

Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C

Francesc Puig-Basagoiti, Xavier Forns, Ivana Furčić, Sergi Ampurdanés, Mireia Giménez-Barcons, Sandra Franco, José M. Sánchez-Tapias and Juan-Carlos Saiz†

Liver Unit, Institut de Malalties Digestives, Departament de Medicina (IMD), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clinic, University of Barcelona, Spain

Correspondence
Juan-Carlos Saiz
jcsaiz@inia.es

The quasispecies nature of hepatitis C virus (HCV) may have important implications concerning resistance to antiviral agents. To determine whether HCV NS5A quasispecies composition and dynamics are related to responsiveness to combined interferon (IFN) and ribavirin therapy, extensive sequence analyses of cloned RT-PCR amplification products of HCV-1b NS5A quasispecies of sequential isolates from 15 treated (nine sustained responders and six non-responders) and three untreated patients were performed. Accumulation of mutations in NS5A during therapy was relatively frequent in the V3 domain, but unusual elsewhere. Amino acid changes were the result of the imposition of minor variants that were already present before treatment and always occurred within the first week of therapy. Before treatment, the complexity and diversity of quasispecies were lower in isolates from responders than in those from non-responders, particularly in the V3 domain, where differences in nucleotide entropy (0.35 vs 0.64, $P=0.003$), genetic distance (0.0145 vs 0.0302, $P=0.05$) and non-synonymous substitutions (0.0102 vs 0.0203, $P=0.036$) were statistically significant. These differences became more apparent during treatment, because complexity and diversity remained stable or tended to increase in non-responders, whereas they tended to decrease in responders. These observations suggest that the composition and dynamics of HCV NS5A quasispecies, particularly in the V3 domain, may play a role in the response to combined IFN/ribavirin therapy.

Received 13 August 2004
Accepted 13 December 2004

INTRODUCTION

About 170 million people worldwide are infected with hepatitis C virus (HCV), which is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (World Health Organization, 1997). HCV, the unique hepacivirus of the family *Flaviviridae*, is an enveloped virus with a positive, single-stranded RNA genome of approximately 9.6 kb, encoding a single polyprotein of about 3000 aa that is co- and post-translationally cleaved by viral and cellular proteases into the structural and non-structural proteins (Forns & Bukh, 1999).

The introduction of alpha interferon (IFN- α) plus ribavirin combination therapy was an important breakthrough in the treatment of chronic HCV infection. However, the rate of sustained virological response is still unsatisfactory (Davis *et al.*, 1998; McHutchison *et al.*, 1998), particularly in patients infected with genotype 1 HCV (Poynard *et al.*, 1998), the most prevalent in our geographical area (López-Labrador *et al.*, 1997). Although viral genotype and viral load, as well as the chronology of serum HCV RNA clearance during therapy, are definitely related to response, further insight into viral factors involved in therapeutic responsiveness is still necessary.

†Present address: Departamento de Biotecnología, Instituto Nacional de Investigaciones Agrarias (INIA), Ctra Coruña km 7.5, 28040 Madrid, Spain.

The GenBank/EMBL/DDBJ accession numbers for the HCV sequences reported in this study are AY378328–AY378694, AY378705–AY379066, AY381300–AY381643 and AY381644–AY381994.

Enomoto *et al.* (1995, 1996) suggested that the genetic heterogeneity of a specific domain of the non-structural 5A (NS5A) region of HCV, termed the IFN sensitivity-determining region (ISDR), was related closely to response in Japanese patients with HCV genotype 1b infection. Similar observations were made in Spain (Sáiz *et al.*, 1998; Puig-Basagoiti *et al.*, 2001) and, although this is a

controversial issue, analysis of the published information supports the hypothesis that a relationship exists between NS5A heterogeneity and response to therapy (Giménez-Barcons *et al.*, 2001; Witherell & Beineke, 2001).

The NS5A region of HCV seems to play a role in virus replication and tumorigenesis, but other *in vivo* functions of this region remain largely unknown (Pawlotsky & Germanidis, 1999; Macdonald & Harris, 2004). Recently, it has been suggested that the region of NS5A that is responsible for resistance to IFN includes the C-terminal 26 aa adjacent to the ISDR. This region has been termed the protein kinase-binding domain (PKR-bd) (Gale *et al.*, 1997). Cellular PKR is an IFN-inducible protein kinase that mediates the antiviral and antiproliferative effects of IFN by phosphorylating eukaryotic initiation factor 2 α (Meurs *et al.*, 1993; Gale *et al.*, 1997). However, PKR-independent effects of NS5A in the IFN response have also been proposed (Macdonald & Harris, 2004). In addition, mutations in the so-called V3 domain of NS5A (Inchauspe *et al.*, 1991), a region of unknown function that appears to be under strong selective pressure, have also been related to response to antiviral therapy (Duverlie *et al.*, 1998; Nousbaum *et al.*, 2000; Murphy *et al.*, 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell *et al.*, 1992; Domingo, 1996). The quasispecies nature of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents (Domingo, 1996; Forns & Bukh, 1999; Forns *et al.*, 1999; Farci & Purcell, 2000). However, previous work on the possible relationship between HCV quasispecies and response to therapy was mainly based on single-strand conformational polymorphism assays, which do not provide precise information about viral quasispecies, whilst more accurate studies based on analysis of cloned viral populations were reported less frequently. On the other hand, most of these studies were carried out in patients treated with IFN- α monotherapy and focused on hypervariable region 1 (HVR-1) of the E2 envelope protein of HCV (Forns *et al.*, 1999; Farci & Purcell, 2000), whereas the NS5A quasispecies dynamics during combined IFN/ribavirin therapy have only been analysed in a small number of HCV-1b patients (Gerotto *et al.*, 1999; Nousbaum *et al.*, 2000).

