Pegylated Interferon and Ribavirin Promote Early Evolution of Nonstructural 5A Protein in Individuals with Hepatitis C Who Demonstrate a Response to Treatment

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Background. Hepatitis C virus (HCV) quasispecies diversity is more likely to affect early viral decline during treatment of hepatitis C than is having human immunodeficiency virus (HIV) infection. We evaluated the influence of HCV therapy on changes in the nonstructural 5A (NS5A) protein.

Methods. Fifteen patients with HCV genotype 1 infection with or without HIV infection were recruited for the present study, and the decrease in the HCV RNA level was measured at early time points. The evolution of HCV NS5A quasispecies within the first week was analyzed by comparing the clones observed at later times in the study with the baseline consensus sequence of individual patients. The response to therapy was defined as an early response (ER; ie, an HCV RNA level <615 IU/mL at week 4) or a slow response (SR; ie, a detectable HCV RNA level at week 4).

Results. HIV infection did not affect early viral kinetics. At baseline, lower diversity was seen in NS5A and in the amino and carboxyl termini of patients with an ER, compared with those with an SR. Rapid evolution of the NS5A genetic region occurred in patients with an ER (P = .01) but not in those with an SR (P = .73). The evolution was the result of an increase in the number of amino acid substitutions in the carboxyl region (P = .02) in patients with an ER.

Conclusions. Selective pressure appears to result in more-marked changes in individuals with an ER than in those with an SR. The carboxyl terminus was subject to the most change and may be an important determinant of phenotypic resistance to interferon-based therapy.

The combination of ribavirin and pegylated interferon is used to treat hepatitis C, but it achieves viral clearance in 19%–52% of patients infected with hepatitis C virus (HCV) genotype 1 and in 76%–80% of patients infected with HCV genotypes 2 and 3 [1–3]. Such host factors as race and infection with human immunodeficiency virus (HIV) have been associated with a poor response to treatment [3–5]. The optimal clinical outcome—a sustained virologic response (SVR)—is closely associated with an early response (ER) at week 4 of treatment [6]. Thus, early changes in the HCV level can give insights as to why some groups have a poor response to anti-HCV treatment. Viral kinetics studies [7–11] have shown a lower effectiveness of interferon in black Americans, compared with white Americans [8], and this lower effectiveness is a possible explanation for the lower SVR encountered among blacks [3]. Similarly, treatment trials involving HIV/HCV-infected patients...

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have also demonstrated a lower SVR [4, 5]. However, whether the presence of HIV alters the early viral kinetics of HCV during interferon therapy remains unclear.

Viral genotype is another important determinant of responsiveness: HCV genotype 1 is less responsive than HCV genotypes 2 and 3, which demonstrate first- and second-phase decreases faster than those observed for genotype 1 [9, 11]. The different viral clones observed within an individual have different pharmacodynamic responses to exogenous interferon. Nonstructural 5A (NS5A) is a viral protein that has been most associated with interferon responsiveness, especially the interferon sensitivity–determining region. NS5A binds and inhibits the protein kinase R–binding domain (PKRbd), attenuating the kinase action in interferon-mediated antiviral responses in a manner that varies among viral quasispecies [9, 11–14]. It has been suggested that variation from an interferon-resistant prototype viral sequence for genotype 1b (ie, HCV-J) is associated with greater likelihood of a response to therapy; however, this suggestion remains controversial [15–22]. We undertook a thorough reexamination of NS5A to determine how variations in this region might contribute to an ER to anti-HCV therapy. We hypothesized that viral factors, such as the composition of NS5A quasispecies and the dynamic changes in NS5A quasispecies during the first week of therapy, may be associated with the virologic response at week 4 (ie, an ER) and an eventual SVR.

