Seroconversion to hepatitis C virus alternate reading frame protein during acute infection.

Yoann Morice, Maxime Ratinier, Ahmed Miladi, Stéphane Chevaliez, Georgios Germanidis, Heiner Wedemeyer, Syria Laperche, Jean-Pierre Lavergne, Jean-Michel Pawlotsky

HEPATOLOGY

SEROCONVERSION TO HEPATITIS C VIRUS ALTERNATE READING FRAME PROTEIN DURING THE ACUTE INFECTION

Journal:	*Hepatology*
Manuscript ID:	HEP-08-0317.R1
Wiley - Manuscript type:	Original
Date Submitted by the Author:	n/a
Complete List of Authors:	Morice, Yoann Ratinier, Maxime Miladi, Ahmed Chevaliez, Stephane; Hopital Henri Mondor, Service de Baceriologie-Virologie Germanidis, Georgios Wedemeyer, Heiner; Hannover Medical School, Dept. of Gastroenterology, Hepatology and Endocrinology Laperche, Syria; Institut National de Transfusion Sanguine Lavergne, Jean-Pierre Pawlotsky, Jean-Michel; Hopital Henri Mondor, Service de Bacteriologie and Virologie
Keywords:	ARFP, antibodies, acute hepatitis C, seroconversion

SEROCONVERSION TO HEPATITIS C VIRUS ALTERNATE READING FRAME PROTEIN DURING THE ACUTE INFECTION

Yoann Morice,[1,2] Maxime Ratinier,[3] Ahmed Miladi,[1,2] Stéphane Chevaliez,[1,2] Georgios Germanidis,[4] Heiner Wedemeyer,[5] Syria Laperche,[6] Jean-Pierre Lavergne,[3] and Jean-Michel Pawlotsky[1,2]*

[1]National Reference Center for Viral Hepatitis B, C and delta, Department of Virology, Henri Mondor Hospital, University of Paris 12, Créteil, France; [2]INSERM U955, Créteil, France; [3]CNRS-UMR 5086, Institut de Biologie et de Chimie des Protéines, IFR 128 Lyon Gerland, Université Lyon I, Lyon, France; [4]Papageorgiou General Hospital, Thessaloniki, Greece; [5]Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Hannover, Germany; [6]National Reference Center for Viral Hepatitis B and C and Human Immunodeficiency Virus in Transfusion, Institut National de la Transfusion Sanguine, Paris, France

Key-words: ARFP, antibodies, acute hepatitis

FOOTNOTE PAGE

Corresponding author: Prof. Jean-Michel Pawlotsky, MD, PhD, Department of Virology, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94010 Créteil, France. Phone: +33-1-4981-2827. Fax: +33-1-4981-4831. E-mail: jean-michel.pawlotsky@hmn.aphp.fr

Abbreviations:

Financial support: This work was supported by Agence Nationale de Recherche sur le SIDA et les Hépatites Virales (ANRS). We are grateful to Erwin Sablon, Geert Maertens and Filip de Kayser (Innogenetics) for preparing the INNO-LIA HCV IV prototype assay.

ABSTRACT

The existence of hepatitis C virus proteins encoded by alternate reading frames overlapping the core-encoding region has been suggested. Several mechanisms of production have been postulated and the function(s) of these proteins in the HCV life cycle remain(s) unknown. We analyzed cases of seroconversion to an alternate reading frame protein in a group of 17 patients infected by the one of two hepatitis C virus genotype 1b strains during an outbreak in a hemodialysis unit. Three patients seroconverted and antibodies were transiently detected in another patient. Three of these patients were infected by one of the two HCV strains, whereas the strain infecting the remaining patient could not be identified. Quasispecies sequence analysis of the core-coding region showed no differences in the core or +1 reading frame sequences that could explain alternate reading frame protein seroconversion in some but not all of the patients infected by one of the HCV strains, and no such difference was found between the two strains. As differences in the structure of RNA elements could play a role in frameshift events, we conducted a predictive analysis of RNA folding by using RNAfold software. No difference was found between the patients who did and did not seroconvert to alternate reading frame protein. **Conclusion:** our findings prove that alternate reading frame proteins can be produced during acute HCV infection. However, seroconversion does not occur in all patients for unknown reasons. Alternate reading frame protein could be generated by minority quasispecies variants or variants that occur transiently.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, has a single-stranded positive RNA genome of approximately 9600 nucleotides. The genome is composed of a 5' non coding region (5'NCR), a long open reading frame (ORF) encoding the precursor polyprotein of about 3000 amino acids, and a 3'NCR. The ORF encodes 10 proteins that are generated through processing of the precursor polyprotein by cellular and viral proteases. HCV proteins comprise structural proteins (core protein and the two envelope glycoproteins E1 and E2) and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) that have various functions in the HCV lifecycle (1, 2).

In 2001, Walewski et al. used computerized sequence analysis to map dual-use regions in HCV coding sequences. The results strongly suggested the existence of a new HCV antigen encoded, at least in part, by an alternate, +1 reading frame overlapping the core-encoding region (3). This was further supported by the observation of a single stop codon in the +1 reading frame of reference HCV strains, at positions that depended on the HCV genotype (4). The detection of specific antibodies targeting this protein in patients with chronic hepatitis C, by both enzyme immunoassay and Western-blot, strongly suggested that the alternate reading frame protein(s) (ARFP) is (are) produced during HCV infection (3, 5).

Since the first report suggesting translation of an ARFP (5), several mechanisms of ARFP production have been postulated (6). The first is a +1 programmed ribosomal frameshift promoted by a cluster of 10 adenines at codons 8-11 of the HCV genotype 1a ORF, leading to the formation of a 16- to 17-kDa protein (called ARFP/F) including the first 10 amino acids of the core protein (3, 5). The adenosine-rich sequence could also induce a -1 programmed ribosomal frameshift

(7). Another potential mechanism in HCV genotype 1b infection is a double-frameshift event. The first (+1) frameshift would occur at codon 42, located in stem-loop VI of the HCV genome, and the second (-1) at codon 144 in the +1 ORF, close to the terminal stem-loops (8). Rephasing back to translation in the HCV ORF leads to production of ARFP-DFC (double frameshift core), whereas interruption of translation after the stop codon leads to the production of ARFP-1b (8). Internal initiation near codons 85-88 or at codon 26 in the core-encoding sequence has also been suggested to generate ARFPs in the +1 reading frame (ARFP-86 and ARFP-26, respectively) (9, 10).

