The Nonstructural 5A Protein of Hepatitis C Virus Genotype 1b Does Not Contain an Interferon Sensitivity–Determining Region

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Background. The nonstructural (NS) 5A protein of hepatitis C virus (HCV) has been suggested to contain an interferon (IFN) sensitivity–determining region (ISDR).

Methods. We studied whether the degree of viral decline on day 1 is associated with differences in NS5A amino acid sequences among patients receiving IFN-α.

Results. Phylogenetic analyses of the full-length protein and of functional domains showed no relationship between the baseline protein sequence and the antiviral response. NS5A quasispecies sequences showed no differences in the number of mutations in the putative ISDR relative to a prototype sequence between responders and nonresponders or according to IFN-α antiviral efficacy. No relationship was found between antiviral efficacy at 24 h and the baseline sequence of any NS5A region. Amino acid changes were observed in a few cases at 24 h in both responders and nonresponders, but no consistent pattern of amino acid shifts was observed, ruling out the possibility that IFN-α selected IFN-resistant variants.

Conclusion. Our findings show that there is no ISDR in the HCV genotype 1 NS5A protein and that the NS5A sequence does not influence the capacity of IFN-α to block viral replication. The findings do not rule out a role for NS5A in subsequent viral clearance.

Hepatitis C virus (HCV) infection afflicts >170 million people worldwide. Chronic hepatitis C frequently leads to cirrhosis and hepatocellular carcinoma. HCV therapy is based on recombinant interferon (IFN)–α, a cytokine with both nonspecific antiviral properties and immunomodulatory effects [1]. The clinical results of IFN-α–based therapy have improved significantly with the use of pegylated forms of IFN-α, which allow the drug to be administered once a week, and by combination with ribavirin, which prevents relapses during and after therapy in patients who respond to the antiviral effect of IFN-α [2]. The main end point of IFN-α–based therapy is the sustained virological response (SVR), defined as undetectable HCV RNA 24 weeks after the end of therapy and corresponding to viral eradication in the vast majority of cases. With the combination of pegylated IFN-α and ribavirin, 42%–52% of patients infected with HCV genotype 1 and 76%–84% of patients infected with HCV genotypes 2 and 3 have an SVR [1, 3–5].

Various explanations have been forwarded for the differences in IFN-α–based therapy outcomes. A number of host parameters, disease characteristics, and virus-related factors play a role in the likelihood of permanent viral clearance after therapy [6, 7]. That specific
HCV proteins play a role is strongly suggested by the fact that patients infected with different genotypes respond differently to therapy [3–5]. Differences in the treatment response among patients infected with the same genotype could be related to differences in the sequence and function of certain viral proteins interacting with IFN-α–induced pathways.

More than 10 years ago, Enomoto et al. [8] reported the identification of variable clusters of amino acids in the C-terminal half of the nonstructural (NS) 5A protein in responders (patients who had an SVR) and nonresponders (patients who did not have an SVR) to standard (nonpegylated) IFN-α. This region, spanning amino acid residues 2209–2248 in the Japanese HCV prototype sequence HCV-J, was designated the “IFN sensitivity–determining region” (ISDR) [8]. However, no experimental evidence was provided to show that this region influenced viral sensitivity to IFN. These authors defined 3 baseline ISDR sequence profiles—wild-type (ISDR sequence identical to that of HCV-J), intermediate (1–3 aa changes), and mutant (4–11 aa changes)—and reported a correlation between the number of ISDR amino acid changes relative to HCV-J and the likelihood of an SVR after IFN-α–based therapy [9]. However, the very existence of an ISDR within the HCV genotype 1 NS5A protein has been controversial from the outset. A large number of articles, mostly from Japan and Asia, supported a link between the number of amino acid changes relative to HCV-J and the final outcome of therapy, whereas a similar body of literature, mainly originating in Europe and the United States, reported no such correlation. A recent meta-analysis showed that the distribution of wild-type, intermediate-type, and mutant-type ISDRs differs significantly between Japanese and European patients and supported the correlation between the number of ISDR mutations and the likelihood of an SVR (even though pretreatment viremia was more predictive of outcome) [10].

