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Hepatitis C Virus: The Role of Molecular Mimicry in Response to Interferon Treatment

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ABSTRACT

Chronic hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease worldwide. In order for HCV to persist, the virus must escape immune recognition or inhibit the host immune response. The NS5A protein contains the interferon sensitivity-determining region (ISDR) and is able to repress dsRNA-dependent protein kinase (PKR) thus influencing the response to interferon (IFN) therapy. Moreover, patients who respond to IFN therapy have stronger antibody reactivity against the NS5A compared to IFN non-responders. Therefore, given the possible role for the ISDR in IFN resistance and differential antibody reactivity, we propose that variation in ISDR may be involved in viral immune escape and development of persistent HCV infection employing aspects of host mimicry.

In this study, pre-treatment samples obtained from HCV infected patients were used to investigate the effect of different NS5A ISDR variants on the IFN antiviral response and their involvement in immune evasion. We have identified NS5A as a homologue of the variable region of immunoglobulins (Ig). The IFN resistant genotypes had higher levels of similarity to Ig compared to IFN sensitive genotypes. Expression of NS5A-6003 (HCV genotype 1b) and NS5A-6074 (HCV genotype 2a) was able to rescue Vesicular Stomatitis Virus (VSV) from IFN inhibition and restore luciferase activity. We have observed a correlation between Ig-like NS5A structure and also antibody response with the outcome of IFN treatment.

KEYWORDS: NS5A protein, HCV persistence, immune evasion, IFN response, antibody reactivity

INTRODUCTION

Since the identification of Hepatitis C virus (HCV) in 1989 (Choo et al., 1989), it has been estimated that more than 170 million people are infected with the virus, corresponding to almost 3% of the world population (WHO, 2002). HCV chronic infection often leads to liver cirrhosis, hepatocellular carcinoma and hepatic failure (Tan and Katze, 2001). With up to 10,000 deaths each year in the United States and an estimated 230,000 new HCV infections annually worldwide, chronic hepatitis C is poised to become a serious global medical problem with considerable burden on the health care system (Lindenbach and Rice, 2001;Tan and Katze, 2001).

Interferons (IFNs) are therapeutically useful for HCV antiviral treatment. The current standard-of-care treatment for patients with chronic HCV infection consists of pegylated IFN alpha (PEG-IFN) in combination with ribavirin, for 24 weeks (genotype 2/3) or 48 weeks (genotype 1); only about half of treated patients achieve a sustained virologic response (SVR) (Fried et al., 2002). Therefore, overcoming IFN resistance remains a major challenge for effective IFN-based therapy and future management of the HCV pandemic. The first hint at a potential molecular mechanism for HCV evasion of IFN response was suggested when clinical observations showed a substantial correlation between mutations in the viral NS5A gene from certain HCV genotypes and response to IFN treatment in HCV-infected patients (Tan and Katze, 2001).

To begin to elucidate the mechanism by which select HCV variants escape the antiviral effects of IFN, Enomoto *et al*. suggested that the genetic heterogeneity within a discrete region of 40 amino acids in the carboxyl half of NS5A (2209 to 2248 aa), termed the IFN sensitivity-determining region (ISDR), was related closely to the response in Japanese patients with HCV

genotype 1b, so that patients with at least four mutations within the ISDR achieved a SVR to IFN therapy (Enomoto et al., 1996). It is known that the PKR (IFN-induced protein kinase R, a primary mediator of the IFN-induced antiviral response) binding domain (ISDR with an additional 26 amino acids) mediates disruption of PKR dimerization resulting in the repression of PKR function and the inhibition of PKR-mediated eIF2α phosphorylation (Gale et al., 1997; Gale et al., 1998). The introduction of multiple mutations within the PKR-binding region, including those within the ISDR, result in different NS5A proteins (corresponding to different HCV genotypes or quasispecies variants) having different capacities to complex with PKR and inhibit its function (Macquillan et al., 2004).

Furthermore, it has been suggested that 95% of patients who respond to therapy have stronger antibody reactivity against NS5A epitopes compared to 13% of non-responders (Frangeul et al., 1998). The loss of the immune recognition is a correlate of host mimicry. Molecular mimicry is one of the immune-evasion mechanisms viruses use to promote survival and persistence. There are several examples of viruses that express proteins that are homologous to human protein sequences involved in the regulation of cell proliferation, intercellular signaling, or immune functions, in order to avoid host defenses (Ploegh, 1998). For example, the HCV E2 protein contains both a PKR and eIF2α phosphorylation homology domain (PePHD) that competes with the genuine eIF2α and PKR to inhibit the antiviral response mediated by IFN (Taylor et al., 1999). Human cytomegalovirus (HCMV) encodes a molecular homologue of major histocompatibility complex 1 (MHC 1) proteins (UL18 gene product) (Beck and Barrell, 1988) that is directly involved in evasion of cellular immune response by inhibiting recognition and attack by natural killer cells (Reyburn et al., 1997). As well, molecular mimicry by herpes simplex virus type 1 (HSV-1) influences the development of autoimmune disease after viral

infection (Zhao et al., 1998). Molecular mimicry therefore represents another potential mechanism that may allow HCV to establish persistent infection and resistance to IFN therapy.

The discovery that the N-terminal region of HCV envelope glycoprotein E2, including the hypervariable region 1 (HVR1), is antigenically and structurally similar to human immunoglobulin (Ig) variable domains (Hu et al., 2005) made us explore the possibility that other instances of molecular mimicry might exist within the HCV genome. We reasoned that NS5A protein could also be employing the strategy of mimicry to aid in the development of HCV persistent infection and immune evasion.

Therefore, given the role for the NS5A ISDR in IFN resistance and differential antibody reactivity, we propose that variation in ISDR may also involve the aspect of mimicry. In the present study, group of ten HCV (genotype 1b) infected patients were used to characterize NS5A ISDR quasispecies and their nucleotide and amino acid sequences. The deduced NS5A ISDR sequences were then used to identify ISDR sequence similarity with other gene sequences on comparison with Ig in the protein structure database. The effect of different NS5A ISDR variants on IFN antiviral activity against the Vesicular Stomatitis Virus (VSV) and the Coxsackievirus B3 (CVB3) replication was also examined.

MATERIALS AND METHODS

Samples. HCV Samples from patients undergoing IFN Treatment: The study group consisted of ten patients chronically infected with HCV genotype 1b from British Columbia, Canada (samples 6003, 6007, 6015, 6017, 6019, 6020, 6023, 6025, 6032 and 6055). A single patient, sample 6074, was infected with HCV genotype 2a. HCV positive sample 6048 (genotype 1b) and sample 6005 (genotype 2a) were used for the amplification of the full-length NS5A gene. In each case, virus positive plasma samples were collected before IFN therapy and the viral load was assessed prior to as well as following IFN therapy using the COBAS Amplicor HCV MonitorTest, v2.0 (Roche Diagnostics, Indianapolis, IN, USA) (Larke et al., 2002). Samples for Assessing Ig Mimicry of NS5A: A complete and unabridged set of 99 published ISDR sequences (HCV genotype 1b) of known IFN sensitivity (49 responders) or resistance (50 non-responders), as published by Watanabe *et al*. (Watanabe et al., 2001) were analyzed with respect to Ig similarity.

