

Mutations in the Protein Kinase–Binding Domain of the NS5A Protein in Patients Infected with Hepatitis C Virus Type 1a Are Associated with Treatment Response

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An interaction of the hepatitis C virus (HCV) NS5A protein with the interferon (IFN)- α -inducible double-stranded RNA-activated protein kinase (PKR) was demonstrated in vitro. The clinical correlation between amino acid mutations within the HCV NS5A region and response to antiviral treatment is controversial. Thirty-two patients chronically infected with HCV-1a, who were treated with IFN- α with or without ribavirin, were studied. The carboxy-terminal half of HCV NS5A was sequenced and was investigated by phylogenetic and conformational analyses. Eight patients achieved a sustained virologic response. An end-of-treatment response but relapse thereafter was observed among 8 patients, whereas 16 patients were nonresponders. The median number of mutations within the PKR-binding domain but not within the previously described IFN sensitivity-determining region was significantly higher for patients with sustained (3 mutations [range, 1–5]) or end-of-treatment (4 mutations [range, 1–5]) virologic response than for nonresponders (2 mutations [range, 0–3]) ($P = .0087$). Phylogenetic and conformational analyses of NS5A sequences allowed no differentiation between sensitive and resistant strains.

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, with frequent progression to liver cirrhosis and an elevated risk for the development of hepatocellular carcinoma [1–3]. Treatment with interferon (IFN)- α leads to sustained virologic response, with undetectable HCV RNA in serum 24 weeks after the end of treatment in <20% of cases [4]; more recently, treatment with IFN- α in combination with ribavirin has been shown to lead to sustained virologic response in 38%–43% of cases [5, 6]. Rates of sustained virologic response to treatment are correlated with different predictive parameters, of which HCV genotype is the strongest. In general, patients infected with HCV-2 and -3 respond better to IFN- α than do patients infected with HCV-1 subtypes [6].

In 1995 and 1996, Enomoto and colleagues [7, 8] demon-

strated that ≥ 4 amino acid mutations between codons 2209 and 2248 of the HCV NS5A protein, compared with HCV-1b prototype HCV-J (mutant type), are correlated with sustained virologic response to IFN- α therapy in Japanese patients infected with HCV-1b isolates. These findings were confirmed by other Japanese investigations [9, 10]. However, the importance of this putative IFN sensitivity-determining region (ISDR) for virologic response was challenged by several studies from Western countries, because the majority of patients with sustained virologic response had <4 amino acid mutations within NS5A_{2209–2248} [11–15]. Recently, we analyzed the NS5A_{2209–2248} region in a larger cohort of HCV-1b-infected patients [16]. In this study, we found higher sustained virologic response rates in European patients infected with NS5A_{2209–2248} mutant type HCV-1b isolates as well. In another study, a higher number of amino acid mutations within complete NS5A protein was observed in IFN- α -treated patients with sustained virologic response than in nonresponders [17]. Moreover, the conformational analysis of NS5A by secondary structure prediction allowed differentiation between most IFN-sensitive and -resistant strains [17].

The effects of IFN- α are mediated through different cellular inducible proteins, including double-stranded RNA-activated protein kinase (PKR) [18]. Recently, Gale et al. [18] demonstrated functional inhibition of PKR by the NS5A protein of HCV genotype 1 isolates, possibly allowing HCV to escape from antiviral effects of IFN- α . Insertion of multiple mutations within the ISDR resulted in an abrogated ability of NS5A to bind to PKR in vitro and to inhibit PKR function in a yeast

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2-hybrid assay. The ISDR (NS5A₂₂₀₉₋₂₂₄₈) was found to be necessary but not sufficient for the interaction between NS5A and PKR, and the region between codons 2209 and 2274 was subsequently identified as the complete PKR-binding domain [19]. These data provided pathophysiologic evidence for the *in vivo*-observed correlation between mutations within NS5A and response to antiviral treatment.

In most studies, only patients infected with HCV-1b were investigated to correlate the antiviral response with the number of mutations within the NS5A protein. Large clinical trials showed no difference between antiviral response rates for patients infected with HCV-1a and HCV-1b [5]. HCV-1a is frequent in Europe and is the most prevalent HCV subtype in the United States [20]. In the present study, we therefore sequenced the carboxy-terminal half of the HCV NS5A gene, comprising the complete PKR-binding domain, in isolates from patients chronically infected with HCV-1a who received IFN- α therapy. Subsequently, phylogenetic analyses were done from the primary nucleotide sequence information and the predicted secondary structure of the resulting protein. Additionally, pretreatment sera were investigated for antibodies directed against the NS5A protein.

Patients and Methods

Patients. In the present study, 32 consecutive patients (15 men, 17 women; mean age, 47 years [range, 25–64]) chronically infected with HCV-1a were enrolled. The diagnosis of chronic hepatitis C was based on elevated serum aminotransferase levels for ≥ 6 months, histologic examination [21], and the consistent detection of serum HCV RNA. Each patient was positive for anti-HCV antibodies (by third-generation ELISA). All patients were negative for hepatitis B surface antigen and antibodies to human immunodeficiency virus types 1 and 2. Eighteen of these patients received 6 MU of recombinant IFN- α 3 times per week subcutaneously for the initial 12 weeks, followed by 3 MU of IFN- α 3 times weekly for a total of 48 weeks (cumulative dose, 540 MU). The remaining 14 patients were treated with 6 MU of IFN- α 3 times weekly for the initial 12 weeks, followed by 3 MU of IFN- α 3 times weekly for 36 weeks (cumulative dose, 540 MU) plus ribavirin orally (1000 mg/day for patients with body weights ≤ 75 kg or 1200 mg/day for patients with body weights > 75 kg).