SVR is the result of a number of successive responses that are related to a variety of different but complementary IFN-α–induced mechanisms that, together, may or may not ultimately lead to viral eradication (reviewed in [6, 7]). IFN-α exerts its direct nonspecific antiviral effect by activating intracellular antiviral effector pathways. The inhibition of HCV replication begins a few hours after IFN-α administration, and its magnitude is reflected by the decline in HCV RNA level at 24 h [11, 12]. A second slope of viral decline then occurs that, mathematically modeling suggests, reflects the gradual clearance and/or cure of infected cells, while viral replication continues to be potently suppressed [11, 12]. The slope of this second decline in viral load is the main predictor of viral eradication in patients who initially respond to IFN-α–based therapy. Not only can it only vary from patient to patient, but it can also be influenced by the type of IFN-α molecule and by the adjuvancy of ribavirin [11–14].

Although cells and bodily systems continue to adapt to IFN-α after the first day of administration, the first slope appears to reflect most of the direct antiviral effect of IFN-α [11, 12]. Any factor influencing the direct antiviral activity of IFN-α on HCV should impact this first slope of viral response. Therefore, if NS5A indeed contains an ISDR, the latter would be expected to mainly impact the initial viral decline, meaning that the degree of viral decline on day 1 should be associated with qualitative or quantitative differences in NS5A functions and, thus, in the amino acid sequences that subsume these functions. Here, we tested this hypothesis by studying patients receiving their first injection of standard IFN-α, the IFN molecule with the most rapid initial antiviral effect.

**PATIENTS, MATERIALS, AND METHODS**

**Patients**

We studied 16 patients (8 men and 8 women; mean ± SD age, 49.8 ± 9.9 years; age range, 32–67 years) with chronic HCV genotype 1b infection who were treated between May 1997 and May 1998. They had elevated alanine aminotransferase activity, detectable HCV RNA in serum, and signs of chronic liver disease on liver biopsy. They all received 1 injection of 3 megaunits of IFN-α2b (Intron-A; Schering-Plough) on the first day of treatment. They were then randomized to receive daily or thrice-weekly injections of 3 megaunits of IFN-α2b, with or without ribavirin. The decline in HCV RNA level (both in percentage and log10 IU per milliliter) was determined exactly 24 h after the injection.

The full-length NS5A quasispecies sequence distribution was determined for each patient at baseline and was interpreted according to the decline in HCV RNA level 24 h after their first IFN-α injection (a measure of the direct effect of IFN-α on HCV replication). The full-length NS5A quasispecies sequence distribution was also determined 24 h after the first IFN-α injection and was compared with the corresponding baseline quasispecies sequence distribution.

**HCV RNA Quantification**

HCV RNA was quantified just before the first IFN-α injection and exactly 24 h later by means of the SuperQuant assay (National Genetics Institute) [15]. The detection limit of this assay is 30 HCV RNA IU/mL (1.5 log10 IU/mL).

**Genetic Characterization of Full-Length NS5A Quasispecies**

**HCV RNA extraction.** HCV RNA was extracted from 200 μL of serum by use of the High Pure viral RNA kit (Roche Applied Science). RNA was reverse transcribed for 40 min at 55°C by use of the ThermoScript reverse-transcriptase polymerase chain reaction (PCR) kit (Invitrogen) with 20 pmol of the external antisense primer R1EAS (5′-GTGGTGACGCAGCAGAGAGT-3′; nucleotide positions 7715–7734).
Table 1. Summary of the principal amino acid changes observed in nonstructural (NS) 5A quasispecies in nonresponders and responders 24 h after the first interferon (IFN–α) injection.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Decline in HCV RNA level, log_{10} IU/mL (%)</th>
<th>Position in polyprotein</th>
<th>Residue</th>
<th>Frequency, %</th>
<th>Position in polyprotein</th>
<th>Residue</th>
<th>Frequency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1</td>
<td>&lt;0.50</td>
<td>nsc</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>NR2</td>
<td>&lt;0.50</td>
<td>nsc</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>NR3</td>
<td>&lt;0.50</td>
<td>2375 D</td>
<td>96</td>
<td>46</td>
<td>N</td>
<td>4</td>
<td>54</td>
</tr>
<tr>
<td>NR4</td>
<td>&lt;0.50</td>
<td>2268 I</td>
<td>92</td>
<td>55</td>
<td>V</td>
<td>8</td>
<td>45</td>
</tr>
<tr>
<td>NR5</td>
<td>&lt;0.50</td>
<td>2187 K</td>
<td>100</td>
<td>52</td>
<td>R</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>A</td>
<td>0.55 (72.0)</td>
<td>2264 G</td>
<td>76</td>
<td>100</td>
<td>E</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0.68 (79.0)</td>
<td>2185 A</td>
<td>100</td>
<td>44</td>
<td>T</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>C</td>
<td>0.80 (84.0)</td>
<td>2356 E</td>
<td>100</td>
<td>44</td>
<td>G</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>D</td>
<td>0.82 (85.0)</td>
<td>2050 K</td>
<td>100</td>
<td>32</td>
<td>R</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>E</td>
<td>0.85 (88.0)</td>
<td>2375 G</td>
<td>66</td>
<td>0</td>
<td>D</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td>F</td>
<td>1.19 (93.5)</td>
<td>nsc</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>G</td>
<td>1.52 (97.0)</td>
<td>nsc</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>H</td>
<td>2.00 (99.0)</td>
<td>nsc</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>I</td>
<td>2.15 (99.3)</td>
<td>2062 V</td>
<td>100</td>
<td>16</td>
<td>I</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>J</td>
<td>2.30 (99.5)</td>
<td>2185 T</td>
<td>100</td>
<td>0</td>
<td>A</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>K</td>
<td>2.70 (99.8)</td>
<td>2050 K</td>
<td>100</td>
<td>60</td>
<td>R</td>
<td>0</td>
<td>49</td>
</tr>
</tbody>
</table>