**METHODS**

**Patient population.** The study included patients who were anti-HCV reactive; 18–65 years of age; infected with HCV genotype 1; of the white race, according to self-identification; and ribavirin and pegylated interferon naïve; and who had a serum HCV RNA level of >1000 IU/mL, as identified by either polymerase chain reaction (PCR) or branched DNA assays. Patients infected with HIV had CD4+ T cell counts of ≥300 cells/mm³ within 12 weeks of study initiation and had no evidence of symptomatic AIDS-defining illness. Subjects could be antiretroviral therapy (ART) naïve, but if they were receiving ART, they needed to have been receiving a stable regimen for 12 weeks before enrollment in the study. Patients receiving ART could not be receiving didanosine, which can cause an adverse reaction when combined with ribavirin [23]. Exclusion criteria included the presence of other liver diseases, use of >50 g of alcohol per day, injection drug use in the 6 months before enrollment, or evidence of hepatic decompensation. The study protocol was approved by the institutional review board at the University of Texas Southwestern Medical Center.

All patients received 180 μg of pegylated interferon-α2a weekly and ribavirin, either 1000 mg (for those with a body weight of ≤75 kg) or 1200 mg (for those with a body weight of >75 kg) given in 2 divided doses daily for a total of 48 weeks. The patients were allowed to use growth factors for anemia and neutropenia.

**Sample collection and laboratory evaluation.** Blood samples were collected at 0, 4, 8, 12, 18, 24, and 32 h and on days 2–4, 7, 9, 11, 14, 21, 28, 42, and 56 after administration of the first dose. Patients were seen at 4- to 6-week intervals until week 48 and at 3 and 6 months after completion of therapy. CD4+ T cell count determination, HIV RNA level quantification, and thyroid-stimulating hormone testing were performed at the time of screening and at 12, 24, 36, 48, 60, and 72 weeks. HCV level quantification for stored serum samples was performed using Versant branched DNA technology (version 3.0; Siemens Medical Solutions Diagnostics).

**Quasispecies analysis.** HCV RNA was extracted from 140 μL of serum by use of the QIAamp Viral RNA Mini Kit (Qiagen). The NS5A region was amplified. The amplification products were visualized on 1% agarose gel. The positive bands were purified using the QIAquick Gel Extraction Kit (Qiagen). The PCR products were ligated into the TA-cloning plasmid pGEM-T Easy (Invitrogen) and cloned independently. A total of 10–12 clones were selected from each sample and were sequenced in the sequence core facility at the University of Texas Southwestern Medical Center.

For each sample, the mean within-sample genetic distance (SGD) between clones, synonymous substitution (dS) rate per synonymous substitution site, and nonsynonymous substitution (dN) rate per nonsynonymous substitution site at baseline and at 24 h, 48 h, and 7 days after treatment initiation were calculated using MEGA software (version 4) [24–26]. Baseline consensus sequences were determined based on 10–12 clones of each subject, and each of the 10–12 clones from later time points was compared to the consensus. The numbers of nucleotide and amino acid substitutions were counted, and then the average numbers of nucleotide and amino acid substitutions were calculated. See the Appendix for further details on quasispecies analysis.