Qualitative measurement of HCV RNA, HCV genotyping, and antibody assay. Serum was prepared in a laminar flow bench and was frozen at -80°C . Qualitative detection of serum HCV RNA was done by reverse transcription-polymerase chain reaction (RT-PCR), as described elsewhere in detail [22]. The lower detection limit of the assay was 1000 copies/mL. Genotyping of HCV (according to the classification of Simmonds et al. [23]) was done by reverse hybridization assay (INNO LiPA HCV-II; Innogenetics, Ghent, Belgium). All contamination prevention measures suggested by Kwok and Higuchi [24] were strictly applied. Anti-HCV antibodies were detected by EIA (INNO-LIA HCV Ab III; Innogenetics). This qualitative assay exposes several specific HCV peptides from 2 core regions (C1 and C2), 1 envelope region (E2), and 3 nonstructural regions (NS3, NS4, and NS5A) of the HCV poly-

protein. The assay was done according to the manufacturer's instructions.

Amplification of HCV NS5A RNA by RT-PCR and sequence analysis. The NS5A region between codons 2188 and 2318 was amplified after RT by seminested PCR. After extraction of HCV RNA from 100 μL of serum, complementary DNA was generated by use of random hexamer oligonucleotides. The first round of PCR was done with external sense primer 1a5A6s (5'-GAGCCCGAACCGGACGT-3'; nt 6828–6844 according to HCV-1 [25]) and antisense primer 1a5A6a (5'-GTGAGGACCACCGT-3'; nt 7325–7338 according to HCV-1). After an initial denaturation step at 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 50 s, and 72°C for 2 min were performed in a PE9700 thermocycler (Perkin-Elmer Cetus, Norwalk, CT). In the second round of the seminested PCR, 35 cycles with internal sense primer 1a5A5s (5'-TCCATGCTCACTGATCC-3'; nt 6858–6874 according to HCV-1) and the antisense primer 1a5A6a were done as described for the first round of the PCR. The resulting amplification product was analyzed on a 2% agarose gel stained with ethidium bromide.

For direct sequencing of the NS5A region (codons 2188–2318), 40 μL of the second-round PCR product was purified with Microcon 100 (Amicon, Witten, Germany). Sequence analysis was done according to the manufacturer's instructions (Big Dye Deoxy Terminators; Applied Biosystems, Weiterstadt, Germany). Sequencing of the plus-strand and the minus-strand was done by an automat (310 DNA Sequencer; Applied Biosystems). The deduced amino acid sequences of the NS5A region (codons 2188–2318) were compared with the NS5A sequences identified in the prototype isolates for HCV-1a (HCV-1 [25]), HCV-1b (HCV-J [26, 27]), and the consensus sequence of all HCV-1a isolates investigated.

Phylogenetic and conformational analysis. Multiple alignment of amino acid sequences and calculation of the consensus sequence were carried out with CLUSTAL X1.64b software (D. Higgins, Heidelberg, Germany) [28]. The phylogenetic tree of NS5A sequences obtained from HCV isolates of the investigated patients was constructed by means of the program Treecon for Windows (version 1.3b; Y. van de Peer, Antwerp, Belgium) [29]. An identity protein weight matrix was used to estimate the evolutionary distance. The correction of Kimura [30] was used to correct for multiple substitutions. The phylogenetic tree was constructed with the neighbor-joining method of Saitou and Nei [31]. Bootstrap resampling (1000 replicates) was used as a pseudoempirical test of reliability of the tree topology [32].

The secondary structure of resulting proteins of the NS5A sequences was predicted with the double prediction method of Deléage and Roux [33] implemented in the ANTHEPROT version 4.2 software (G. Deléage, Lyon, France) [34]. This method is a combination of protein class predictions according to Nakashima et al. [35] and Chou and Fasman [36]. Multiple alignment of predicted secondary structures was done and an identity comparison matrix was calculated, both by use of CLUSTAL X1.64b [28], whereas a tree based on the differences in secondary structure of the proteins was drawn with *njplot* [37].

Data presentation. Clinical and biochemical characteristics of patients are expressed as mean \pm SD or median and range, as appropriate. Distributions of continuous variables were analyzed by the Mann-Whitney *U* test, and qualitative data were compared with Fisher's 2-tailed exact test. $P < .05$ was considered significant.

Results

In the present study, the NS5A region of HCV isolates from 32 patients chronically infected with HCV-1a was investigated. All patients received antiviral therapy for 48 weeks. Eight patients achieved a sustained virologic response (sustained responders), with undetectable HCV RNA 24 weeks after termination of therapy. In 8 patients, a virologic response with negative HCV RNA at the end of treatment but relapse thereafter was observed (end-of-treatment responders). Sixteen patients revealed no virologic response to antiviral therapy (nonresponders).

Pretreatment amino acid sequences of the NS5A region. Amino acid changes in the carboxy-terminal, but not the amino-terminal, region of NS5A have been correlated with virologic treatment response [7, 17]. The carboxy-terminus of NS5A is highly variable (V3 region, codons 2356–2379), indicating no functional conservation [7, 17, 25, 26]. Recently, the NS5A region from codon 2209 through codon 2275 was described as the complete PKR-binding domain [19]. However, the secondary structure of the PKR-binding region is most likely also dependent on the flanking amino acids. Therefore, the amino acid sequence of the PKR-binding region and the flanking sequences within the carboxy-terminal part of the NS5A region (codons 2188–2318) were investigated in the present study (figures 1 and 2).