**NOTE.** Only changes affecting residues observed in >30% of the variants of each patient’s quasispecies are reported. Frequencies indicate the proportion of variants within the quasispecies harboring the specific amino acid at 0 h (before the first IFN-α injection) and at 24 h after the injection. The 5 nonresponder patients (those without a response 24 h after the first IFN-α injection) are designated NR1–NR5, and the responders (A–K) are classified from the lowest to the highest decline in log_{10} hepatitis C virus (HCV) RNA level (0.55–2.70 log_{10} IU/mL). nsc, no specific amino acid change.

**NSSA PCR amplification.** The first nested PCR round was performed with 10 pmol of the external sense primer R1ES (5′-CAGCTCACCATCCTCAGC-3′; nucleotide positions 6189–6208), 10 pmol of the external antisense primer R1AS, and Pwo high-fidelity DNA polymerase (Roche Molecular Biochemicals). Initial denaturation for 5 min at 94°C was followed by 30 cycles (94°C for 30 s, 60°C for 1 min, and 72°C for 2 min) and by a final extension step at 72°C for 5 min. The second nested PCR round was performed with 10 pmol of the internal sense primer R2IS (5′-ATCGGATCCTTCTAGAGATTGAGCAGCAGAAGGA-3′; nucleotide positions 7622–7653), and Pwo high-fidelity DNA polymerase. Denaturation for 5 min at 94°C was followed by 30 cycles (94°C for 30 s, 65°C for 1 min, and 72°C for 90 s) and by a final extension step at 72°C for 5 min. Amplified products were analyzed by electrophoresis and staining with ethidium bromide.

**Cloning and sequencing of PCR products.** PCR products were purified using Microcon 100 centrifugal filter devices (Millipore). Purified products (50 ng) were ligated into the pCR4-TOPO vector (TOPO TA cloning kit for sequencing; Invitro-
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Figure 1. Phylogenetic tree plotted on the basis of the full-length nonstructural (NS) 5A protein sequences of quasispecies variants isolated from 16 patients before administration of interferon (IFN)-α. Each distinct cluster of sequences on the tree corresponds to quasispecies variants from a single patient. The 11 patients who responded at 24 h (decline in hepatitis C virus [HCV] RNA level of >0.50 log10 IU/mL) are designated by a letter (Pt A–K, as in table 1), and the corresponding reduction is given in parenthesis (in log10 IU/mL) for each. The 5 nonresponder patients (those without a response 24 h after the first IFN-α injection) are designated NR1–NR5.