**Statistical analysis: viral kinetics and sequence diversity analysis.** For continuous variables at baseline, 2 group comparisons were made using the Wilcoxon rank sum test. Categorical variables were compared using Fisher’s exact test. Nonlinear regression models were used to estimate parameters on the basis of the Neumann model [10]. Only the 14 subjects who were infected with HCV genotype 1a were included in the analyses that incorporated genetic diversity variables. Spearman correlation coefficients were used to evaluate the association between viral kinetic parameters and sequence diversity variables quantified by SGD, dS, or dN substitutions. To compare longitudinal changes in genetic diversity over time, a repeated-measures analysis-of-variance model using mixed-model methodology was constructed. These linear models had a repeated factor for the measurements obtained at baseline and on days...
**Table 1. Viral Kinetic Parameters and 95% Confidence Intervals for Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>HIV status</th>
<th>( V_{0} ) log IU/mL</th>
<th>( T_{0} ) days</th>
<th>( c )</th>
<th>( e )</th>
<th>( \delta )</th>
<th>( \lambda_1 )</th>
<th>( \lambda_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>6.76 (6.61-6.87)</td>
<td>0.63 (0.48-0.79)</td>
<td>7.62  (2.08-13.15)</td>
<td>0.95  (0.92-0.98)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>6.13 (6.09-6.17)</td>
<td>0.53 (0.07-0.99)</td>
<td>1.42  (1.11-3.95)</td>
<td>0.57  (0.25-0.88)</td>
<td>0.05   (0.02-0.07)</td>
<td>1.44</td>
<td>.03</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>4.25 (3.14-4.53)</td>
<td>0.50 (0.28-0.38)</td>
<td>4.25  (1.46-7.04)</td>
<td>0.97  (0.93-1.01)</td>
<td>0</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>6.14 (5.97-6.25)</td>
<td>0.33 (0.32-0.35)</td>
<td>UE</td>
<td>0.37   (0.09-0.65)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>6.21 (5.91-6.38)</td>
<td>0.33 (0.11-0.56)</td>
<td>3.84  (0.29-7.38)</td>
<td>0.90  (0.77-1.04)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>5.74 (5.68-5.81)</td>
<td>0.64 (0.47-0.82)</td>
<td>3.37  (1.07-5.67)</td>
<td>0.75  (0.66-0.84)</td>
<td>0.40   (0.37-0.42)</td>
<td>3.48</td>
<td>.29</td>
</tr>
<tr>
<td>16</td>
<td>+</td>
<td>5.76 (5.58-5.89)</td>
<td>0.48 (0.38-0.58)</td>
<td>7.58  (2.75-12.85)</td>
<td>0.92  (0.87-0.96)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>6.83 (6.73-6.91)</td>
<td>0.58 (0.30-0.86)</td>
<td>2.08  (0.66-3.49)</td>
<td>0.96  (0.90-1.09)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>26</td>
<td>+</td>
<td>5.20 (5.09-5.30)</td>
<td>0.67 (0.63-0.71)</td>
<td>14.96 (11.97-17.94)</td>
<td>0.996 (0.996-0.998)</td>
<td>0</td>
<td>14.95</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>6.18 (6.06-6.27)</td>
<td>0.21 (0.2 to 0.61)</td>
<td>1.67  (0.34-3.69)</td>
<td>0.70  (0.48-0.91)</td>
<td>0.03   (0.01 to 0.07)</td>
<td>1.68</td>
<td>.02</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>5.56 (5.44-5.66)</td>
<td>0.32 (0.26-0.37)</td>
<td>UE</td>
<td>0.28   (0.05-0.52)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>5.84 (5.51-6.03)</td>
<td>0.74 (0.11-1.37)</td>
<td>1.94  (0.81 to 4.68)</td>
<td>0.91  (0.74-1.07)</td>
<td>0.48   (0.37-0.60)</td>
<td>2.00</td>
<td>.42</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>4.87 (4.62-5.02)</td>
<td>0.70 (0.48-0.91)</td>
<td>5.76  (0.08 to 11.61)</td>
<td>0.92  (0.85-0.99)</td>
<td>0.22   (0.17-0.27)</td>
<td>5.78</td>
<td>.2</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>5.75 (5.54-5.89)</td>
<td>0.11 (0.01-0.21)</td>
<td>12.0  (0.10 to 25.15)</td>
<td>0.79  (0.69-0.88)</td>
<td>0.01   (0.05 to 0.06)</td>
<td>12.0</td>
<td>.005</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>6.51 (6.35-6.62)</td>
<td>0.63 (0.35-0.91)</td>
<td>4.22  (0.14 to 8.58)</td>
<td>0.85  (0.74-0.95)</td>
<td>UE</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

**NOTE:** \( e \), percent (%) efficacy of treatment in blocking viral production; \( \delta \), infected cell death rate per day; \( \lambda_1 \), the slope of the first-phase decrease per day; \( \lambda_2 \), the slope of the second-phase decrease per day; \( C \), clearance rate of free virus per day; HIV, human immunodeficiency virus; \( T_0 \), delay (expressed in days) between the start of treatment and the onset of action; UE, unable to estimate; \( V_0 \), virus level at baseline.

1, 2, and 7 after treatment initiation and a between-group factor for comparison of the groups (patients with and without HIV infection) or comparison of patients who had an early response (ER; ie, <615 IU/mL at week 4) with those who had a slow response (SR; ie, a detectable HCV RNA level at week 4). In the linear model, a significant (\( P < .05 \)) interaction between group and time indicated a differential response over time between patients with an ER and those with an SR. Statistical analysis was performed using SAS software (version 9.1.3; SAS Institute).