ARFP/F can be expressed *in vitro*. It is present as a highly basic protein in the cytoplasm of transfected cultured cells, sometimes with a perinuclear distribution (11). ARFP/F is unstable, however, and is degraded by the proteasome complex rapidly after its synthesis (half-life about 10 min) (5, 11). There is some evidence that ARFPs are produced during HCV infection. Indeed, anti-ARFP antibodies have been detected with specific assays in up to 40% of patients with chronic HCV infection, and ARFP-specific T- and B-cell-mediated responses have been documented (5, 8, 12-14). However, the frequency and timing of ARFP production during infection, as well as the role of ARFPs in the HCV lifecycle and pathogenicity, are totally unknown. ARFP does not appear to play a role in viral replication, as subgenomic replicons lacking the full structural region of the genome successfully replicate *in vitro* (15). The basic nature and cytoplasmic localization of ARFP are compatible with an interaction with HCV RNA. The possible role of ARFP in the establishment of acute and chronic HCV infection is unknown.

Here we analyzed cases of seroconversion to ARFP in a group of patients infected by the one of two HCV genotype 1b strains during an outbreak in a

hemodialysis unit. Our findings prove that ARFP can be produced during acute HCV infection, but not all patients or even all patients infected by a given strain are able to mount a humoral response against it if produced.

MATERIALS AND METHODS

Patients

Six groups of patients were studied (Table 1). Group I consisted of a cohort of 17 patients (6 males and 11 females, mean age 64 years) who developed acute hepatitis C during an outbreak in a Greek hemodialysis unit (16). The route of transmission was not identified. The patients were infected with one of two distinct HCV genotype 1b strains, nine patients with strain A and seven with strain B (one patient's strain could not be genotyped) (16). The patients were monitored without treatment during the first 6 months, and blood samples were taken frequently (on average, 15±4 samples per patient) and stored at -80°C. Alanine aminotransferase (ALT) and HCV RNA levels were measured in all samples. The seroconversion profiles were characterized by using a semi-quantitative line immunoassay (Inno-LIA HCV IV prototype assay, Innogenetics, Ghent, Belgium) that semi-quantifies antibodies against eight HCV epitopes present in the core, E1, E2, NS3, NS4 and NS5 protein sequences.

Group II consisted of 24 patients with acute viral hepatitis unrelated to HCV: 9 patients had acute hepatitis A, characterized by the presence of anti hepatitis A virus IgM; 15 patients had acute hepatitis B, characterized by a seroconversion to HBs antigen (including 7 patients who presented with symptoms of acute hepatitis and 8

regular blood donors who seroconverted between two donations). Group III consisted of a cohort of 216 patients chronically infected by HCV genotype 1 strains and managed in the Hepatology Department of Henri Mondor hospital, France.

Three control groups of subjects with no markers of HCV infection were used to assess the specificity of our anti-ARFP ELISA. Group IV consisted of 187 patients managed in medical departments of Henri Mondor hospital who had no serological markers of common viral infections. Group V consisted of 122 patients infected by human immunodeficiency virus (HIV) and Group VI consisted of 93 patients infected by hepatitis B virus (HBV).

Study design

We developed and characterized a sensitive ELISA method for anti-ARFP (43-141) antibody detection. It was used to detect anti-ARFP antibodies in all available serum samples, including serial samples from Group I patients and single samples from Group II and III patients. The nucleotide and deduced amino acid sequences of the HCV core region were determined in all Group I patients. Multiple clones were generated to characterize the quasispecies distribution of the analyzed region. The ARFP sequences were deduced from the core protein-coding sequences and compared between the patients who did and did not seroconvert to ARFP. The core protein-coding sequence contains highly conserved structural RNA elements, including two consecutive stem-loops (V and VI), and two consecutive terminal stem-loops (17). In order to determine whether the production of anti-ARFP antibodies during acute HCV infection in some patients but not in others was related to structural differences in the core protein-coding sequence of the corresponding HCV

strains, we examined sequence variations and predicted their impact on the secondary structure of the core protein-coding region. The prevalence of anti-ARFP antibodies was also measured with our ELISA method in the 24 Group II patients with acute hepatitis A or B in order to assess the specificity of anti-ARFP antibody detection for HCV-related acute hepatitis and in the 216 Group III patients with chronic HCV genotype 1 infection, in order to compare it with the incidence of anti-ARFP seroconversion during acute infection. The specificity of anti-ARFP antibody detection was assessed by testing the patients in Groups IV to VI.

Anti-ARFP (43-141) antibody ELISA

For ELISA detection of anti-ARFP (43-141) antibodies, we used the synthetic peptide G97A, composed of 99 amino acids encoded by codons 43-141 and derived from the +1 ORF of the core protein sequence of HCV genotype 1b isolate HCV-JA (12). The wells of microtiter plates were coated with 200 ng per well of purified G97A peptide (10 µg/mL) by overnight incubation at room temperature in sodium carbonate buffer (50 mmol, pH 9.5). Anti-ARFP (43-141) monoclonal antibodies raised in mice immunized with the same peptide were used as a positive control. The wells were washed three times with PBS-Tween and incubated with 200 µL of blocking buffer (200 mmol/L Tris, 200 mmol/L maleic acid, 0.9 g/L sodium azid, 5 g/L bovine serumalbumin, 1% goat seru, pH 6.2) for 2 hours at 37℃. After washing, coated plates were incubated with 200 µL of human serum diluted to 1:10 with Diasorin sample diluent (Saluggia, Italy) at 37℃ for 1 hour. After washing, the wells were incubated for 1 hour at 37℃ with 200 µL of a peroxidase-conjugated anti-human IgG (Diasorin) or anti-mouse IgG antibody for the clinical specimens or positive controls,

respectively. After washing, the reaction was developed by adding a tetramethylbenzidine/hydrogen peroxide mix and incubating the plate at 37℃ for 40 min in the dark. After adding 100 µL of 0.4 N sulfuric acid, absorbance was read at 450/620 nm. The cut-off value was determined as the mean optical density value obtained with three sera from HCV-uninfected blood donors plus three standard deviations plus 0.1. A gray zone of ±20% was defined around the cutoff value.

