Clinical Significance of In Vitro Replication–Enhancing Mutations of the Hepatitis C Virus (HCV) Replicon in Patients with Chronic HCV Infection

Christoph Sarrazin, Ulrike Mihm, Eva Herrmann, Christoph Welsch, Mario Albrecht, Ulrike Sarrazin, Stella Traver, Thomas Lengauer, and Stefan Zeuzem

1Klinik für Innere Medizin II, Universität des Saarlandes, Homburg/Saar, 2Fachbereich Mathematik der Technischen Universität, Darmstadt, and 3Max-Planck Institut für Informatik, Abteilung Bioinformatik und Angewandte Algorithmik, Saarbrücken, Germany

Background. Mutations in nonstructural (NS) hepatitis C virus (HCV) proteins enhance replication in HCV-1a/b replicons. The prevalence of such mutations and their clinical significance in vivo are unknown.

Methods. Parts of HCV NS3 and NS4B–NS5B genes that included 31 in vitro replication–enhancing sites were sequenced for 26 patients with chronic HCV genotype 1 infection.

Results. Five patients showed specific mutations within NS3 at sites enhancing replication in the replicon. Those mutations were associated with a slower decrease in HCV RNA concentration during interferon (IFN–α)–based therapy (P = .007). Neither specific nor other mutations within NS3 and NS4B–NS5B were associated with baseline HCV RNA concentrations. Within NS5A, fewer mutations in the major HCV strain (P = .001) and increased quasi-species complexity (P = .02) and diversity (P = .02) correlated with increasing baseline HCV RNA concentrations. In silico analyses of NS3 protein structures suggested that the majority of observed mutations did not lead to major conformational changes.

Conclusions. Specific mutations leading to enhanced replication in the replicon system were detected in 5 of 26 patients in vivo and were not associated with baseline HCV RNA concentrations but were associated with a slower decrease in HCV RNA concentration during IFN–α–based therapy. Quasi-species heterogeneity of NS5A correlated with baseline HCV RNA concentrations.

Infection with hepatitis C virus (HCV) is characterized by a high rate of chronicity and the risk of the development of liver cirrhosis and hepatocellular carcinoma [1–3]. The outcome of the current standard therapy—interferon (IFN)–α and ribavirin—is still unsatisfying, with sustained virologic response (SR) rates of ~50% for patients infected with HCV genotype 1 [4–6]. Among other parameters, the pretreatment viral load is known to have a large impact on treatment outcome in patients infected with HCV genotype 1 [7–9]. The underlying mechanisms for widely different HCV RNA concentrations in patients with chronic HCV infection are unknown.

Recently, stable replication of selectable subgenomic HCV RNA replicons was established by Lohmann et al. in a hepatoma cell line [10]. More recently, several single and combined mutations within the nonstructural (NS) protein 3, NS4B, NS5A, and NS5B genes of the original replicon were described, which were associated in part with dramatically increased viral replication [11–21].

The observation of specific mutations that are critical for the efficiency of replication in the replicon system raises the question whether—similar to the in vitro situation—a virus-innate higher replicative potential that depends on specific amino acid residues might contribute to different HCV RNA concentrations and/or viral persistence in vivo, despite IFN-based antiviral therapy. Furthermore, it has to be assumed that HCV genomic regions with in vitro replication–enhancing sites may be critical for HCV replication efficiency in
Table 1. Epidemiological and virologic characteristics of 26 patients with chronic hepatitis C subtype 1 infection.

