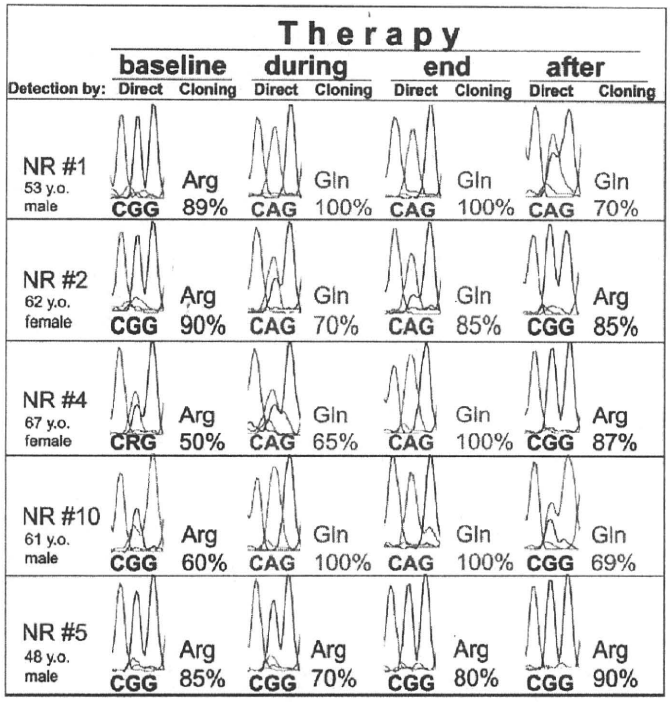

Figure 2. Changes in the proportion of clones with the 70Q substitution relative to wild type (70R) in patients with nonresponse (NR), as determined by both direct sequencing and cloning at different time points during treatment with pegylated interferon plus ribavirin. Each row summarizes the findings for a single patient identified by a unique ID, age, and sex. The direct sequencing results are demonstrated by 5′–3′ nucleotide sequence electrophoregrams. The cloning results are given as the translated amino acid (glutamine or arginine) from the dominant clone, and the proportion of the dominant clone is evaluated by the percentage of the total clones (15–20) sequenced at the respective time point.

a weekly basis. After inoculation, the HCV RNA titer increased rapidly in all mice, and by week 2 after inoculation the mean HCV RNA titers of the nonresponsive strains plateaued at ~7.6 log copies/mL. The HCV-1b RNA level in the mice inoculated with the sample from the SVR group increased to 8.5 log copies/mL until week 3 and then reached a plateau of ~8.0 log copies/mL (Figure 3). These 5 animals were subsequently treated with 4 different regimens, as outlined in Materials and Methods, and viral kinetics were described in detail in another report on the interferon sensitivity of the strains [10]. Briefly, no reduction of the HCV RNA titer was observed after administration of 3 injections of pegylated interferon alone at the clinical dosage (3 μg/kg) in the mice inoculated with a sample from the nonresponse group, whereas the mice inoculated with a sample from the SVR group achieved a 0.5 log copies/mL reduction in the HCV RNA titer. Administration of the 10-fold dose of interferon (30 μg/kg) induced reductions in the HCV RNA titer of 1.0 and 2.0 log copies/mL for the nonresponse and SVR groups, respectively ($P < .02$). No statistically significant difference was observed among the groups after administration of ribavirin in standard or 5-fold clinical doses. However, administration of the pegylated interferon in combination with ribavirin resulted in reductions of 0.9 and 0.2 log copies/mL in the SVR and nonresponse groups, respectively ($P < .02$). Mice serum samples collected at 4 time points, as indicated in Figure 3, were subjected to cloning. A total of 320 clones (15–20 for each time point) were analyzed. The results are summarized in Figure 3. The proportion of 70Q clones relative to 70R clones differed in the 2 mice infected with a nonresponse sample; after administration of 10-fold pegylated interferon (point 1), the proportion of 70Q clones was 15% in serum samples from mouse 5-1 and 90% in serum samples from mouse 5-2. Seven weeks later (point 2), the predominance of 70R clones was restored in both animals. After administration of pegylated interferon with ribavirin, 70Q was detected in the majority of clones retrieved from both mice (point 3), but 2