**RESULTS**

**Response to Treatment: Monoinfected versus Coinfected Patients**

ER is an early indicator of eventual response [6] and was used as an end point in this study. ER was achieved in 3 HIV/HCV-infected patients and in 2 patients infected with HCV alone. An early virologic response (EVR), which was defined as a >2-log decrease in the HCV RNA level at week 12, was achieved in 7 HIV/HCV-infected patients and in 5 patients infected with HCV alone. SVR, which is considered to denote clinical cure and is defined as the absence of detectable virus (<50 IU/mL) 6 months after completion of therapy, was achieved in 3 HIV/HCV-infected patients and in 3 patients with HCV monoinfection. Two HIV/HCV-infected patients and 1 HCV-infected patient did not achieve an EVR and were considered to have no response. One patient who was infected with HCV alone had an EVR but was withdrawn from the study because of drug-related thrombocytopenia and was considered to have treatment failure. Four HIV/HCV-infected patients and 1 HCV-infected patient had an EVR, but they had detectable HCV RNA later in the study and were considered to have experienced a relapse. Seven patients (5 HIV/HCV-infected patients and 2 HCV-infected patients) had viral rebound, which was defined as an increase in the log HCV RNA level at day 7, compared with the lowest value noted during days 1–4.

**Early Viral Kinetics**

An initial steep decline in the HCV RNA level occurs within 24–48 h after treatment (ie, phase 1) and is estimated by use of the parameter \( e \) (ie, efficacy of treatment in blocking viral production); it is followed by a slower decline (ie, phase 2), which is estimated by the parameter \( \delta \) (infected cell death rate per day), as is shown for individual patients in table 1. Phase 1 reflects the inhibition of viral production or release of virus from infected cells and is a function of interferon effectiveness (as estimated by log \( -[1 - e] \)), whereas phase 2 is attributed to the rate of loss of infected hepatocytes [10]. Similar \( e \) rates were noted for HIV–HCV–coinfected and HCV-monoinfected patients (data not shown). However, \( \delta \) could not be estimated for several patients because of viral rebound. Thus, the Neumann model may not accurately estimate all viral kinetic parameters [27]. We therefore also used the decrease in the absolute log HCV RNA level from baseline to day 7, to examine the antiviral effect of the first dose of pegylated interferon, as well as that from day 7 to day 14 and that from day 7 to day 28, to examine the effect of subsequent doses (data not shown).

No difference in early response rates and viral kinetics was noted between HIV/HCV-infected and HCV-infected patients. When we compared the genetic diversity noted at baseline with...
that noted over time, there were no significant differences between the 2 groups. Therefore, in subsequent analysis, patients were grouped together regardless of their HIV infection status.

Viral kinetic parameters were compared between patients with an ER (n = 5) and those with an SR (n = 10). Compared with patients with an SR, those with an ER had a lower median log HCV RNA level at baseline (4.91 vs 6.13 IU/mL; \( P = .005 \)) but a similar median value (0.92 vs 0.82; \( P = .14 \)), delay (expressed in days) between the start of treatment and the onset of action (\( T_0 \)) (0.67 days vs 0.41 days; \( P = .08 \)), and clearance rate of free virus (4.25 per day vs 4.03 per day; \( P = .72 \)). The \( \delta \) value could be estimated for 3 (60%) of 5 patients with an ER, compared with 3 (30%) of 10 patients with an SR. The median log decrease in the HCV RNA level occurred more rapidly in patients with an ER than in patients with an SR, at day 7 (1.65 vs 0.46 IU/mL; \( P < .001 \)), day 14 (0.67 vs 0.20 IU/mL; \( P < .001 \)), and day 28 (0.67 vs 0.24 IU/mL; \( P = .01 \)). Viral rebound was associated with failure to achieve an ER (\( P = .03 \)). Patients with an ER were also more likely to achieve an SVR (prevalence, 80% vs 10%; \( P = .002 \)).