RNA extraction and PCR. Viral RNA was extracted from patient sera using QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany) as described in the manufacturer's instructions. Viral cDNA synthesis for RT-PCR was done using random hexamer, $pd(N)_6$, and the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ, USA). Nested PCR was used to amplify the ISDR. For genotype 1b, the primary amplification was carried out with primers ISDR-F1 (sense: 5'-CACAGGTACGCTCCGGCGTGC-3') and ISDR-R1 (antisense: 5'-GCACCCGTGTACCACCGGAGGG-3'). Forty-cycle PCR amplification was performed at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 60 seconds and one cycle final elongation step at 72°C for 7 minutes. Secondary amplification was carried out with primers ISDR-F2 (sense: 5'-CCCGGTTGGGTCACAGCTCCC-3') and ISDR-R2 (antisense: 5'-

GTGGAGGGTTGTAATCCGGGCG-3') and the same cycling conditions as for primary amplification. For the ISDR region of NS5A, genotype 2a, the primary amplification was carried out with primers ISDR2a-F1 (sense: 5'-GCCGTTTTTCCGGGATGAGGT-3') and ISDR2a-R1 (anti-sense: 5'-CATCTCCTATGGTGCTCTC-3'). Secondary amplification was carried out with primers ISDR2a-F2 (sense: 5'-GTCGTCGGGTCTGACCTTCC-3') and ISDR2a-R2 (antisense: 5'-CACTGTCCGGCGTCTCCTTG-3'). The cycling conditions for primary and secondary amplification were the same as for genotype 1b. For the full-length NS5A, genotype 1b, the primary amplification was carried out with primers F1:HCV-wholeNS5A-1b (sense: 5'-GGCTG ATAGCGTTCGCTTCG-3') and R1:HCV-wholeNS5A-1b (antisense: 5'-CCTTCATCTCCTTG AGCACG-3'). Secondary amplification was carried out with primers F2:HCV-wholeNS5A-1b (sense: 5'-TATGTGCCTGAGAGCGACGCTGC-3') and R2:HCV-wholeNS5A-1b (antisense: 5'-GTCCATGTGTAGGACATCG-3'). For the full-length NS5A, genotype 2a, the primary amplification was carried out with primers F1:HCV-wholeNS5A-2a (sense: 5'-GGTCCAATGG ATGAACAGGC-3') and R1:HCV-whole NS5A-2a (antisense: 5'-GCCGCTAGCTTGATGTC CTTAAG-3'). Secondary amplification was carried out with primers F2:HCV-wholeNS5A-2a (sense: 5'-TTCCAGAGGAAACCACGTCGC-3') and R2:HCV-wholeNS5A-2a (antisense: 5'-T ATGACATGGAGCAGCACACG-3'). Amplified PCR products were analyzed on a 1.5% agarose gel and visualized in the presence of ethidium bromide under the ultraviolet (UV) transillumination. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Manufacturer's instructions were followed for purification.

Cloning and sequencing. PCR products were cloned into the TOPO TA vector using TOPO TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA, USA). The ISDR sequences were amplified from positive colonies of *E.coli* using M13 primers (Pharmacia Biotech, Uppsala,

Sweden). The ISDR amplified PCR products were sequenced using Cy5.5 labeled M13 primers and the Open Gene automated DNA sequencing system (Visible Genetics Inc., Toronto, ON, Canada).

Computer analysis of nucleotide sequences. Nucleotide sequences were compared to an online database using BLAST (Basic Local Alignment Search Tool) program found on the NCBI (National Center for Biotechnology Information) site (http://ncbi.nlm.nih.gov). Nucleotide sequences were then translated into amino acid sequences using the Translator-online tool found on the JustBio home page (http://justbio.com). The deduced amino acid sequences were compared to an online database for the best possible match using SSEARCH program found on the NPS@ (Network Protein Sequence Analysis) home page (http://npsa-pbil.ibcp.fr).

Computer Analysis of ISDR Sequences. HCV ISDR sequences were derived from patient samples, GenBank, published study, or the LANL (Los Alamos National Laboratory) HCV database (http://hcv.lanl.gov). Immunoglobulin sequences were obtained from the NREF (Non-redundant REFerence) sequence database. Sequences were aligned using BLAST v2.2.6 and ClustalW v1.82 followed by manual alignment according to the IMGT (Immunogenetics) numbering system (Lefranc et al., 2003). ISDR sequences were tested for Ig similarity using a scoring system based on a position specific scoring matrix that quantifies the physical and chemical properties of amino acids. Specifically, each amino acid of a given HCV sequence was the average of similarity scores (Dayhoff matrix) (Feng et al., 1984;Jones et al., 1992) derived on comparison with each amino acid of Ig genes using files of 111 to 1212 proteins for each Ig class [variable domain of human heavy (n=984), light kappa (n=843), light lambda (n=1212), TCR alpha (n=111), and TCR beta (n=346)]; and summed for each region of comparison.

Expression of 6xHis-tagged ISDR proteins. Carboxy terminal regions of NS5A gene (aa 2156-2295, including ISDR) from two HCV-1b NS5A variants (6003 and 6007) and HCV-2a NS5A variant (6074) were amplified using forward primers that contained an NheI restriction enzyme site (shown in italics), followed by an initiator codon ATG (underlined), 6xHis codon sequence (shown in bold), and sequence complementary to the 5' end of NS5A ISDR variant. Each reverse primer contained a sequence complementary to the 3' end of NS5A ISDR variant, stop codon TAG (underlined), and an EcoRI restriction enzyme site (shown in italics). For samples 6003 and 6007 PCR amplification was carried out using the forward primer F:HCV1b-ISDR-NheI 5'-ATTA*GCTAGC*ATG**CATCACCATCACCATCAC**CCGGTTGGGTCACAGC TCCCATGC-3' and reverse primer R:HCV1b-ISDR-EcoRI 5'-CATG*GAATTC*CTATGGAGG GTTGTAATCCGGGCG-3'. For sample 6074 PCR amplification was carried out using the forward primer F:HCV2a-ISDR-NheI 5'-ATTA*GCTAGC*ATG**CATCACCATCACCATCAC** GTCGTCGGGTCTCAGCTTCC-3' and reverse primer R:HCV2a-ISDR-EcoRI 5'-CATG*GAAT TC*CTACGGCGGATTGTAGTCGGGCCG-3'. The thermal cycler conditions for PCR amplification were: 40-cycles at 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 1 min and one cycle of final elongation step at 72°C for 7 minutes. The purified 6xHis-tagged NS5A ISDR fragments were sub-cloned into NheI and EcoRI sites of pET-17b vector (Novagen, Madison, WI, USA). The sequences of cloned genes were confirmed prior to expression. Three 6xHis-tagged recombinant proteins were expressed in *E.coli* BL21 competent cells. The recombinant protein expression was induced with 1mM IPTG (isopropyl-β-D-thio-galactoside).

