Iron Regulates Hepatitis C Virus Translation via Stimulation of Expression of Translation Initiation Factor 3

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Background. Although the response to treatment with interferon-α in individuals with chronic hepatitis C virus (HCV) infection is negatively associated with increased liver iron stores, the underlying mechanisms at work remain elusive to date. The translation initiation factor 3 (eIF3) is essential for HCV translation, and thus the effects that iron perturbations have on eIF3 expression and HCV translation were studied here.

Methods. eIF3 expression was analyzed by TaqMan polymerase chain reaction, Northern and Western blot analysis of HepG2 cells, and liver biopsies. Functional effects of iron on HCV mRNA translation were estimated by use of transient transfection experiments with bicistronic vectors.

Results. Iron treatment of HepG2 cells increased eIF3 mRNA and protein expression, whereas iron chelation reduced it. Accordingly, iron-dependent stimulation of eIF3 specifically induced the expression of reporter genes under the control of regulatory HCV mRNA stem-loop structures. Moreover, a positive association between liver iron levels, eIF3 expression, and HCV expression was found when liver-biopsy samples from HCV-infected patients were analyzed.

Conclusion. Iron promotes the translation of HCV by stimulating the expression of eIF3, which may be one reason for the negative association between liver iron overload and HCV infection. Modulation of the affinity of eIF3 to bind to HCV mRNA may be a promising target for the treatment of chronic HCV infection.

Evidence has accumulated that iron homeostasis may have an effect on the clinical course of hepatitis C virus (HCV) infection. This notion is supported by the observation that hepatic iron overload in HCV-infected patients predicts a poor response to treatment with interferon (IFN)-α [1–7]. Iron may thus modulate the course of HCV infection by at least 3 mechanisms. First, iron is known to interact directly with cell-mediated immune pathways, thereby weakening Th1-mediated effector mechanisms, such as nitric oxide formation, major histocompatibility complex class II expression, and tumor necrosis factor-α production [8–10]. Thus, iron-loaded macrophages have reduced cytotoxic potential toward various intracellular pathogens, including viruses [11, 12]. Moreover, weakening IFN-γ activity and Th1-mediated effector mechanisms induces the expression of anti-inflammatory cytokines by Th2 cells, which is an unfavorable condition for fighting infectious diseases, including HCV infection [13, 14].

Second, iron may worsen the clinical course of HCV infection by promoting tissue damage, the cause of which can be traced to the potential of the metal to catalyze the formation of highly toxic hydroxyl radicals by the Fenton reaction [15], leading to progressive liver fibrosis. Third, that iron is an essential nutrient for all cells suggests that it may also affect replication of HCV. This notion is supported by the recent finding that stimulation of an HCV-infected human hepatocyte cell line with different concentrations of iron salts significantly increases HCV RNA levels [16].

The expression of HCV is prominently controlled at the translational level, which involves the interaction
of cellular translation initiation factors and a specific mRNA stem-loop structure—the internal ribosome entry site (IRES)—within the 5’ untranslated region (5’UTR) (for review, see [17]). The native 40S ribosomal subunit directly binds to the IRES to form a stable binary complex in which the initiation codon is located at the ribosomal P site.

The translation initiation factor 3 (eIF3) is a crucial protein synthesis initiation factor consisting of at least 10 polypeptide chains (p170, p116, p110, p110, p66, p48, p47, p44, p40, p36, and p35) that have a total molecular weight of 650 kDa [18, 19]. The eIF3 complex directly binds to the IRES and may thereby modify the conformation of the 40S complex in a way that improves the joining of the 60S subunit. Thus, eIF3 is an indispensable factor for the initiation of HCV RNA translation [20–24].

A recent study has demonstrated that another metal, cadmium, can up-regulate the expression of the mouse initiation factor 3, which shows significant similarity to human eIF3 [25]. In light of this observation and because of the described interaction between iron and the clinical course of HCV infection, we investigated in the present study whether iron might have an effect on eIF3 expression and whether this would have any implications for the translation of HCV.