<table>
<thead>
<tr>
<th>Patient, sex</th>
<th>Age, years</th>
<th>Therapy</th>
<th>HCV RNA concentration, IU/mL</th>
<th>Virologic response</th>
<th>Mutations at in vitro replication–enhancing sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, M</td>
<td>35</td>
<td>Stand</td>
<td>168,410</td>
<td>8140</td>
<td>SR S1496R</td>
</tr>
<tr>
<td>2, M</td>
<td>36</td>
<td>Stand</td>
<td>297,199</td>
<td>&lt;615</td>
<td>SR P1112Q, R1283K, S1496G</td>
</tr>
<tr>
<td>3, F</td>
<td>45</td>
<td>Stand</td>
<td>314,743</td>
<td>&lt;615</td>
<td>SR P1112Q, S1496R</td>
</tr>
<tr>
<td>4, M</td>
<td>65</td>
<td>Stand</td>
<td>355,007</td>
<td>&lt;615</td>
<td>SR P1112Q, S1496R, S1950G, V1896I</td>
</tr>
<tr>
<td>5, F</td>
<td>32</td>
<td>Ind</td>
<td>375,383</td>
<td>107,558</td>
<td>ETR P1112Q</td>
</tr>
<tr>
<td>6, M</td>
<td>25</td>
<td>Stand</td>
<td>479,286</td>
<td>1047</td>
<td>NR P1115Q, S1496P</td>
</tr>
<tr>
<td>7, M</td>
<td>36</td>
<td>Stand</td>
<td>499,967</td>
<td>11,628</td>
<td>SR S1496R</td>
</tr>
<tr>
<td>8, F</td>
<td>44</td>
<td>Stand</td>
<td>741,216</td>
<td>406,977</td>
<td>NR R1283G, S1496R</td>
</tr>
<tr>
<td>9, M</td>
<td>47</td>
<td>Ind</td>
<td>771,092</td>
<td>&lt;615</td>
<td>ETR P1112Q, S1496A</td>
</tr>
<tr>
<td>10, M</td>
<td>59</td>
<td>Ind</td>
<td>886,818</td>
<td>&lt;615</td>
<td>SR P1115Q, S1496P</td>
</tr>
<tr>
<td>11, M</td>
<td>45</td>
<td>Stand</td>
<td>943,600</td>
<td>698</td>
<td>NR P1112Q, S1496R</td>
</tr>
<tr>
<td>12, F</td>
<td>38</td>
<td>Ind</td>
<td>949,135</td>
<td>7558</td>
<td>NR P1115Q, S1496P</td>
</tr>
<tr>
<td>13, M</td>
<td>39</td>
<td>Ind</td>
<td>962,121</td>
<td>&lt;615</td>
<td>SR S1496P, S1950N</td>
</tr>
<tr>
<td>14, M</td>
<td>30</td>
<td>Stand</td>
<td>1,055,220</td>
<td>267,442</td>
<td>NR P1115Q, R1283G, S1496P</td>
</tr>
<tr>
<td>15, M</td>
<td>37</td>
<td>Ind</td>
<td>1,107,160</td>
<td>&lt;615</td>
<td>ETR P1112Q, S1496R</td>
</tr>
<tr>
<td>16, F</td>
<td>59</td>
<td>Stand</td>
<td>1,161,990</td>
<td>13,372</td>
<td>ETR P1112Q, R1283G, S1496V</td>
</tr>
<tr>
<td>17, M</td>
<td>66</td>
<td>Ind</td>
<td>1,208,270</td>
<td>148,256</td>
<td>ETR P1112R</td>
</tr>
<tr>
<td>18, F</td>
<td>64</td>
<td>Stand</td>
<td>1,630,340</td>
<td>116,279</td>
<td>NR P1112Q, S1496N, S1950N</td>
</tr>
<tr>
<td>19, F</td>
<td>52</td>
<td>Ind</td>
<td>1,669,930</td>
<td>1395</td>
<td>SR P1112Q, S1496A, S1950N</td>
</tr>
<tr>
<td>20, M</td>
<td>63</td>
<td>Stand</td>
<td>1,754,703</td>
<td>93,023</td>
<td>NR P1112Q, S1496R</td>
</tr>
<tr>
<td>21, M</td>
<td>54</td>
<td>Stand</td>
<td>1,915,990</td>
<td>79,942</td>
<td>NR P1112Q, S1496R</td>
</tr>
<tr>
<td>22, F</td>
<td>56</td>
<td>Ind</td>
<td>2,662,170</td>
<td>281,977</td>
<td>NR S1496T, S1950N</td>
</tr>
<tr>
<td>23, M</td>
<td>27</td>
<td>Ind</td>
<td>3,189,200</td>
<td>6686</td>
<td>SR S1496P</td>
</tr>
<tr>
<td>24, M</td>
<td>60</td>
<td>Ind</td>
<td>3,368,634</td>
<td>21,802</td>
<td>NR P1112Q, S1496A, K1846R</td>
</tr>
<tr>
<td>25, M</td>
<td>45</td>
<td>Stand</td>
<td>4,070,700</td>
<td>162,791</td>
<td>NR P1112Q, S1496G</td>
</tr>
<tr>
<td>26, M</td>
<td>52</td>
<td>Ind</td>
<td>7,024,590</td>
<td>&lt;615</td>
<td>SR P1115G, S1496R</td>
</tr>
</tbody>
</table>