Core and ARFP quasispecies sequence analysis

In order to generate core and ARFP quasispecies sequences, viral RNA was extracted from 200 µL of serum with the High Pure kit (Roche Applied Science, Indianapolis, France) and recovered in 50 µL of RNase-free water. After 15 min of denaturation at 75℃, 5 µl of viral RNA was reverse transcribed at 53℃ for 1 hour using 10 pmol of antisense primer CORE-AS1 (5'-CCGAACACAGGGCACGCACC-3') and 200 U of Superscript III reverse transcriptase (Invitrogen, Carlsbad, California). Samples were heated for 15 min at 70℃ to inactivate the reaction. The core protein-coding region was amplified by means of nested polymerase chain reaction (PCR) with outer primers CORE-S1 (sense, 5'-CTAGCCGAGTAGTGTTGGG-3') and CORE-AS1 (antisense) and inner primers CORE-S2 (sense, 5'-CTGATAGGGTGCTTGCGAG-3') and CORE-AS2 (antisense) (5'-TCCGCTGCCTCATACACAATGC-3'). The two PCR rounds were carried out with the Advantage 2 Polymerase Mix (BD, Palo Alto, California). The first round consisted of 5 min of denaturation at 95℃, followed by 40 PCR cycles with denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 68℃ for 45 s, and a final extension step at 68℃ for 5 min. Two microliters of the first-round

PCR product was used as a template for the second round, which consisted of 5 min of denaturation at 95°C, followed by 35 PCR cycles with denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 45 s, and a final extension step at 68°C for 5 min.

PCR amplicons were purified before ligation into the pCR4.0 plasmid (Invitrogen). On average, 24 clones per sample were sequenced. Nucleotide and amino acid sequences were aligned with the CLUSTAL W program (18). ARFP sequences were deduced from core protein sequence alignments. Sequence data from this study have been deposited in the EMBL Nucleotide Sequence Database under accession numbers AM285684 to AM286184 and AM397933 to AM397949.

Predictive analysis of conserved secondary structures in core/ARFP-coding sequences

All analyses were performed using the European HCV Database website facilities (http://euhcvdb.ibcp.fr) (19), the Clustal W program (18) and the Vienna RNA secondary structure server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (20). We predicted the impact of core protein-coding sequence variations on the RNA secondary structure by using the RNAfold program. Two HCV genotype 1b reference genome sequences from the European HCV database were used (numbered D90208 and M58335). The regions selected to generate stem-loops V and VI and the terminal stem-loops spanned nucleotides 43 to 169 and 438 to 518, respectively, using the A of the AUG initiation codon as position 1. Only nonredundant sequences were analyzed in each patient. In order to eliminate misincorporation errors at the

PCR stage, sequences harboring a nucleotide substitution at a position that was fully conserved in all the other sequences were excluded.

RESULTS

Performance of the anti-ARFP (43-141) ELISA

We evaluated the analytical sensitivity of our anti-ARFP (43-141) ELISA by testing several dilutions of an anti-ARFP (43-141) monoclonal antibody produced in mice immunized with the G97A peptide (initial concentration: 2.8 mg/mL; range of dilutions: 1/1000 to 1/128 000). The ELISA started to detect anti-ARFP (43-141) at a concentration of 2.2×10^{-2} ng/µL. By comparison, commercial anti-HCV assays can detect anti-HCV antibody concentrations of about 10^{-3} ng/µL.

The specificity of our anti-ARFP (43-141) ELISA was 99.4% (95% confidence interval: 99.3%-99.5%) when tested on samples from Group IV patients, who had no markers of common viral infections (one of the 187 patients fell in the gray zone) (Table 2). None of the 122 HIV-infected patients in Group V had anti-ARFP (43-141) antibodies. In contrast, one (1.1%) of the 93 HBs antigen-positive patients in Group VI was positive and seven (7.5%) fell in the gray zone (Table 2), suggesting cross-reactivity between the two viruses, as previously reported with another ELISA assay using a similar peptide (13).

The intra- and inter-assay coefficients of variation were determined by testing 15 sera five times each in five different runs. They ranged from 2.5% to 8.1%, suggesting that our ELISA had excellent precision and reproducibility.

Seroconversion to ARFP (43-141) in the 17 Group I patients with acute HCV infection

Seroconversion to ARFP (43-141) was studied in the 17 Group I patients who were infected by one of two distinct HCV genotype 1b strains during an outbreak in a hemodialysis unit (16). The seroconversion profiles were compared for ARFP (43-141) and for structural and nonstructural HCV proteins in 82 serum samples from the 17 patients, who were sampled roughly every month during the 6-month follow-up period.

Figure 1 shows the dynamics of anti-ARFP (43-141) antibody titers in the 17 patients. Three patients seroconverted to ARFP (43-141) during the study period (patients 1, 6 and 14), and anti-ARFP (43-141) antibodies were transiently detected in another patient (patient 3). Patients 1 and 6 were both infected by HCV strain A, whereas the strain infecting patient 14 could not be identified. Patient 3 was also infected by HCV strain A. Figure 2 shows anti-ARFP (43-141) antibody seroconversion dynamics relative to alanine aminotransferase (ALT) and HCV RNA dynamics in these three patients. Seroconversion occurred respectively 4 and 8 weeks after the ALT peak in patients 1 and 6, and at the time of the ALT peak in patient 14.

Figure 3 shows the profiles of seroconversion to ARFP (43-141) and to eight structural or nonstructural HCV epitopes of the core, E1, E2, NS3, NS4 and NS5 proteins. Fifteen patients seroconverted to the core and nonstructural proteins, while patients 13 and 16 had incomplete seroconversion profiles. Thus, the failure to detect anti-ARFP antibodies in 14 patients was not related to an inability to mount HCV-specific humoral responses.

Analysis of the nucleotide sequence of the core-coding region in the 17 Group I patients with acute HCV infection

In order to assess whether seroconversion to ARFP (43-141) was associated with specific nucleotide sequence motifs in the core-coding region that could explain the occurrence of frameshift events, we characterized the core-coding region quasispecies distribution in the 17 patients with acute HCV infection. Twenty-four clones per sample were generated and their sequence was determined at baseline (earliest available sample) in all the patients except two, for whom PCR amplification of the core protein-coding region was not possible (a patient infected with strain B who did not seroconvert to ARFP, and the patient infected by an unidentified strain who seroconverted to ARFP). As two patients infected with HCV strain A infection seroconverted to ARFP (43-141) during follow-up, quasispecies sequence analysis was repeated on the latest available sample in all the patients infected by HCV strain A (except for one patient who did not seroconvert to ARFP and in whom PCR amplification of the target region was not possible). A total of 504 full-length core protein-coding region sequences were analyzed.