We report here the results of an extensive analysis of the structure and evolution of NS5A quasispecies that was carried out in representative serial serum samples from patients with genotype 1b chronic hepatitis. Responders to IFN/ribavirin combination therapy, non-responders to this therapy and untreated patients were included in the study.

METHODS

Patients and treatment. Fifteen treated patients, including nine with a sustained virological response (patients 1–9) and six non-responders

(patients 10–15), and three untreated patients (patients 16–18) with chronic hepatitis C were studied. All had genotype 1b infection and were retrieved from a large cohort of patients seen at our unit. Treatment consisted of the administration of IFN- α -2b (3 MU three times weekly) and ribavirin (1–1.2 g day⁻¹, according to body weight) for 48 weeks. Patients were followed for 24 weeks after treatment withdrawal. Sustained response was defined by normal alanine aminotransferase (ALT) levels and undetectable HCV RNA at the end of therapy and at the end of post-treatment follow-up. No response was defined by elevated ALT levels and detectable HCV RNA during therapy and at the end of follow-up. No patient was alcoholic or co-infected with hepatitis B virus or human immunodeficiency virus. Causes of chronic liver disease other than chronic HCV infection were carefully ruled out. All participating patients gave their informed consent and the Ethics Committee of our institution approved the study.

Detection and amplification of HCV RNA. HCV detection (ELISA-HCV third generation; Ortho Diagnostics Systems) and qualitative determination and quantification of HCV RNA (Amplicor HCV-RNA and COBAS Amplicor HCV Monitor test 2.0; Roche Diagnostics) were carried out with commercial reagents. HCV RNA extraction and specific amplification of the 5' non-coding (5'NC) and NS5A regions by RT-PCR were carried out as described previously (Sáiz *et al.*, 1997, 1998; Giménez-Barcons *et al.*, 2001) using a proof-reading polymerase (Expand; Roche Molecular Biochemicals). To minimize experimental error and under-representation of minor variants within a given quasispecies, three independent RT-nested PCRs were carried out on each sample. HCV genotype was determined by restriction fragment length polymorphism analysis of the 5'NC region, as described previously (López-Labrador *et al.*, 1997).

Cloning and sequencing. RT-PCR-amplified products of 762 bp encompassing the PKR-bd, which included the ISDR, and V3 regions of the NS5A protein were purified, ligated to the TA-cloning plasmid pGEM-T Easy (Promega) and cloned independently, as described previously (Sánchez-Fueyo *et al.*, 2001). On average, 20 (17–25) randomly selected clones from each serum sample were analysed (775 clones, 5.5 \times 10⁵ nt). Clones were sequenced with a DyeDeoxy Terminator cycle sequencing kit (Perkin Elmer Applied Biosystems) in a 310 DNA sequencer (Applied Biosystems). Electropherograms were analysed by using Sequence Navigator software (Perkin Elmer).

Sequences of all clones were aligned with the CLUSTAL W 1.6 program (Thompson *et al.*, 1994) and the within-consensus sequence of each sample (WTH-CONS) and the between-consensus sequence of all analysed clones (BTW-CONS) were obtained.

Statistical and phylogenetic analyses. Shannon entropy (Pawlotsky *et al.*, 1998), which is defined as the different sequences or clusters of sequences that appear at a given time point, was calculated at the nucleotide and amino acid levels as follows: $S = -\sum (p_i \ln p_i)$, where p_i is the frequency of each sequence in the viral population. Entropy was normalized (S_n) as $S_n = S/\ln n$, where n is the total number of sequences analysed in each sample.

Genetic distance between pairs of sequences and phylogenetic trees were computed with the PHYLIP package (Felsenstein, 1993). K_a and K_s , where K_s is the frequency of synonymous substitutions per synonymous site and K_a is the frequency of non-synonymous substitutions per non-synonymous site, were calculated by pairwise comparison of every single sequence using the MEGA program (Kumar *et al.*, 1994), which was also applied to calculate the mutation rate (no. nucleotide substitutions per nucleotide site per year) for each isolate.

Comparisons between groups were made by using χ^2 or Fischer's tests for categorical variables and the Wilcoxon test (paired samples) or the Mann-Whitney test (unpaired samples) for quantitative variables. Values of $P < 0.05$ were considered significant.