Transformation of recombinant plasmid DNA into *Escherichia coli*-competent cells was performed, and transformants were grown on ampicillin plates. Cloned DNA was reamplified with the M13 plasmid universal primers M13R (5′-CAGGAAA-CAGCTATGAC-3′) and M13F (5′-GTAAAACGACGGCCAG-3′) and *Pwo* DNA polymerase. After purification with Microcon 100 centrifugal filter devices, a 5-min denaturation step at 94°C was followed by 30 cycles (94°C for 30 s, 55°C for 1 min, and 72°C for 90 s) and by a final extension step at 72°C for 5 min. Thirty clones per sample per patient were sequenced with the Long-Read Tower sequencer and the Cy5/Cy5.5 dye primer cycle sequencing kit (Visible Genetics). The sequencing primers were the Cy5-labeled sense and antisense primers M13R and M13F.

Genetic and Phylogenetic Analysis of NS5A Quasispecies Sequences

In total, 960 full-length NS5A quasispecies variant sequences were generated in this study. Nucleotide sequences were aligned by use of the Clustal X program (version 1.8) [16]. The PHYLIP program (version 3.6a3α) was used to construct phylogenetic trees by means of the neighbor-joining method with a sequence matrix determined by the Jones-Taylor-Thornton method. Trees were constructed for each patient with the PHYLIP DRAWTREE module. Sequence data from this article have been deposited in GenBank under accession numbers AM399655–AM400224.

Protein Sequence Analysis

All analyses were performed using the European HCV database Web site facilities (euHCVdb; available at: http://euhcvdb.ibcp.fr/) [17]. The NS5A sequence of the HCV-J strain, a prototype strain of HCV genotype 1b, was used as the reference (EMBL accession number D90208) [18]. Multiple sequence alignments were done using the Clustal X program [16]. Visualization of sequence alignments and plotting of the most frequent amino acid residues at each position were performed using the NPS@ Web site facilities (available at: http://npsa-phil.ibcp.fr/) [19].

RESULTS

Antiviral response to IFN-α. Five of the 16 patients did not respond to the first IFN-α injection (their plasma HCV RNA
levels did not decline by $>0.50 \log_{10}$ IU/mL 24 h after the injection. The small number of true nonresponders in this study reflects the fact that they are rare in the population of HCV-infected patients. The percentage decline in HCV RNA level ranged from 72.0% to 99.8% in the other 11 patients, corresponding to a decrease of 0.55 to 2.70 $\log_{10}$ IU/mL (table 1).

**Phylogenetic analysis of the relationship between the baseline NS5A sequence and the antiviral response to IFN-α.** Figure 1 shows the phylogenetic tree plotted with full-length NS5A quasispecies sequences from the 16 patients before therapy. As expected, each patient’s quasispecies variants clustered closely in the tree, separately from those of the other patients. No distinctive clustering was seen according to the response to IFN-α at 24 h. Likewise, among the 11 responders, no distinctive clustering was seen according to the magnitude of the response at 24 h. Thus, phylogenetic analysis showed no relation between the pretreatment full-length NS5A sequences and the virological response 24 h after a single injection of IFN-α.

Because phylogenetic analysis of the full-length NS5A sequence might have missed a relationship between antiviral efficacy and the sequences of particular NS5A functional domains, we separately analyzed known and putative functional domains of the NS5A protein (figure 2, adapted from Polyak [20], and table 2, adapted from Macdonald and Harris [21]), including the putative, double-stranded, RNA-activated, IFN-induced protein kinase (PKR)–binding domain with the putative ISDR; the putative apolipoprotein A1–binding domain; the putative core-binding region; the NS5A transcriptional activation domain; the growth factor receptor–binding protein 2–interacting domain; the putative NS4 protein–binding region; the phosphorylation and hyperphosphorylation sites; variable regions V3 and V4; and the N-terminal NS5A anchor region (figure 2 and table 2). A phylogenetic tree was plotted for each functional region (data not shown). No distinctive clustering of functional NS5A sequences was observed according to the antiviral response 24 h after a single injection of IFN-α.

