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IRES Complexity before IFN-alpha treatment and Evolution of the Viral Load at the Early Stage of Treatment in Peripheral Blood Mononuclear Cells from Chronic Hepatitis C Patients.

MA Thélu⁽¹⁻²⁾, V Leroy⁽¹⁻²⁾, M Ramzan⁽²⁾, T Dufeu-Duchesne⁽¹⁻²⁾, PN Marche⁽¹⁻²⁾, JP Zarski⁽¹⁻²⁾.

(1) *Département d'Hépatogastroentérologie, Hôpital Albert Michallon ; Université Joseph Fourier, Grenoble, France*

(2) *Institut National de la Santé et de la Recherche Médicale U548 ; Université Joseph Fourier Grenoble, France*

Abstract

At the early stage of treatment, IFN alpha-2a induces inhibition of HCV replication. The viral load mainly reflects the degradation rate of the viruses. However, differences in the behaviour of the viral population depend on changes which occurred in the HCV-IRES genome. In this study, cloning and sequencing strategies have allowed to generate a large number of IRES sequences from the PBMCs of eighteen patients (5 women, 13 men) with chronic hepatitis C. The HCV IRES appeared to be highly conserved structurally. However, some variability was found between the different isolates obtained: 467 substitutions with a median of 7 variants/patients. No relationship was observed between pre-treatment IRES complexity and the viral load at the beginning. However, as we considered the evolution of viral load in the PBMCs during the first 3 days of IFN alpha-2a treatment, patients could be classified into 2 groups: Group1, in which viral population continued to replicate and the Group 2, in which the viral load decreased significantly ($p=0.01727$). Positioning of the mutations on the predicted IRES secondary structure showed that the distribution of the mutations and their apparition frequency were different between the two groups. At the early stage of treatment, IFN alpha-2a was efficient to reduce the viral replication in a significant number of cases; mechanisms of response might affect the virus directly. However, pre-treatment genomic variations observed in the 5'NCR of HCV were not a parameter of later response to antiviral therapy in chronic hepatitis C patients. (244)

KEYS WORDS: HCV- IRES, PBMCs, Quasi-species, variants, viral load.

INTRODUCTION

Hepatitis C Virus (HCV) is one of the major causative agent of chronic hepatitis, which can result in cirrhosis and ultimately in hepatocellular carcinoma. The cell's first-line of defence is the IFN-induced antiviral response (Pawlotsky., 2003b). However, the antiviral mechanisms by which IFN- α induces modifications of specific immune responses and the establishment of non specific immune antiviral state in the infected cells is unknown. It has been suggested that it may act at various stages of the viral cycle and was likely mediated by several biochemical pathways involving various proteins: 2'-5'-oligoadenylate synthetase (2-5 OAS), the Mx proteins and the double-stranded RNA-activated protein kinase RNA-dependent (dsRNA-PKR) (Giannelli et al., 1993; He & Katze., 2002; Pawlotsky et al., 1995; Pawlotsky et al., 1996; Samuel., 1998; Samuel., 2001; Shimazaki et al., 2002; Tan & Katze., 2001; Taylor et al., 2005).

The basis of the treatment of chronic hepatitis C is interferon- α (IFN- α) which is currently used in combination with ribavirine, a molecule that potentiates its antiviral effects. Nevertheless, therapeutic failure is frequent and the outcome of antiviral treatment seems to depend on many factors. Indeed, many viruses possess strategies to evade the IFN responses and some virological factors are well known to play a key role in the failure of IFN treatment. Amongst them, it is known that the high rate of virus mutations leads to the generation of a mixture of closely related genomes, the quasispecies, which persist and evolved in the infected patients (Pawlotsky., 2003a; Pawlotsky., 2003c; Rumin et al., 1999; Soler et al., 2002; von Wagner et al., 2003; Vuillermoz et al., 2004).