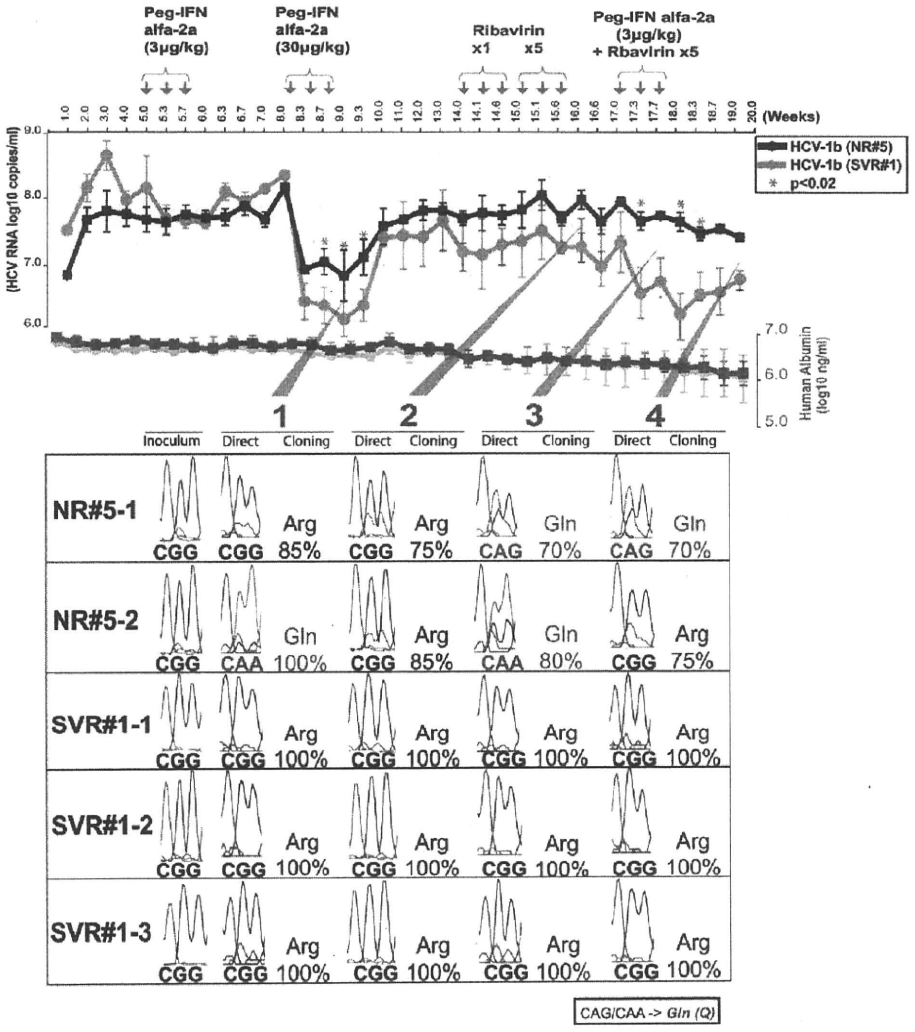

Figure 3. Changes in the proportion of core protein 70Q to 70R coding clones in chimeric mice infected with hepatitis C virus (HCV) by inoculation of serum samples from patient 5, who experienced nonresponse (NR; 2 mice), or from patient 1, who achieved sustained virologic response (SVR; 3 mice), as determined by both direct sequencing and cloning at 4 different time points during the course of postinfection follow-up. The changes in the mean HCV RNA levels estimated for mice grouped according to inoculation source are graphed on the top (*left scale*), and corresponding human albumin levels are graphed on the bottom (*right scale*). Times of administration of drugs are indicated by arrows. Each row summarizes findings for a single mouse, identified by inoculation source patient ID. The direct sequencing results are demonstrated by 5′–3′ nucleotide sequence electropherograms. The cloning results are given as the translated amino acid (glutamine or arginine) from the dominant clone, and the proportion of the dominant clone is evaluated by the percentage of the total clones (15–20) sequenced at the respective time point.