Within the putative ISDR (NS5A_{2209–2248}), comparison of the

HCV-1a prototype sequence HCV-1 [25] with the consensus sequence of the 32 isolates showed only 1 amino acid change (2217T→A). Two different amino acid changes were observed within the ISDR between the HCV-1a consensus sequence and the HCV-1b prototype sequence HCV-J [26] (2218N→H and 2225E→D). Compared with the consensus sequence of the ISDR, 6 of 32 patients showed no amino acid change, 26 patients had 1–3 changes, and no patient had >3 amino acid changes (figure 2). The differences between the median number of mutations in sustained responders (1 [range, 0–3]), end-of-treatment responders (2 [range, 1–2]), and nonresponders (1 [range, 0–3]) were not statistically significant (table 1). Comparison of the 32 HCV-1a isolates with the HCV-1b prototype sequence HCV-J showed similar results (sustained responders, 3 [range, 2–5]; end-of-treatment responders, 2 [range, 0–4]; nonresponders, 3 [range, 2–4]), with no significant differences between the groups.

Extending the analysis to the PKR-binding domain (codons 2209–2274) revealed no additional differences between the HCV-1a prototype sequence HCV-1 [25] and the consensus sequence. Comparison of the consensus sequence with the HCV-1b prototype sequence HCV-J [26] showed 3 additional amino acid changes between codons 2249 and 2274 (2259L→I, 2260V→R, and 2261E→V) (figure 2). Within the complete PKR-binding domain (codons 2209–2274), the median number of mutations, compared with the consensus sequence, was 3

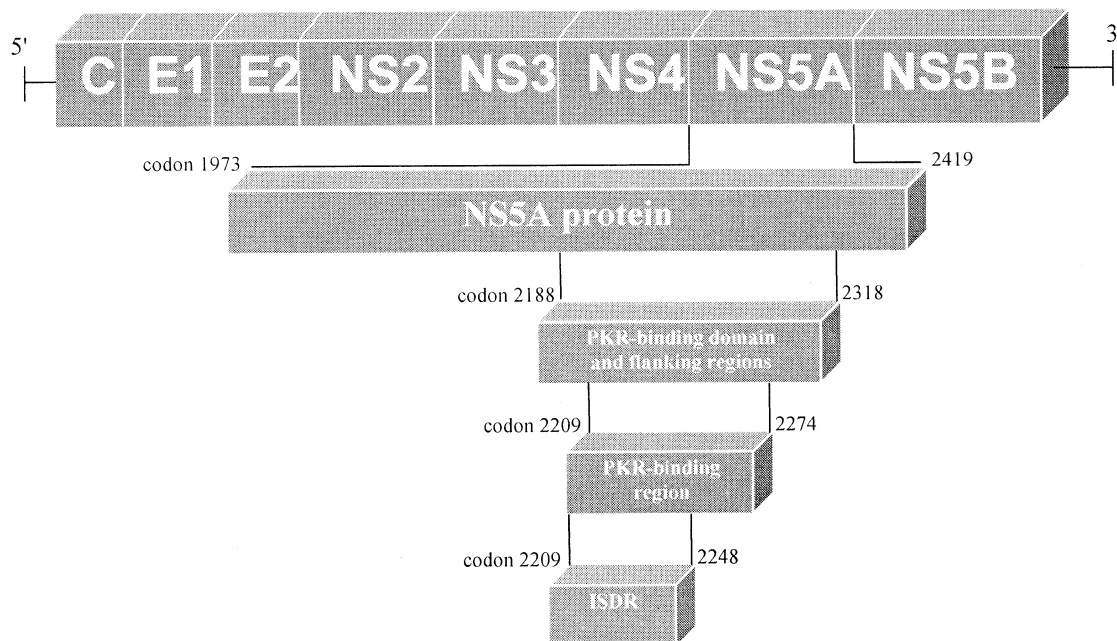


Figure 1. Location of interferon (IFN) sensitivity-determining region (ISDR) and protein kinase (PKR)-binding domain within hepatitis C virus (HCV) NS5A and open-reading frame of HCV polyprotein. NS5A protein (codons 1973–2419), PKR-binding domain flanking region (codons 2188–2318), PKR-binding domain (codons 2209–2274), and ISDR (codons 2209–2248) are indicated according to numbering system of HCV-1b prototype HCV-J [26].