Expression of N-terminal FLAG-tagged full-length NS5A. Due to the lack of RNA for samples 6003 and 6007 (genotype 1b) and 6074 (genotype 2a) the full-length NS5A could not be amplified. To overcome this problem, the ISDR region of samples 6048 (genotype 1b) and 6005

(genotype 2a) was replaced with the corresponding ISDR regions of samples 6003, 6007, and 6074, respectively. Ligation-independent cloning strategy was used where PCR amplified ISDR insert and PCR amplified ISDR-deleted NS5A containing vector had identical 5' end sequence and a different identical 3' end sequence. For the amplification of FLAG-tagged full-length NS5A (genotype 1b-6003 and 6007; genotype 2a-6074) each forward primer contained an EcoRI restriction enzyme site (shown in italics), followed by a Kozak sequence (underlined), FLAG codon sequence (shown in bold), and sequence complementary to the 5' end of NS5A variant. Each reverse primer contained a sequence complementary to the 3' end of NS5A variant, stop codon TAG (underlined), and an XbaI restriction enzyme site (shown in italics). For samples 6003 and 6007, PCR amplification was carried out using the forward primer F:HCV1b-FLAG-NS5A(EcoRI) (sense: 5'-ATTA*GAATTC*GCCACCATG**GATTACAAGGATGACGACGATA AG**TTCGGCTCGTGGCTAAGG-3') and reverse primer R:HCV1b-FLAG-NS5A (XbaI) (antisense: 5'-CATG*TCTAGA*CTAGCAGCAGACGACGTCCTC-3'). For sample 6074, PCR amplification was carried out using the forward primer F:HCV2a-FLAG-NS5A (EcoRI) (sense: 5'-ATTA*GAATTC*GCCACCATG**GATTACAAGGATGACGACGATAAG**GGCGGCTCGTG GCTCCGC-3') and reverse primer R:HCV2a-FLAG-NS5A(XbaI) (antisense: 5'-CATG*TCTAG A*CTAGCAGCACACGACGGAGTC-3'). The thermal cycler conditions for PCR amplification were: 94°C for 2 min, 40-cycles at 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 min, and one cycle of final elongation step at 72°C for 10 minutes. The purified FLAG-tagged full-length NS5A proteins were sub-cloned into EcoRI and XbaI sites of pCI-neo plasmid. Three FLAG-tagged full-length NS5A recombinant proteins were expressed in mammalian cell line Cos-1, maintained in 1x Minimum Essential Medium [1x MEM; 3% NaHCO3, 1% penicillin/streptomycin, 1% L-glutamine, and 10% fetal bovine serum (FBS)] by lipofection

using Lipofectamine™ 2000 (Invitrogen Corporation, Carlsbad, CA, USA) reagent according to the manufacturer's protocol.

Western Blot. NS5A ISDR recombinant protein expression levels were assessed using 6xHis monoclonal antibody (1:5000 dilution; BD Biosciences, Franklin Lakes, NJ, USA) and anti-mouse polyvalent Ig-alkaline phosphatase (AP) conjugated secondary antibody (1:6000 dilution; Sigma, St. Louis, MO, USA). The blots were calorimetrically developed by AP Conjugate Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer' instructions. The antigenic cross-reactivity between recombinant ISDR proteins and human IgG was detected using goat anti-human IgG-AP conjugated antibody (Fab specific; 1:15000 dilution; Sigma, St. Louis, MO, USA). Full-length NS5A recombinant protein expression was assessed using mouse anti-FLAG M2 monoclonal antibody (1:500 dilution; Sigma, St. Louis, MO, USA) and anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (1:8,000 dilution; Sigma, St. Louis, MO, USA). The protein bands were visualized by an enhanced chemiluminescence method using Western Lightning™ Chemiluminescence Reagent Plus kit (PerkinElmer, Waltham, MA, USA) according to the manufacturer' instructions.

IFN antiviral activity assay. Cos-1 cells were seeded in 6-well tissue culture plate and transfected with pCI-NS5A plasmids by lipofection (described previously). After incubation for 24 hours at 37°C CO_2 incubator, cells were treated with recombinant human IFN-α2b (Schering Canada Inc., Pointe-Claire, QC, Canada) at concentration of 400U per well or left untreated for another 24 hours. The cells were then washed two times with 1xPBS, infected with VSV or CVB3 at the multiplicity of infection (MOI) of 0.5, and incubated at 37°C CO_2 incubator for 1 hour with gentle rocking of the plate every 15 minutes. After further two washes, 2ml of complete 1xMEM (for VSV) or serum-free 1xMEM (for CVB3) was added to each well and the

cells were incubated at 37°C CO_2 incubator for further 24 hours. The supernatant was then collected and stored at -80°C.

Plaque Assay. Twenty four hours prior to the assay, L cells (for VSV) or Vero cells (for CVB3) were seeded in a 6-well tissue culture plate at a density of $7.5x10^5$ cells per well so that they were 90% confluent the following day. On the day of the assay, serial dilutions of the virus were prepared ranging from 10^{-2} to 10^{-7}. The cells were washed two times with 1xPBS and 100µl of each viral dilution per well was added to duplicate 6-well tissue culture plates. The plates were incubated at 37°C CO_2 incubator for 1 hour with intermittent rocking. After 1 hour, each well was overlaid with 3ml of 2% Agar (ONBIO Inc., Richmond Hill, ON, Canada) diluted in 2xMEM (6% $NaHCO_3$). The agar was allowed to solidify for 20 min after which the cells were incubated at 37°C CO_2 incubator for 24 hours (for VSV) or 72 hours (for CVB3). The cells where then fixed with Carnoy's Fixative (3:1 methanol to acetic acid) for 45 minutes and stained with 1% Crystal Violet dye to facilitate visualization of plaques. The number of plaques in IFN-treated cells was compared with the number of plaques in untreated cells.

Reporter gene expression system. 293T cells, maintained in 1x MEM, were seeded in a 96-well tissue culture plate and were transfected with constant amounts of the purified pCI-NS5A expression plasmids (100ng), pGL3-Control Luciferase Reporter Vector (10ng), and pRL-SV40 Vector encoding Renilla luciferase (10ng) using Lipofectamine™ 2000 reagent to a total of 120ng DNA per well. Another set of 293T cells were transfected with the pcDNA3.1-PKR (100ng) in addition to the same combination of DNA plasmids to a total of 220ng DNA per well. Twenty four hours post transfection, the cells were treated with recombinant human IFN-α2b at concentration of 20U per well or left untreated for another 24 hours.

Luciferase Assay. Twenty four hours post IFN treatment, Dual-Glo™ Luciferase Assay System (Promega Corporation, Madison, WI, USA) was used to measure luciferase activity in 293T cells following manufacturer's instructions. Briefly, a 75µl of Dual-Glo™ Luciferase Reagent was added to each well containing 75µl of the culture medium and mixed on a rocking platform for 10 minutes. After 10 min incubation, 75µl of Dual-Glo™ Stop & Glo® Reagent was added to each well and mixed on a rocking platform for 10 minutes. The firefly luminescence in Relative Light Units (RLU) was measured using luminometer (Glomax Multidetection System, Promega Corporation, Madison, WI, USA).

RESULTS

The NS5A ISDR region shares similarity with Ig variable regions. HCV genotype 1b positive plasma samples obtained from a group of ten patients prior to IFN treatment were used to assess the sequenced relationship of the ISDR region relative to IFN response. Plasma samples were also collected to assess the viral load at weekly intervals after the onset of IFN therapy. Although the majority of patients experienced a reduction in viral load, only five of ten showed this response to IFN treatment during the early stage of IFN therapy (6019, 6020, 6023, 6025 and 6055); the other five showed some resistance to the IFN treatment (6003, 6007, 6015, 6017 and 6032) (Figure 1). This observation was consistent with the pattern normally seen to result from IFN treatment where about 50% of patients are resistant to the treatment (Fried et al., 2002;Ghany et al., 2009). Among the group with higher viral load, patient 6003 was the most resistant to the initial IFN treatment, possessing the highest viral load before IFN treatment (860,000 IU/ml) that remained unchanged through eight weeks of IFN treatment (Figure 1). Six to ten molecular clones of the ISDR region derived from each patient's sample were sequenced prior to IFN treatment. Surprisingly, comparison of sample 6003 ISDR amino acid sequence with the prototype HCV 1b ISDR amino acid sequence (HCV-J) revealed one amino acid substitution at position 2218 where histidine was replaced with cysteine (H→C) in 100% of the clones (10/10), while the other samples contained multiple mutations within this region (Table I).