Table 1. Characteristics of HCV quasispecies in pre-treatment isolates from sustained responders, non-responders and untreated patients

Values were calculated as described in Methods. Only *P* values lower than 0.05 were considered to indicate significant differences.

Region and patient type	No. amino acid mutations	Shannon entropy		Genetic distance	K_s	K_a
		Nucleotide	Amino acid			
NS5A						
Sustained responders	11.4	0.85	0.62	0.0128	0.0323	0.0057
Non-responders	9.3	0.95	0.87	0.0165	0.0421	0.0071
Untreated	13	0.90	0.80	0.0166	0.0474	0.0055
PKR-bd						
Sustained responders	2.8	0.57	0.26	0.0140	0.0375	0.0064
Non-responders	1.6	0.69	0.37	0.0146	0.0427	0.0054
Untreated	4.3	0.68	0.37	0.0176	0.0491	0.0073
ISDR						
Sustained responders	1.5	0.38	0.17	0.0116	0.0255	0.0070
Non-responders	0.6	0.44	0.20	0.0101	0.0289	0.0040
Untreated	1	0.60	0.30	0.0173	0.0507	0.0063
V3						
Sustained responders	3.2	0.35 ^a	0.21	0.0145 ^b	0.0250	0.0102 ^c
Non-responders	2.1	0.64	0.38	0.0302	0.0541	0.0203
Untreated	3.6	0.45	0.22	0.0163	0.0396	0.0076

$P=0.003$ (a), $P=0.05$ (b) and $P=0.036$ (c) when sustained responders were compared with non-responders.

RESULTS

At baseline, a significantly higher ALT level (175.5 vs 71.6 IU l^{-1} , $P=0.019$) and a lower viral load (1.039×10^3 vs 2.018×10^3 copies ml^{-1} , $P=0.005$) were observed in responders compared with non-responders. As shown in Table 1, when compared with the consensus sequence derived from every clone of all basal samples, the number of amino acid changes observed in the deduced WTH-CONS sequence obtained from all clones of each basal sample was higher in HCV isolates from responders than in those from non-responders, either in the whole NS5A region analysed (11.4 vs 9.3) or in the different domains: ISDR (1.5 vs 0.6), PKR-bd (2.8 vs 1.6) and V3 (3.2 vs 2.1).

According to the chronology of HCV RNA clearance during therapy, sustained responders were separated into three subgroups of three patients each: very rapid responders (patients 1–3), rapid responders (patients 4–6) and slow responders (patients 7–9), in whom HCV RNA became undetectable by qualitative PCR (detection limit, 50 IU ml^{-1}) after 2, 4 or 12 weeks on therapy, respectively. In non-responders, HCV RNA remained detectable during the whole study period. Three sequential serum samples from each of the three untreated patients, obtained at baseline and 3 and 12 months thereafter, were also analysed.

Fig. 1 shows a schematic representation of the deduced amino acid changes that were detected in the different samples analysed. Mutations were scattered throughout the NS5A region and no specific amino acids or motifs related

to a particular type of response to therapy were observed in any of the regions analysed.

Fig. 2 shows the deduced WTH-CONS amino acid sequence of the PKR-bd, which includes the ISDR, obtained upon alignment of all clones from each sample. Despite using different sets of primers and different reaction conditions, NS5A could not be amplified in three HCV RNA-positive samples (patient 5 at week 1 and patients 4 and 8 at week 2). The derived BTW-CONS amino acid sequence of these regions, obtained after alignment of all sequenced clones from every sample, was representative of the HCV-1b strains of our geographical area, as it matched that obtained upon alignment of over 300 isolates sequenced previously at our institution (Sáiz *et al.*, 1998; Soguero *et al.*, 2000; Giménez-Barcons *et al.*, 2001; Puig-Basagoiti *et al.*, 2001; Franco *et al.*, 2003). The WTH-CONS sequence of each isolate remained invariable throughout the study, except in two patients (4 and 7). In both cases, changes were detected during the first week of treatment and they were the result of the imposition of minor variants (representing 4–10% of the total basal population) that were already present within the quasispecies in baseline samples (data not shown). In one case (patient 6), a detailed analysis of the quasispecies structure showed that the patient was co-infected with two different HCV-1b strains (see below).

Fig. 3 displays the deduced WTH-CONS amino acid sequence of the V3 domain of every sample analysed. In this region, changes in the consensus sequences were detected