**Association of HCV Quasispecies Diversity with Viral Kinetic Parameters**

Determination of the sequence of full-length NSSA (FL-NSSA) from clones was performed at baseline (n = 162), at 24 h (n = 156), at 48 h (n = 167), and at 7 days (n = 159). No significant correlation was apparent between SGD and the HCV RNA level at baseline (r = 0.47). A higher \( \epsilon \) value correlated with a lower baseline SGD for FL-NSSA (figure 1) and with the number of dS (\( \rho = -0.65; P = .01 \)) but not with the number of dN (\( \rho = -0.28; P = .38 \)). A faster decrease in the HCV RNA level was noted in patients who had a more homogeneous quasispecies population at baseline.

To better define the region of NSSA that correlated with a decrease in the HCV RNA level, we examined the following regions: the membrane attachment region (amino acid [aa] 1–236); the carboxyl region (aa 237–448); and the regions within the carboxyl end, such as PKRbd (aa 237–302), variable region 4 (V4; aa 310–330), variable region 3 (V3; aa 381–409), the region between V4 and V3 (aa 331–380), and the downstream region of V3 (aa 410–448) (figure 2). At the amino terminus,
Figure 3. Phylogenetic trees with clonal distribution, by treatment day, for representative patients. The bootstrap method was used to calculate the reliability of each branch point. A, Patient 3 (who was infected with human immunodeficiency virus [HIV] and hepatitis C virus [HCV]) had undetectable HCV at 4 week of treatment and a sustained virologic response (SVR; clinical response and undetectable HCV 6 months after completion of treatment). B, Patient 12 (who was infected with HIV and HCV) had detectable HCV RNA at week 4 but had an SVR. C, Patient 20 (who was infected with HCV) had detectable virus at week 4 and did not achieve an early virologic response (EVR; undetectable HCV or a >2-log decrease in HCV RNA at week 12 of treatment) or an SVR. D, Patient 7 (who was infected with HIV and HCV) had detectable virus at week 4 and did not achieve an EVR/SVR.

Figure 4. Changes in NS5A over time, by response. 

Sequence Analysis of FL-NS5A at Baseline, by Response

Patients with an ER had a lower SGD at baseline (0.008) than did those with an SR (0.025; P<.001) within FL-NS5A. A significantly lower SGD at baseline was also seen in both the amino and carboxyl ends of NS5A, including the subregions PKRbd, V4, aa 331–380, V3, and aa 410–448, in patients with an ER, compared with patients with an SR. At the protein level, a lower dN rate at baseline was found in both FL-NS5A and the amino and carboxyl termini. Similarly, lower dS values were seen. Only the V3 region had a significantly lower dN rate, among patients with ERs (figure 4).

Changes in NS5A over Time, by Response

Assessment of SD, dS, and dN: cross-sectional analysis. The average dS rates were significantly lower in patients with ERs than in those with SRs, for FL-NS5A, the amino and carboxyl termini, and each of the subregions over the first 7 days (data not shown). At the protein level, there were significantly fewer numbers of dNs in FL-NS5A and in the amino and carboxyl termini of patients with ERs (data not shown). However, only V3 and 410–448 regions had significantly lower dN rates at all time points, among patients with ERs (data not shown).
Figure 4. Comparison of the within-sample genetic distance (SGD) at baseline (A), synonymous substitution (dS) rate (B), and nonsynonymous mutation (dN) rate (C). Patients with an early response (ie, a hepatitis C virus [HCV] RNA level ≤615 IU/mL at week 4 after treatment initiation) are denoted by a black bar, and patients with a slow response (ie, detectable HCV RNA level at week 4) are denoted by a white bar. *Significant differences; all comparisons were significant in A and B. aa, amino acid; amino, amino terminus; carboxyl, carboxyl terminus; NS5A, full-length nonstructural protein 5A; PKRbd, protein kinase R–binding domain; V3, variable region 3; V4, variable region 4.

Quasispecies of patients with an ER had more homogeneity and less evidence of selective pressure, especially in the terminal carboxyl region, at all 4 time points assessed during the first week of treatment.