Similar to coding regions, the 5' Non Coding Region (5'NCR) is distributed as a quasispecies. Previous studies have shown that this part of HCV genome might accumulate nucleotide substitutions, (Vizmanos et al., 1998) but it appears to be subjected strong conservatory constraints than the other regions of the HCV genome. This is probably due to the need for structural conservation of the mediated translational element: the Internal Ribosome Entry Site (IRES) located in the 5'NC region. This segment (nt 40-372), highly conserved between

isolates (Soler et al., 2002), has the potential to form a stable secondary and tertiary structure. Changes in the secondary or tertiary structure of IRES as well as changes in primary nucleotide sequence result in a decrease of efficiency of protein translation (Collier et al., 2002; Honda et al., 1999b; Kieft et al., 2001; Laporte et al., 2003; Lerat et al., 2000; Luo et al., 2003).

HCV is assumed to replicate in PBMCs on the basis of detection of HCV-RNA negative-strand by RT-PCR and in *in situ* hybridization (Moldvay et al., 1994). Interference with the cells of the immune system is a known mechanism by which viruses evade the host response and become chronically infective (Oldstone., 1997). Infection of extrahepatic tissues is supported by the finding that HCV quasispecies composition differs according to the sample type, i.e., liver, serum, or PBMCs (Cabot et al., 1997; Di Liberto et al., 2006; Fujii et al., 1996; Maggi et al., 1997; Navas et al., 1998). However, the notion of the infection in extrahepatic tissues by HCV is still controversial. Indeed, detection of HCV RNA in the PBMCs might be due to the simple adsorption of viral particles.

The aim of this study is to generate a large number of IRES sequences from PBMCs of the patients with chronic hepatitis C, in order to assess whether quasispecies distribution can be evidenced within the IRES sequences found by using cloning and sequencing strategies. Then, the IRES complexity and its relationship with viral load before IFN alpha-2a treatment will be investigated in order to know if that relationship is a prognostic factor for the response to the treatment. Finally, the relationship between IRES variability before IFN alpha-2a treatment and the evolution of viral load at the early stage of the therapy will be investigated.

PATIENTS, MATERIAL AND METHODS

Patients. Eighteen chronic HCV-infected patients were enrolled in CHU de Grenoble (France). All were included in a randomised clinical trial for testing the safety and the efficacy of high daily doses of interferon alpha-2a (Laroféron, Roche) in combination with Ribavirine. At the time of inclusion, each patient had HCV antibodies (ELISA 3), HCV RNA (PCR Monitor, Roche) positive in serum, ALT serum levels more than the upper limit of normal, and the liver biopsy compatible with the diagnosis of chronic viral hepatitis C. Genotyping was carried out after standardized RT-PCR using biotinylated primers (Amplicor, Roche). Among the eighteen HCV genotypes studied, a majority of patients were found to be infected with HCV genotype 1 [1a (n=6) -1a/b (n=1) and 1b (n=7)], 1 patient with HCV genotype 2a/2c, 1 patient with genotype 3a and 2 patients with genotype 4c/d. The main characteristics of patients are summarized in

Table I.

TABLE I. Clinical and Virological Characteristics of the Patients at Entry

Patients N = 18		
Age (years)*		44.1 ± 7.9
Sex (no of men)		13
Mode of transmission	Blood Transfusion	4
	Intravenous drug use	7
	Others / unknown	3 / 5
Histological Metavir index**	Activity/Fibrosis	2.0 ± 0.8 / 2.0 ± 0.9
	Genotype	1a -1a/1b -1b -2c - 3a - 4c/d
ALAT level*		2.3 ± 1.8
Serum Viral load (UI/mL) (log)*		6.2 ± 6.2
PBMCs viral load (UI/mL) (log)*		3.2 ± 3.2

* mean ± one standard deviation

** median

Treatment. Patients were randomised to receive an induction course of IFN alpha-2a (Laroféron, Roche) subcutaneously 18MUI/d for 2 days followed by 9MUI/d for 12 days. In addition, all patients received 3 MUI/d of IFN alpha-2a for 20 weeks followed by 3 MUI

twice a week for 24 weeks. Ribavirin (1000-1200 mg) was given in association with IFN alpha-2a for 48 weeks to all patients.

Serum viral load was determined at the start of the treatment i.e.:D0, then D2, D3, D4, W2, W4 and W12.

Response to therapy. Eighteen patients with chronic hepatitis C were strictly selected and paired according to age, sex, mode of infection and duration of disease. Then they were separated into two groups according to their response to antiviral therapy. Patients with undetectable serum HCV RNA after six-month follow-up were classified as sustained responders (SR = 9/18). Patients with continuously positive HCV RNA in the serum were considered as non-responders (NR = 9/18).