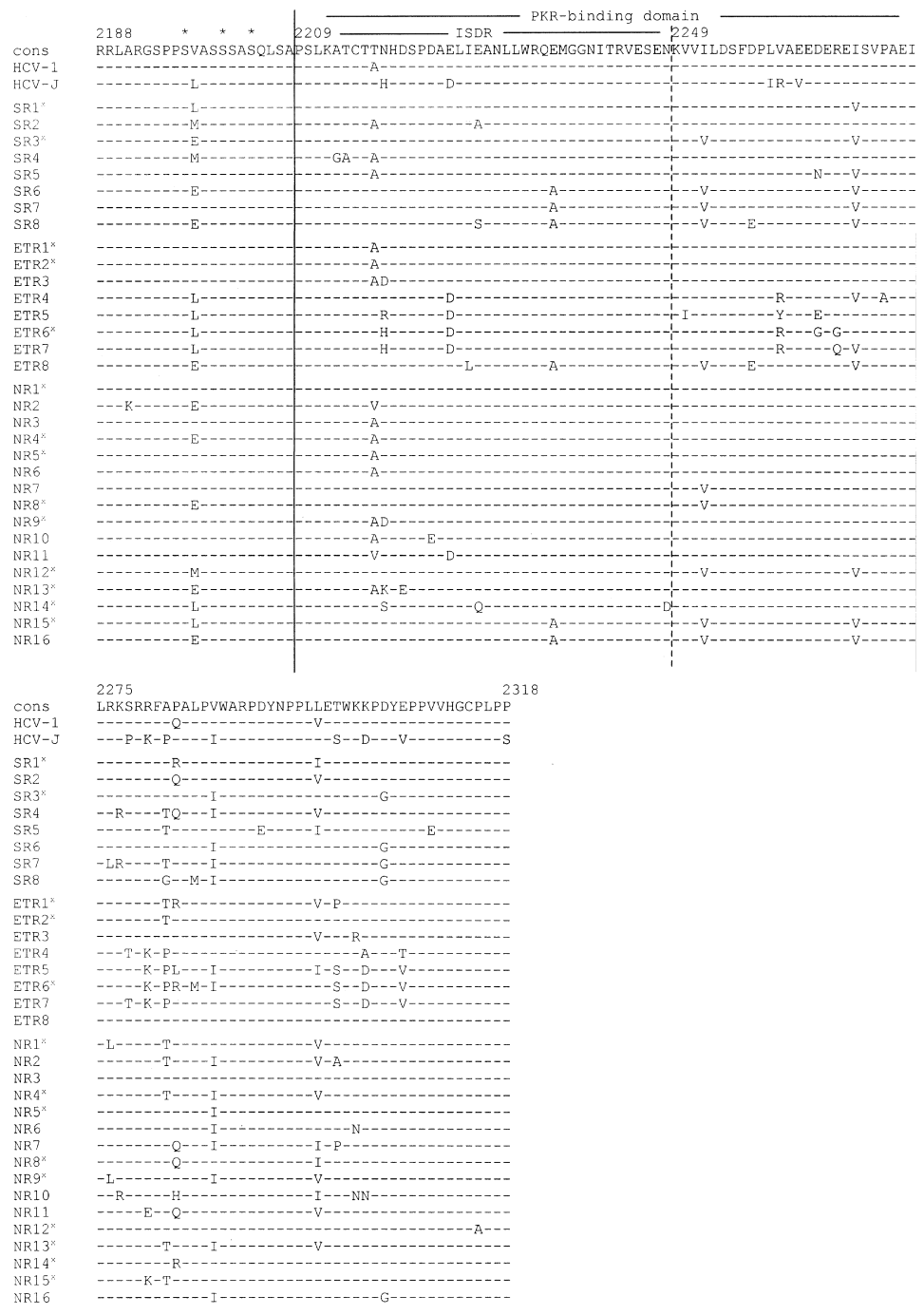


Figure 2. Sequence alignment of amino acid residues 2188–2318 in NS5A protein in pretreatment isolates of patients infected with hepatitis C virus (HCV)–1a. Amino acid residues are indicated by standard single-letter codes. Dashes indicate residues identical to consensus sequence calculated from all 32 isolates investigated (cons). Additionally, sequences of HCV-1 [25] and HCV-J [26] are shown as reference sequences. Vertical lines indicate putative interferon (IFN) sensitivity–determining region (ISDR; *dashed vertical line*) [8] and protein kinase (PKR)–binding domain (*unbroken vertical line*) [19]. SR1–8, strains of patients with sustained virologic response to antiviral therapy; ETR1–8, strains of patients with relapse after therapy; NR1–16, strains of patients without virologic response to antiviral treatment. *, hyperphosphorylation sites; ×, patients who received combination therapy with IFN-α plus ribavirin.

Table 1. Mutations in different parts of NS5A_{2188–2318} region in patients infected with hepatitis C virus (HCV)–1a.

Response group (<i>n</i>)	ISDR ^a	PKR-binding domain ^b	NS5A _{2188–2318} ^c
Sustained responders (8)	1 ± 1 (0–3)	3 ± 1 (1–5)	7 ± 2 (4–10)
End-of-treatment responders (8)	2 ± 1 (1–2) ^d	4 ± 2 (1–5) ^d	8 ± 4 (2–14) ^d
Nonresponders (16)	1 ± 1 (0–3) ^e	2 ± 1 (0–3) ^f	5 ± 2 (1–7) ^g

NOTE. Data are median no. ± SD (range) of mutations compared with consensus sequence. Sustained responders: HCV RNA undetectable 24 weeks after termination of therapy; end-of-treatment responders: HCV RNA undetectable at end of treatment but relapse thereafter; nonresponders: HCV RNA–positive during treatment and thereafter.

^a Interferon sensitivity–determining region [8], NS5A codons 2209–2248 according to HCV-J [26].

^b Protein kinase (PKR)–binding region [19], NS5A codons 2209–2274 according to HCV-J [26].

^c Carboxy-terminal part of NS5A region, including PKR-binding domain and 5' and 3' flanking regions, codons 2188–2318 according to HCV-J [26].

^d Not significant compared with sustained responders.

^e Not significant compared with virologic responders (sustained and end-of-treatment responders).

^f *P* = .0087 compared with virologic responders (sustained and end-of-treatment responders).

^g *P* = .023 compared with virologic responders (sustained and end-of-treatment responders).