NOTE. Patients whose isolates underwent HCV nonstructural (NS) protein 5A quasi-species analyses are underlined. Mutations specific for enhancing replication in the replicon system according to figure 1 are shown in bold type. The reference sequence is HCV-1b prototype HCV-J. ETR, virologic end-of-treatment response with relapse; Ind, induction interferon (IFN)–α–based therapy with 10, 5, or 3 MU daily for 24 weeks, followed by 3 × 3 MU for the remaining 24 weeks; NR, virologic non-responder; SR, sustained virologic response; Stand, standard interferon-α therapy, 3 MU 3 times/week for 48 weeks.

* All patients received ribavirin (1000 mg/day in patients weighing ≤75 kg and 1200 mg/day in patients weighing >75 kg).

* Mutations in HCV isolates from patients with chronic HCV infection at in vitro replication–enhancing sites.

vivo as well. Therefore, within these regions, as-yet-unknown mutations may be located that cause alterations in the in vivo replication efficiency.

For the present study, we sequenced parts of the NS3, NS4B, NS5A, and NS5B genes containing the sites of 31 different in vitro replication–enhancing mutations from 26 patients chronically infected with HCV genotype 1. Furthermore, the NS5A region from aa 2186 to 2248 (according to HCV-1b prototype HCV-J), which includes the IFN sensitivity-determining region (ISDR) and harbors the most-efficient adaptive mutations in the replicon system, was cloned and sequenced for quasi-species analyses. The overall importance of mutations within the NS3–NS5B genes for HCV replication in vivo was estimated on the basis of smoothed mutational analyses [22]. This procedure allows an efficient comparison, even for small to moderate sample sizes. The presence or absence of mutations was correlated with baseline HCV RNA concentration and the response to IFN-α–based antiviral therapy. Moreover, mutations within the NS3 protein were analyzed on the basis of the known crystallographic secondary structures of HCV protease/helicase and related enzymes for the estimation of potential conformational and functional alterations resulting from the mutations observed in vitro and in vivo.

PATIENTS, MATERIALS, AND METHODS

Patients. In the present study, 26 patients chronically infected with HCV subtype 1a/b were enrolled (table 1). Ten patients achieved an SR with undetectable HCV RNA concentrations in serum at least 24 weeks after the end of treatment. Five patients had an end-of-treatment response with relapse (ETR) thereafter, and 11 patients had no virologic response (NR) to
antiviral treatment with IFN-α plus ribavirin (table 1). Chronic HCV infection was diagnosed by the presence of anti-HCV antibodies, elevated serum aminotransferase levels for at least 6 months, histological examination of the liver [23], and consistent detection of HCV RNA in serum. All patients were negative for hepatitis B surface antigen and antibodies to HIV types 1 and 2.

Quantification of HCV RNA and HCV genotyping. HCV RNA was quantified retrospectively in pretreatment serum samples (−80°C) by branched DNA assay (Versant HCV Quantitative Assay 3.0 [bDNA]; Bayer Diagnostics). Genotyping of HCV according to the classification of Simmonds et al. [24] was performed by a reverse-hybridization assay (INNO LiPA HCV-II; Innogenetics). All contamination prevention measures suggested by Kwok and Higuchi [25] were strictly applied.