Preparation of serums and PBMCs. Peripheral venous blood was collected in EDTA-treated tubes. Serum samples were obtained by centrifugation (10 min-1400 rpm - 4°C) of the blood samples at the start of the treatment i.e.:D0. The HCV concentration was then measured in these samples by using the Amplicor Monitor Test Kit (Hoffmann-La Roche AG).

The PBMCs were isolated by Ficoll density gradient centrifugation at the start of the treatment and 72h after the start of the treatment (D4) and were stored in liquid nitrogen in RPMI medium containing 10%DMSO (3 aliquots of $>10^7$ cells for each patient).

In vivo cells culture. The frozen PBMCs pellets ($>10^7$ cells) were incubated at 37°C for 10 min with 1mL RPMI supplemented with 20% Human AB serum and the mixture was centrifuged for 10 min at 1400 rpm. PBMCs adjusted to 1×10^6 cells/well in PBS were plated in duplicate in 24-well plate and cultured at 37°C in a humidified atmosphere with 50mL/L CO₂ for 30 min. Then samples were pooled in order to obtain 2×10^6 cells.

In order to eliminate the extra cellular HCV possibly adsorbed on the mononuclear cells surface, an adapted assay was performed (Cribier et al., 1995). Each sample was treated with trypsin (final concentration 0.05%) and EDTA (final concentration 0.02%) before incubation.

Trypsin activity was then blocked by the addition of foetal calf serum (Invitrogen) (final concentration 10%) and the pellets were washed in PBS. Before manual extraction, the pellet was resuspended in 100 μ L of PBS followed by quantification with COBAS Amplicor.

In vitro cells culture. The frozen PBMCs pellets ($>10^7$ cells) were defrosted in the same way as mentioned above (10 min with 1mL RPMI supplemented with 20% Human AB serum - centrifuged for 10 min at 1400 rpm - . adjusted to 1×10^6 cells/well in PBS - plated in duplicate in 24-well plate). Then, they were cultured for 6 hours, at 37°C in a humidified atmosphere with 50mL/L CO₂ in the absence or presence of IFN at a concentration of 100 UI/10⁶ cells or presence of IFN (100 UI/10⁶cells) plus Ribavirine at a concentration of 600ng/10⁶ cells. Then the samples were pooled in order to obtain 2×10^6 cells and underwent for the trypsin treatment before quantification of the viral load.

Quantification of HCV in the PBMCs : In order to adapt the standardised method for quantification of HCV in the PBMCs (i.e.: The COBAS AMPLICOR HCV MONITOR v 2.0 standard serum protocol provides manual HCV RNA extraction, uses an automated RT-PCR to co-amplify an internal RNA quantification calibrator and the viral quantification was achieved by comparison to the internal RNA quantification calibrator), the following assays were performed: i) determination of the optimal cell number for the assay by testing 1×10^6 , 2×10^6 , 5×10^6 isolated PBMCs, 2×10^6 cells were retained; ii) the pellet was resuspended from 1 ml to 100 μ l, which represented a ten-fold increment in the target RNA to be amplified and would consequently increase the assay sensibility. Taking these modifications under consideration, the formula calculated was:

$$\frac{\text{Adsorbance HCV} \times \text{dilution factor} \times 200 \times 2 \times \text{number of cells}}{\text{Adsorbance IQS} \times \text{dilution factor}}$$

The linearity of the assay range was between 50 and 5.10^5 RNA copies / 2.10^6 cells. The results regarding the viral load were expressed as log of multiples of UI/mL (= 2.7Copies/mL).