**Relationship between baseline NS5A amino acid sequences and antiviral responses to IFN-α.** Phylogenetic analysis may fail to show the influence of small motifs or individual amino acid residues that have important functional roles. We therefore analyzed NS5A quasispecies sequences amino acid by amino acid and compared the physicochemical properties of the proteins both between responders and nonresponders and according to the magnitude of the antiviral response, using the protein analysis tools available in the European HCV database euHCVdb [17, 19]. Examination of baseline quasispecies sequences showed occasional single-amino-acid differences in a given patient. Most of the differences relative to the most frequent amino acid sequence in a given patient involved 1 or a

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**Figure 2.** Principal known and putative functional regions in the nonstructural (NS) 5A protein, including the NS4A-binding region, hyperphosphorylation sites (PO$_4$), the N-terminal amphipathic helix that serves as a membrane anchor region (anchor), the interferon (IFN)–induced protein kinase (PKR)–binding domain spanning the putative IFN sensitivity–determining region (ISDR), the nuclear localization signal (NLS), the apolipoprotein A1 (apoA1)–binding region, the growth factor receptor–binding protein 2 (Grb2)–binding region, the V3 and V4 variable regions, the core-binding domain (core), and the transcriptional activation domain. Adapted from Polyak [20].
few minor quasispecies variants, regardless of the virological response to IFN-α at 24 h (data not shown).

We then examined domains previously suggested to influence the antiviral response to IFN-α-based therapy, including the putative PKR-binding domain and its putative ISDR as well as the variable region V3. Figure 3 shows the baseline sequences of the putative PKR-binding domain in the most frequent quasispecies variants from nonresponders and responders, on the basis of the log$_{10}$ HCV RNA decline at 24 h (the putative ISDR is highlighted). The sequences showed no obvious differences in the number of mutations relative to the prototype HCV-J sequence between responders and nonresponders or according to IFN-α antiviral efficacy. The most variable position, 2218, bore histidine, arginine, or glutamine residues in the most frequent sequences from both nonresponders and responders (figure 3) as well as in the corresponding quasispecies variant sequences before IFN-α administration. Three positions within the C-terminal part of the PKR-binding domain (2259, 2262, and 2268) harbored amino acids different from those of the HCV-J sequence, but they were almost fully conserved in both nonresponders and responders. Other patient-specific single-amino-acid variations were conservative or nonconservative in physicochemical terms but did not discriminate between nonresponders and responders. Overall, no relationship was found between the amino acid sequence of the putative PKR-binding domain (or that of the putative ISDR) and the antiviral effect of IFN-α on day 1 of therapy (figure 3). Similarly, no relationship was found between antiviral efficacy at 24 h and the sequence of the V3 region (figure 4). Finally, no relationship was found between antiviral efficacy at 24 h and the baseline sequence of any other NS5A region (figure 4).

### NS5A quasispecies sequence changes 24 h after a single IFN-α injection.

Amino acid changes were found at specific positions in 3 nonresponders (patients NR3, NR4, and NR5) and in 6 responders (patients A, B, D, I, J, and K) (summarized in table 1 and shown in figure 4). These subjects had 1–3 aa changes in the NS5A sequence, except for patient I, who had 10 aa changes. Some of these changes were true shifts (all quasispecies variants bore the same substitution at 24 h), but, in most cases, the residues were already present in some quasispecies variants at baseline. Changes were observed at various amino acid positions, especially in the V3 region. No changes were observed in the putative ISDR. Importantly, no consistent pattern of amino acid shifts was observed; in other words, when changes did occur, they involved different amino acids at different positions in the different patients, except for 3 positions that were each changed in 2 patients (position 2050 in patients D and K, position 2185 in patients B and J, and position 2375 in patients D and NR3). However, these latter changes either were conservative or were not of the same type in the two

### Table 2. Cellular proteins reported to interact with nonstructural (NS) 5A protein and corresponding putative interaction regions in the NS5A sequence.