weeks after the treatment (point 4) the 70R clone majority was restored again in mouse 5-2. The last sample available for mouse 5-1 was taken 2 days after the last administration of medication; 70Q was detected in 70% of the clones sequenced at that time point (data not shown). No clone with 70Q was identified at any of the 4 points in the 3 mice infected with the SVR serum sample.

No changes were observed in the core 91 region (leucine)

and ISDR of the nonresponse group (resistant type; 0 substitutions) and SVR group (mutant type; 8 substitutions). These results suggest that the 70Q genotype is positively selected during pegylated interferon with ribavirin treatment and that detectable 70Q at baseline can be an important predictive marker of potential treatment failure.

Statistical evaluation of the 70Q substitution. We performed univariate analysis including the following variables: greater age (cutoff at median, 56 years), female sex, HCV RNA level of >1500 kIU/L, no early virologic response (EVR; absence of a reduction of 2 log copies/mL by week 12 of treatment), alanine transaminase level of ≥48 IU/L, γ glutamyl transferase level of >40 IU/L, low-density lipoprotein cholesterol level of >86.0 mg/dL, platelet count of $>14.6 \times 10^4$ platelets/μL, glucose level of ≥95 mg/dL, presence of ISDR, presence of the 70Q substitution (including patients in whom it was detected as a minor clone), and core 91M substitution. The analysis indicated that non-EVR and presence of the 70Q substitution were statistically significant factors associated with treatment failure (data not shown).

Statistical significance of the 70Q substitution was further assessed by logistic regression analysis. Non-EVR was not included, and only baseline factors were analyzed. Sixteen patients with nonresponse were compared with 50 patients with response by means of the backward stepwise likelihood ratio logistic regression method; estimated odds ratio (OR) coefficients, their 95% confidence interval ranges, and *P* values are summarized in Table 3 for the variables that remained in equitation at the last step. The predictive value in the multivariate analysis was assessed for the parameters detected at baseline. Detectable 70Q-possessing clones were counted. It was found that the 70Q substitution was statistically significantly associated with nonresponse (OR, 8.7; $P = .007$). High γ glutamyl transferase levels (>40 IU/L) and low alanine aminotransferase levels (<48 IU/L) also had weak association with nonresponse (OR for high γ glutamyl transferase levels, 7.08; $P = .043$; OR for low alanine aminotransferase levels, 0.12; $P = .035$). The positive predictive value of detecting 70Q for nonresponse was 50% (10 of 20 patients) in this cohort. The positive predictive value of detecting 70Q for failure to achieve SVR was 75% (15 of 20 patients). The positive predictive value of detecting 70Q together with a resistant ISDR sequence for failure to achieve SVR was 87.5% (14 of 16 patients).

Table 3. Logistic Regression Analysis of Variables Contributing to Resistance to Treatment with Pegylated Interferon Alfa-2b Plus Ribavirin

Variable	OR (95% CI)	*P*
Core substitution 70Q[a]	15.11 (2.436–93.702)	.004
Age, ≥56 years	4.94 (0.973–25.087)	.054
Viral load, >1500 kIU/L	4.37 (0.798–23.962)	.089
Platelet count, $>14.6 \times 10^4$ platelets/μL	5.396 (0.77–38.08)	.091
γ-GTP level, >40 IU/L	7.27 (1.11–47.648)	.039
Glucose level, ≥95 mg/dL	0.182 (0.33–1.01)	.052
ALT level, ≥48 IU/L	8.711 (1.21–62.816)	.032

NOTE. For each variable except the core substitution, the median value was used as a grouping cutoff. ALT, alanine aminotransferase; CI, confidence interval; γ-GTP, γ glutamyl transferase; OR, odds ratio.