Detection of 5'NC variants in PBMCs by cloning and sequencing. HCV specific RNA was extracted from PBMCs (100 µL) using QIAamp viral RNA Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. The 5'NCR was amplified by RT/ PCR by using the set of primers designed from the IRES region (*IRES forward (nt 21-40): gac act cca cca tga atc ac, IRES reverse (nt 360-374): gtt ttt ctt tga ggt tta gg*). PCR was carried out for 40 cycles (94°C-30 sec, 55°C-15 sec, 72°C-30sec). The final PCR products of 354 bp were analysed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under UV light. These PCR products were purified with QIAquick PCR purification kit (Qiagen) as specified by the manufacturer and quantified by UV Spectrophotometry. Amplicons were directly ligated into pCR2.1-TOPO vector (Invitrogen, San Diego, CA), 1 min at room temperature. Recombinant plasmids were used to transform *Escherichia coli* competent cells (Invitrogen, San Diego, CA). Transformants were grown on Luria-Bertani Broth agar plates containing ampicillin (100mg/ml) and incubated overnight at 37°C. More than twenty four independently isolated cDNA clones from PCR products were selected. Plasmid DNAs containing 5'NCR sequences were extracted by the alkaline lysis method with the Concert Rapid Plasmid Purification System (Life technologies, GIBCO BRL products). A total of 352/456 clones were quantified by spectrophotometry and sequenced bidirectionally by the ABI PRISM automated sequencer by using M13 universal primers. Electrophoresis data obtained by the automatic sequencer were analysed by using the sequences navigator BLAST search. Artefactual quasispecies could result from polymerase-derived errors introduced during amplification or from selection during amplification and cloning procedures. A thermostable polymerase with proof-reading functions (BRL Life Technologies, Gaithersburg, MD) was used in order to minimise these artefacts. Moreover, if incorporation errors from polymerase were implicated, an increment in mutations from single mutant to multiple mutants should have been observed, which was not the case. These two points

argued in favour of the reliability of our sequence data. Sequences were aligned and compared with the sequence prototypes or the nearest prototype (PBIL, IBCP, Lyon). Sequence alignments were reviewed manually; mutation sites were identified by the CLUSTAL alignment multiple software and edited prior to phylogenetic analysis. Sequence gaps were excluded from the analysis. The non random distribution of the observed substitutions was incompatible with artefacts related to errors made by DNA polymerase during PCR reaction. In some patients, the HCV-IRESs appeared chimeric, with successive blocks of sequence reminiscent of different genotypes. This may suggest the multiple recombination events.

Phylogenetic tree. Viral sequences were aligned by using CLUSTAL X software version 1.8. Neighbor joining analyses were performed by using DNADIST and NEIGHBOR in the PHYLIP package, version 3.572, with pairwise distances estimated by using Kimura two parameters distances. We analysed the robustness of different branches by bootstrapping (103 replicates). Tree was visualized with Tree View v32.

Statistical analysis. Results were presented as means \pm standard error of the mean or as percentages. Differences between the 2 groups were assessed by Spearman's correlation and coefficient test, Kruskal-Wallis test and Wilcoxon test. Factors with $P < 0.05$ were considered as significant.

RESULTS

I - Quasispecies distribution of the HCV-5'NCR in the PBMCs of patients.

The PBMCs from 18 patients (5 women, 13 men) with chronic HCV diseases were collected before treatment initiation. Quantification of HCV in the PBMCs was assayed according to adaptive modification of the standard method. A fragment of 341 nt-long 5'Non-coding region (5'NCR) including the IRES was analysed. After cloning in *Escherichia coli*, an average of 19 clones from each patient was sequenced and each IRES sequence was aligned with the reference sequence strands: HCV-J4 for genotype 1b (Okamoto et al., 1992), HCV NZL1 for genotype 3a (Okamoto et al., 1993) and HCV-S83 or HCV CH-563 for genotype 2 or with the closest prototype reference strains after BLAST research. Although the 5'NC region appeared to be highly conserved structurally; a quasispecies distribution was found between the different isolates obtained. Indeed, 467 substitutions distributed all along the IRES were included in 131 variants (>7 variants/patients). These variants were characterised by the presence of both major variants; without any nucleotidic changes, accounting for 1/3 of the clones studied and, the minor variants; accounting for 2/3 of the clones which undergo changes.

II - Location of the mutations on the predicted secondary and tertiary structures of HCV-IRES.