MATERIALS AND METHODS

Cell culture techniques. HepG2 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, at 37°C in humidified air containing 5% CO₂. For experiments, cells were stimulated either with the iron chelator desferrioxamine (DFO; final concentration, 100 μmol/L; Sigma) or with ferric chloride (50 μmol/L; Sigma) for 48 h or were left untreated. Neither DFO nor iron treatment resulted in a significant change in cell viability over the 48-h incubation period, as determined by trypan blue exclusion.

RNA extraction and reverse transcription. Total RNA was extracted from nitrogen-frozen tissue and HepG2 cells by a guanidinium-isothiocyanate-phenol-chloroform–based procedure. Liver-biopsy samples were homogenized in RNA-Clean (Angewandte Gentechnologie Systeme). After the addition of chloroform/isoamylalcohol, the lysates were mixed, were incubated on ice, and were centrifuged. The RNA was precipitated with isopropanol, followed by 2 washing steps with 70% ethanol. Reverse transcription was performed with 1 μg of total RNA, random hexamer primers (5 μmol/L) and dNTPs (62.5 μmol/L; Roche), and 200 U of Molony murine leukemia virus reverse transcriptase (Gibco), in 1× reverse transcription buffer for 1.5 h at 37°C.

Northern blot analysis. Northern blot analysis was performed as described elsewhere [8]. In brief, 10 μg of total RNA was separated on 1% agarose/2.2 mol/L formaldehyde gels, and RNA was blotted onto Duralon-UV membranes (Stratagene Europe). After UV cross-linking and prehybridization for 6–8 h at 65°C, blots were hybridized overnight with 10 cpm/mL of α[32P]-dCTP–radiolabeled p170 and p110 cDNA probes at 65°C. The primers used for cDNA synthesis were p170/150 (sense, 5’-AGCCTGCTCTGGATGTTCTT-3’; antisense, 5’-AGACGATGGAGTGATAGTGAC-3’) and p110 (sense, 5’-GAGCGAGGAAGACTCTTG- antisense, 5’-ACCAGGTACCTTTCAACAG-3’). Co-hybridization of blots with the cDNA for the housekeeping gene β-actin was used as an internal control.

TaqMan real-time polymerase chain reaction (PCR). For p170 cDNA quantification, we used the Assays-on-Demand System, according to the manufacturer’s instructions (Applied Biosystems). PCR was performed by use of the MX4000 Multiplex Quantitative PCR System (Stratagene Europe). Amplification conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles for 15 s at 95°C and 1 min at 60°C. Data were analyzed by use of MX4000 software (version 4.00).

Measurement of hepatic HCV RNA. HCV RNA was quantified by use of a commercially available test, bDNA (Roche), as described elsewhere [26]. RNA preparation was completed
Figure 2. Effect that iron perturbation has on translation efficiency of hepatitis C virus (HCV) subtypes 1b, 2b, and 6a in HepG2 cells. Cells were transiently transfected with bicistronic vectors containing the untranslated regions of either HCV subtype 1b, 2b, or 6a and then were treated with iron or desferrioxamine (DFO) or were left untreated (control) for another 24 h, as described in detail in Materials and Methods. Data are shown as means ± SD normalized to control, which are estimated to be 1 for 6 independent experiments performed in duplicate. * and **, for comparison of cells treated with iron (white bars) and DFO (black bars) in the respective virus-transfected subgroups.

as described above. HCV RNA was measured by use of the bDNA assay, with results calculated as equivalents per gram of net tissue weight.

**Western blot analysis.** To prepare cellular protein extracts, cells were washed twice in PBS and were lysed in ice-cold lysis buffer (0.01 mmol/L Tris-HCl [pH 7.5], 0.5% NP40, and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]), followed by centrifugation at 15,000 g at 4°C. The supernatant was mixed with 1 volume of 2× sample buffer containing 0.1 mol/L Tris-HCl (pH 6.8), 4% SDS, and 20% glycerol. This mixture was incubated for 30 min at 56°C and was centrifuged at 15,000 g for 10 min. The protein concentration in supernatants was determined by use of the BCA protein assay kit (Pierce).