The G97A peptide used to detect anti-ARFP (43-141) antibodies in our ELISA showed a high percentage of homology with the putative ARFP sequences deduced from the core protein-coding sequence in the patients, ranging from 88.5% to 91.0% (99 amino acids). In patients infected by HCV strain A, only patient 1's major quasispecies variant harbored a single nucleotide variation (C to U at position 93) relative to the strain A consensus sequence. The major variant of all strain B-infected patients had the same sequence as the consensus strain B sequence.

Several mechanisms have been forwarded to explain the production of ARFP proteins, including +1 ribosomal frameshifts at various positions of the ORF (codons 8-11, 42 and 144), and internal initiations of translation at codons 26 or 86-88 in the +1 reading frame. All of these positions were carefully examined in the viral sequences of the patients with and without anti-ARFP (43-141) antibody seroconversion (Figure 4). (i) No AGA to AAA substitution at codon 9 and no ACC to AAC substitution at codon 11, that would have created a polyA stretch (5, 21), was observed in any variant from any patient, regardless of ARFP (43-141) seroconversion (Figure 4A). (ii) The regions surrounding codons 42 and 144, which have been reported to harbor a double frameshift event (8), were highly conserved and did not differ between the patients who seronconverted to ARFP (43-141) and those who did not (Figures 4B and 4C). (iii) Codon 26 in the +1 reading frame, which has been described as a potential site for internal initiation of translation of ARFP proteins (9), had GTG and GCG sequences in 99.2% and 0.8% of analyzed sequences, respectively (Figure 4D). The sequence did not therefore differ between the patients with and without ARFP (43-141) seroconversion, and nor did the sequence spanning codons 28-35, that has been suggested to contain a signal that enhances the selection of codon 26 for initiation of translation (Figure 4D). (iv) Strong conservation was also observed in the region spanning codons 81-88, a suggested region of translation initiation in the +1 reading frame (10), and particularly the ATG sequence of codons 86 and 88. A few variations were observed in minor quasispecies variants, that did not differ according to ARFP (43-141) seroconversion status.

Overall, no differences in the core or +1 reading frame sequences were found that could explain ARFP (43-141) seroconversion in two of the patients infected by

HCV strain A, and no such difference was found between strains A and B. The studied regions were highly conserved and no sequence known to be potentially associated with a +1 frameshift was found in any variant from any patient.

Predictive analysis of conserved secondary structures in the core protein coding sequence

As differences in the structure of RNA elements could play a role in frameshift events and may not be readily identified by sequence analysis of the core protein coding region, we conducted a predictive analysis of RNA folding by using RNAfold software. The regions spanning nucleotides 43-169 and 438-518 were used to predict the structures of stem-loops V and VI and of the terminal stem-loops, respectively. Only non-redundant sequences from each patient were included in the analysis, and sequences harboring one nucleotide substitution at a fully conserved position among all the other sequences were removed in order to exclude potential PCR errors. A final set of 76 sequences spanning stem-loops V and VI (53 and 23 sequences from patients infected by strains A and B, respectively) and 69 sequences spanning the terminal loops (51 and 18 sequences from patients infected by strains A and B, respectively) was analyzed in comparison with two reference HCV genotype 1b strains.

We observed strong conservation of predicted structural elements in the core coding sequence, with only a minority of sequences generating different RNA folding predictions (5% for stem-loops V and VI, and 2.2% for the terminal stem-loops). Figure 5 shows the predicted secondary structures of stem-loops V and VI. Four folding predictions were generated, with the strain A consensus sequence, patient 1's

major quasispecies sequence, the strain B consensus sequence, and the reference sequence M58335, respectively. Stem-loop V successively includes a long 10-base-pair stem followed by a conserved bulge, a 4-base-pair stem and the 5-nucleotide loop. This structure was conserved in the HCV strain A consensus sequence, whereas an additional bulge at the beginning of the long stem was predicted in the HCV strain B consensus sequence (Figure 5). Stem-loop VI successively includes a 7-base-pair stem followed by a conserved bulge, a 7-base-pair stem, two conserved excluded adenines, a variable 10- to 13-base-pair stem, another conserved bulge, a 4-base-pair stem, and the final loop (6 nucleotides) (Figure 5).

The strain A consensus sequence structure differed from that of the reference strains and strain B by a variation in the first bulge of stem-loop VI (Figure 5). However, this difference was not found in the major quasispecies variant from patient 1, as a C to U substitution at position 93 restored the reference secondary structure (Figure 5). Thus, patients 1 and 6, who both seroconverted to ARFP (43-141), had different predicted stem-loops VI, whereas stem-loop VI was identical in patient 6 and in the patients infected by strain A who did not seroconvert to ARFP (43-141). Only one substitution distinguished patients 1 and 6 from the other patients infected by strain A, namely an A to G substitution at position 59, which altered the conserved bulge in stem-loop V and added one base pair to the stem (Figure 5). Other point substitutions were found in patient 1 and patient 6 (Figure 5).

The terminal stem-loops were also highly conserved among the different patients, as a result of strong conservatory constraints on the sequence of the core protein. No difference was found between the patients infected by strain A who did and did not seroconvert to ARFP (43-141). Rare substitutions sometimes affected the

number of predicted stem-loops (data not shown), but they bore no relationship to ARFP (43-141) seroconversion.

Prevalence of anti-ARFP (43-141) antibodies in patients with acute hepatitis A or B (Group II)

None of the 9 patients with acute hepatitis A had detectable anti-ARFP (43-141) antibodies. One blood donor with an HBs antigen seroconversion (out of 15 patients with acute hepatitis B) was found to be positive in our anti-ARFP (43-141) ELISA but was negative on repeat testing, confirming possible cross-reactivity between the two viruses.

Prevalence of anti-ARFP (43-141) antibodies in patients with chronic HCV genotype 1 infection (Group III)

Twenty-six (12.3%) of the 216 patients with chronic HCV genotype 1 infection (Group II) had detectable anti-ARFP (43-141) antibodies in our ELISA.