[a] Including patients with a detected minor clone.

DISCUSSION

A substantial proportion of HCV-infected patients do not have an optimum response to current pegylated interferon with ribavirin treatment regimens [11]. Individualization of therapy would offer the possibility of tailoring treatment to particular patients and selecting the treatment duration that ensures the best chance of achieving SVR while preventing overtreatment. Effective identification of potentially nonresponding patients would be very useful in the context of the rather costly treatment with pegylated interferon–ribavirin, which is accompanied by many adverse effects [2]. A few recent studies reported an arginine-to-glutamine amino acid substitution at position 70 of the HCV core protein (70Q) is associated with poor response in patients infected with HCV-1b [6, 12]. In the present study, comparison between the nonresponse and the response groups also indicated statistically significant differences for the ISDR and core amino acid patterns. Interestingly, the 70Q substitution was detected in the studied cohort at a higher rate during and after treatment, compared with baseline. Patients in whom there was heterogeneity of nucleotide sequence coding of the amino acid at position 70 of the core protein were further investigated for quasispecies composition at different time points. Selection of 70Q-coding clones during treatment was indicated in 4 (25%) of 16 patients with nonresponse. Additionally, a sample obtained from a patient during treatment, who had a minority of core 70Q clones before, during, and after treatment, was inoculated into 2 chimeric mice that were consecutively treated with various regimens of pegylated interferon and ribavirin. After treatment with pegylated interferon and ribavirin, 70Q was detected in a majority of clones retrieved from both mice. The discrepancy between the clinical case and the mouse model may be associated with different dosages, different regimens, different drug metabolisms, and/or different contributions of host factors, which require further study. This study, by demonstrating the positive selection of the 70Q viral strains, provides additional evidence for an association between the amino acid substitution in the core protein of the infecting HCV and the response to antiviral treatment. Hence, outlined findings indicate that the presence of a 70Q clone at baseline (including patients with a detectable minor quasispecies population) may indicate potential failure in achieving virologic response or SVR in patients with chronic HCV-1b infection. In the present study, a 70Q clone was de-

Table 4. Prevalence of the Core 70Q Substitution in Hepatitis C Virus Core Protein among DDBJ, EMBL, and GenBank Entries

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tected at baseline in 43.8% of patients who had nonresponse and 17.5% of patients who achieved SVR (similar to the rates of 47.7% and 18.2%, respectively, that were found in another study [13]), indicating that the positive predictive value of detecting 70Q for nonresponse was 50%. The positive predictive values of detecting 70Q alone or together with resistant ISDR for failing to achieve SVR was 75% and 87.5%, respectively. Furthermore, the difference in the proportion of 70Q detected in 2 chimeric mice infected with a nonresponse strain indicated the contribution of host genetic factors [14].

Other reports investigating the baseline prevalence of core amino acid patterns indicated that both 70Q and 91M were associated with nonresponse among treated patients [6, 7]; however, in the cohort studied here, the baseline prevalence of 91M was relatively higher in the SVR group (44.8%) than in the nonresponse and posttreatment relapse groups (25% and 23.8%, respectively). Furthermore, the prevalence of 91M during treatment and at the end of treatment was stable in the studied cohort, which suggests that it was not associated with selection of resistant strains. A study investigating the evolution of the NS5A region during treatment (with pegylated interferon and ribavirin) in patients with HCV genotype 1 infection indicated that an absence of changes in the ISDR pattern was associated with treatment outcome [15]. In the present study, samples from only 1 patient (who was in the nonresponse group) demonstrated a change in the amino acid pattern from sensitive to resistant (from 2 mutations before treatment to 1 mutation after treatment). Although the number of patients was relatively small in this study, the prevalence of the 70Q substitutions and resistant ISDR patterns concurred with those in other published studies [4, 7, 15–17]; 18 (27%) of 66 patients had a sensitive ISDR pattern, and the positive predictive value of presence of the sensitive ISDR pattern for achieving SVR was 89% (16 of 18 patients). Among the remaining 48 (73%) patients, who had the resistant ISDR pattern, 16 (33%) had detectable 70Q in the core protein. Presence of the resistant-type ISDR and detectable 70Q together had a positive predictive value for failing to achieve SVR of 87.5% (14 of 16 patients) in this cohort.