Amplification of HCV RNA by reverse-transcription polymerase chain reaction (RT-PCR) and sequence analysis. After the extraction of HCV RNA from 100 µL of serum, complementary DNA was generated using random hexamer oligonucleotides or antisense primers. The regions of the HCV NS genes (NS3, NS4B, NS5A, and NS5B), which include 31 described replication-enhancing mutations in the replicon system (figure 1), were amplified by nested PCR (primer details available on request from the corresponding author).

After initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 50 s, and 72°C for 1–2 min were performed for the first and second rounds of PCR in a PE9700 thermocycler (PerkinElmer Cetus). The amplification product was analyzed on a 1.5%–2% agarose gel stained with ethidium bromide.

For sequencing of the different parts of the NS3, NS4B, NS5A, and NS5B genes, 40 µL of the second-round PCR products were purified with Microcon 100 (Amicon). PCR products were labeled for sequencing (Big Dye Deoxy Terminators; Applied Biosystems), and automated sequencing of positive and negative strands was performed (310 DNA Sequencer; Applied Biosystems).

For quasi-species analyses, the region of the NS5A gene (aa 2186–2248) that included the most-efficient adaptive mutations in the replicon system (S2197P, del2202S, and S2204R/I) and the ISDR was selected [12, 14]. Primers for RT-PCR were as follows: external sense, 1b5A2s (5′-CAGTGCTCATTCCATGTCTA-3′; nt 6835); external antisense, 1b5A4a (5′-ACGGATACTTCTCTCATCC-3′; nt 7138); internal sense, 1b5A4s (5′-ACCCCTCCACATTACAGCAG-3′; nt 6859); and internal antisense, 1b5A2a (5′-CCGAAGCGGATCGAAGAgTGCC-3′; nt 7087). Purified nested PCR products were ligated with the pCR 2.1 vector (Invitrogen) and were transformed into Escherichia coli–competent cells in accordance with the manufacturer’s instructions. Twenty individual clones from each sam-

Figure 1. Location of in vitro replication–enhancing sites within the nonstructural (NS) protein 3, NS4B, NS5A, and NS5B genes of the hepatitis C virus (HCV) genome. Single amino acid substitutions with a pronounced effect on enhancement of replication are shown in bold type. Numbering and reference of amino acid mutations is according to the open-reading frame of HCV-1b prototype HCV-J [27].
ple were sequenced using internal sense and antisense primers. For quasi-species analysis, 6 patients with SR and 6 patients with NR were selected (table 1).

The deduced amino acid sequences of the NS3, NS4B, NS5A, and NS5B regions (EMBL Nucleotide Sequence Database accession numbers AM055836–AM055939) were compared with the respective sequences identified in the prototype isolates for HCV-1a (HCV-1 [26]) and HCV-1b (HCV-J [27]), the consensus sequence of all HCV-1b isolates (cons), and replicon isolates RBcon1, HCV-BK, HCV-N, and HCV-H77 [10, 13, 17, 18]. Unless indicated otherwise, numbering and reference for mutations of amino acids is according to the open-reading frame of HCV-1b prototype HCV-J.

**Quasi-species heterogeneity.** For the determination of genetic complexity and genetic diversity within the NSSA region (aa 2186–2248 according to HCV-1b prototype HCV-J [27]), the normalized Shannon entropy $(S_n)$ value and the Hamming distance were calculated, respectively, as described elsewhere [28]. The $S_n$ and mean Hamming distances were calculated at the amino acid level on the basis of 20 HCV clones isolated from pretreatment serum samples from the 12 patients selected for NSSA quasi-species analyses.