The observed 467 mutations were then positioned on the predicted IRES secondary structure (Honda et al., 1999b) (Figure 1) and out of the 341 nucleotides forming the IRES sequence, only 102 nucleotidic sites underwent mutations. It means that the nucleotidic change observed on a particular site will be found on the numerous other clones of the same patient. These particular sites, gathering numerous substitutions (> 2% of mutations), were called "clustering position". Alignment of sequences confirmed that these clustering sites did not result by cross contaminations. Indeed, in each patient, numerous sequences presented both clustering positions and minor mutations and these minor mutations were different among

isolates of the same patient. Nine clustering positions gathering 3/4 (70 % - 332/467) of the total mutations were observed. These 9 clustering positions were evident for nucleotidic sites 78, 104, 107 in domain II, gathered 35.4% of the changes (166/467); sites 183, 204, 206 in domain IIIb gathered 15.4% of the mutations (72/467); position 243 in domain IIIc and 259 in domain IIIId gathered 4.5% (21/467) and 2.5% of the mutations (12/467) respectively. In the smaller stem-loop IV; 13.1% (61/467) of mutations on position 340 were found. Out of the 467 mutations, only one mutation (U to C) on nt 343 inside the AUG-4 initiator codon was observed. As the paired regions without compensation were very important for the stability of the structure, a majority of mutations occurred in the paired regions were such that maintained the base-pairing. Out of the 467 changes observed in the viral population, only a minority (21.5%) would be expected to result in a loss of base-pairing, i.e. an alteration of the IRES structure. Changes on some positions were transferred in a concomitant way at numerous clones (29%). They acted on positions 78-104-206 and 259 and on positions 107, 183 and 340. Finally, the GGG triplet between nucleotides 266 and 268 of the hexanucleotide apical loop of stem loop III was conserved in all of the observed sequences except the clones found in two patients for which 2 mutations of nt 267 (G to C) and two deletions of nt G in position 268 have been found.

III –Relationship between pre-treatment IRES complexity and the viral load

As the global viral population infecting PBMCs was considered at the outset, the viral load was on average of $1\,591 \pm 1\,637$ UI/mL ($3.2 \log_{10} \pm 3.2$ UI/mL), whereas, the number of viral genomes in the serum was on average of $1\,660\,712 \pm 1\,617\,850$ UI/mL ($6.2 \log_{10} \pm 6.2$ UI/mL).

No relationship was observed between pre-treatment IRES variability and the viral load at Day 0 (data not shown).

IV –Evolution of viral load at the early stage of treatment

During the first three days of treatment with IFN alpha-2a, the viral load in PBMCs decreased by 50.3% ($2.9 \log_{10} \pm 3.1$ vs $3.2 \log_{10} \pm 3.2$ UI/mL - $p = 0.1341$) and by 88% ($5.3 \log_{10} \pm 5.3$ vs $6.2 \log_{10} \pm 6.2$ UI/mL - $p=0.0001322$) in the serum. A decrease occurred significantly in the serum, whereas, a decreasing tendency was noted in the PBMCs.

In parallel, the study of the evolution of viral load in *ex vivo* 6 hours cultured cells showed that these values decreased by 94.5% ($1.9 \log_{10} \pm 2.2$ vs $3.2 \log_{10} \pm 3.2$ UI/mL - $p=0.00003642$) when the cells were treated with IFN alpha-2a alone and by 94.2% ($2.0 \log_{10} \pm 2.3$ vs $3.2 \log_{10} \pm 3.2$ UI/mL - $p=0.00001015$) when the cells were treated with IFN alpha-2a plus ribavirine.

Decrease of viral load in PBMCs was more important in *ex vivo* experiment than in *in vivo* treatment. Addition of ribavirine did not affect the *ex vivo* response. No relationship was observed between pre-treatment IRES variability and the viral load at the early stage of the treatment.

The evolution of viral load during the first three days of treatment was considered and the patients might be classified into two groups:

- Group 1: included 1/3 of the patients (N=6), whose viral load in the PBMCs did not decrease during the therapy (increasing by 104%) ($3.2 \log_{10} \pm 3.3$ vs $2.9 \log_{10} \pm 3.0$ UI/mL - $p=0.4848$). In the serum, a significant decrease of viral load by 82% was noted ($5.4 \log_{10} \pm 6.1$ vs $6.2 \log_{10} \pm 6.3$ UI/mL - $p=0.09307$). As the later response to the treatment (more than 6 months) was considered, it should be notice that 4 out of 6 patients included in this group were non responders.