Examination of clonal evolution during the first week of therapy: longitudinal analysis of mutations in FL-NS5A. The NS5A clones from 24 and 48 h and from day 7 after treatment initiation were compared with the consensus sequence at baseline to determine whether new variations (mutations) occurred. At the protein level (figure 5), there were fewer substitutions at baseline in patients with ERs than in those with SRs (median number of substitutions, 2.2 vs 4.69; P < .001) (figure 5A), and there was a difference in the number of amino acid substitutions, with the patients with ERs—but not the patients with SRs—experiencing an increase in the number of substitutions over time (P = .01) (figure 5A) for FL-NS5A. In the NS5A amino terminus, fewer numbers of amino acid substitutions were present at baseline in patients with ERs than in patients with SRs (mean number of changes, 1.3 vs 2.3 changes; P = .007), but there were differences in the number of substitutions between the 2 groups (P = .02, analysis of variance) (figure 5C), with patients with ERs experiencing an increase in the average number of substitutions over time. This finding was not evident in the patients with SRs, who did not experience the same increase. The nucleotide substitutions for FL-NS5A and for the amino and carboxyl regions (data not shown) were similar to the amino acid substitutions for these areas.

Within the carboxyl region, the median number of nucleotide substitutions at baseline was lower in PKRbd, V4, aa 331–380, V3, and aa 410–448 in patients with ERs than in patients with SRs. Furthermore, an increase in the number of nucleotide substitutions was observed in the PKRbd, V4, and aa 331–338 regions in the patients with ERs but not in the patients with SRs. However, at the protein level (figure 5), only V3 had a lower number of substitutions at baseline (P = .002) (figure 5G). Furthermore, patients with ERs had no significant increase in the number of amino acid substitutions over time in any of the subregions of the carboxyl terminus. The high level of variability in the PKRbd, V4, and aa 331–380 regions makes the NS5A amino terminus and the V3 and aa 410–448 regions appear to be more conserved under interferon drug pressure.
Figure 5. Longitudinal analysis of evolution of nonstructural protein 5A (NS5A) and subregions. Median no. of amino acid mutations (y-axis) from the baseline consensus sequence in patients with an early response (ER; ie, a hepatitis C virus [HCV] RNA level <615 IU/mL at week 4) (black circles), compared with patients with a slow response (SR; ie, detectable HCV RNA level at week 4) (white circles) over time (x-axis) for full-length NS5A. Error bars denote the 25th and 75th percentiles. *Significant differences between patients with an ER and those with an SR, across the 4 time points (ie, interaction between group and day) (P < .05). A–C, Patients with an SR had a significantly lower no. of amino acid mutations in full-length NS5A and the amino and carboxyl termini. A significant increase in the no. of mutations occurred over time in full-length NS5A (A) and the carboxyl terminus (C). D–F, No significant difference was seen between the no. of amino acid mutations in the protein kinase R–binding domain (PKRbd), variable region 4 (V4), and amino acids (aa) 331–380 in patients with an ER. G and H, A significant difference in the no. of amino acid mutations, compared with baseline, was seen in patients with an ER, for variable region 3 (V3) and aa 410–448. There was no significant increase or decrease in the no. of mutations noted compared with baseline, over time.

in patients with ERs. Thus, in our patients, the changes in NS5A under drug pressure appeared to be focused on the carboxyl terminus and within the PKRbd, V4, and aa 331–380 regions in individuals with ERs.

Changes in genetic diversity in individual patients. Individual data on changes in the HCV level and changes in genetic diversity over time were examined (representative graphs are shown in figure 6). Among patients with ERs who had an SVR, the HCV level was lower, and SGD appeared to be low and remain low during the first 7 days of treatment. More variability was seen in the average dS rate, however. Among patients who experienced relapses and among those without a response, V3 appeared to have variability higher than that observed in patients with ERs. Thus, patients with an SR had more diversity in V3, which may play a role in mediating resistance.