**Statistical analyses.** Data were analyzed by the Mann-Whitney U test, Spearman’s rank correlation, and Pearson’s correlation. All tests were 2-tailed. $P<.05$ was considered to be significant. For the presentation of mutational frequencies within NS3, NS4B, NS5A, and NS5B, smoothed values were obtained using Gaussian kernel weights with a bandwidth of 3 aa [29]. Smoothed mutational frequencies of NS3, NS4B, NS5A, and NS5B were calculated for different serum HCV RNA concentrations at baseline (≤10$^{5}$, 5 × 10$^{5}$–1 × 10$^{6}$, 1 × 10$^{6}$–1.5 × 10$^{6}$, and >1.5 × 10$^{6}$ IU/mL). Furthermore, a specific functional data-analysis procedure was used to test whether local accumulations of mutations or enhanced mutational frequencies occur in sequences from patients responding or not responding to treatment, as described elsewhere [22]. The same test was used to compare mutational frequencies in sequences from patients with baseline serum HCV RNA concentrations < or >10$^6$ IU/mL.

**In silico analyses.** For in silico analyses, 3-dimensional structures of the HCV protease and helicase domains were downloaded from the protein data bank (PDB) [30]. Secondary structure assignment was retrieved from the dictionary of protein secondary structure (DSSP) database [31], and domain boundaries were obtained from the Structural Classification of Proteins database [32]. Protein structure pictures were illustrated using Accelrys Discovery Studio Viewer Lite.

The ProSup Web server [33] was used to superpose the HCV helicase domains of the PDB structures 1A1V (chain A) and 1CU1 (chain A), which resulted in a low root-mean-square deviation of 1.58 Å, with 397 identical amino acids of 423 residues. Because not all available HCV helicase domain structures are bound to a nucleotide such as ATP, we examined the relevant nucleotide-binding P-loop of other closely related helicase domains that were crystallized together with a bound ATP molecule and Mg. The P-loop conformation of the nucleotide excision repair enzyme UvrB (PDB identifier 1D9Z) was very similar to the P-loop of HCV helicase 1A1V. Therefore, we superposed the corresponding P-loops of 1A1V and 1D9Z (from PDB chains A) using a transformation matrix derived from the 4 Cα atoms of the catalytic phosphate-binding lysine and the 3 preceding amino acids. The described superpositions of 1A1V with 1CU1 and 1D9Z provided an overall model of the HCV helicase domain bound to single-stranded RNA (from 1A1V) and to ATP-Mg (from 1D9Z).

To compute a rough measurement of the relative solvent accessibility of each amino acid in the HCV helicase domains, we divided the absolute residue solvent-exposed area taken from the DSSP database for 1CU1 (chain A) by the maximum reference value [34] of the corresponding amino acid type. The degree of conservation at each amino acid position in the HCV protease and helicase domains was investigated on the basis of ConSurf conservation scores (ConSurf online server) [35]. PDB structure 1CU1 (chain A) was submitted to this server for 3 PSI-BLAST iterations with an E-value cutoff of 0.001 by the maximum-likelihood method, which resulted in a multiple sequence alignment of 253 unique homologous sequences.

**RESULTS**

**Specific in vitro replication–enhancing mutations detected in vivo.** Within NS3, no mutations were detected at 11 of 15 amino acid sites that caused enhanced in vitro replication in the replicon (V1133I, E1202G, A1226D, T1261S, M1268V, T1280I, T1287A, G1304S, E1383A, I1452L, and K1609E) (table 1 and figures 2 and 3). At the remaining 4 in vitro replication–enhancing amino acid sites, 13 different mutations were observed. Altogether, 5 isolates (ETR4, ETR5, NR2, NR5, and NR6) showed 3 different mutations specific for enhanced in vitro replication in the subgenomic replicon system (P1112R, R1283G, and S1496M) (table 1 and figures 2 and 3). The mean baseline HCV RNA concentration, however, was not significantly different in the 5 patients with specific mutations for in vitro–enhanced replication and in the remaining 21 patients (1.2 × 10$^6$ and 1.6 × 10$^6$ IU/mL, respectively). However, of the 5 patients with specific in vitro replication–enhancing mutations within the HCV NS3 gene, none achieved SR. Furthermore, a smaller log decrease in HCV RNA concentration after 2 weeks of antiviral therapy was observed in these patients, compared with those in other groups ($P = .007$) (table 1). This difference remained significant when patients who had received...
IFN-α-based induction therapy were excluded from the analysis ($P = .03$). Within the NS4B, NS5A, and NS5B proteins at 5, 10, and 1 in vitro replication–enhancing amino acids sites, respectively, no specific mutations were observed within the 26 HCV-1b isolates.