DISCUSSION

Our observation of seroconversion to ARFP (43-141) in three patients and transient detection in one patient with dated acute HCV infection proves that ARFPs are produced during HCV infection, that they are expressed early in the acute phase of infection, and that their production can trigger a specific humoral response. In two of the three patients who seroconverted, anti-ARFP (43-141) antibodies emerged

several weeks after antibodies against the main ORF proteins, both structural and nonstructural. This may point to late ARFP production during infection, or may simply reflect the slightly lower sensitivity of our anti-ARFP (43-141) antibody ELISA relative to commercial anti-HCV assays. In the third patient anti-ARFP (43-141) antibodies were detected one week before anti-core antibodies, and no other antibodies emerged during the short follow-up period of a few months. Our anti-ARFP (43-141) antibody assay was highly specific, virtually ruling out false-positive results. Importantly, anti-ARFP (43-141) antibodies were not found in acute viral hepatitis of other causes.

Our study population was exceptional, because the patients with acute hepatitis C were infected by one of only two HCV genotype 1b strains. This situation is ideal for studying the respective roles of viral and host factors. Indeed, two patients infected by strain A, but none of the patients infected by strain B, developed anti-ARFP (43-141) antibodies, suggesting that some HCV strains but not others express ARFP. However, the number of patients was relatively small, and a third patient with an untypeable strain also developed anti-ARFP (43-141) antibodies. More interesting is the fact that only two of the nine patients infected by the same HCV strain A experienced anti-ARFP (43-141) antibody seroconversion. In the patients who did not have detectable anti-ARFP (43-141) antibody seroconversion, it was not possible to know if ARFP was not produced, or was produced but the patients failed to mount a detectable anti-ARFP (43-141) antibody response, or the antibody response was not detectable with our assay. In this respect, it is of interest that 10 out of our 17 patients had no evidence of anti-NS5B antibodies, as shown in Figure 3, whereas it is obvious that they all produced the NS5B RNA-dependent RNA polymerase, the viral enzyme needed for replication. We were not able to study the possible role of

host factors in this variability, or the underlying mechanisms. It also remains to be determined whether or not anti-ARFP (43-141) antibody seroconversion can occur later in patients who develop chronic HCV infection. This could not be studied, as our patients were treated when the acute infection did not resolve spontaneously after 6 months of follow-up.

The prevalence of anti-ARFP (43-141) antibodies was 12.6% in our patients with chronic HCV genotype 1 infection, a rate lower than in another study (13). The difference could be due to a cohort effect (only patients infected with HCV genotype 1a were tested in the other study) or to the use of a longer antigen (160 amino acids instead of 99 in our assay) and/or a less stringent cut-off in the latter study (13). Contrary to the acutely infected patients in our study, our chronically infected patients were unrelated and were infected by different HCV genotype 1 strains. Two factors may have played a role in the production of anti-ARFP (43-141) antibodies in these patients, namely the intrinsic capacity of the infecting viral strain to produce ARFP, and host factors regulating ARFP production and/or the humoral response to ARFP.

In an attempt to identify viral characteristics potentially explaining why only some patients seroconverted to ARFP (43-141), we analyzed the sequence of the core protein-coding region quasispecies. The aim was to detect sequences known to be associated with ARFP production in major or minor quasispecies populations. We focused on sites previously implicated in ARFP production, such as the adenine cluster at codons 8-11, the frameshift codons 42 and 144, and the internal initiation codons 26 and 85-88 (3, 5, 7-10). No sequences potentially involved in ARFP production were found and no difference was seen between strains A and B, or between the HCV strains infecting patients who seroconverted and those who did not. Likewise, predictive structural analysis of the core protein-coding sequence

showed no differences between the patients who seroconverted and those who did not. These results suggest that ARFPs are probably encoded by minority variants that cannot be detected by clonal sequence analysis of serum samples. Alternatively, the expression of ARFP-encoding variants may be transient, leading to brief ARFP production that is sufficient to trigger a humoral response in some patients but not in others. The lack of seroconversion could be due to a lack of ARFP production, excessively brief ARFP expression, an expression level too low to elicit specific antibodies, or an inability of our assay to detect low levels of these antibodies. The observed conservation of predicted ARFP sequences in our patients rules out a failure of our assay to detect antibodies directed at different antigenic sequences.

In conclusion, our observation of anti-ARFP (43-141) antibody production in some hemodialysis patients acutely infected with one of two HCV genotype 1b strains during a nosocomial outbreak is the strongest argument to date that ARFPs are produced during HCV infection. Our findings also indicate that ARFP production occurs early during infection. ARFP could be generated by minority quasispecies variants or variants that occur transiently at the beginning of infection. Our findings throw no light on the possible function of ARFP. The patients who did and did not seroconvert did not differ by their clinical outcomes, severity of infection, or seroconversion profiles. The role of ARFP in the replicative cycle and pathogenicity of HCV remains to be unraveled.

ACKNOWLEDGMENTS

This work was supported by Agence Nationale de Recherche sur le SIDA et les Hépatites Virales (ANRS). We are grateful to Erwin Sablon, Geert Maertens and

Filip de Kayser (Innogenetics) for preparing the INNO-LIA HCV IV prototype assay.

REFERENCES

1. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol 2007;5:453-463.
2. Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 2007;132:1979-1998.
3. Walewski JL, Keller TR, Stump DD, Branch AD. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. RNA 2001;7:710-721.
4. Cristina J, Lopez F, Moratorio G, Lopez L, Vasquez S, Garcia-Aguirre L, Chunga A. Hepatitis C virus F protein sequence reveals a lack of functional constraints and a variable pattern of amino acid substitution. J Gen Virol 2005;86:115-120.
5. Xu Z, Choi J, Yen TS, Lu W, Strohecker A, Govindarajan S, Chien D, et al. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. EMBO J 2001;20:3840-3848.
6. Branch AD, Stump DD, Gutierrez JA, Eng F, Walewski JL. The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. Semin Liver Dis 2005;25:105-117.
7. Choi J, Xu Z, Ou JH. Triple decoding of hepatitis C virus RNA by programmed translational frameshifting. Mol Cell Biol 2003;23:1489-1497.
8. Boulant S, Becchi M, Penin F, Lavergne JP. Unusual multiple recoding events leading to alternative forms of hepatitis C virus core protein from genotype 1b. J Biol Chem 2003;278:45785-45792.

9. Baril M, Brakier-Gingras L. Translation of the F protein of hepatitis C virus is initiated at a non-AUG codon in a +1 reading frame relative to the polyprotein. Nucleic Acids Res 2005;33:1474-1486.