HCV genotype 2a positive plasma sample (6074) was also obtained prior to IFN treatment. As it was the case with HCV genotype 1b samples, this sample was also collected at weekly intervals to evaluate the viral load after the start of IFN therapy. In contrast to HCV genotype 1b samples, sample 6074 showed complete response to IFN treatment only one week after the onset of therapy that was sustained throughout eight weeks of IFN treatment (Table I).

Molecular clones of the ISDR region sequenced prior to IFN treatment revealed three amino acid substitutions when compared to prototype HCV 2a ISDR amino acid sequence (HCV-J6) (Table I). However, compared to prototype HCV 1b ISDR amino acid sequence this sample contained numerous amino acid substitutions in addition to a deletion of four amino acids. Therefore, given the complete initial response to IFN treatment, sample 6074 was treated as IFN sensitive HCV genotype 2a variant in subsequent experiments.

To begin to assess the function of the ISDR, the NPS@ SSEARCH sequence alignment program was used to identify ISDR sequence similarity with other gene sequences in the protein structure database. Among the ISDR sequences of ten patients, sample 6003 amino acid sequence was detected to share high similarity (37.5% aa identity) with IgG kappa light chain variable region (IgVLκ) (Figure 2A). When sequence gaps were optimized and alignment employed a panel of representative kappa light chains the amino acid identity increased to 47.5% (Figure 2B). The H2218→C change makes the ISDR sequence converge with an invariant C in Ig that may contribute to an Ig-like function.

Sequence alignment analysis of NS5A with Ig and T cell receptors. To examine the similarity between NS5A and Ig, we aligned the C-terminal sections of NS5A sequences representing the six major HCV genotypes with sequences of various Ig types. The computer generated alignments using BLAST identified the regions of highest sequence similarity to the variable region of Ig molecules to be within a region of 104 amino acids of NS5A, including the ISDR, spanning amino acids 2193-2295.

To better align these sequences, the IMGT (Immunogenetics) unique numbering system was used because it allows comparison among members of the Ig superfamily (Lefranc et al., 2003). This numbering is derived from the sequence alignment and 3-D structural comparison of

more than 5,000 sequences of the Ig superfamily members and provides a definition of the highly conserved framework regions (FR) that support the antigen binding site formed by complementarity determining regions (CDR). Therefore, in the IMGT unique numbering system, conserved amino acids from FR always have the same number whatever the Ig or T cell receptor (TCR) chain type, whatever the domain (variable or constant) and whatever species they come from. Examples of conserved amino acids are: cysteine at the position 23 (C_{23}), tryptophan at the position 41 (W_{41}) and leucine at the position 89 (L_{89}).

Using the IMGT sequence alignment system we were able to identify twelve completely conserved amino acids (S_9, S_{10}, S_{12}, L_{19}, C_{23}, L_{39}, W_{41}, R_{43}, Q_{44}, G_{47}, S_{83}, and A_{100}) among NS5A and Ig types (Figure 3). These NS5A positions were then used as references to manually complete the IMGT alignment with the FR of Ig and TCR. This allowed us to align FR1, FR2 and FR3 as well as CDR1 and CDR2 of Ig and TCR with corresponding regions in NS5A.

The majority of amino acid positions in NS5A corresponded to identical amino acids found among Ig members (all masked amino acids in Figure 3A) and most of the highly conserved Ig amino acids were maintained in NS5A (masked red amino acids in Figure 3A). Most importantly, 17 amino acid positions were highly conserved ($\geq$ 70% shared identity) among NS5A sequences and all or individual Ig groups including the light (kappa and lambda) and heavy as well as TCR (alpha and beta) chains (red bars in Figure 3B). The highly conserved site at amino acid position 53 contained an alternative amino acid of similar chemistry that was in common with Ig and TCR groups (masked blue amino acid in Figure 3A).

Quantification of NS5A, Ig and TCR sequence similarity. To determine a numeric measure of amino acid similarity between Ig groups (light kappa, light lambda, heavy, TCR-alpha and TCR-beta chains) and NS5A, the Dayhoff matrix that quantifies the physical and

chemical properties of amino acids was used. An algorithm was employed where the identical amino acids were given a score of 1,500 (1,000 x identity score of 1.5) while dissimilar amino acids were given a score proportionate to their similarity scores. As a result, using this scoring system we were able to determine the similarity score for each of the 673 NS5A sequences (genotype 1 to 7) when compared to Ig groups (Figure 4). The IMGT similarity scores were significantly higher for Ig heavy chain variable region compared to other Ig classes ($p<0.0001$) suggesting specific rather than random relationship of NS5A to Ig types. The sequence similarity of NS5A to each of the Ig groups was also significantly higher than sequence similarity calculated for random sequences (random model; $p<0.0001$) and most importantly randomized Ig (light kappa chain) and NS5A consensus sequences ($p<0.0001$, respectively) (Figure 4).

We also analyzed the alignment of NS5A and the variable region of human Ig and TCR genes with respect to different HCV genotypes. The similarity scores were higher for the resistant genotype, genotype 1a and 1b, compared to the sensitive genotype, genotype 2 (data not shown).

NS5A sequence similarity analysis with respect to IFN treatment. It has been shown that only 13% of IFN non-responders (NR) have antibody reactivity to NS5A compared to 95% of IFN responders (R) (Frangeul et al., 1998). We hypothesized that NS5A protein possessing ISDR regions that have sufficient similarity to Ig are not recognized by the immune response by virtue of their similarity to host proteins. As host proteins are not recognized by the immune response due to tolerance mechanisms it would be predicted that NS5A would benefit from a lack of immune recognition. Given this hypothesis and the fact that 13% of IFN NR HCV 1b NS5A is immunogenic but that this proportion increases to 95% for IFN R, we assessed the

changes in Ig similarity for the ISDR in IFN R. The mutations in ISDR of IFN R could increase, decrease or not change with respect to Ig similarity.

This published data is supported by our alignment analysis where ISDR region of 50 HCV genotype 1b IFN NR and 49 HCV genotype 1b IFN R was aligned with the variable region of human Ig and TCR genes (Figure 5). Interferon NR that were less immunogenic had a significantly higher similarity scores compared to IFN R which were more immunogenic and had lower similarity scores ($p<0.0001$). Alignment with Ig heavy chain variable region had a higher similarity scores compared to alignments with other Ig groups or all of them combined.

To determine the predicted percentage of NS5A reactivity of IFN R for total or each Ig group, we used 13% of reactivity of IFN NR (Figure 6A) as a base value, assuming that reduced similarity relative to IFN NR became serologically responsive, in addition to the proportion of IFN R samples that were less similar to Ig groups relative to the score of IFN NR. The data in Figure 6B indicates that IFN R with lower similarity scores were more similar to the proportion that would be expected to be immunogenic. The reduced similarity score averaged from all Ig groups was predicted to result in increased immunoreactivity by 88.51% (75.51% added to the baseline level of 13%) which is similar to the observed level of 95.0%. The predicted immunoreactivities of lambda, TCR-alpha and TCR-beta Ig groups were most similar (96.67%, 98.71% and 88.51%, respectively) to the observed level of 95.0% with lesser predicted immunoreactivity levels for kappa and heavy chain Ig groups (64.02% and 82.39%, respectively).

NS5A is structurally similar to IgG. Given the sequence similarity of NS5A to Ig and observed differential similarity between resistant and sensitive genotypes (genotypes 1 and 2,

respectively), we wanted to determine whether recombinant NS5A proteins were recognized by anti-IgG antibody and to what degree.