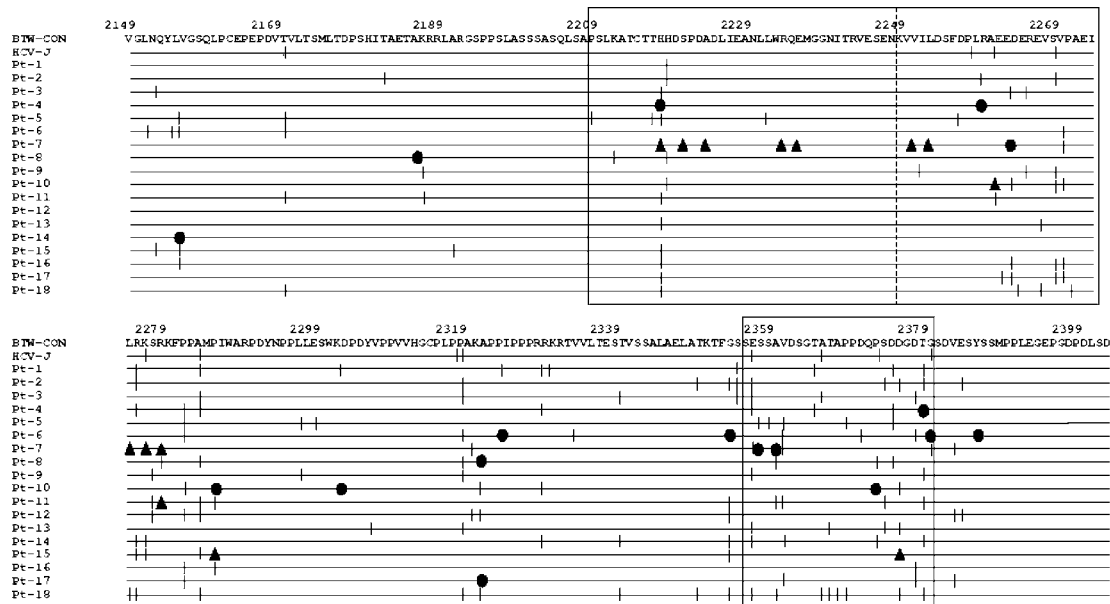


Fig. 1. Schematic representation of the deduced amino acid sequence of the amplified NS5A fragments. The BTW-CONS sequence obtained after alignment of every sequenced clone and the HCV-J prototype (Kato *et al.*, 1990), to which residue positions are referred, are shown for comparison. Sequence representation of isolates from sustained responders (patients 1–9), non-responder patients (10–15) and untreated patients (16–18) are depicted. Vertical lines represent amino acid differences from the BTW-CONS sequence. Circles and triangles represent amino acid changes that appeared or disappeared, respectively, during therapy. The PKR-bd (positions 2208–2274), which included the ISDR (positions 2209–2248), and the V3 domain (positions 2356–2379) are shown in boxes.

in five cases (patients 4, 6, 7, 10 and 15). Thus, two isolates (from patients 4 and 7) experienced changes both in the PKR-bd and in V3. In responders, amino acid substitutions in the V3 domain also developed within 1 week of therapy and were a consequence of the imposition of minor variants that were already present before therapy and represented 4–35% of the basal viral population (data not shown). As observed in the PKR-bd, two different HCV strains were also detected in the V3 domain of samples from patient 6, the minor strain being completely eliminated shortly after the beginning of treatment (data not shown). The presence of two co-infecting strains in samples from patient 6 was confirmed by pairwise analysis of the genetic distances within its clones: the genetic distance between the basal clones from this patient was 0.0588, a value similar to that obtained when the genetic distances between the clones of isolates from different patients were compared with each other (median 0.0922, range 0.0419–0.1397). However, when within-clones genetic distances were calculated independently for the clones of each co-infecting strain, their respective values were 0.0158 and 0.0168, values similar to those obtained within the clones of any other single sample analysed (Table 1).

The results of a detailed analysis of the complexity and diversity of HCV quasispecies in pre-treatment serum samples are summarized in Table 1. In all regions studied, when compared with the BTW-CONS sequence of all

analysed samples, the median number of mutations was higher in isolates from sustained responders than in those from non-responders, whilst the nucleotide and amino acid Shannon entropy, the genetic distance, the number of synonymous substitutions per synonymous site and the number of non-synonymous substitutions per non-synonymous site were usually lower. These differences were more marked in the V3 domain where, despite the relatively small number of patients analysed, the differences between sustained responders and non-responders, either in nucleotide normalized Shannon entropy ($P=0.003$), genetic distance ($P=0.05$) or number of non-synonymous substitutions per non-synonymous site ($P=0.036$), were statistically significant. Although the small number of patients included in each subgroup of responder patients (very rapid, rapid and slow responders) did not permit an adequate statistical analysis, clear differences between these subgroups in the different variables analysed were not observed (data not shown).