**Nonspecific mutations detected at in vitro replication–enhancing sites.** In addition to the 3 mutations specific for enhanced in vitro replication in the subgenomic replicon system, 10 nonspecific mutations were observed at NS3 amino acid sites for in vitro replication–enhancing mutations, with the majority of mutations located at codon 1496 (P1112Q, P1115Q, P1115G, R1283K, S1496R, S1496P, S1496A, S1496G, S1496V, and S1496T) (table 1 and figures 2 and 3). Within NS4B, NS5A, and NS5B, at 13 of 16 amino acid sites with in vitro-enhanced replication, no mutations were detectable in the 26 HCV-1b isolates. At the remaining 3 amino acid sites, 4 nonspecific mutations within the NS4B protein (K1846R, V1896I, S1950G, and S1950N) were detected in 6 isolates (table 1 and figures 2 and 3). None of the nonspecific mutations at in vitro replication–enhancing sites was associated with HCV RNA concentrations at baseline or with the initial decrease in HCV RNA concentration or overall virologic response.

Analyses of NS3, NS4B, NS5A, and NS5B mutations outside of the in vitro replication–enhancing sites showed low mutational variability within the different HCV genes (figure 4). No correlation of individual mutations or the number of mutations with the pretreatment HCV RNA concentration (figure 4) or with the initial decrease in HCV RNA concentration or overall virologic response was observed within NS3, NS4B, or NS5B. However, within NS5A, fewer mutations, with respect to the consensus sequence, were detected in sequences from patients with baseline HCV RNA concentrations >10⁶ IU/mL, compared with sequences from patients with baseline HCV RNA concentrations <10⁶ IU/mL ($P = .001$). This difference was associated with differences in local accumulations of mutations within the ISDR ($P = .01$).

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

---

**Figure 1.** Mutations at in vitro replication–enhancing sites in hepatitis C virus (HCV)–1 isolates from 26 patients with chronic HCV infection within the nonstructural (NS) protein 3, NS4B, NS5A, and NS5B proteins, compared with HCV-1b prototype HCV-J, according to the data in figure 1. Mutations are shown as different colored columns. The relative frequency of mutations is shown by the height of the respective column. Mutations specific for enhancing replication in the replicon system are shown in dark brown. Numbering of amino acid positions is according to HCV-1b prototype HCV-J [27].

**Figure 2.** Sequence alignment of the pretreatment amino acid residues of different hepatitis C virus (HCV) regions nonstructural (NS) protein 3, NS4B, NS5A, and NS5B of HCV-1 isolates from 26 patients with chronic HCV infection. The figure and legend are available in their entirety in the online edition of the *Journal of Infectious Diseases*.
NS5A quasi-species analyses. The most efficient in vitro replication–enhancing mutations are located within NS5A up-stream of the ISDR (i.e., S2204I/R) [12, 14]. Furthermore, an association of an ISDR deletion mutant with enhancement of in vitro replication in the HCV-1b replicon system has been described in a previous investigation [12]. Therefore, in the present study, the NS5A region from aa 2186 to 2248, which includes the ISDR, was amplified, cloned, and sequenced for quasi-species analyses (20 clones/patient) in 12 HCV isolates. Interestingly, an increasing number of different isolates (quasi-species complexity calculated as Shannon entropy), as well as an increasing number of mutations in the isolates (quasi-species diversity calculated as Hamming distances) of HCV quasi species was correlated with increasing HCV RNA concentrations at baseline (P = .02 for both) (figure 5).