In parallel, a significant decrease by 83% of the viral load occurred in *ex vivo* study ($2.1 \log_{10} \pm 2.3$ vs $2.9 \log_{10} \pm 3.0$ UI/mL - $p=0.1797$) when the cells were cultured with IFN alpha-2a for 6 hours. Obviously, in *ex vivo* experiment, viral resistance to treatment has disappeared.

- Group 2: This group included the remaining 2/3 of the patients (N=12) with a significant decrease of the viral load by 77% in the PBMCs ($2.7 \log_{10} \pm 2.7$ vs $3.3 \log_{10} \pm 3.2$ UI/mL – $p=0.01727$) and by 90% ($5.2 \log_{10} \pm 5.3$ vs $6.2 \log_{10} \pm 6.1$ UI/mL – $p=0.000656$) in the serum during the first three days of the therapy. However, the later response to treatment (more than 6 months) has shown that 3 out of 12 patients of this group were non responders.

In addition, during the first 6 hours of cultured cells with IFN alpha-2a, viral load decreased by 97% ($1.8 \log_{10} \pm 2.1$ vs $3.3 \log_{10} \pm 3.2$ UI/mL – $p=0.0001955$). It was noticed that even in this group, the viral resistance mechanisms might have disappeared in the cultured cells but the differences between the evolution of viral load values in *in vivo* and *ex vivo* experiments were less notable (77% vs 97%).

Briefly, i) *in vivo*: viral load decreased in the serum of both groups (decreasing 82% and 90% respectively), but the evolution of viral load in the PBMCs of both groups was different: increasing by 104% in group 1 and decreasing by 77% in group 2; ii) *ex vivo*: as significant decreases of viral load in PBMCs occurred in both groups, however, addition of IFN alpha-2a in the cultured PBMCs of group1 was less effective than in the group 2 (83% vs 97%).

V – Analysis of relationship between viral load and IRES complexity in both groups:

Comparison between the average PBMCs viral load from group 1 and 2 at the beginning of therapy showed no significant difference ($2.9 \log_{10} \pm 3.0$ vs $3.3 \log_{10} \pm 3.2$ UI/mL – $p=0.09182$). However, in group 1: the corresponding viral population gathered 37% of the total quasispecies (49/131). The 303 mutations (303/467 – 65%) observed in these variants were mainly (254/303 – 84%) distributed on 7 clustering sites 78, 104, 107, 183, 206, 243 and 340 (Figure 2); in group 2: the corresponding viral population included 63% of the variants (82/131) which concentrated 164 mutations (164/467 – 35%). Out of these, 81/164 (49%) mutations which

composed of these quasispecies appeared on 6 clustering sites such as 78, 104, 107, 204, 206 and 259, whereas, changes on site 183, 243, and 340 were undetectable (Figure 3).

It was noted that in group1, less variant was observed than in group 2, but it gathered more changes (303 and 164 respectively - $p=0.001045$). Significant difference between group1 and group 2 for the number of clustering substitutions (254 vs 81 clustering mutations – $p= 0.0006438$) was observed. In both groups, mutations were distributed all along the IRES genome; however in group 1, majority of them were concentrated on few nucleotidic sites.

Some positions (78, 104, 107 and 206) were retrieved in both groups but mutations on these sites were encountered more frequently in group1 than in group 2 (Fig 2-3): (i) in stem-loop II, U to C at nt 78 (4.3% vs 1.5%), C to U at nt 104 (4.3% vs 1.3%), G to A at nt 107 (17.6% vs 6.6%); (ii) in stem-loop III, nucleotide C insertion at 206 (4.3% vs 1.3%). In addition, mutations C to U (6.2%) at position 183 in domain III, G to A (4.5%) at nt 243 in stem-loop IIIc and C to U (13.1%) at position 340 in stem-loop IV, occurred only in group1. In contrast, mutations C to A (3.6%) at position 204 in bulge IIIb and mutations U to C at nt 259 (2.5%) in domain IIIId were observed only in the patients of group 2.