Analyses of these variables in samples taken serially during therapy showed a trend similar to that observed in baseline samples. In non-responders, genetic distances and K_s and K_a values were higher than in responders and remained stable or tended to increase during therapy, whereas a trend to decrease was observed in responders. Differences between responders and non-responders were statistically significant at several time points, particularly in the V3

	2356	2379		2356	2379		
BTW-CONS	ESSAVDSGTA	TAPPDQPSDD	GDTG	BTW-CONS	ESSAVDSGTA	TAPPDQPSDD	GDTG
HCV-J	G.....	.G....A...	..K.	HCV-J	G.....	.G....A...	..K.
Sustained responders				Non-responders			
Pt. 1 W-0V.N.	..A.	Pt. 10 W-0G..
Pt. 1 W-1V.N.	..A.	Pt. 10 W-1G..
				Pt. 10 W-2G..
				Pt. 10 W-4G..
Pt. 2 W-0	G.....S.S.	..A.	Pt. 10 W-72G..
Pt. 2 W-1	G.....S.S.	..A.				
				Pt. 11 W-0	..IA.....E.	..A.
Pt. 3 W-0	G.....A...	.G..	Pt. 11 W-1	..IA.....E.	..A.
Pt. 3 W-1	G.....A...	.G..	Pt. 11 W-2	..IA.....E.	..A.
				Pt. 11 W-4	..IA.....E.	..A.
				Pt. 11 W-72	..IA.....E.	..A.
Pt. 4 W-0	G.....	S.....N.				
Pt. 4 W-1	G.....	S.....N.	..A.	Pt. 12 W-0
				Pt. 12 W-1
Pt. 5 W-0	.PP.A.....	..S.....N	Pt. 12 W-2
Pt. 5 W-2	.PP.A.....	..S.....N	Pt. 12 W-4
				Pt. 12 W-72
Pt. 6 W-0	...A.....	...E.....	.G..				
Pt. 6 W-1	...A.....	...E.....	.G.S	Pt. 13 W-0	G.....	S.....P.N
Pt. 6 W-2	...A.....	...E.....	.G.S	Pt. 13 W-1	G.....	S.....P.N
				Pt. 13 W-2	G.....	S.....P.N
				Pt. 13 W-4	G.....	S.....P.N
				Pt. 13 W-72	G.....	S.....P.N
Pt. 7 W-0	G...A.....R					
Pt. 7 W-1	GL.SA.....R		Pt. 14 W-0	G...I.....	...T....	..A.
Pt. 7 W-2	GL.SA.....R		Pt. 14 W-1	G...I.....	...T....	..A.
Pt. 7 W-4	GL.SA.....R		Pt. 14 W-2	G...I.....	...T....	..A.
				Pt. 14 W-4	G...I.....	...T....	..A.
				Pt. 14 W-72	G...I.....	...T....	..A.
Pt. 8 W-0	...G.....	...A..G				
Pt. 8 W-1	...G.....	...A..G	Pt. 15 W-0
Pt. 8 W-4	...G.....	...A..G	Pt. 15 W-1
				Pt. 15 W-2
Pt. 9 W-0	G.....A.	Pt. 15 W-4
Pt. 9 W-1	G.....A.	Pt. 15 W-72
Pt. 9 W-2	G.....A.				
Pt. 9 W-4	G.....A.	Untreated			
				Pt. 16 W-0G..
				Pt. 16 W-12G..
				Pt. 16 W-48G..
				Pt. 17 W-0	...A.....G..
				Pt. 17 W-12	...A.....G..
				Pt. 17 W-72	...A.....G..
				Pt. 18 W-0	T..G.....	V PDC.....	..N .F.
				Pt. 18 W-12	T..G.....	V PDC.....	..N .F.
				Pt. 18 W-48	T..G.....	V PDC.....	..N .F.

Fig. 3. Alignment of the deduced amino acid consensus sequences of the V3 domain (2356–2379) of sequential isolates obtained from each HCV-1b-infected patient analysed. BTW-CONS represents the deduced between-consensus amino acid sequence obtained after alignment of every sequenced clone. Amino acid residues are indicated with standard single-letter codes and dots indicate residues identical to those in the BTW-CONS sequence. Residue positions are referred to the HCV-J prototype sequence (Kato *et al.*, 1990), which is shown for comparison. Sequences corresponding to isolates from sustained-responder patients: 1–3 (very rapid responders), 4–6 (rapid responders) and 7–9 (slow responders), non-responder patients (10–15) and untreated patients (16–18) are followed by W and a number that represents the week at which the sample was obtained.

E2 gene (Forns & Bukh, 1999; Forns *et al.*, 1999; Farci & Purcell, 2000). Closer investigation of other HCV regions, such as NS5A, may be relevant, because amino acid sequence heterogeneity of this region before treatment, particularly of the ISDR, PKR-bd and V3 domains, has been related to IFN-monotherapy responsiveness and, so far, few studies have analysed it during combined treatment in small series of HCV-1b-infected patients (Gerotto *et al.*, 1999; Nousbaum *et al.*, 2000).

In the present study, and in agreement with previous observations (Enomoto *et al.*, 1996; Duverlie *et al.*, 1998; Sáiz *et al.*, 1998; Pawlotsky & Germanidis, 1999; Nousbaum *et al.*, 2000; Puig-Basagoiti *et al.*, 2001; Murphy *et al.*, 2002), no single amino acid position or motif was associated with different responses to therapy in any of the NS5A regions analysed. Furthermore, phylogenetic analyses did not show clustering of NS5A variants in relation to different responses to therapy.