Out of the original ten samples, three NS5A ISDR variants (genotype 1b - 6003 and 6007; genotype 2a - 6074) were expressed in *E.coli*. All three ISDR variants were seen to bind antibody to human IgG Fab fragment, however to different extents (Figure 7B). ISDR-6003 recombinant protein with significant initial IFN resistance reacted more strongly than ISDR-6007 (lower initial IFN resistance) and ISDR-6074 recombinant protein that was initially IFN sensitive as well. This observation suggest structural similarity between NS5A and human Ig molecules and demonstrates the Ig-like nature of NS5A ISDR since both Ig and NS5A are bound by a common ligand, specifically anti-IgG antibody (Cohen I.R., 2004).

Effects of NS5A ISDR variants on the IFN antiviral activity. One of the characteristics of HCV is resistance to the antiviral action of IFN, but this resistance can vary among isolates. To measure the possible inhibitory effects of NS5A on the antiviral activity of IFN, we analyzed whether the expression of NS5A proteins containing ISDR variants from samples 6003, 6007 or 6074 in Cos-1 cells interfered with the action of IFN on the replication of two different IFN-sensitive viruses, VSV and CVB3. Cos-1 cells were transfected with NS5A variants under control of the CMV promoter, empty pCI-neo plasmid or non-transfected and then treated with IFN or left untreated for 24 hours to confer the antiviral state. The cells were then infected at an MOI of 0.5pfu/cell of VSV (Figure 8 – A and B) or CVB3 (Figure 8 – C and D). The yield of infectious virus was determined by plaque assay to measure the antiviral activity of IFN by comparison with non-treated controls (cells and pCI-neo).

As shown in Figure 8A, IFN treatment prior to VSV infection suppressed virus production in non-expressing control cells (cells and pCI-neo) and thus only showed 14.3% and

14.5% VSV replication, respectively. In contrast, VSV replicated to 38.8% of the untreated level in the NS5A-6003 expressing cells therefore indicating a two-fold increase of plaque formation compared to non-expressing control cells ($p<0.05$) (Figure 8B). The enhanced replication due to NS5A gene expression in IFN treated cells was evident on comparison between pCI-neo vector control or on comparison of IFN treated to untreated samples (Figure 8A). This increase could be clearly attributed to a specific inhibition of the antiviral action of IFN, since no increase in VSV titer was observed in Cos-1 cells as a result of the sole expression of NS5A proteins. IFN-mediated repression of virus production was also inhibited by expression of NS5A-6074 (about three-fold) ($p<0.05$). In contrast, expression of NS5A-6007 did not repress IFN-mediated inhibition of virus production. Induction of NS5A proteins (6003, 6007 and 6074) had no significant effect on virus production in untreated cells.

No difference in viral replication could be seen for CVB3 (Figure 8 - C and D). This data indicated that expression of NS5A gene of HCV 1b resistant genotype was able to suppress IFN inhibition and result in enhanced viral replication where the IFN resistant NS5A of 6007 did not enhance replication; however expression of NS5A from an HCV genotype 2a isolate (6074) was also able to rescue the virus.

Effect of NS5A ISDR variants on protein synthesis. The effects of NS5A ISDR variants on PKR activity were measured by quantifying the translation product from luciferase reporter gene. A Dual-Glo™ Luciferase Assay System, consisting of two luciferase reporter genes, was used to monitor NS5A ISDR's translation stimulatory effect. 293T cells transiently transfected with NS5A ISDR variants (+/- PKR) were simultaneously transfected with the two luciferase reporter plasmids and, after IFN treatment, firefly luciferase luminescence [shown as relative firefly luciferase units (RLU)] was measured using luminometer.

The transfection efficiency of 293T cells was monitored by detecting the expression of the green fluorescent protein (GFP). The 293T cells were transfected with the GFP and its expression was detected by a fluorescent microscope (Olympus BX50) (Figure 9C).

The expression of NS5A-6003 and NS5A-6074 showed only a slight reduction of luciferase activity in PKR(-) IFN(-) cells compared to control pCI-neo cells (Figure 9A). In contrast, expression of NS5A-6007 and Reovirus Type 3 Dearing S4 gene (T3DS4) (positive control) resulted in an increase in luciferase activity (Figure 9A). The 293T cells that were transfected with PKR and treated with IFN [PKR(+) IFN(+)] resulted in a further decrease in the luciferase activity compared to PKR(-) IFN(-) cells. The luciferase activity was restored by the expression of NS5A-6003 and NS5A-6074 ($p<0.05$, respectively), which was repressed by PKR(+) IFN(+) treatment (Figure 9B). The expression of T3DS4 also restored luciferase activity ($p<0.05$). The expression of NS5A-6007 did not however restore luciferase activity relative to untreated samples indicating that the NS5A-6007 gene increased protein synthesis in the absence of IFN but not in the presence of IFN.

DISCUSSION

High prevalence of HCV persistence after viral infection and the efficient replication of HCV in hosts after seroconversion indicate that HCV possesses properties for evading the host's natural immune responses. The IFN response is one of the body's first defense mechanism to limit or prevent the establishment of viral infection (Sen and Ransohoff, 1993). The ability of a given virus to establish a productive infection thus depends in part on the ability of the virus to circumvent the IFN response, which includes the transcriptional induction and functional activation of PKR. In many cases, the relative ability of a virus to establish a productive infection correlates well with its ability to repress PKR function (Katze, 1995). In addition, escape from the host immunosurveillance system is another possible mechanism underlying persistent HCV infection. Accumulated data suggest that HVR1, located at the amino terminus of E2 protein, is a critical neutralization domain of HCV (Farci et al., 2000;Kato, 2001). During HCV infection, antigenic drift of HVR1 results in antibody escape mutants that are not recognized by the immune response and are involved in the establishment of persistent infection. Moreover, the amino terminal region of E2 was shown to share similarity with Ig variable domains and the degree of similarity was correlated with viral immune escape and persistence consistent with a model of immune evasion by host mimicry.

In the present study, we investigated the effect of different NS5A ISDR variants on the IFN antiviral activity. We also explored the possibility that NS5A protein may also be involved in immune evasion and development of persistent HCV infection.

Immunoglobulin mimicry by NS5A. We have identified, here in this study, the HCV NS5A protein as a homologue of the variable region of Ig. We showed that the carboxy terminal region of NS5A, encompassing a region of 103 amino acids (aa 2193-2295) including the ISDR

(aa 2209-2248), is structurally and antigenically similar to the human Ig variable domains. Furthermore, both NS5A and Ig were shown to bind anti-IgG antibodies demonstrating the Ig-like nature of the NS5A and therefore satisfying the operational definition proposed for molecular mimicry where both molecules were bound by a common ligand (Cohen I.R., 2004). We also showed that HCV genotypes that are IFN sensitive possessed lower levels of similarity compared to IFN resistant genotypes that had higher levels of similarity. Furthermore, IFN sensitivity of HCV 1b IFN treatment NR correlated with the degree of similarity, where mutations that increased Ig similarity had increased IFN resistance and conversely, mutations that decreased similarity were associated with decreased resistance to IFN. Taken together these data indicate a conserved structure among NS5A and Ig-like genes rather than a chance association of amino acid in the variable CDR. Several panels of random sequences as well as randomized consensus sequences demonstrated that the levels of Ig group similarities were statistically significant relative to the control groups.