Study of stem-loops showed minor other differences between the 2 groups. Indeed, two of the five short ORFs (Open Reading Frame) which may encode very small peptides (Han et al., 1991; Inchauspe et al., 1991) located in domain II, start at positions 85-87 (AUG-1) and 96-98 (AUG-2). Mutation on nt 87 (G to C) inside AUG-1, on positions 96 (A to G) and 97 (U to C) inside AUG-2 were encountered only in viral population from group 1. In the smaller bulge IV, mutations at nt 340 and 350, flanking both sides of the AUG-4 initiator codon were found. Upstream mutations frequency of this initiator codon was present only in group 1. In addition, one mutation was observed at nt 343 inside the AUG-4 initiator codon in group 2.

DISCUSSION

The presence of negative-strand RNA in PBMCs from infected patients has been demonstrated (Ducoulombier et al., 2004; Lanford et al., 1995; Laskus et al., 2000; Lerat et al., 2000; Moldvay et al., 1994) which suggests the existence of a potentially important extrahepatic site of HCV replication (Bain et al., 2001; Laporte et al., 2003; Roque Afonso et al., 1999; Roque-Afonso et al., 2005). Many viruses possess strategies for evading or limiting the interferon-induced antiviral response and lead to progression of a disease. HCV-IRES variability is one of the mechanisms by which the virus might escape the treatment. Indeed, strong selection pressure against any mutations in this region alters the structure of the HCV-IRES (Soler et al., 2002). Moreover, previous works (Gale et al., 1998a; Gale et al., 1998b; Odreman-Macchioli et al., 2001; Odreman-Macchioli et al., 2000) have demonstrated that correct RNA-protein interactions involving the HCV-IRES are highly dependent on both the primary nucleotide sequence and its ability to form complex of secondary and tertiary RNA structures. Recently, Dash et al (2005) have showed that IFN-alpha, -beta, and gamma inhibit replication of HCV in a cell culture model by directly inhibiting the internal translation site of HCV-IRES.

Clustering sites observed in HCV-IRES sequences were mainly located in domains II (sites 78,104,107 - 35%), III (sites 183,204,206,243,259 - 22%) and IV (site 340 - 13%) (Fig 1) and mutations on positions 107, 204 and 243 might reflect a viral phenotype that was particularly well adapted for replication in the lymphoid cells with a specifically enhance HCV translation in these cells (Lerat et al., 2000; Maggi et al., 1997; Nakajima et al., 1996; Navas et al., 1998; Shimizu et al., 1997). In spite of that, no significant difference in viral load (reflecting enhance of translational activity) was related to these nucleotidic sites in this study. These results agreed with what it was mentioned previously (Yamamoto et al., 1997): sequence variability of HCV-IRES was not appearing to correlate with any difference in HCV-RNA concentration in the PBMCs which

reflects the level of HCV replication. Obviously, viral population has established an equilibrium between the quasispecies and immune constraints.

In parallel of this quasispecies characterization, viral load of the individuals who carried these variants was studied. Before treatment, viral load in the serum was 3 log upper than in the PBMCs (6.2 log vs 3.2 log). During the first three days of *in vivo* treatment, viral load decreased more significantly in the serum than in the PBMCs (88% vs 50%). These results agreed with authors who indicated that human lymphoid cells were permissive for replication of HCV but at low-level ([Shimizu et al., 1997](#); [Shimizu et al., 1993](#)), nevertheless, HCV-RNA level in the serum remained high (5.3 log vs 6.2 log). Obviously, IFN treatment has an impact on the circulating viral particles, but the intra cellular viruses seemed to be affected in a less significant manner. Then the evolution of *in vivo* viral load during the first three days of therapy was observed, and the results allowed the patients to be classified into two groups. These two groups behaved differently according to the *in vivo* or *ex vivo* context. Indeed, during *in vivo* treatment, viral load was affected by both mechanisms: one by which the viral population escape from the treatment and continued to replicate and another by which IFN alpha-2a has an impact on the viral genome. In *ex vivo* treatment, out of the context, HCV might not replicate and might not evade the treatment or the immune pressure, as the viral load decreased significantly. These results suggested that at the early stage of the treatment, mechanisms of response affected directly on the capacity of the virus to replicate, whereas, the adaptive immunological response did not occur yet. As viral replication depends on the IRES integrity, it might be suggest that IRES complexity play a key role in this phenomenon.