Although the number of cases analysed was relatively small, there were no significant differences in the mutation rate of the different regions analysed between non-responder

and untreated patients. In fact, in these patients, mutation rates were closely similar to those observed previously in a large number of untreated patients with chronic hepatitis C of variable severity seen at our institution (Soguero *et al.*, 2000; Giménez-Barcons *et al.*, 2001; Franco *et al.*, 2003). In contrast, the median rate of fixation of mutations observed in sustained responders was 30–70 times greater, depending on the region evaluated, and led to a high number of amino acid changes during therapy, suggesting that, in responder patients, treatment exerts a selective pressure over this region.

Overall, the NS5A region remained quite stable during combined antiviral therapy and no specific amino acid or combination of amino acid changes could be related to the type of response to treatment.

In the PKR-bd, imposition of amino acid changes was quite infrequent during antiviral therapy and, when detected, they were observed exclusively in a minority of isolates from sustained responders. Thus, accumulation of nucleotide changes in the NS5A region, including the PKR-bd and the ISDR, was not usually reflected in the imposition of amino

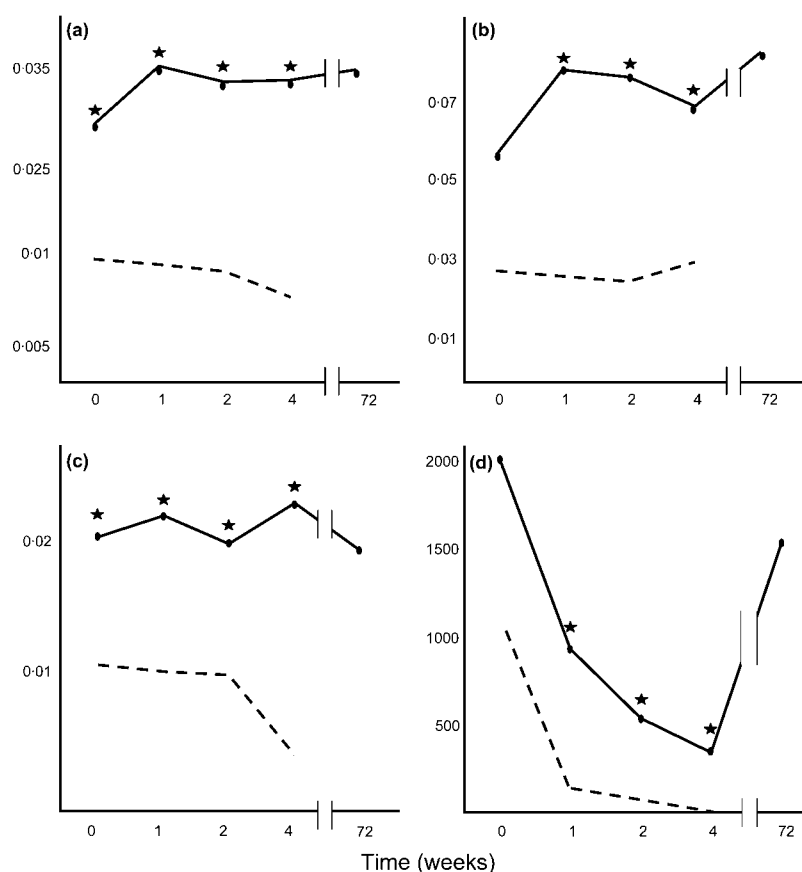


Fig. 4. V3 domain kinetics during and after therapy of genetic distance (a), K_s (b), K_a (c) and viral load (copies $\times 10^3$ ml $^{-1}$) (d). Solid and dashed lines represent mean values of non-responders and sustained responders, respectively. Statistically significant differences are indicated by an asterisk.

acid changes, indicating that these domains remain mainly stable during antiviral therapy. Similar observations have been described in untreated patients during the natural course of infection (Franco *et al.*, 2003). Previous studies have indicated that strains bearing an ISDR with a so-called 'wild-type' sequence (Enomoto *et al.*, 1996) similar to that of prototype HCV-J (Kato *et al.*, 1990) represent the sequence with the highest affinity to cellular PKR (Gale *et al.*, 1997); however, in our study, whilst one of the strains that arose during treatment (patient 7) presented a sequence with a putative higher capacity to interact with PKR, the other (patient 4) showed a more divergent sequence. Thus, our data indicate that amino acid changes in the PKR-bd alone are not necessarily responsible for the different sensitivities to antiviral therapy. In fact, it has recently been described that not only mutations in this

domain, but also changes located outside the PKR-bd, or a combination of both, affect NS5A–PKR interaction (Macdonald & Harris, 2004) and it is thus possible that mutations in NS5A regions other than the PKR-bd also contribute to decreasing the capacity of different HCV strains to interact with PKR or with some other cellular pathway, making the arising viral population more susceptible to drugs. In any case, our study could not assign any mutation or combination of mutations to such a capacity.