Our findings constitute further evidence of ISDR involvement in viral IFN resistance and also provide insight into the structural basis for the function and biochemical interactions of the HCV NS5A protein in virus evasion of the IFN response. Mutations that change Ig similarity had a significant impact on the response to IFN treatment. Therefore, using Ig similarity as a scale, the outcome of IFN treatment could be predicted in the majority of instances. However the occurrence of high similarity of ISDR to Ig was not predictive of IFN resistance in some HCV 1b IFN treatment R. If ISDR function was due to a binding specificity that is controlled by Ig similarity, then it would be expected that in addition to maintenance of conserved features of Ig a particular unique sequence embodied in the HCV 1b genotype is required to maintain binding properties and that such mutations may not be seen to change the similarity to the consensus

sequence of Ig. Thus in addition to the conserved features of Ig, other amino acids that correspond to variable sequences of the antibody binding site will be expected to contribute to binding to specific ligands. Further work is needed to characterize the role of mutations in affecting the Ig-like nature and function of the ISDR.

Inhibition of the IFN response by NS5A. Although IFN resistance is stronger in patients infected with HCV genotype 1 than those infected with genotype 2, the underlying mechanism remain poorly understood. The ISDR region was originally identified by genomic sequencing as a site of NS5A sequence heterogeneity within IFN sensitive HCV genotype 1b viruses (Tan and Katze, 2001). Whereas expression of the entire HCV polyprotein effectively inhibits IFN induced JAK-STAT signaling in cultured cells, NS5A expression is only partially effective at blocking the antiviral effect of IFN (Khabar and Polyak, 2002). Previous studies have shown that expression of genotype 1b NS5A protein is sufficient to partially inhibit the antiviral activity of IFN (Song et al., 1999;Polyak et al., 2001). In these reports, the inhibitory effect of NS5A on IFN antiviral activity was strong against EMCV (Encephalomyocarditis virus) and only partial against VSV replication.

To characterize how NS5A affects the antiviral activity of IFN, the effects of NS5A ISDR variants (genotype 1b - 6003 and 6007; genotype 2a - 6074) on antiviral activity of IFN against VSV and CVB3 infection were examined. IFN treatment of Cos-1 cells reduced the yield of infectious virus following VSV infection in non-NS5A-expressing cells, indicating the establishment of an antiviral state in these cells. We observed that expression of NS5A proteins had no effect on CVB3 growth, whereas expression of NS5A-6003 and NS5A-6074 increased the replicative efficiency (and thus yield) of VSV by two-fold and three-fold, respectively. The expression of NS5A-6007 had no effect on VSV yield. A possible explanation for this difference

is that NS5A may inhibit IFN activity by multiple mechanisms and it employs different domains to affect different aspects of IFN function. As such, HCV genotype 1b NS5A-6003 and genotype 2a NS5A-6074 may exert differential inhibitory effects on the IFN-stimulated effectors that inhibit VSV, but exhibit similar inhibitory effects on the effectors that inhibit CVB3. The increase in VSV yields by NS5A-6003 and NS5A-6074 was a result of the inhibition of the antiviral action of IFN, since we observed no increase in VSV by the expression of NS5A proteins alone. In actual fact, VSV yields were slightly lower in the non-IFN treated cells expressing NS5A proteins compared to non-expressing control cells. The reason for this is not known, but may be related to the observation that high level expression of NS5A proteins can affect cell metabolism (Macdonald and Harris, 2004). High-level expression of HCV proteins might be toxic to cells; cells were unable to grow and died when cultured in the presence of HCV proteins.

Inhibition of IFN-mediated antiviral activity by NS5A was not observed when CVB3 was used as a challenge virus. The reason why VSV, but not CVB3 showed suppressive results is not clear. Different virus families have different means of counteracting IFN such that addition of NS5A may be beneficial to one system but not another. Therefore, there are two possible explanations for this observation: (i) IFN inducible antiviral signaling pathways that can be inhibited by NS5A do not play an important role in establishing an antiviral state against CVB3; or (ii) CVB3 protein(s) may interfere with the antiviral activity of IFN through the same mechanism as HCV NS5A therefore masking its effect.

PKR inhibition in HCV. It has been reported that the PKR binding domain, including the ISDR, can interact with and inhibit PKR activity, which provides a possible explanation for the NS5A-mediated IFN antagonism (Gale, Jr. et al., 1997;Gale, Jr. et al., 1998). NS5A isolated

from patients who responded well to IFN therapy had a lesser tendency to interact with PKR than that from patients resistant to IFN. In contrast, other studies also demonstrated that HCV NS5A could inhibit IFN antiviral responses in a PKR-independent manner, arguing against the importance of PKR in the NS5A-mediated IFN inhibition (Francois et al., 2000;Podevin et al., 2001).

Another possibility is that other variable regions of NS5A, distinct from the ISDR, may be involved in the regulation of IFN sensitivity. The amino acid sequence of the variable stretch (V3) located in the C-terminal region of NS5A was shown to be correlated more closely to IFN sensitivity than sequence variation in ISDR (Duverlie et al., 1998;Nousbaum et al., 2000). The V3 region appeared to be well conserved in HCV strains resistant to IFN treatment, while being highly variable in IFN sensitive strains. In addition, other HCV proteins such as core, E2 and NS3/NS4A may also function together to block IFN activity (Gale, Jr. and Foy, 2005;Thimme et al., 2006). In particular, an additional viral genetic factor within the E2 protein, called the PKR-eIF2α phosphorylation homology domain (PePHD) is proposed to be involved in the response to IFN treatment (Taylor et al., 1999). PePHD sequences of IFN resistant HCV 1a and 1b viruses possess high homology to PKR dependent phosphorylation domains in PKR and eIF2α. The virus is thus using the PePHD, a structural homologue of both the activation domain of PKR and the eIF2α target domain of the activated protein, to competitively inhibit both aspects of PKR function. In contrast, E2 sequences with low homology to PKR and eIF2α are found in the less resistant HCV genotypes (2a, 2b, and 3a). Interestingly specific PePHD variations in HCV 2a and 2b viruses correlated with increased resistance to IFN therapy (Saito et al., 2003). This correlation is similar to Ig similarity among HCV genotypes that showed that the more resistant genotypes (1a and 1b) shared a higher degree of similarity, while the more sensitive genotypes

(2a and 2b) shared a lower degree of similarity. Thus there are now two PKR interaction sites that mimic host structures and that display higher activities for strains of higher similarity to host proteins.

We also sought to determine if expression of NS5A ISDR variants resulted in an inhibition of PKR function in 293T cells. Through repression of the eIF2α-phosphorylating activity of PKR, transient inhibition of PKR results in a net increase in the level of protein synthesis. Reflecting this effect of PKR inhibition, negative regulators of PKR can stimulate protein synthesis above basal levels when introduced into mammalian cells (Seliger et al., 1992;Tang et al., 1996). To determine if NS5A expression could alter the level of protein synthesis, we tested the ability of NS5A ISDR variants to stimulate protein synthesis in transfected 293T cells using a luciferase reporter assay. Reporter activity decreased on treatment with IFN and transfection with PKR, PKR(+) IFN(+). The expression of NS5A-6003 and NS5A-6074 restored the activity of the reporter gene following PKR(+) IFN(+) pretreatment. In contrast, the expression of NS5A-6007 did not show such activity. As IFN treatment stimulates the activation of PKR, reduction of luciferase activity might be mediated at least in part by activation of PKR. Furthermore, restoration of luciferase activity with PKR(+) IFN(+) by NS5A-6003 and NS5A-6074 was consistent with the data obtained with VSV viral rescue where NS5A-6003 and NS5A-6074 were able to rescue VSV, while NS5A-6007 did not.