To determine eIF3 expression in liver-biopsy samples (which had been previously collected for diagnostic evaluation of HCV-related hepatopathy) from HCV-infected subjects, the samples were suspended in sodium Tris EDTA buffer (0.1 mol/L NaCl, 0.01 mol/L Tris-HCl [pH 7.6], and 1 mmol/L EDTA) in the presence of protease inhibitors aprotinin (2 μg/mL), leupeptin (1 μg/mL), and PMSF (1 mmol/L) (all from Sigma) and were homogenized by use of a Polytron homogenizer. The homogenates were centrifuged at 1000 g for 15 min and were further treated as described above for cellular extracts.

Informed consent was obtained to use the liver-biopsy samples from HCV-infected subjects previously collected for diagnostic evaluation of HCV-related hepatopathy for additional scientific investigation. Five control persons and 10 HCV-infected patients were studied; 5 of the latter group were infected with HCV subtype 1b, and 5 were infected with HCV subtype 2b. Among the patients infected with subtype 1b, 1 had previously undergone treatment with INF-α, which was terminated 14 months before he underwent a liver biopsy, the sample from which was used in the present study. We used specimens from patients who had undergone liver biopsies for diagnostic evaluation of steatohepatitis as controls. All patients provided written, informed consent.

Thirty micrograms of cellular or tissue protein extracts was subjected to polyacrylamide gel electrophoresis in the presence of 0.1% SDS. After electrophoresis, the protein was transferred onto a polyvinylidene difluoride membrane at 1 mA/cm² for 90 min (Amersham Pharmacia Biotech). The membranes were treated with blocking solution (0.1 mol/L Tris-HCl [pH 7.4], 0.1 mol/L MgCl₂, 0.5% Tween, 1% Triton X-100, 1% bovine serum albumin, and 5% FCS) for 30 min at room temperature, were washed with blocking solution, and were then incubated with a goat anti–chicken peroxidase–coupled secondary antibody (1:2000; Southern Biotechnology Associates) for 1 h at room temperature. Finally, the protein-antibody complex was visualized by use of Super Signal West Pico Chemiluminescent Substrate (Pierce), according to the manufacturer’s instructions.

**Transient transfection of cultured cells.** Subconfluent cell monolayers were infected with vTF7-3, a recombinant vaccinia virus expressing T7 RNA polymerase, at a concentration of 5 pfu/cell in 500 μL of serum-free medium (OptiMEM; Gibco BRL) for 30 min at 37°C. The inoculum was then removed, and the cells were washed twice with OptiMEM. Cells were then transiently transfected with 3 different mutants of the bicistronic, dual-luciferase reporter vector, pRL, containing the 5’UTR of HCV subtype 1b, 2b, or 6a [28], by use of a lipofection method with the Cytofectene Transfection Reagent (Bio-Rad Laboratories).

Two replicate wells were transfected with each construct, and
Figure 3. Levels of translation initiation factor 3 (eIF3) mRNA (A), eIF3 protein (B), and liver iron (C) in liver-biopsy samples from individuals infected with different hepatitis C virus (HCV) subtypes. Liver tissue was subjected to RNA preparation, and mRNA levels were estimated by use of TaqMan polymerase chain reaction; liver tissue also was subjected to protein extraction, and protein levels were estimated by use of Western blot assays. Tissue iron was measured by atomic absorption. Data are shown as means ± SD for 4–6 patients/group. *P < .05; **P < .005, compared with control (Student’s t test).

RESULTS

Effects that iron perturbations have on eIF3 expression in HepG2 cells. As shown in figure 1A, mRNA levels of eIF3 subunits p170 and p116 are regulated in response to iron treatment in HepG2 cells. p170 mRNA and p116 mRNA levels increased over time in cells treated with ferric chloride and decreased in cells treated with DFO in a time-dependent manner.

Moreover, Western blots confirmed that iron-dependent changes in eIF3 mRNA expression also affect protein expression in the same way (as shown in figure 1B), meaning that iron treatment of HepG2 cells increased p170 protein expression. This observation was further supported by immunocytochemistry demonstrating increased cellular expression of eIF3 p170 protein in HepG2 cells treated with iron, compared with cells stimulated with DFO (data not shown).