10. Vassilaki N, Mavromara P. Two alternative translation mechanisms are responsible for the expression of the HCV ARFP/F/core+1 coding open reading frame. J Biol Chem 2003;278:40503-40513.

11. Roussel J, Pillez A, Montpellier C, Duverlie G, Cahour A, Dubuisson J, Wychowski C. Characterization of the expression of the hepatitis C virus F protein. J Gen Virol 2003;84:1751-1759.

12. Bain C, Parroche P, Lavergne JP, Duverger B, Vieux C, Dubois V, Komurian-Pradel F, et al. Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection. J Virol 2004;78:10460-10469.

13. Komurian-Pradel F, Rajoharison A, Berland JL, Khouri V, Perret M, Van Roosmalen M, Pol S, et al. Antigenic relevance of F protein in chronic hepatitis C virus infection. Hepatology 2004;40:900-909.

14. Fiorucci M, Boulant S, Fournillier A, Abraham JD, Lavergne JP, Paranhos-Baccala G, Inchauspe G, et al. Expression of the alternative reading frame protein of Hepatitis C virus induces cytokines involved in hepatic injuries. J Gen Virol 2007;88:1149-1162.

15. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110-113.

16. Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, et al. Human serum facilitates hepatitis C virus infection, and

neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J Virol 2005;79:6023-6034.

17. Walewski JL, Gutierrez JA, Branch-Elliman W, Stump DD, Keller TR, Rodriguez A, Benson G, et al. Mutation Master: profiles of substitutions in hepatitis C virus RNA of the core, alternate reading frame, and NS2 coding regions. RNA 2002;8:557-571.

18. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-4680.

19. Combet C, Garnier N, Charavay C, Grando D, Crisan D, Lopez J, Dehne-Garcia A, et al. euHCVdb: the European hepatitis C virus database. Nucleic Acids Res 2007;35:D363-366.

20. Hofacker IL. Vienna RNA secondary structure server. Nucleic Acids Res 2003;31:3429-3431.

21. Varaklioti A, Vassilaki N, Georgopoulou U, Mavromara P. Alternate translation occurs within the core coding region of the hepatitis C viral genome. J Biol Chem 2002;277:17713-17721.

FIGURE LEGENDS

Figure 1. Kinetics of anti-ARFP (43-141) antibodies during the acute phase of HCV infection in a cohort of 17 patients infected with one of two genotype 1b strains during an outbreak in a hemodialysis unit. For each patient, optical density in the enzyme immunoassay is shown at the different sampling times. The dotted line indicates the positivity cut-off. Three patients (patients 1, 6 and 14) seroconverted, and patient 3 had transiently detectable anti-ARFP (43-141) antibodies.

Figure 2. Respective kinetics of HCV RNA (black circles), alanine aminotransferase (ALT, white squares) and anti-ARFP (43-141) antibodies (gray arrow) in the three patients who seroconverted to ARFP (43-141).

Figure 3. Seroconversion profiles in the 17 patients infected with one of two genotype 1b strains during an HCV outbreak in a hemodialysis unit. The blue bars indicate the detection of anti-ARFP (43-141) antibodies in our enzyme immunoassay; the other bars indicate the detection of antibodies directed at antigens encoded by the main HCV ORF in a prototype line immunoassay (yellow: low titer; orange: medium titer; brown: high titer).

Figure 4. Summary of quasispecies sequence analysis in the 17 patients infected by one of two genotype 1b strains during an HCV outbreak in a hemodialysis unit. Each different variant sequence is reported, with its frequency among the 504 analyzed sequences on the right. Five genomic regions that might be involved in frameshift events were studied (see Introduction and Results): (A) Codons 8-11; the HCV-1

strain known to possess a poly(A) stretch is shown for comparison; (B) sequence spanning codon 42; (C) sequence spanning codon 144; (D) sequence spanning codon 26 and codons 28-35; (E) sequence spanning codons 86-88.

Figure 5. Prediction of stem-loop V and VI secondary structures. Numbering starts at the A of the AUG initiation codon. Variable positions are indicated in bold. (A) Predicted secondary RNA structure of the consensus strain A sequence. Differences relative to the consensus sequence observed in the patients who seroconverted (patients 1 and 6) are indicated by the bold patient's number. The circled U was found in the major quasispecies variant of patient 1. Positions highlighted in gray correspond to substitutions found in both patients 1 and 6. Squared differences relative to the consensus sequence were found either in patient 1 or in patient 6. (B) Predicted secondary RNA structure of patient 1's major quasispecies sequence, which was identical to that of reference strain D90208. (C) Predicted secondary RNA structure of the consensus of strain B sequences. (D) Predicted secondary RNA structure of reference strain M58335.

Table 1. Study groups.

Groups	N	Characteristics
Group I	17	Acute HCV infection with one of two HCV genotype 1b strains (strain A and strain B) during an HCV outbreak in a hemodialysis unit
Group II	24	HCV-negative acute hepatitis A (n=9) or B (n=15)
Group III	216	Chronic HCV genotype 1 infection
Group IV	187	Patients with no markers of common chronic viral infections
Group V	122	HIV-infected patients
Group VI	93	Patients with chronic HBV infection (HBsAg-positive)

Table 2. Specificity of anti-ARFP (43-141) antibody detection with our ELISA assay in subjects with no markers of viral infection (Group III) and in patients infected by HIV (Group IV) or HBV (Group V).