In silico analyses. For further investigation of the relevance of the replication-enhancing mutations of the replicon system and the hot-spot mutation (codon 1496) observed within the NS3 gene in vivo in the present study, the mutations were analyzed for potential conformational and functional modulation of the NS3 protease/helicase on the basis of the known crystallographic 3-dimensional structure of the NS3 protein. All mutations, except for amino acid positions 1133, 1261, 1280, and 1452, (1) were solvent exposed and located on the surface of the NS3 protein, (2) presumably did not lead to major alterations in protein structure, and (3) were not located within one of the functional NS3 helicase motifs. Furthermore, analyses of aligned homologous helicases (using the ConSurf online server) showed a high natural variability in amino acid types at corresponding amino acid positions, which indicates a low functional importance.

In the present study, aa 1496 within the NS3 protein was found to be highly variable (aa 470 of NS3 protein, according to 1CU1). Moreover, different mutations at aa 1496 have been shown to be critical for replication of the in vitro replicon system (S1496L/M) (figure 1) for subtype HCV-1a and HCV-1b isolates, respectively [13, 18]. The Y-shaped HCV helicase is located in the COOH-terminal part of the NS3 protein and consists of 6 motifs that form 3 domains for RNA binding and unwinding. In the present study, 8 different amino acids were present at aa 1496, which is located in a surface loop of domain 2 downstream of motif VI of the NS3 helicase domain (figure 6). Because of the large distance (∼16 Å) between aa 1496 within domain 2 and the ATP bound by domain 1, a direct interaction is unlikely to occur (figure 6). A superposition of 2 very similar HCV helicase structures (PDB identifiers 1A1V and 1CU1) with different amino acids, KP and RR, at positions aa 1495 and 1496 did not reveal a major conformational change within the loop and indicated no significant effect on the flanking secondary structure elements, the NH2-terminal α-helix and the COOH-terminal β-strand (figure 6, inset). Structural analysis demonstrated that aa 1496 residue solvent exposed. In accordance with this finding, in an additional analysis that was based on phylogenetic information assembled by the ConSurf online server, at least 10 different amino acids (A, E, G, K, L, N, P, Q, R, and S) at the corresponding positions in a nonredundant multiple sequence alignment of 253 helicase homologues were observed.
Figure 5. Quasi-species analyses of the nonstructural (NS) protein 5A gene (aa 2186–2248) of hepatitis C virus (HCV) isolates from 12 selected patients with chronic HCV infection at baseline, in correlation with baseline HCV RNA concentration. Black columns show the HCV RNA concentration in serum at baseline (1, corresponding to $1 \times 10^7$ IU/mL). Gray columns show the quasi-species complexity (1, normalized Shannon entropy $S_p = 1$). Light gray columns indicate quasi-species diversity (1, Hamming distance $= 100$). NR, strains from patients without virologic response to antiviral treatment; SR, strains from patients with sustained virologic response to antiviral therapy.

DISCUSSION

Although the phenomenon of replication-enhancing mutations has been well evaluated in the HCV replicon system in vitro [11–21], to our knowledge, the present study is the first to have investigated the existence of replication-enhancing mutations in human in vivo infections and to have examined the clinical relevance of such mutations for viral replication in terms of the HCV RNA concentration at baseline and during IFN-α-based therapy in patients with chronic HCV infection. Of course, baseline HCV RNA concentrations and decreases during therapy depend not only on HCV replication efficiency but also on other parameters, such as the immune system of the host and the number and host-dependent characteristics of infected cells. However, because a direct analysis of the replication efficiency can hardly be done in vivo, baseline HCV RNA concentrations and decreases during therapy were used as the best available surrogate parameters for HCV replication efficiency. Overall, amino acid positions with replication-enhancing mutations in vitro are highly conserved in vivo. Mutations were detected in vivo at only 7 of 31 amino acid positions of in vitro replication–enhancing sites.