<table>
<thead>
<tr>
<th>Cellular protein</th>
<th>Position of the NS5A interaction region, aa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-stranded, RNA-activated, interferon-induced protein kinase</td>
<td>2209–2274</td>
</tr>
<tr>
<td>Growth factor receptor–binding protein 2</td>
<td>2323–2329</td>
</tr>
<tr>
<td>Phosphoinositide-3-kinase p85 subunit</td>
<td>2243–2272</td>
</tr>
<tr>
<td>Amphiphysin 2</td>
<td>2322–2328</td>
</tr>
<tr>
<td>Src-family kinases Fyn, Lck, and Hck</td>
<td>2322–2328</td>
</tr>
<tr>
<td>Src-family kinase Lyn</td>
<td>2315–2328</td>
</tr>
<tr>
<td>Transcription factor SRCAP</td>
<td>NI</td>
</tr>
<tr>
<td>Karyopherin β3</td>
<td>1973–2172</td>
</tr>
<tr>
<td>Apolipoprotein A1</td>
<td>1973–2197</td>
</tr>
<tr>
<td>hVAP-33 (SNARE-like protein)</td>
<td>NI</td>
</tr>
<tr>
<td>p53</td>
<td>NI</td>
</tr>
<tr>
<td>Bax</td>
<td>2232–2247</td>
</tr>
<tr>
<td>TNF-R1–associated death domain protein</td>
<td>NI</td>
</tr>
<tr>
<td>Cyclin-dependent kinase 1</td>
<td>NI</td>
</tr>
<tr>
<td>Homeodomain protein PTX1</td>
<td>2103–2190</td>
</tr>
<tr>
<td>TNF-R–associated factor 2</td>
<td>2118–2271</td>
</tr>
<tr>
<td>hTAFII32</td>
<td>2147–2151</td>
</tr>
<tr>
<td>TATA box–binding protein</td>
<td>2180–2251</td>
</tr>
<tr>
<td>La autoantigen</td>
<td>2337–2419</td>
</tr>
</tbody>
</table>

**NOTE.** Adapted from Macdonald and Harris [21]. hTAF, human TATA box–binding protein–associated factor; hVAP, human vesicle–associated membrane protein–associated protein; NI, not identified; PTX, pituitary homeobox protein; SNARE, combinatorial soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SRCAP, SNF2-related CBP activator protein; TFN-R, tumor necrosis factor receptor.
Figure 3. Alignment of the amino acid sequences of the putative interferon (IFN) sensitivity–determining region (ISDR) and the IFN–induced protein kinase (PKR)-binding domain of the nonstructural (NS) 5A protein, before (A) and 24 h after (B) the first IFN-α injection. The 5 nonresponder patients (those without a response 24 h after the first IFN-α injection) are designated NR1–NR5, and the responders are classified from the lowest to the highest decline in log 10 hepatitis C virus (HCV) RNA level (0.55–2.70 log 10 IU/mL) (table 1). The sequences are numbered relative to the HCV-J prototype polyprotein. Amino acid positions at which residues different from those of the HCV-J sequence were observed in the majority of each patient’s sequences are underscored in the HCV-J sequence. Dashes indicate amino acids identical to those observed at the same positions of the HCV-J sequence.

A, Most frequent sequence in the viral quasispecies from each patient before the IFN-α injection (h0). In the homology line, identical, strongly conservative, and weakly conservative amino acids, with respect to the protein conformation, are indicated by stars, colons, and dots, respectively, in accordance with the Clustal X convention [16].

B, Most frequent quasispecies sequence 24 h after the first IFN-α injection (h24) in the 2 patients with amino acid changes relative to the h0 sequence. The residues that changed at 24 h are boxed both at baseline and at 24 h; gray boxes indicate amino acid changes toward residues observed in all nonresponders’ sequences.

patients. These results, together with the presence of amino acid changes at 24 h in both responders and nonresponders, ruled out the possibility that IFN-α administration selected IFN-resistant variants with specific NS5A sequences. Phylogenetic analysis of the viral quasispecies at baseline and 24 h after a single IFN-α injection confirmed the lack of a genetic shift (data not shown).

DISCUSSION

The present study shows that the antiviral efficacy that a single injection of IFN-α has on HCV genotype 1 replication is not influenced by the full-length NS5A amino acid sequence, analyzed either globally or at each individual position. In addition, the number of mutations in the putative ISDR did not differ
between patients who responded to IFN-α and those who did not or according to the magnitude of the antiviral response at 24 h. Finally, in contrast to the findings of a previous report [22], we found no relationship between the number of mutations in the V3 region and the antiviral efficacy of IFN-α on day 1. Together, these findings suggest that the NS5A protein does not contain an ISDR. These results are in keeping with data from experiments with transfected Huh7 cells harboring chimeric HCV subgenomic replicons into which NS5A sequences from IFN responders and nonresponders had been inserted; these experiments showed no significant difference in the sensitivity of HCV replication to IFN-α [23].