DISCUSSION
In the present study, we examined 3 characteristics that might influence HCV responsiveness to interferon: HIV infection, diversity in NS5A, and the dynamic changes that occur after receipt of 1 dose of pegylated interferon. Previous studies involving patients infected with HIV and HCV were limited be-
NS5A Evolution during HCV Therapy

Figure 6. Individual graphs of the decrease in the log hepatitis C virus (HCV) RNA level (top line; y-axis), with changes in genetic diversity (z-axis) at different time points (x-axis) in nonstructural protein 5A (NS5A). A, Patients with an early viral response at week 4 of treatment and nonsynonymous substitutions by full-length NS5A and subregions in patients 3, 9, and 12. The no. of nonsynonymous substitutions (dNs) is low and does not increase significantly over time. B, Patients who did not achieve an undetectable virus level at week 4 but were considered to have a relapse. A higher no. of dNs was seen in variable region 3 (V3) in patients 1, 16, and 24. C, Patients who had a detectable virus level and no response. Higher nos. of dNs are seen in V3 in patients 7 and 20. Patient 10 has an HCV 1b genotype and does not have the higher variability in V3. PKRbd, protein kinase R–binding domain.

cause of either a lack of control subjects [28] or mixed racial and genotypic groups [29, 30]. We found that HIV infection does not appear to substantially affect the initial antiviral response of interferon-based therapy in white patients. The lower SVR seen in clinical trials of HIV-infected patients may be the result of impaired immune clearance, a higher HCV level at baseline, or a higher proportion of blacks with coinfection [4, 5].

We did, however, find that a number of our patients experienced viral rebound during the first week of therapy. Viral rebound was associated with a lack of response. We found no correlation between ε and viral rebound, but we were unable to assess the association of viral rebound with other viral kinetic parameters (such as δ). Furthermore, we were unable to examine the association of the CD4 cell count or other HIV-related factors with rebound, because of the small number of patients. The current models used for viral kinetic modeling are insufficient, and a new model incorporating changing drug levels may be more appropriate [31]. Further studies are needed to determine whether pharmacokinetic parameters differ in HIV/HCV-coinfected patients, compared with HCV-infected patients.

Because the variable rate of decrease in the HCV RNA level is not affected by HIV status, we wanted to further examine the genetic composition of the HCV quasispecies and determine how its evolution was associated with the development of an ER, rather than an SR, in some patients. We found that a more homogeneous NS5A was associated with an ER. Other investigators have shown that higher diversity in the PKRbd or V3 is associated with response [32, 33], but these studies compared viral quasispecies with a reference strain, which may be the reason why discrepant results were obtained. We found no
differences when we compared our clones with a reference strain (data not shown). In patients infected with HCV genotype 1b, an alanine at position 2360 and, to a lesser extent, a threonine at position 2378 have been associated with response [33], but we did not find these associations in our patients who were infected with HCV genotype 1a. Our findings were similar to those of studies in which genetic diversity was evaluated without comparison with a reference strain [34–37]. In the present study, we went on to show that lower diversity and less complexity correlated with a greater decrease in the HCV RNA level and ER. The initial decrease in the virus level may be the result of a direct effect of interferon on HCV protein synthesis and replication [38]. Thus, our study is unique in evaluating the effect of quasispecies diversity on the initial antiviral effect of interferon.

Viral evolution within NS5A has not been adequately evaluated in past studies. Two studies have evaluated changes in NS5A at either 24 h or 7 days after treatment initiation and have failed to detect any changes [36, 39]. Failure to detect differences may have occurred because clonal analysis was not used [36] and because changes in the dominant quasispecies may take >24 h to detect [39]; we also did not detect any viral evolution at 24 h.

We examined viral evolution by using a sample (obtained from each patient at baseline) as the motif against which subsequent samples from the same patient were compared. Patients with an ER demonstrated a greater number of substitutions at the nucleotide and amino acid levels in NS5A during interferon therapy, indicating viral evolution of this population under strong drug pressure. Patients with an SR did not demonstrate further changes in such substitutions during the early stages of interferon treatment, implying a lack of selective pressure to the effect of interferon.

When we analyzed various subregions of NS5A, the carboxyl terminal coding region stood out as showing the most prominent differences between patients with ERs and SRs and selective selection. Specifically, the number of substitutions from baseline clones in patients with ERs was lower in V3, and this region appeared to be conserved over time (during the initial phase of treatment). In contrast, in the regions of PKRbd, V4, and aa 331–380, no differences were apparent in both groups; however, we observed an increased number of nucleotide substitutions over time, albeit with few changes noted at the protein level. Selective pressure in the carboxyl region occurred during the first week of treatment, which helps to narrow the area within NS5A that is affected by interferon.