On the other hand, and in contrast to other regions of the NS5A, in the V3 domain, mutations were more common and occurred in isolates from responders and non-responders. Even more, analysis of sequential samples showed that, overall, the rate of fixation of mutations during therapy was higher in V3 than in other viral regions, supporting the hypothesis that this domain has a higher capacity to accumulate mutations (Inchauspe *et al.*, 1991; Nousbaum *et al.*, 2000). However, until the biological function of the V3 domain is completely elucidated, it is not possible to establish whether the mutations that arose during antiviral therapy were merely a reflection of the high capacity of this domain to accept amino acid changes with preservations of its functional role, or whether they represent a direct consequence of the pressure exerted on the V3 domain by antiviral therapy.

Table 2. Median rate of fixation of mutations in different viral regions (nucleotide substitution per nucleotide site per year)

Patient type	NS5A	PKR-bd	ISDR	V3
Sustained responders	0.3311	0.3344	0.2655	0.4611
Non-responders	0.0068	0.0053	0.0050	0.0100
Untreated	0.0046	0.0060	0.0060	<0.0150

Our results also indicate that, in treated patients, mutations were always detected during the first week of therapy and were due to the imposition of minor variants that were already present prior to treatment, strongly supporting the hypothesis that antiviral drugs do exert pressure over specific HCV NS5A variants, as suggested previously (Pawlotsky *et al.*, 1998; Polyak *et al.*, 1998; Sáiz *et al.*, 1998; Noursbaum *et al.*, 2000). For instance, in patient 6, a patient co-infected with two different HCV-1b strains, the variant represented less at baseline disappeared after 1 week of treatment. However, it should be noted that clearance of one of the co-infecting variants has also been observed during the natural course of the disease in the absence of antiviral therapy (Franco *et al.*, 2003) and, thus, imposition of minor variants and/or strains cannot be attributed exclusively to the action of the drugs in treated patients.

The pre-therapy complexity and diversity of NS5A quasi-species observed in this study were higher in non-responders than in responders, particularly in the V3 domain. Differences became more apparent during therapy, as the diversity and complexity of the quasispecies remained stable or tended to increase in non-responders, but tended to decrease in sustained responders. It is therefore possible that greater complexity and diversity of quasispecies offer the virus a better chance of having subpopulations that are able to elude antiviral therapy.

In summary, according to the observations made in the current study, fixation of mutations in the NS5A region induced by IFN and ribavirin combination therapy is uncommon, although it is more frequent in the V3 domain. Fixation of mutations occurs very early during therapy and is due to the imposition of minor variants that are already present prior to therapy. Overall, the complexity and diversity of quasispecies are lower and tend to decrease in sustained responders, whereas they tend to remain stable in non-responders. These differences are more marked in the V3 domain. Therefore, the quasispecies composition and dynamics of HCV NS5A seem to be related to responsiveness to combined antiviral therapy of patients with chronic hepatitis C.

ACKNOWLEDGEMENTS

Supported in part by a grant from Ministerio de Ciencia y Tecnología (SAF01-1997) to J.-C.S. M.G.-B. and F.P.-B. were supported by scholarships from Instituto de Salud Carlos III (ICIII) and I.F. by Asociación Española de Cooperación Internacional (AECI).

REFERENCES

Davis, G. L., Esteban-Mur, R., Rustgi, V. & 8 other authors (1998). Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. *N Engl J Med* **339**, 1493–1499.

Domingo, E. (1996). Biological significance of viral quasispecies. *Viral Hepatitis Rev* **2**, 247–261.

Duverlie, G., Khorsi, H., Castelain, S., Jaillon, O., Izopet, J., Lunel, F., Eb, F., Penin, F. & Wychowski, C. (1998). Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity. *J Gen Virol* **79**, 1373–1381.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Izumi, N., Marumo, F. & Sato, C. (1995). Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b: sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* **96**, 224–230.

Enomoto, N., Sakuma, I., Asahina, Y. & 7 other authors (1996). Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* **334**, 77–81.

Farci, P. & Purcell, R. H. (2000). Clinical significance of hepatitis C virus genotypes and quasispecies. *Semin Liver Dis* **20**, 103–126.

Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, USA.

Forns, X. & Bukh, J. (1999). The molecular biology of hepatitis C virus. Genotypes and quasispecies. *Clin Liver Dis* **3**, 693–716.

Forns, X., Purcell, R. H. & Bukh, J. (1999). Quasispecies in viral persistence and pathogenesis of hepatitis C virus. *Trends Microbiol* **7**, 402–410.

Franco, S., Giménez-Barcons, M., Puig-Basagoiti, F., Furčić, I., Sánchez-Tapias, J.-M., Rodés, J. & Sáiz, J.-C. (2003). Characterization and evolution of NS5A quasispecies of hepatitis C virus genotype 1b in patients with different stages of liver disease. *J Med Virol* **71**, 195–204.

Gale, M. J., Jr, Korth, M. J., Tang, N. M., Tan, S.-L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R. & Katze, M. G. (1997). Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* **230**, 217–227.

Gerotto, M., Sullivan, D. G., Polyak, S. J., Chemello, L., Cavalletto, L., Pontisso, P., Alberti, A. & Gretch, D. R. (1999). Effect of retreatment with interferon alone or interferon plus ribavirin on hepatitis C virus quasispecies diversification in nonresponder patients with chronic hepatitis C. *J Virol* **73**, 7241–7247.