(range, 1–5) for sustained responders, 4 (range, 1–5) for end-of-treatment responders, and 2 (range, 0–3) for nonresponders. The difference between virologic responders (sustained responders and end-of-treatment responders) and nonresponders was statistically significant (*P* = .0087; table 1), as were the differences between sustained responders and nonresponders (*P* = .037) and between end-of-treatment responders and nonresponders (*P* = .026). The median number of mutations of the 32 HCV-1a isolates, compared with the HCV-1b prototype sequence HCV-J, was 8 (range, 6–10) for sustained responders, 6 (range, 4–10) for end-of-treatment responders, and 6 (range, 5–8) for nonresponders. The differences between sustained responders and nonresponders and between end-of-treatment responders and nonresponders were significant (*P* = .0072 and *P* = .0034, respectively).

In the NS5A region, including the PKR-binding domain and the 5' and 3' flanking regions (codons 2188–2318), 2 additional amino acid changes were observed between the consensus sequence and the HCV-1a prototype sequence HCV-1 (2283P→Q, and 2298L→V). Comparison of the consensus sequence with the HCV-1b prototype sequence HCV-J showed 9 amino acid changes in the 5' and 3' flanking regions (2198V→L, 2278S→P, 2280R→K, 2282A→P, 2287V→I, 2300T→S, 2303K→D, 2207E→V, and 2318P→S) (figure 2). Compared with the consensus sequence, the median numbers of mutations for sustained responders, end-of-treatment responders, and nonresponders were 7 (range, 4–10), 8 (range, 2–14), and 5 (range, 1–7), respectively. The difference between virologic responders (sustained responders and end-of-treatment responders) and nonresponders was statistically significant (*P* = .023; table 1), as were the differences between sustained responders and nonresponders (*P* = .043) and between end-of-treatment responders and nonresponders (*P* = .047). The median numbers of amino acid changes of the 32 HCV-1a isolates, compared with the HCV-1b prototype sequence HCV-J, were 18 (range, 16–20) for

sustained responders, 13 (range, 7–19) for end-of-treatment responders, and 16 (range, 14–20) for nonresponders. Differences between sustained responders and nonresponders were significant (*P* = .0071).

Hyperphosphorylation sites have been described upstream of the putative ISDR at codons 2197, 2201, and 2204 [38]. At these positions, no mutations, compared with the consensus sequence or the HCV-1a and -1b prototype sequences (HCV-1, HCV-J), were observed in any of the 32 patients studied (figure 2).

Phylogenetic analysis of NS5A sequences. Evolutionary distance calculation based on the predicted NS5A amino acid sequences (codons 2188–2318) was performed for phylogenetic analysis. Patients were assigned to groups according to their virologic response to treatment. Several HCV strains of sustained virologic responders and end-of-treatment responders were located relatively close together (strains SR3, SR6, SR7, and SR8; SR5 and ETR2), but no uniform cluster was observed, because isolates of nonresponders were located throughout the phylogenetic tree (figure 3). Restricting the analysis to the ISDR or the PKR-binding domain showed similar results.

Conformational analyses of the NS5A region. To further investigate potential differences between sensitive and resistant HCV strains, a secondary-structure conformational analysis was done. The secondary structure of each strain (codons 2188–2318) was calculated by the double prediction method of Deléage and Roux [33]. In this method, 4 possibilities for each amino acid residue are given: helix, β-sheet, turn, and coil. The variation from the predicted secondary structure is shown in comparison with the secondary structure of the consensus sequence for all 32 HCV-1a isolates (figure 4). The majority of amino acid mutations observed in isolates of sustained responders, end-of-treatment responders, and nonresponders were conservative (58%, 67%, and 56%, respectively). Few mutations of hydrophobic amino acids, mainly located in the hy-