Effect that iron has on IRES-dependent translation in different HCV subtypes. To study the effect that iron-dependent changes in eIF3 expression have on HCV mRNA translation, we used bicistronic reporter expression vectors (pRL) containing the 5′UTR of 3 representative subtypes (1b, 2b, and 6a) of HCV, which have been used in other studies [28]. The 5′UTR of HCV RNA is highly conserved; however, the location and conformation of hairpin IIIb, which contains the binding sites for the 2 large subunits of eIF3, p116 and p170, are different among subtypes 1b, 2b, and especially 6a.

As shown in figure 2, iron perturbation of transiently transfected HepG2 cells results in a highly significant increase in relative luciferase activity, compared with treatment with DFO, when a bicistronic vector containing the 5′UTR of subtype 1b is used (P = .005). The same also holds true for the transient transfection assay when a subtype 2b–specific vector is used (P = .05). In contrast, no significant difference between the luciferase activity of iron-depleted HepG2 cells and that of iron-repleted HepG2 cells could be detected when a vector containing the 5′UTR of subtype 6a—which is known to have a modified eIF3 binding site within hairpin III, compared with subtype 1b—was used.

p170 expression in HCV-infected livers and its association with iron parameters and HCV RNA levels. To see whether the association among iron perturbation, eIF3 expression, and HCV translation also holds true in vivo, we performed TaqMan real-time PCR and Western blot assays for eIF3 on extracts derived from liver-biopsy samples, previously obtained for routine examination, from HCV-infected patients. As can be seen in figure 3A, a significantly increased expression of eIF3 mRNA was found in liver-biopsy samples from patients infected with...
Table 1. Correlations between liver translation initiation factor 3 (eIF3) mRNA levels, serum and hepatic hepatitis C virus (HCV) RNA levels, and different serum iron parameters, in patients infected with HCV subtypes 1b and 2b.

<table>
<thead>
<tr>
<th>Parameter, value</th>
<th>Serum ferritin</th>
<th>Liver iron</th>
<th>Serum HCV RNA</th>
<th>Hepatic HCV RNA</th>
<th>Liver eIF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum transferrin saturation (n = 8)</td>
<td>0.783</td>
<td>0.522</td>
<td>0.029</td>
<td>-0.116</td>
<td>0.116</td>
</tr>
<tr>
<td>r</td>
<td>0.528</td>
<td>0.957</td>
<td>0.827</td>
<td>0.927</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>.066</td>
<td>.288</td>
<td>.957</td>
<td>.827</td>
<td></td>
</tr>
<tr>
<td>Serum ferritin (n = 10)</td>
<td>...</td>
<td>0.810</td>
<td>0.500</td>
<td>0.491</td>
<td>0.619</td>
</tr>
<tr>
<td>r</td>
<td>...</td>
<td>0.116</td>
<td>0.116</td>
<td>.957</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>...</td>
<td>.288</td>
<td>.288</td>
<td>.827</td>
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</tr>
<tr>
<td>Liver iron (n = 10)</td>
<td>...</td>
<td>...</td>
<td>0.500</td>
<td>0.683</td>
<td>0.690</td>
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<tr>
<td>r</td>
<td>...</td>
<td>...</td>
<td>0.500</td>
<td>0.683</td>
<td></td>
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<tr>
<td>P</td>
<td>...</td>
<td>...</td>
<td>0.207</td>
<td>0.062</td>
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<tr>
<td>Serum HCV RNA (n = 10)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.683</td>
<td>0.571</td>
</tr>
<tr>
<td>r</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.683</td>
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<tr>
<td>P</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.062</td>
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</tr>
<tr>
<td>Hepatic HCV RNA (n = 10)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.898</td>
</tr>
<tr>
<td>r</td>
<td>...</td>
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<td>...</td>
<td>0.898</td>
<td></td>
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<tr>
<td>P</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. r, Spearman’s rank correlation coefficients.

HCV subtypes 1b and 2b, compared with control samples. Accordingly, these changes were paralleled by increased eIF3 protein levels in these patients, compared with those in uninfected control persons (figure 3B). As described elsewhere [28], we found increased liver iron levels in patients infected with subtype 1b (P<.05) (figure 3C).