Patients	N	Anti-ARF (43-141) Negative Response: n (%)	Gray Zone	Anti-ARF (43-141) Positive Response: n (%)
Group III (no infection)	187	186 (99.5%)	1 (0.5%)	0 (0%)
Group IV (HIV infection)	122	120 (98.4%)	2 (1.6%)	0 (0%)
Group V (HBV infection)	93	85 (91.4%)	7 (7.5%)	1 (1.1%)

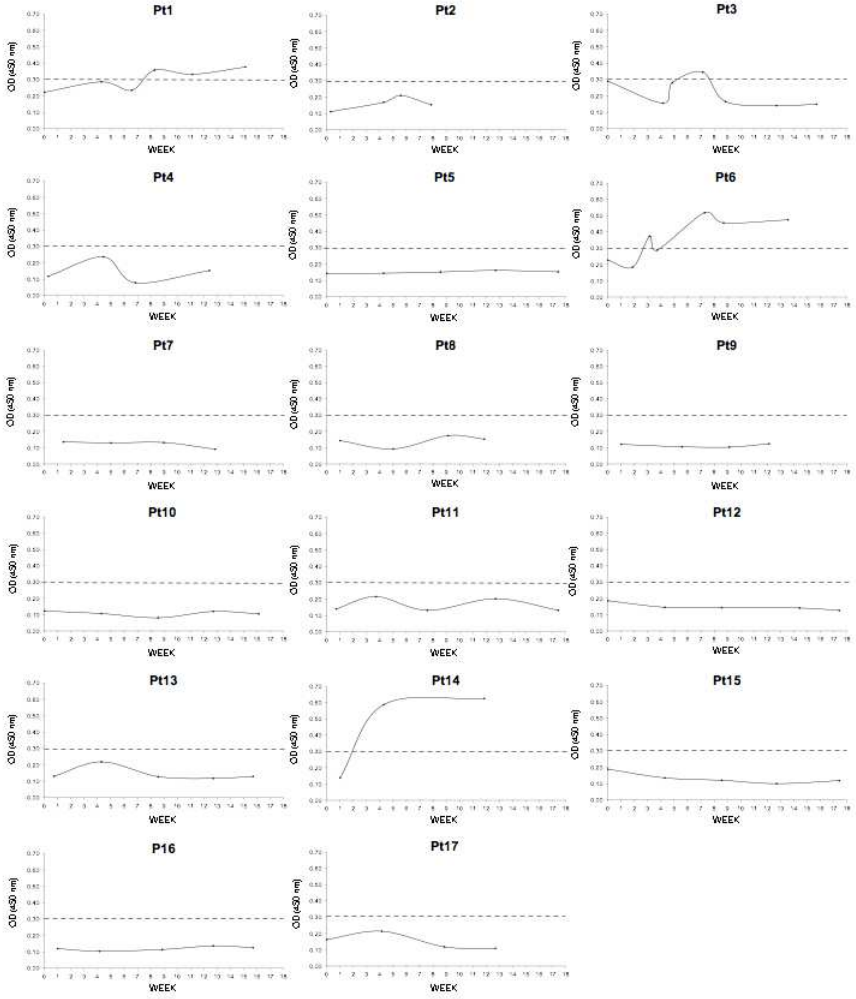
Pt1
Pt2
Pt3
Pt4
Pt5
Pt6
Pt7
Pt8
Pt9
Pt10
Pt11
Pt12
Pt13
Pt14
Pt15
P16
Pt17
OD (450 nm)
WEEK

Figure 1

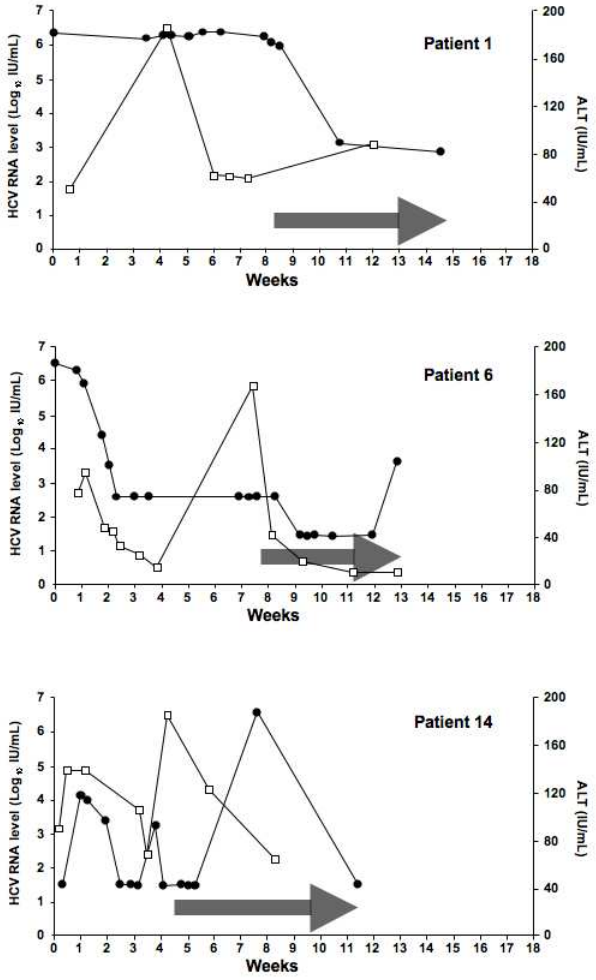
Patient 1
Patient 6
Patient 14
HCV RNA level (Log10 IU/mL)
ALT (IU/mL)
Weeks