Immunogenicity of NS5A. The evasion of immune recognition is another correlate of host mimicry. In this regard, the majority of IFN R have been shown to produce anti-NS5A antibody (95%) in contrast to most IFN resistant HCV strains that do not induce an anti-NS5A immune response (13% of patients induce anti-NS5A); relative to 63% of patients with partial, end-of-treatment, responses (Frangeul et al., 1998). Therefore, we propose that high host

similarity (in conjunction with IFN treatment) is associated with low immunogenicity and the converse, loss of similarity, is associated with higher immunogenicity in IFN sensitive strains (Figure 10). Although NS5A is a non-structural intracellular protein, its immunogenicity may be important for avoiding a cytotoxic cellular immune response as occurs during HCV infection due to NS5A expression (Reyes, 2002;Pavio, 2003). Moreover, we believe that the nature of NS5A ISDR mutations affects protein's function and that a prime consideration is maintenance of Ig-like structure and function that may mediate evasion from authentic immune response molecules (Figure 10).

Immunoglobulin mimicry and autoimmune disease. Molecular mimicry as a pathogenic mechanism for understanding and developing insights into therapies for autoimmune disease was first presented in the early 1980s (Oldstone, 1987). The mechanism of molecular mimicry by pathogens allows immune evasion but also may result in overcoming immunological tolerance to host proteins. This raises the possibility that a virus can present amino acid sequences similar to those of antigens of the host, with the consequence that the immunological response of the host is directed not only against the infectious agent but also against "self" proteins. This cross-reactivity between foreign antigens and host tissue would be the basis of the attack by humoral and cellular immune effectors and therefore development of various autoimmune diseases. Seventy five percent of chronic HCV infections were shown to be associated with immune-mediated pathologies, such as type II mixed cryoglobulinemia (MC) (Dammacco et al., 2000).

Furthermore, several epidemiological groups have indicated an association between HCV and B-cell non-Hodgkin's lymphoma (Zignego et al., 1997;Dammacco et al., 2000). The discovery of E2 and now NS5A protein domains with similarity to Ig provides the identification

of shared antigenic structures which may, through chronic antigen stimulation be responsible for the induction of autoimmune diseases.

Our findings in this study along with the previous reports of E2 similarity to Ig and identification of PePHD function suggest a common viral strategy: use of molecular mimicry to establish resistance to antiviral functions, allowing virus to evade the host immune response and establish successful infections. A broader analysis of NS5A proteins from defined IFN-resistant and IFN-sensitive HCV strains would be required to correlate Ig-like ISDR structure with the ability of the virus to establish persistence and resistance to IFN treatment.

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REFERENCES

Beck S and Barrell BG. 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. Nature 331:269-272.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359-364.

Cohen IR. 2004. Principles of molecular mimicry and autoimmune diesease. In: Cunningham MW and Fujinami RS, editors. Molecular Mimicry, Microbes and Autoimmunity. ASM Press. pp. 17-26.

Dammacco F, Sansonno D, Piccoli C, Racanelli V, D'Amore FP, Lauletta G. 2000. The lymphoid system in hepatitis C virus infection: autoimmunity, mixed cryoglobulinemia, and Overt B-cell malignancy. Semin Liver Dis 20:143-157.

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 (Pt 6):1373-1381.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 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, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, Strazzera A, Chien DY, Munoz SJ, Balestrieri A, Purcell RH, Alter HJ. 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 288:339-344.

Feng DF, Johnson MS, Doolittle RF. 1984. Aligning amino acid sequences: comparison of commonly used methods. J Mol Evol 21:112-125.

Francois C, Duverlie G, Rebouillat D, Khorsi H, Castelain S, Blum HE, Gatignol A, Wychowski C, Moradpour D, Meurs EF. 2000. Expression of hepatitis C virus proteins interferes with the antiviral action of interferon independently of PKR-mediated control of protein synthesis. J Virol 74:5587-5596.

Frangeul L, Cresta P, Perrin M, Duverlie G, Khorsi H, Musset L, Opolon P, Huraux JM, Lunel F. 1998. Pattern of HCV antibodies with special reference to NS5A reactivity in HCV-infected patients: relation to viral genotype, cryoglobulinemia and response to interferon. J Hepatol 28:538-543.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975-982.

Gale M Jr, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. 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.

Gale M Jr, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. 1998. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol Cell Biol 18:5208-5218.

Gale M Jr, Foy EM. 2005. Evasion of intracellular host defence by hepatitis C virus. Nature 436:939-945.

Ghany MG, Strader DB, Thomas DL, Seeff LB. 2009. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 49:1335-1374.

Hu YW, Rocheleau L, Larke B, Chui L, Lee B, Ma M, Liu S, Omlin T, Pelchat M, Brown EG. 2005. Immunoglobulin mimicry by Hepatitis C Virus envelope protein E2. Virology 332:538-49.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275-282.

Kato N. 2001. Molecular virology of hepatitis C virus. Acta Med Okayama 55:133-159.

Katze MG. 1995. Regulation of the interferon-induced PKR: can viruses cope? Trends Microbiol 3:75-78.

Khabar KS, Polyak SJ. 2002. Hepatitis C virus-host interactions: the NS5A protein and the interferon/chemokine systems. J Interferon Cytokine Res 22:1005-1012.

Larke B, Hu YW, Krajden M, Scalia V, Byrne SK, Boychuk LR, Klein J. 2002. Acute nosocomial HCV infection detected by NAT of a regular blood donor. Transfusion 42:759-765.

Lefranc MP, Pommie C, Ruiz M, Giudicelli V, Foulquier E, Truong L, Thouvenin-Contet V, Lefranc G. 2003. IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains. Dev Comp Immunol 27:55-77.

Lindenbach BD, Rice CM. 2001. Fields Virology. Lippincott, Williams & Wilkins, Philadelphia. pp. 991-1041.

Macdonald A, Harris M. 2004. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 85:2485-2502.

Macquillan GC, Niu X, Speers D, English S, Garas G, Harnett GB, Reed WD, Allan JE, Jeffrey GP. 2004. Does sequencing the PKRBD of hepatitis C virus NS5A predict therapeutic

response to combination therapy in an Australian population? J Gastroenterol Hepatol 19:551-557.

Nousbaum J, Polyak SJ, Ray SC, Sullivan DG, Larson AM, Carithers RL Jr, Gretch DR. 2000. Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy. J Virol 74:9028-9038.

Oldstone MB. 1987. Molecular mimicry and autoimmune disease. Cell 50:819-820.

Pavio N. 2003. The hepatitis C virus persistence: how to evade the immune system? J Biosci 28:287-304.

Ploegh HL. 1998. Viral strategies of immune evasion. Science 280:248-253.

Podevin P, Sabile A, Gajardo R, Delhem N, Abadie A, Lozach PY, Beretta L, Brechot C. 2001. Expression of hepatitis C virus NS5A natural mutants in a hepatocytic cell line inhibits the antiviral effect of interferon in a PKR-independent manner. Hepatology 33:1503-1511.

Polyak SJ, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, Levy DE, Mukaida N, Gretch DR. 2001. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. J Virol 75:6095-6106.

Reyburn HT, Mandelboim O, Vales-Gomez M, Davis DM, Pazmany L, Strominger JL. 1997. The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells. Nature 386:514-517.

Reyes GR. 2002. The nonstructural NS5A protein of hepatitis C virus: an expanding, multifunctional role in enhancing hepatitis C virus pathogenesis. J Biomed Sci 9:187-197.