In agreement with our in vitro data, increased eIF3 mRNA expression was positively associated with liver iron concentration (r = 0.690; P = .058, Spearman’s rank correlation). Interestingly, eIF3 mRNA expression correlated significantly with hepatic HCV RNA levels (r = 0.898; P = .002). In addition, hepatic HCV RNA levels showed a positive association with liver iron concentration (r = 0.683; P = .062) (table 1).

**DISCUSSION**

In recent years, the interaction among body iron status, the clinical course of HCV infection, and resistance to treatment with IFN-α has attracted increasing attention [16, 29, 30]. Here, we have shown for the first time that iron has a direct effect on HCV translation via induction of eIF3 expression, an indispensable factor for effective initiation of HCV RNA translation [20–24]. This result is in accordance with a previous study demonstrating that iron supplementation of an HCV-infected human hepatocyte cell line stimulates HCV expression up to 10-fold, compared with that of untreated cells [16].

The specificity of our observed interaction among iron, eIF3, and HCV infection was underlined by our finding that the translation of HCV subtypes bearing a functional eIF3 binding site within its IRES responded to iron perturbations, a finding that paralleled the effects that iron has on eIF3 expression. Finally, a positive association among levels of circulating HCV subtype 1b, liver iron status, and eIF3 expression in the liver was found in biopsy samples. A similar association was also seen with regard to HCV subtype 2b; however, the liver iron levels in these specimens were not significantly higher than those in control specimens (which may be due to the small number of subjects [n = 5] investigated), although the mean liver iron concentration was twice as great in HCV-2b–infected specimens than in control specimens (figure 3C).

Thus, iron may be detrimental to HCV infection not only by reducing the responsiveness to IFN-α but also by stimulating HCV translation and by catalyzing radical formation, leading to cell death and damage. [31]. In addition, iron is a profibrogenic factor, acting as an activator of both hepatic stellate cells and Kupffer cells [15]. Moreover, the reason why IFN-α is less effective in patients with a high iron burden [4, 32] has partly been referred to the fact that iron overload reduces the number of functional helper precursor cells [33, 34], impairs the generation of cytotoxic T cells with expansion of suppressor T cell activity [33, 34], and weakens the immune potential of macrophages against viruses by directing the immune response from a Th1 to a Th2 pattern [13].

Therefore, various studies have been initiated to find a means of decreasing the amount of circulating and liver iron. Phlebotomy therapy improved serum aminotransferase levels in chronic active hepatitis C [6, 35–37]. However, discrepancy exists whether phlebotomy also reduces HCV RNA levels or indeed improves the response to treatment with IFN-α. Some studies have found that retreatment of previous nonresponders to IFN with a combination of IFN-α and phlebotomy does not improve the rate of sustained response [37, 38], whereas other trials treating naïve patients [39] or nonresponders [40, 41] have suggested that pretreatment iron-reduction therapy may improve responsiveness to IFN-α and subsequent clinical, histi-
tolological, and virological outcome, compared with treatment with IFN-α alone.

These differences in outcome in the various trials suggest that additional factors may exist that modulate the effectiveness of iron reduction on HCV expression; such factors include genetic variations in iron homeostasis due to homozygosity or heterozygosity in the HFE gene [42]; environmental, racial, and dietary factors, including alcohol consumption [43], timing and amount of iron reduction, and polymorphism of immune effectors pathways; and, finally, genetic variations in the various HCV subtypes, leading to different responsiveness to iron perturbations. The last factor became evident in the present study, which showed iron-dependent translation via eIF3 induction in subtype 1b, whereas subtype 6a was unresponsive. This result is in accordance with the finding that subtype 6a has a much lower basal translation efficiency than does subtype 1b [28], and subtype 6a differs significantly from subtype 1b, especially in hairpin IIIb, the binding region of p170 and p116, which further supports our theory.

Moreover, it has been reported that patients infected with subtype 1b have higher hepatic iron levels than do patients infected with 2a or 2b [30], which underlines our data that show that the translation of subtype 1b is significantly influenced by iron, at least in vitro. In the same study, it was pointed out that, in patients with subtype 1b infection, hepatic iron content in responders is significantly lower than that in non-responders, whereas, in patients infected with viral genotype 2a + 2b, no statistically significant difference in iron content of the liver. J Hepatol 1994; 20:410–5.