Our results are not particularly surprising, because Enomoto et al.’s data were essentially misinterpreted. Indeed, the existence of the ISDR was postulated on the basis of a relationship between a global number of amino acid changes relative to the Japanese prototype sequence HCV-J, not a signature sequence or motif, and permanent HCV eradication after IFN-α-based therapy rather than the antiviral efficacy of IFN [8, 9]. Thus, our findings are not incompatible with the conclusions of a recent meta-analysis confirming the relationship between the number of amino acid changes in the putative ISDR relative to HCV-J and the likelihood of an SVR in patients with HCV genotype 1 infection [10]. Similar results have been reported in regions other than the ISDR, such as the V3 and V4 regions, and could simply witness the global level of genetic variability of the HCV strain in regions that are tolerant of amino acid changes, a parameter that could influence the response to therapy [24, 25]. It may yet be found that NS5A plays a role in the final outcome of IFN-α-based therapy, but not by primarily influencing the antiviral efficacy of IFN. Alternatively, the observed sequence changes may be a surrogate marker of unidentified virus or host properties that influence the chances of viral eradication after IFN-α-based therapy.

Various interactions between NS5A and cellular proteins or pathways have been described in vitro (summarized in table 2), and some have been suggested to mediate intrinsic HCV resistance to IFN-α [26–32]. However, NS5A sequences are highly conserved among the different HCV genotype 1 strains, and the functional properties of NS5A have not been shown to differ, either qualitatively or quantitatively, according to IFN-α antiviral efficacy in vivo. Thus, any NS5A antagonism of the antiviral properties of IFN-α would probably play a modest role in the overwhelming presence of exogenous IFN-α. In contrast, together with other viral proteins potentially involved in “IFN resistance” [33], NS5A may play an important role in antagonizing natural type 1 IFN responses during the initial stages of HCV infection, when viral replication triggers the host’s innate response, thereby allowing the virus to become established.

Therefore, the respective roles played by viral and host factors in the response of HCV genotype 1 to IFN-α–based therapy—and the underlying mechanisms—remain to be determined. HCV genotype 1 is intrinsically more resistant to the antiviral action of IFN-α than are genotypes 2 and 3 [12, 34]. HCV proteins other than NS5A have been reported to antagonize the antiviral activity of IFN-α in vitro [35–41], but there is no firm evidence that these proteins (or their functions) are involved in the differing susceptibilities to IFN-α between genotype 1 and other genotypes. Pharmacodynamics and individual host factors appear to play an important role in the response to IFN-α–based therapy. Expression profiling in liver biopsy specimens obtained before treatment has identified IFN-inducible genes whose expression differs significantly between responders and nonresponders, regardless of HCV load, disease activity, and fibrosis [42]. Additional factors, such as ribavirin administration, may subsequently influence the likelihood that patients who respond to IFN-α will have an SVR [43]. The second slope of viral decline in patients who initially respond to IFN-α is significantly shallower in genotype 1 infection than in genotype 2 or 3 infection [12, 34], suggesting that genotype–1–infected cells have a longer half-life. This slope influences the ultimate outcome of therapy, and the role played by viral proteins such as NS5A in this secondary decline deserves to be explored, especially given Enomoto et al.’s initial report [8, 9], confirmed by the recent meta-analysis [10], that NS5A sequence variability influences the likelihood of an SVR after IFN-α–based therapy.

Some amino acid changes did occur during the 24 h after the first IFN-α injection in the present study, but no common pattern was found, showing that IFN-α does not rapidly select HCV variants via specific amino acid substitutions in NS5A that confer IFN-α resistance. This was not surprising, given that IFN-α does not inhibit HCV replication by directly interacting with a viral component but rather by creating a virus-hostile environment. However, all the quasispecies variants from certain patients harbored the same amino acid change, suggesting that an early selection process had occurred. This is in keeping with an unpublished observation of very early quasispecies changes in both NS5A and E2 envelope glycoprotein hypervariable region 1 in patients receiving IFN-α–based ther-
apy (authors' unpublished data). These changes could create a fitter virus in the IFN-modified host environment, irrespective of resistance to the antiviral effects of IFN-α.

In conclusion, we show that there is no ISDR in the NS5A protein of HCV genotype 1 and that there is no relationship between the NS5A sequence and the capacity of IFN-α to block HCV replication after subcutaneous administration. These results do not rule out a role for NS5A—or a role for combinations of changes in different domains of the protein—in subsequent viral clearance, through effects that are unrelated to IFN resistance.

References

38. Foy E, Li K, Sumpter R Jr, et al. Control of antiviral defenses through