Giménez-Barcons, M., Franco, S., Suárez, Y. & 10 other authors (2001). High amino acid variability within the NS5A of hepatitis C virus (HCV) is associated with hepatocellular carcinoma in patients with HCV-1b-related cirrhosis. *Hepatology* **34**, 158–167.

Inchauspe, G., Zebedee, S., Lee, D.-H., Sugitani, M., Nasoff, M. & Prince, A. M. (1991). Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. *Proc Natl Acad Sci U S A* **88**, 10292–10296.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. & Shimotohno, K. (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* **87**, 9524–9528.

Kumar, S., Tamura, K. & Nei, M. (1994). MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Comput Appl Biosci* **10**, 189–191.

López-Labrador, F. X., Ampurdanés, S., Forns, X. & 7 other authors (1997). Hepatitis C virus (HCV) genotypes in Spanish patients with HCV infection: relationship between HCV genotype 1b, cirrhosis and hepatocellular carcinoma. *J Hepatol* **27**, 959–965.

Macdonald, A. & Harris, M. (2004). Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* **85**, 2485–2502.

Martell, M., Esteban, J. I., Quer, J., Genescà, J., Weiner, A., Esteban, R., Guardia, J. & Gómez, J. (1992). Hepatitis C virus (HCV) circulates as a population of different but closely related

- genomes: quasispecies nature of HCV genome distribution. *J Virol* **66**, 3225–3229.
- McHutchison, J. G., Gordon, S. C., Schiff, E. R. & 7 other authors (1998)**. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N Engl J Med* **339**, 1485–1492.
- Meurs, E. F., Galabru, J., Barber, G. N., Katze, M. G. & Hovanessian, A. G. (1993)**. Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A* **90**, 232–236.
- Murphy, M. D., Rosen, H. R., Marousek, G. I. & Chou, S. (2002)**. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. *Dig Dis Sci* **47**, 1195–1205.
- Nousbaum, J.-B., Polyak, S. J., Ray, S. C., Sullivan, D. G., Larson, A. M., Carithers, R. L., Jr & Gretch, D. R. (2000)**. Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy. *J Virol* **74**, 9028–9038.
- Pawlotsky, J.-M. & Germanidis, G. (1999)**. The non-structural 5A protein of hepatitis C virus. *J Viral Hepat* **6**, 343–356.
- Pawlotsky, J.-M., Germanidis, G., Neumann, A. U., Pellerin, M., Frainais, P. O. & Dhumeaux, D. (1998)**. Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasispecies mutations. *J Virol* **72**, 2795–2805.
- Polyak, S. J., McArdle, S., Liu, S.-L. & 8 other authors (1998)**. Evolution of hepatitis C virus quasispecies in hypervariable region 1 and the putative interferon sensitivity-determining region during interferon therapy and natural infection. *J Virol* **72**, 4288–4296.
- Poynard, T., Marcellin, P., Lee, S. S. & 8 other authors (1998)**. Randomised trial of interferon α 2b plus ribavirin for 48 weeks or for 24 weeks versus interferon α 2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. *Lancet* **352**, 1426–1432.
- Puig-Basagoiti, F., Sáiz, J.-C., Forns, X. & 7 other authors (2001)**. Influence of the genetic heterogeneity of the ISDR and PePHD regions of hepatitis C virus on the response to interferon therapy in chronic hepatitis C. *J Med Virol* **65**, 35–44.
- Sáiz, J. C., Ampurdanés, S., Olmedo, E. & 8 other authors (1997)**. Hepatitis G virus infection in chronic hepatitis C: frequency, features and response to interferon therapy. *J Hepatol* **26**, 787–793.
- Sáiz, J.-C., López-Labrador, F.-X., Ampurdanés, S., Dopazo, J., Forns, X., Sánchez-Tapias, J.-M. & Rodés, J. (1998)**. The prognostic relevance of the nonstructural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. *J Infect Dis* **177**, 839–847.
- Sánchez-Fueyo, A., Giménez-Barcons, M., Puig-Basagoiti, F., Rimola, A., Sánchez-Tapias, J. M., Sáiz, J. C. & Rodés, J. (2001)**. Influence of the dynamics of the hypervariable region 1 of hepatitis C virus (HCV) on the histological severity of HCV recurrence after liver transplantation. *J Med Virol* **65**, 266–275.
- Soguero, C., Campo, E., Ribalta, T., Sánchez-Tapias, J. M., Sáiz, J. C. & Bruguera, M. (2000)**. Assessment of genotype and molecular evolution of hepatitis C virus in formalin-fixed paraffin-embedded liver tissue from patients with chronic hepatitis C virus infection. *Lab Invest* **80**, 851–856.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994)**. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Witherell, G. W. & Beineke, P. (2001)**. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. *J Med Virol* **63**, 8–16.
- World Health Organization (1997)**. Hepatitis C: global prevalence. *Wkly Epidemiol Rec* **72**, 65–72.