Table 5. Geographical Distribution of Hepatitis C Virus Genotype 1b Strains with the 70Q Substitution

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Inspection of 4933 HCV-1b strains currently available in the DDBJ, EMBL, and GenBank genetic databases indicated that the 70Q substitution is present in 54% of HCV-1b isolates; other genotypes in which 70Q is common are 5a (84.4%) and 6 (58.3%), whereas only 8.6% of genotype 3a and none of genotype 2 isolates had this amino acid in this position (Table 4). The geographical distribution of genotype 1b strains with 70Q was substantially different, with 44.3% of strains in Asia, 58.9% in Europe, and 63.7% in America ($P < .001$) (Table 5). Because complete clinical background data were not available for all entries in the database, further studies are needed to confirm the clinical impact of core 70Q in each geographic region.

In conclusion, in this study both clinical material and an in vivo model were used to provide further evidence of an association between the 70Q substitution in the HCV core protein and treatment response by detecting positive selection by treatment with pegylated interferon–ribavirin treatment. Further studies are required to investigate the mechanism of this association.

References

1. Ray Kim W. Global epidemiology and burden of hepatitis C. Microbes Infect **2002**; 4:1219–1225.
2. Fried MW. Side effects of therapy of hepatitis C and their management. Hepatology **2002**; 36:S237–S244.
3. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med **1996**; 334:77–81.
4. Pascu M, Martus P, Hohne M, et al. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. Gut **2004**; 53:1345–1351.
5. Shirakawa H, Matsumoto A, Joshita S, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology **2008**; 48: 1753–1760.
6. Akuta N, Suzuki F, Kawamura Y, et al. Prediction of response to pegylated interferon and ribavirin in hepatitis C by polymorphisms in the viral core protein and very early dynamics of viremia. Intervirology **2007**; 50:361–368.
7. Mori N, Imamura M, Kawakami Y, et al. Randomized trial of high-dose interferon-α-2b combined with ribavirin in patients with chronic hepatitis C: Correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol **2009**; 81:640–649.
8. Kuiken C, Combet C, Bukh J, et al. A comprehensive system for consistent numbering of HCV sequences, proteins and epitopes. Hepatology **2006**; 44:1355–1361.
9. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol **2004**; 165:901–912.
10. Kurbanov F, Tanaka Y, Chub E, et al. Molecular epidemiology and interferon susceptibility of the natural recombinant hepatitis C virus strain RF1_2k/1b. J Infect Dis **2008**; 198:1448–1456.

11. Manns MP, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in HCV treatment—finding the right path. Nat Rev Drug Discov **2007**; 6:991–1000.
12. Akuta N, Suzuki F, Kawamura Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol **2007**; 46:403–410.
13. Akuta N, Suzuki F, Sezaki H, et al. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol **2006**; 78:83–90.
14. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of *IL28B* with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet **2009**; 41:1105–1109.
15. Cuevas JM, Torres-Puente M, Jimenez-Hernandez N, et al. Genetic variability of hepatitis C virus before and after combined therapy of interferon plus ribavirin. PLoS One **2008**; 3:e3058.
16. Akuta N, Suzuki F, Hirakawa M, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. J Med Virol **2009**; 81:452–428.
17. Yoshioka K, Ito H, Watanabe K, et al. Interferon sensitivity-determining region of nonstructural region 5A of hepatitis C virus genotype 1b correlates with serum alanine aminotransferase levels in chronic infection. J Viral Hepat **2005**; 12:139–145.