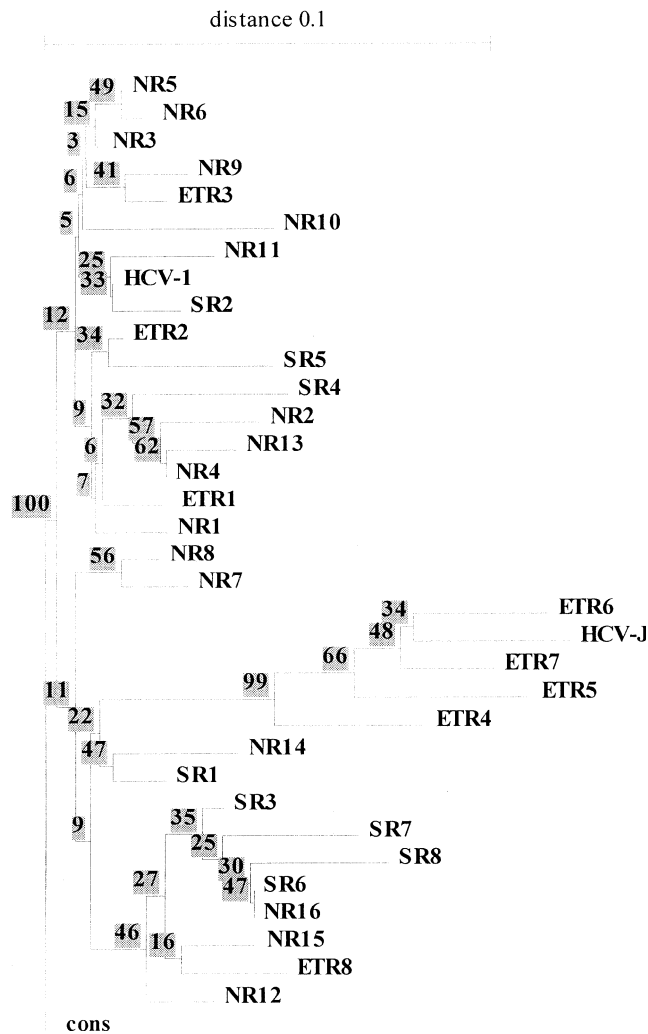


Figure 3. Phylogenetic tree of hepatitis C virus (HCV)-1a isolates. Consensus sequence calculated from all 32 isolates investigated (cons) is shown as reference sequence. SR1–8, strains of patients with sustained virologic response to antiviral therapy; ETR1–8, strains of patients with relapse after therapy; NR1–16, strains of patients without response to antiviral treatment; HCV-1, prototype sequence of HCV-1a subtype; HCV-J, prototype sequence of HCV-1b subtype. Distance scale: 10% sequence dissimilarity. Internal node numbers represent bootstrap values.

drophobic core of the resulting protein, were observed in the 3 groups (14%, 14%, and 18%, respectively). The rate of mutations for polar and charged amino acids was slightly higher in strains obtained from sustained responders (28%) than in isolates from end-of-treatment responders (19%) and non-responders (16%). These differences did not achieve statistical significance. The conformational similarities were calculated by use of an identity matrix, and a tree was drawn to show the relationship of the different strains based on their secondary structures (figure 5). Some isolates of sustained and end-of-treatment responders were closely located together in the con-

formational tree (strains SR2 and SR4; SR3, SR6, SR8, and ETR8; and SR7, ETR4, ETR5, ETR6, and ETR7). However, uniform clusters of isolates from sustained responders and end-of-treatment responders or from nonresponders were not observed.

Anti-HCV NS5A antibodies. Recently, a correlation between the pretreatment reactivity of anti-HCV NS5A antibodies and the antiviral response to IFN- α treatment was described in patients chronically infected with HCV-1–5 isolates [39, 40]. In the present study, anti-NS5A antibodies were detected in pretreatment serum samples of all 8 patients who achieved a sustained virologic response. In 3 (38%) of 8 patients with end-of-treatment response and in 9 (56%) of 16 patients with no virologic response to treatment, anti-HCV NS5A antibodies were found. The differences between sustained responders or end-of-treatment responders and nonresponders were statistically not significant. No correlation with the presence of antibodies to other HCV proteins (C1, C2, E2, NS3, or NS4) was observed.

Discussion

Studies from Japan showed that HCV-1b isolates with at least 4 amino acid changes within NS5A_{2209–2248} (ISDR), compared with the prototype sequence HCV-J, are more sensitive to IFN than are isolates with the prototype sequence HCV-J [7–10]. However, the results were not unequivocally confirmed in studies from Europe and the United States [11–15]. Data of a recent European study suggested that these discrepancies are related to the efficacy of antiviral treatment and to the low prevalence of mutant type HCV-1b isolates in Western countries [16]. In that study, a correlation between mutant type HCV-1b isolates and sustained virologic response was also observed in European patients treated with combination therapy with IFN- α and ribavirin. However, the majority of patients with sustained virologic response showed only 1–3 amino acid changes within ISDR [16].

In general, the virologic response rates to IFN- α treatment are lower for patients infected with HCV genotype 1 than for patients infected with types 2 and 3 isolates. Within genotype HCV-1, subtypes HCV-1a and HCV-1b are most prevalent; however, large geographic variations exist. HCV-1a isolates are virtually not present in Japan but are common in Western countries. In the United States and Canada, HCV-1a is even more prevalent than HCV-1b [20]. Previous studies investigating the correlation between NS5A sequences and sustained virologic response have almost exclusively focused on subtype HCV-1b. Because the prevalence of HCV-1a in Western countries is high, and the virologic response rates are not different between patients infected with HCV-1a and HCV-1b [5], we performed sequence and phylogenetic analyses in patients infected with subtype HCV-1a. As shown in the present study, no correlation was observed between the number of amino acid changes in