Figure 2

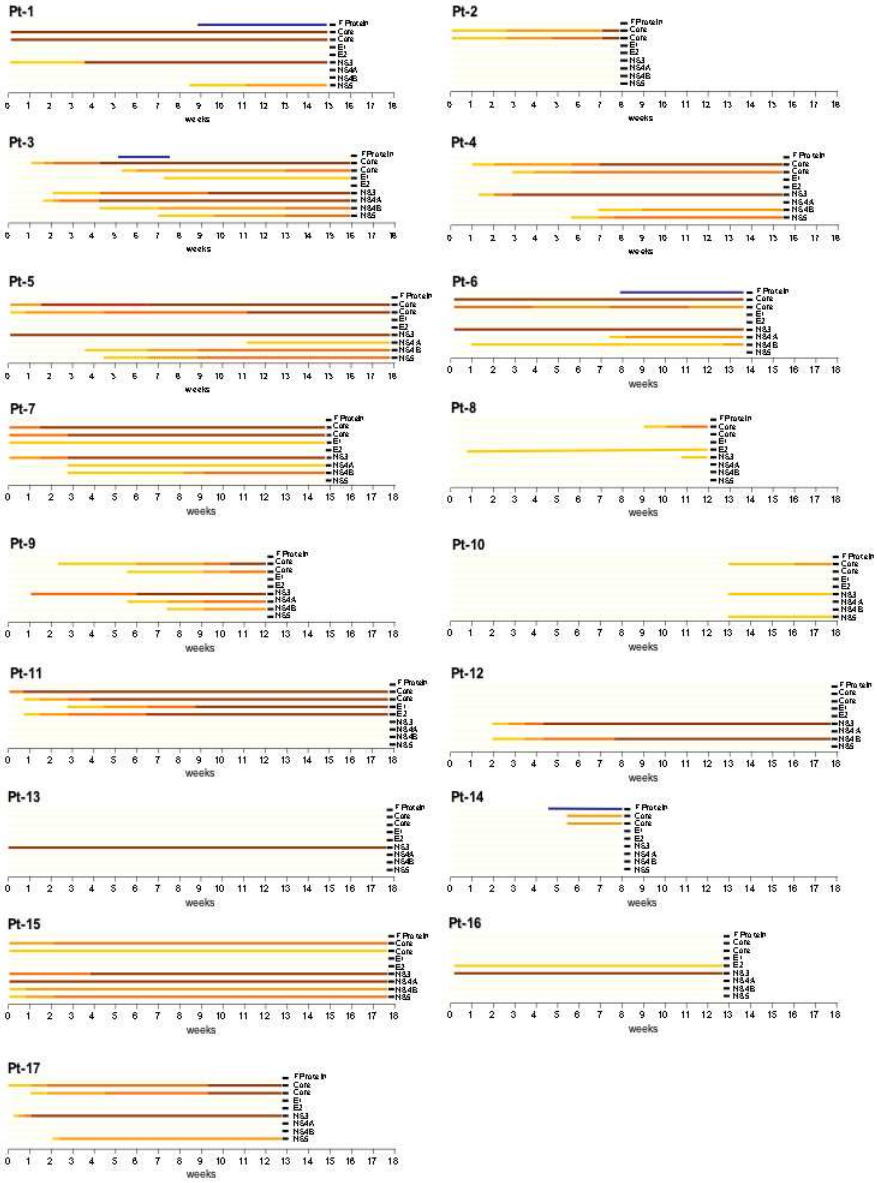

Figure 3
264x352mm (72 x 72 DPI)

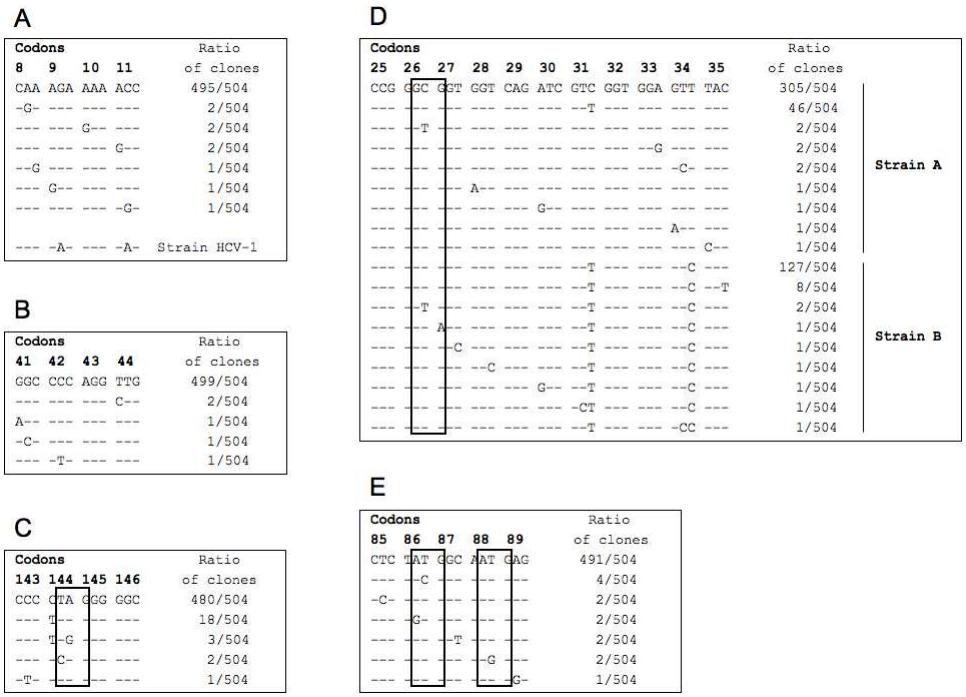

Figure 4

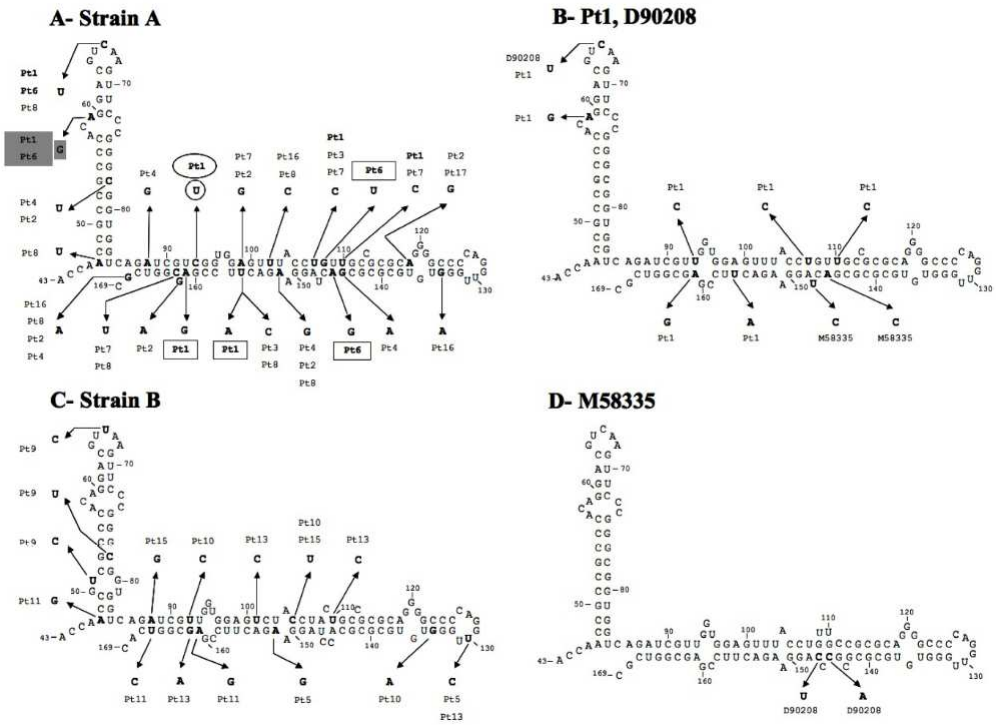

Figure 5