Previous results ([Laporte et al., 2000](#)) have demonstrated that IRES activity was independent of the number of additional mutations detected but that the nucleotide location which played the most important role for IRES efficiency. So location and frequency of the

clustering mutations observed before therapy were investigated in order to understand the behaviour of the viral population.

The HCV-IRES domain II induces conformational changes on the ribosome which have been implicated in the decoding process (Kalliampakou et al., 2002). The introduction of mutations in this domain can cause a moderate or total loss of translational ability (Kalliampakou et al., 2002; Kim et al., 2003). In this study, three clustering sites (78-104-107) located in base paired region on the stem-loop II gathered numerous changes (35.4%). These mutations were higher in patients among whom virus continued to replicate than in patients with decrease of viral population. Viral load which reflect the ability of HCV to replicate was not affected by these mutations. However, the importance of stem-loop II for IRES function has been discussed controversially and the changes that occurred in the base-paired regions have not been demonstrated as affecting the translational activity of HCV-IRES (Da Rocha Gomes et al., 2004; Honda et al., 1999a; Kalliampakou et al., 2002; Odreman-Macchioli et al., 2001; Reynolds et al., 1996; Rijnbrand et al., 1995).

The apical stem-loop IIIb has been shown to bind initiation factor eIF3. Disruption of the secondary structure of this region results in the loss of both eIF3 binding and IRES activity (Collier et al., 2002; Odreman-Macchioli et al., 2001). In this study, changes on position 183, located in the stem loop IIIb, were often associated with the changes on positions 107 and 340 in group 1. These latest mutations observed only in the group of patients whose viral load did not change could lead to stabilisation of the IRES structure. In the same way, insertion on site 206 was associated with mutations on position 204 in group 1 and, might likely contribute to stabilise the IRES complexity.

The correct secondary structure of IIIId has also been suggested to be important for the binding of ribosomal protein S9. Indeed, mutations within the IIIId stem-loop abolish IRES activity (Kieft et al., 2002). In this study, the mutated site, gathering the most frequent changes located on the stem-loop IIIId, was on position 259 found only in the patients with decreasing

viral population. Consequently, it is suggested that the mutation encountered in this region did not allow the three-dimensional structure to be conserved between isolates of varying primary sequence and could impair viral replication. Finally, the changes observed on position 243 (likely important for maintain the IRES structure) encountered only in the patients with stabilisation or increase of viral load, might serve the virus to escape from the treatment, whereas, mutations on site 259 could expose the virus to IFN action.

The mutations observed at positions 340 and 350, flanking both sides of the AUG-4 initiator codon, inside the smaller stem-loop IV which modulated the translation during virus replication (Collier et al., 2002), were only found in the patients with increasing viral population. This result was agreed with Honda et al., (1996) who have demonstrated that mutations which destabilise the stem-loop IV restored translation to normal.

Study of the later response to treatment showed that even in group 2 with a significant decrease of the viral load at the start, some patients did not respond to the therapy at the later stage. IFN alpha-2a induced inhibition definitively affected the virus only in *ex vivo* replication. In contrast, in the group in which the virus continued to replicate during the first three days of treatment, some individuals respond in the later stage of therapy. These results might suggest that, at the early stage of therapy, the virus alone was the key of the response, and that the HCV complexity influenced the response to treatment. In contrast, all along the treatment, other mechanisms implying host immune defences were set up. These observations agreed with authors (von Wagner et al., 2003) who concluded that although quasispecies evolution during IFN therapy is common, it occurs after a wide range of time intervals after initiation of therapy. However in some cases, at the early stage of treatment, escape mechanisms were set up quickly, reflecting a fast adaptation of the virus to its environment.

Obviously some viral population are more sensitive to the therapy than others but these mechanisms remain to be explored. Although, IFN alpha-2a was efficient to reduce the

viral replication in a significant number of cases at the early stage of therapy, non responsiveness to IFN cannot be explained exclusively by the changes in quasispecies. Consequently, pre-treatment genomic variations observed in the 5'NCR of HCV are not parameters of later response to the antiviral therapy in chronic hepatitis C patients.

Several genomes and/or viral protein functions and their interactions with numerous host cell functions are likely involved in HCV resistance to the non specific antiviral action of IFN alpha-2a in the infected cells.

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