Individual patient data showed the V3 region to have a higher dN rate in patients with no response than in those with an SVR. Taken together, these findings suggest that an increase in heterogeneity in V3 (and, perhaps, in aa 410–448) may be associated with resistance to interferon. The carboxyl terminus, when deleted, has not been found to directly affect HCV replication [40–42], but this region may mediate other functions, such as regulation of production of infectious virus particles [43].

We conclude that the initial antiviral effect of interferon is determined at least in part by the genetic composition of the viral quasispecies at the time of initiation of therapy. Our study demonstrates that differential selection of HCV quasispecies by drug pressure occurs within 1 week in patients who have an ER. However, pharmacokinetic studies will be needed to exclude the possibility that viral rebound of HCV RNA is a result of a decrease in drug concentrations [31] versus our proposed increase in resistant clones. Patients who demonstrate high viral diversity at baseline are less likely to be responsive to interferon and ribavirin therapy. The new antiviral therapies that are currently in development, which target different components of the HCV replication complex, may be needed to achieve viral clearance in patients with higher viral diversity.

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APPENDIX

QUASISPECIES ANALYSIS

Amplification and cloning of the HCV NS5A region. The HCV NS5A region was amplified as described by Paterson et al [44] with modifications. First-round amplification was performed using the One-Step RT-PCR Kit (Qiagen) with outer sense primer 5′-CAGTGGATGAACCGGTRATA-3′ (R = A or G; nucleotides [nt] 6081–6101 in strain H77 [GenBank accession no. AF009606]) and antisense primer 5′-TGTGGTGACGGTAGCAACGAGTTGCT-3′ (nt 7680–7704). Reverse-transcriptase PCR conditions were as follows: 50°C for 30 min and 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, at 49°C for 1 min, and at 72°C for 1.5 min, with a final extension at 72°C for 10 min. Second-round PCR was performed using high-fidelity polymerase (Platinum Taq DNA Polymerase High Fidelity; Invitrogen) with sense primer 5′-TCCGGTTTCTGGCTAAGRGA-3′ (nt 6258–6277) and nest antisense primer 5′-CAGGATAGACATTTGACGACGAC-3′ (nt 7595–7618). The PCR conditions were 35 cycles at 94°C for 30 s, at 49°C for 1 min, and at 72°C for 1.5 min, with a final extension at 72°C for 10 min.

Sequence analysis. The sequences of all clones were edited and aligned using Vector NTI software (version 9.0; Invitrogen). The NS5A sequence was classified into 6 fragments according...
to potential functional domains, including the interferon sensitivity–determining region/protein kinase R–binding domain, V4, and V3 (figure 2) [45]. The SGD, ds, and dN values for each region at baseline, at 24 and 48 h, and on day 7 were calculated using MEGA software (version 4). The average numbers of nucleotide and amino acid substitutions were calculated for each fragment.

**Phylogenetic tree analysis.** Evolutionary history was inferred using the neighbor-joining method [46]. The optimal tree with the sum of branch lengths was estimated. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to each branch [47]. The tree is drawn to scale, and branch lengths expressed in the same units used for evolutionary distances were used to infer the phylogenetic tree (figure 3). The evolutionary distances were computed using the maximum composite likelihood method [48] and are expressed as the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (ie, the complete deletion option). There were a total of 1344 positions in the final data set. Phylogenetic analyses were conducted using Mega software (version 4) [26].

**Comparison of clinical strains with HCV-1.** We also examined our isolates in comparison to the reference HCV-1 strain for all patients infected with HCV genotype 1a, examining the full-length NSSA, the amino and carboxyl termini, and all subregions, including PKRbd, V3, V4, and regions encoding aa 331–380 and aa 410–448. We found no differences between patients with ERs and SRs with regard to the number of mutations (both at the nucleotide level and the amino acid level) away from the HCV-1 strain, either at baseline or over time.


**References**