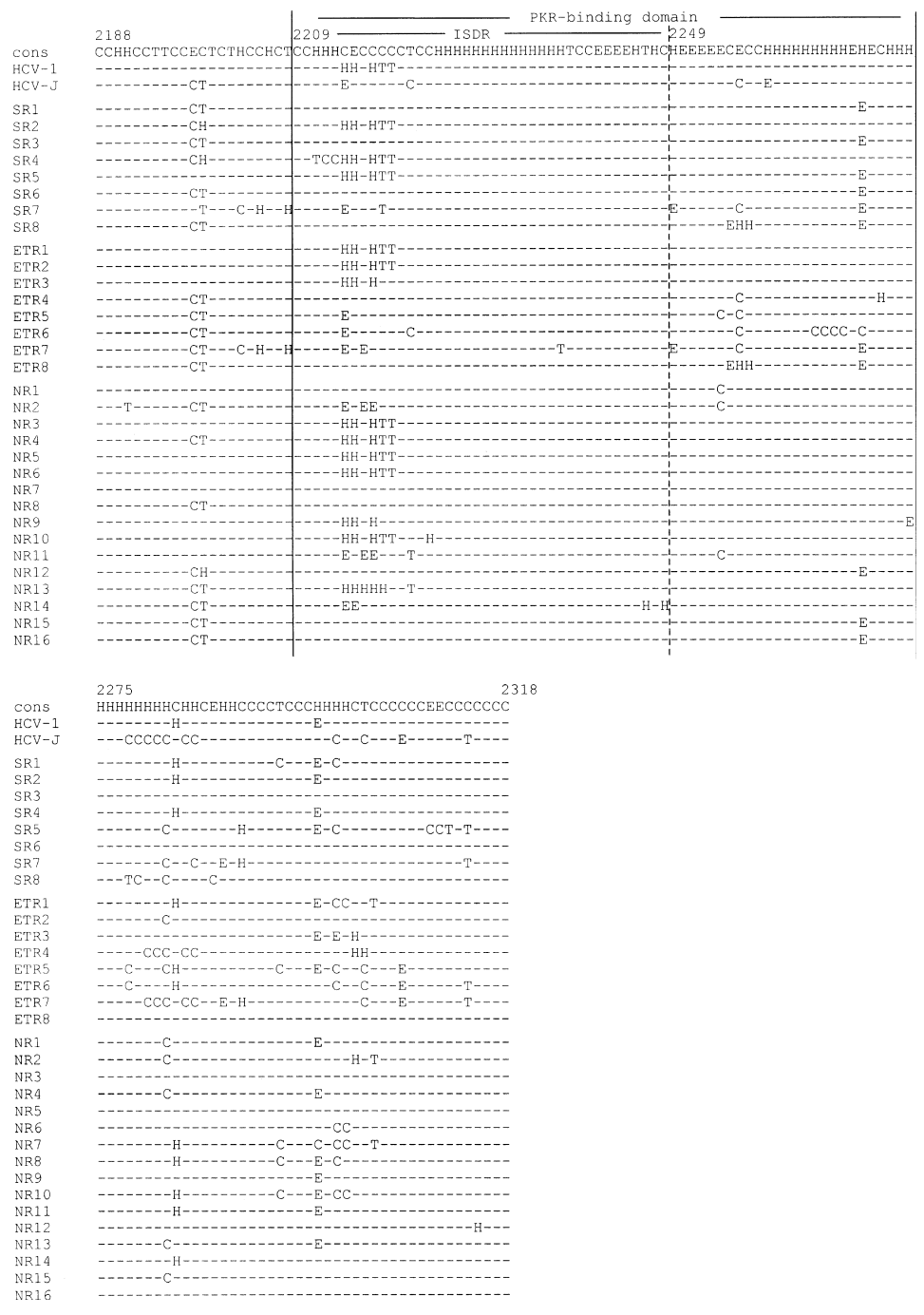


Figure 4. Predicted secondary structure of corresponding sequences (figure 1) of codons 2188–2318 of NS5A protein. Prediction was made by double-prediction method [33]. H, helix; E, β -sheet; T, turn; C, coil. Dashes indicate identical conformation to consensus sequence calculated from all 32 isolates investigated (cons). Additionally, secondary structure prediction of hepatitis C virus (HCV)–1 [25] and HCV-J [26] is shown. Vertical lines indicate putative interferon sensitivity-determining region (ISDR; *dashed vertical line*) and protein kinase (PKR)–binding region (*unbroken vertical line*). SR1–8, strains of patients with sustained virologic response to antiviral therapy; ETR1–8, strains of patients with relapse after therapy; NR1–16, strains of patients without response to antiviral treatment; HCV-1, prototype sequence of HCV-1a subtype; HCV-J, prototype sequence of HCV-1b subtype.

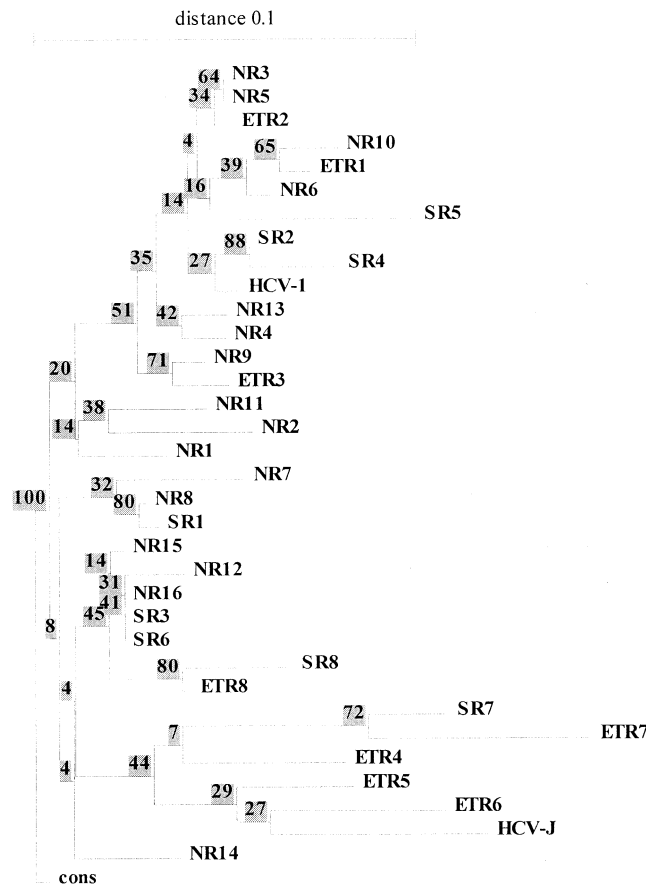


Figure 5. Tree of conformational similarities of hepatitis C virus (HCV)-1a isolates sequenced based on secondary structure prediction made in figure 4. Conformational analysis of the consensus sequence calculated from all 32 isolates investigated is shown as reference. SR1-8, strains of patients with sustained virologic response to antiviral therapy; ETR1-8, strains of patients with relapse after therapy; NR1-16, strains of patients without response to antiviral treatment; HCV-1, prototype sequence of HCV-1a subtype; HCV-J, prototype sequence of HCV-1b subtype. Internal node numbers represent bootstrap values.

the putative ISDR (NS5A₂₂₀₂₋₂₂₄₈) and the antiviral response in patients infected with HCV-1a.

