal 21 amino acids. The truncated NS5B/H900421 retains the catalytic properties of full-length NS5B (38). Experiments with endogenous tryptophan fluorescence emission spectroscopy demonstrate that both Fe²⁺ (FeSO₄) and Fe³⁺ (FeCl₃) ions bind directly to NS5B/H900421 in a saturable manner (Figs. 6 and 7). This powerful technique was previously employed to monitor the metal binding properties of full-length NS5B (30). We show that the apparent Kd for Fe²⁺ is 613.2 M, and the apparent Kd for Fe³⁺ is 62.6 M (Table I). Thus, Fe²⁺ binds to NS5BΔ21 with ~500- and ~50-fold higher affinities than Mg²⁺ and Mn²⁺, respectively. Conversely, Fe³⁺...
CNS5B activity is indeed illustrated by the data in Fig. 8, the RdRp activity of NS5B. A direct iron-mediated inhibition suggests that there is a cross-talk between the levels of body iron and antiviral immune responses. Increasing evidence supports the role of iron in the control of HCV replication and may aid in the design of antiviral therapies.

Modulation of HCV Subgenomic Replicon by Iron

![Diagram of HCV replication](http://example.com/diagram.png)

**Fig. 9.** Iron inhibits NS5B activity in crude extracts of replicon Huh7 cells. Polymerization assays in crude lysates from parent or replicon Huh7 cells in the absence (lanes 1 and 3) or presence of 5 mM MgCl2 (lanes 2 and 4–7) and the indicated concentrations of Fe2+ (FeSO4) (lanes 5–7). RNA products were purified, separated by denaturing formaldehyde/agarose gel, and visualized by autoradiography. The position of the reaction products is indicated on the left. Data from two independent experiments were quantified by phosphorimaging; the percentage of inhibition in enzymatic activity (mean ± S.D.) is plotted against the Fe2+ concentrations (right panel).

Iron inhibits NS5B activity in crude extracts of replicon Huh7 cells. A dual ligand titration assay (Fig. 8A) using Fe2+ as ionb and Mg2+ as iono offers compelling evidence that Fe2+ and Mg2+ compete for binding to the same site. Because the binding of Mg2+ (and/or Mn2+?) to NS5B is indispensable for its structural stabilization (42) and for catalysis (30, 38, 39), the displacement of Mg2+ (or Mn2+) by iron can explain the inhibition of the RdRp activity of NS5B. A direct iron-mediated inhibition of NS5B activity is indeed illustrated by the data in Fig. 8, B and C. It should be noted that the IC50 value (7.5 μM) for Fe2+ in the RdRp enzymatic assay is in good agreement with the binding affinity of Fe2+ to NS5B (apparent Kd, 6 μM). Importantly, the dose-dependent inhibition of RdRp activity by iron observed with purified NS5B is quantitatively recapitulated (with an IC50 of ~5 μM) in crude lysates of replicon Huh7 cells (Fig. 9). This finding provides a link between the data obtained with replicon cells and purified NS5B and strongly suggests that the profound iron-dependent decrease in replicon RNA expression (Figs. 4 and 5) is due to inactivation of NS5B by iron.

Interestingly, other divalent metal cations, such as Cu2+, Zn2+, Co2+, and Ni2+, have also been reported to inhibit NS5B in vitro (30, 38, 39). Thus, one would expect that the treatment of replicon cells with Cu2+, Zn2+, Co2+, or Ni2+ salts might also impair the expression of viral proteins, such as NS5A. However, the results in Fig. 2 directly show that this does not occur. An inefficient uptake of these metals could provide a reasonable explanation, but the treatment with Mn2+ appears to stimulate NS5A expression (Fig. 2A, lane 4). Moreover, the administration of Cu2+ or Zn2+ to replicon cells is associated with a profound increase in the mRNA levels of endogenous Mt-2, which is transcriptionally activated by these metals (35). Thus, an alternative scenario would be that the failure of Cu2+, Zn2+, or Co2+ to efficiently inhibit viral replication in replicon cells may be related to lower affinities for NS5B compared with iron. This is in fact the case for Co2+, which has an apparent Kd of 35 mM (30). A testable prediction is that Cu2+ and Zn2+ may also have relatively high dissociation constants.

Collectively, this work demonstrates that iron can impair HCV viral replication. In light of clinical data supporting a view that iron constitutes an unfavorable risk factor in chronic hepatitis C (5) and of biochemical data suggesting that iron enhances HCV translation via induction of translation initiation factor 3 (eIF3) (43), the results presented here are unexpected and uncover a more complex role of iron in HCV biology than previously anticipated. It is conceivable that the negative clinical effects of iron in the course of HCV infection are indirect and primarily linked to long-term iron-dependent attenuation of antiviral immune responses. Increasing evidence suggests that there is a cross-talk between the levels of body iron and functions of the immune system (44). For example, iron overload promotes the expansion of CD8+ suppressor T cells and a decrease in CD4+/CD8+ ratios (45). Furthermore, iron shifts the balance between CD4+ T-helper cells of types 1 (Th1) and 2 (Th2) toward a Th2 response pattern, which is believed to be unfavorable in combating viral (or bacterial) infection (46). In addition, iron impairs interferon-γ signaling in macrophages (46) and compromises the ability of these cells to generate NO by the inducible nitric-oxide synthase (47), which is involved, among others, in antiviral defense mechanisms (48).

Based on clinical and epidemiological observations, iron would have been predicted to be favorable or at least neutral to HCV replication. This work reveals, however, that low micromolar concentrations of iron can strongly inhibit HCV replicon activity and identifies the viral RNA polymerase NS5B as the likely molecular target. These findings have implications for the control of HCV replication and may aid in the design of antiviral therapies.

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Iron Inactivates the RNA Polymerase NS5B and Suppresses Subgenomic Replication of Hepatitis C Virus

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