Altogether, in 5 patients, 3 different mutations specific for enhanced in vitro replication in the replicon system were detected within the NS3 gene. However, no correlation was observed with serum baseline HCV RNA concentration and the presence or absence of these mutations. A previous article reported a negative influence of in vitro replication–enhancing mutations on viral infectivity and replication in chimpanzees [36]. These discrepancies between in vitro and in vivo findings may reflect the specificity of viral adaptation to different conditions in different host systems. However, the 5 patients with specific mutations at in vitro replication–enhancing sites showed a slower decrease in HCV RNA concentrations during IFN-α–based therapy, compared with patients without specific in vitro replication–enhancing mutations. Notably, none of the 5 patients with specific in vitro replication–enhancing mutations achieved SR. Thus, a potentially higher replication efficiency of HCV isolates containing replication-enhancing mutations may be uncovered only during enhanced antiviral and or immunological pressure due to IFN-α–based antiviral therapy.

In addition to the 3 specific mutations, 10 nonspecific mutations at NS3 amino acid sites for in vitro replication enhancement were detected. On the basis of the known crystallographic 3-dimensional structure of the NS3 protein, analyses of the potential functional and conformational changes due to in vivo and in vitro mutations at replication-enhancing sites were performed. Sites of mutations were mostly located on the surface of the protein, and the comparison with homologous helicases showed a relatively high natural variability at these amino acid positions but very similar conformations of the different helicase homologues. Interestingly, although the majority of amino acid sites within NS3 were highly conserved,
Mutations Enhancing HCV Replication

Multiple mutations were detected at aa 1496 in the 26 isolates from patients with chronic HCV infection, which suggests immunological pressure on this region of NS3 helicase. The superposition of HCV helicase structures with different amino acids at this position showed no relevant conformational changes within the loop and no significant effect on the flanking secondary structure elements. Thus, no direct functional changes due to different amino acid residues at aa 1496 were visible on the basis of in silico structure analysis of the NS3 helicase. Because mutations at this position of the NS3 helicase have been associated with significant differences in replication efficiency in the in vitro replicon system, the functional relevance of the mutations may depend on additional mutations of the NS3 and/or other HCV proteins or on changed interactions with host proteins. This hypothesis is supported by the fact that, despite a high sequence homology of the NS3 proteins within genotypes, treatment with a specific protease inhibitor (BILN 2061) was less effective in HCV genotype 3 infection than in HCV genotype 1 infection [37]. None of the unspecific mutations within NS3 and no mutation within the NS4B and NS5B genes were associated with baseline HCV RNA concentrations.

Interestingly, by direct sequencing, lower mutation rates within the NS5A-ISDR region of the major HCV strain were observed in patients with higher baseline HCV RNA concentrations, as has been reported elsewhere [38]. This finding indicates a potential role of the ISDR amino acid sequence in the replication efficiency of HCV. When HCV quasi species of the NS5A region—including the most important in vitro replication–enhancing sites—and the ISDR were analyzed, an increasing quasi-species complexity and diversity was associated with higher serum HCV RNA concentrations. Independent of the number of mutations in the major HCV strain, a higher quasi-species heterogeneity may lead to a higher replication efficiency in the different compartments of the host. Indeed, different HCV quasi species are detectable in different cell com-
partments of a single host [39–41], and different needs for an efficient replication of HCV in different compartments are feasible [42]. This hypothesis is corroborated by the in vitro observation that transfection of the HCV replicon to different cell types leads to differences in the efficiency of replication [43].

In conclusion, specific mutations associated with enhanced replication of HCV in the in vitro replicon system were detected in 5 of 26 patients in vivo, and these did not correlate with baseline serum HCV RNA concentrations. In vitro replication-enhancing effects of single mutations within NS3 cannot be explained by direct changes in the structure of the protein. However, in accordance with the replication-enhancing effects in vitro, specific mutations at replication-enhancing sites were associated with a slower initial decrease in HCV RNA concentrations during IFN-α–based antiviral therapy in vivo. Moreover, quasi-species heterogeneity of NSSA, including the most-efficient in vitro replication–enhancing sites and the ISDR, correlated with baseline HCV RNA concentrations, which suggests a key role of the NSSA protein in viral replication efficiency in vitro and in vivo.

Acknowledgment
We are grateful to Ralf Bartenschlager for critically reading the manuscript and for helpful discussions.

References