The NS5A protein has been implicated in repression of the double-stranded RNA-activated PKR, which is an important antiviral effector protein of IFN- α . The ISDR was found to be necessary but not sufficient for the interaction between NS5A and PKR, and the region between codons 2209 and 2274 was identified as the complete PKR-binding domain. In the present study, we have therefore extended the sequence analysis to the carboxy-terminal half of the NS5A protein, including the complete PKR-binding region.

The number of amino acid changes within the PKR-binding domain, compared with the consensus sequence of all isolates investigated, was significantly higher for patients with virologic response (sustained or end-of-treatment responders) than for

patients who were nonresponders. The differences between sustained and end-of-treatment responders were not significant. These results are in accordance with the concept of functional interaction between NS5A protein and the double-stranded RNA-activated PKR, a major target protein of the IFN- α signal transduction pathway. Multiple mutations within the NS5A PKR-binding region may impair the interaction between NS5A and PKR, which subsequently could lead to enhanced inhibition of viral replication by IFN- α . However, no specific mutation or a minimal number of mutations within the PKR-binding region (codons 2209-2274) correlated with virologic response. Therefore, other response factors, such as mutations within HCV quasispecies or mutations in other regions of HCV, may be involved in response to antiviral treatment [41].

Recently, sequence analyses of the complete NS5A gene of 19 HCV-1b isolates from patients treated with IFN- α were done. In IFN- α -sensitive strains, amino acid changes were more frequent throughout NS5A than they were in IFN- α -resistant strains. The amino acid differences between sensitive and resistant strains were mainly observed for polar and charged amino acids located at the presumed surface of the protein. Conformational analysis of the carboxy-terminal half of the NS5A protein by secondary structure prediction allowed the differentiation between most sensitive and resistant strains [17]. In the present study, analysis of NS5A in patients infected with HCV-1a revealed mainly conservative amino acid changes, compared with the prototype sequence. The rate of polar and charged amino acid mutations was only slightly higher in strains from patients with sustained response (28%) than in strains from end-of-treatment responders (19%) or nonresponders (16%). Conformational analysis of NS5A by the secondary prediction method allowed no differentiation between isolates of sustained responders, end-of-treatment responders, and nonresponders. In the present study, 16 patients with sustained or end-of-treatment virologic response and 16 patients with no response were analyzed, whereas in the study of Duverlie et al. [17], 11 resistant strains were compared with 8 sensitive strains. Extending the number of resistant strains appears to increase the variability of mutations detected.

To further elucidate the importance of NS5A mutations in the IFN- α signal-transduction pathway, functional analyses of NS5A proteins are required. In a recent study, HCV-1b but not HCV-1a NS5A proteins expressed in human cell lines were able to rescue encephalomyocarditis virus replication during IFN- α challenge [42]. Thus, especially for HCV-1a NS5A proteins, further sequence and functional analyses are needed to specify the relevance during IFN- α treatment.

Antibodies directed against the NS5A protein are present in almost all patients infected with different HCV genotypes who achieved a sustained virologic response to IFN- α therapy [39, 40]. In a recent study, anti-NS5A antibodies were detectable in 95% of patients with sustained virologic response but in only 13% of patients without virologic response and in 63% of pa-

tients with end-of-treatment virologic response [39, 40]. In the present study, anti-NS5A antibodies were detected in all patients with a sustained virologic response but also in many patients without antiviral response or an end-of-treatment response to IFN- α therapy. These findings reinforce a potential importance of mutations within *NS5A* for immunogenicity of the encoded protein.

In a recent study, a PKR-eIF2 α phosphorylation homology domain within E2 (envelope) protein of HCV-1a and -1b genotypes was demonstrated to interact with PKR and eIF2 α , leading to inhibition of PKR function in vitro. Interestingly, within HCV-2a, -2b, and -3a prototype isolates, mutations were found within the PKR-eIF2 α phosphorylation homology domain of HCV E2 protein, abolishing the PKR-E2 interaction. Therefore, the authors concluded that the PKR-eIF2 α phosphorylation homology domain of HCV-1a and -1b genotypes might potentially be responsible for the relative IFN- α resistance of HCV-1a and -1b genotypes, compared with that of HCV-2 and -3 genotypes [43]. Additional studies need to investigate whether mutations within the PKR-eIF2 α phosphorylation homology domain are clinically associated with virologic response to antiviral therapy and need to compare the predictive value of the PKR-eIF2 α phosphorylation homology domain versus the PKR-binding domain within NS5A.

In conclusion, we have demonstrated in patients infected with HCV-1a that the number of amino acid changes within the PKR-binding region of the NS5A protein, compared with the consensus sequence, is significantly higher for those with a sustained or end-of-treatment virologic response than for those who were nonresponders. Analysis of NS5A_{2209–2248} (ISDR) only was not sufficient to detect a significant difference in the number of mutations between the 3 groups. No specific mutation or minimum number of mutations was associated with sustained virologic response, which would have allowed individual prediction of treatment outcome. Sensitive and resistant HCV-1a isolates could not be differentiated by secondary protein structure prediction.

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