Saito T, Ito T, Ishiko H, Yonaha M, Morikawa K, Miyokawa A, Mitamura K. 2003. Sequence analysis of PePHD within HCV E2 region and correlation with resistance of interferon

therapy in Japanese patients infected with HCV genotypes 2a and 2b. Am J Gastroenterol 98:1377-1383.

Seliger LS, Giantini M, Shatkin AJ. 1992. Translational effects and sequence comparisons of the three serotypes of the reovirus S4 gene. Virology 187:202-210.

Sen GC, Ransohoff RM. 1993. Interferon-induced antiviral actions and their regulation. Adv Virus Res 42:57-102.

Song J, Fujii M, Wang F, Itoh M, Hotta H. 1999. The NS5A protein of hepatitis C virus partially inhibits the antiviral activity of interferon. J Gen Virol 80 (Pt 4):879-886.

Tan SL, Katze MG. 2001. How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. Virology 284:1-12.

Tang NM, Ho CY, Katze MG. 1996. The 58-kDa cellular inhibitor of the double stranded RNA-dependent protein kinase requires the tetratricopeptide repeat 6 and DnaJ motifs to stimulate protein synthesis in vivo. J Biol Chem 271:28660-28666.

Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285:107-110.

Thimme R, Lohmann V, Weber F. 2006. A target on the move: innate and adaptive immune escape strategies of hepatitis C virus. Antiviral Res 69:129-141.

Watanabe H, Enomoto N, Nagayama K, Izumi N, Marumo F, Sato C, Watanabe M. 2001. Number and position of mutations in the interferon (IFN) sensitivity-determining region of the gene for nonstructural protein 5A correlate with IFN efficacy in hepatitis C virus genotype 1b infection. J Infect Dis 183:1195-1203.

World Health Organization. Hepatitis C. 2002. Geneva: WHO; 2002. Available at: http://www.who.int/entity/csr/disease/hepatitis/whocdscsrlyo2003/en/index.html.

Zhao ZS, Granucci F, Yeh L, Schaffer PA, Cantor H. 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. Science 279:1344-1347.

Zignego AL, Ferri C, Giannini C, La Civita L, Careccia G, Longombardo G, Bellesi G, Caracciolo F, Thiers V, Gentilini P. 1997. Hepatitis C virus infection in mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma: evidence for a pathogenetic role. Arch Virol 142:545-555.

FIGURE LEGEND

Figure 1. HCV viral load at early stages of IFN treatment. Plasma samples (HCV genotype 1b) were collected before and during the early phase of IFN therapy. All samples show initial inhibition by IFN except the virus of sample 6003.

Figure 2. NS5A ISDR similarity to IgG. A) Sequence similarity between NS5A ISDR 6003 (genotype 1b) and the Ig kappa light chain variable region (IgVLκ). B) Sequence alignment of a panel of representative IgVLκ regions with NS5A ISDR 6003. Identical amino acids are shown in bold.

Figure 3. Immunogenetics (IMGT) sequence alignment of the HCV NS5A (104 amino acids including ISDR; aa 2193-2295) and the variable region of human Ig and T cell receptor genes. A) Groups of 10 sequences representing the major genotypes of NS5A, as well as groups of 5 expressed and 5 germ line variable gene sequences are shown for each set of light kappa, light lambda, heavy, T cell receptor alpha and T cell receptor beta v-genes. The location of the NS5A ISDR is shown at the top. The location of the framework (FR) and complementarity determining regions (CDR) are shown within the IMGT numbered region at the bottom of the alignment. Amino acid sites with ≥ 70% shared identity between both NS5A and any or all Ig groups are masked in red with alternative common substitutions at these sites (similar chemistry) masked in blue. All other instances of shared amino acid identity with NS5A are masked in black. B) The

percent identity to NS5A shown by the bar graph with those amino acid sites that share ≥ 70% identity within NS5A and any Ig group are shown in red.

Figure 4. Similarity scores of the HCV NS5A and the variable region of human Ig and T cell receptor genes. Immunogenetics sequence alignment was used to analyze 673 NS5A protein sequences with respect to similarity for each Ig group or each of random models (random light chain kappa, random NS5A and random no model).

Similarity scores: Each amino acid of a given HCV sequence is the average of similarity scores (Dayhoff matrix) derived on comparison with each amino acid of Ig genes using files of 111 to 1212 proteins for each Ig class; and summed for each region of comparison.

Figure 5. Similarity scores of IFN non-responders (NR) and IFN responders (R). Immunogenetics sequence alignment of the HCV NS5A ISDR and the variable region of human Ig and T cell receptor genes was used to analyze sequences of 50 IFN NR and 49 IFN R with respect to similarity for total Igs or each Ig group.

Figure 6. Predicted NS5A immunoreactivity from change in Ig similarity. A) The NS5A reactivity of IFN non-responders (NR) and IFN responders (R) (Frangeul et al., 1998). B) Expected level of NS5A reactivity (percentage) for 49 IFN R determined for total Ig or each Ig group assuming that reduced similarity relative to IFN NR become serologically responsive [NR level 13% + percentage of isolates that are reduced in Ig class relative to NR (R proportion < NR) = expected].

Figure 7. Anti-human IgG binds HCV NS5A. A) ISDR amino acid sequence alignment of samples 6003 and 6007 (genotype 1b) and sample 6074 (genotype 2a) with a panel of representative IgVLκ region. Identical amino acids are shown in bold. B) ISDR region of 6003, 6007 and 6074 expressed in *E.coli*. Top panel shows anti-human IgG binding and bottom panel shows the loading control detected by anti-His antibody.

Figure 8. Effect of NS5A ISDR variants on VSV and CVB3 challenge in Cos-1 cells. Cos-1 cells expressing NS5A ISDR variants (genotype 1b - 6003 and 6007; genotype 2a - 6074), as well as non-expressing controls (cells and pCI-neo), were left untreated or treated with IFNα (200U/mL) for 24h before infection with VSV (A) or CVB3 (C) at multiplicity of infection of 0.5pfu/mL. Effect of NS5A ISDR variants on VSV (B) and CVB3 (D) replication represented as relative values for (Treated/Untreated). E) Cos-1 cells expressing FLAG-tagged 6003, 6007, and 6074 NS5A variants (anti-FLAG antibody). Bars represent the mean ± SEM of 4 independent experiments; * $p<0.05$, significant difference compared to non-expressing controls.

Figure 9. Effects of NS5A ISDR variants on protein synthesis in 293T cells. 293T cells expressing NS5A ISDR variants (genotype 1b - 6003 and 6007; genotype 2a - 6074), as well as non-expressing controls (cells and pCI-neo), were left untreated [PKR(-) IFN(-)] or were transfected with PKR and treated with IFN [PKR(+) IFN(+)]. A) Luciferase activity (RLU-relative luciferase unit) measured for PKR(-) IFN(-) and PKR(+) IFN(+) cells. B) Changes in luciferase activity represented as relative values for PKR(+) IFN(+)/(PKR(-) IFN(-). C) Transfection efficiency of 293T cells monitored by GFP

detection. Bars represent the mean ± SEM of 4 independent experiments; * $p<0.05$, significant difference compared to non-expressing controls.

Figure 10. Model of NS5A immunogenicity. Mutations in NS5A ISDR of IFN resistant variants that result in high host similarity are associated with low NS5A immunogenicity and lack of immune response. On the other hand, IFN sensitive variants that share low similarity with a host are more immunogenic and elicit the immune response. In addition, the mutations in the ISDR may have an impact on the NS5A's binding ability to PKR and in turn affect the response to IFN treatment.

FIGURES

Figure 1.

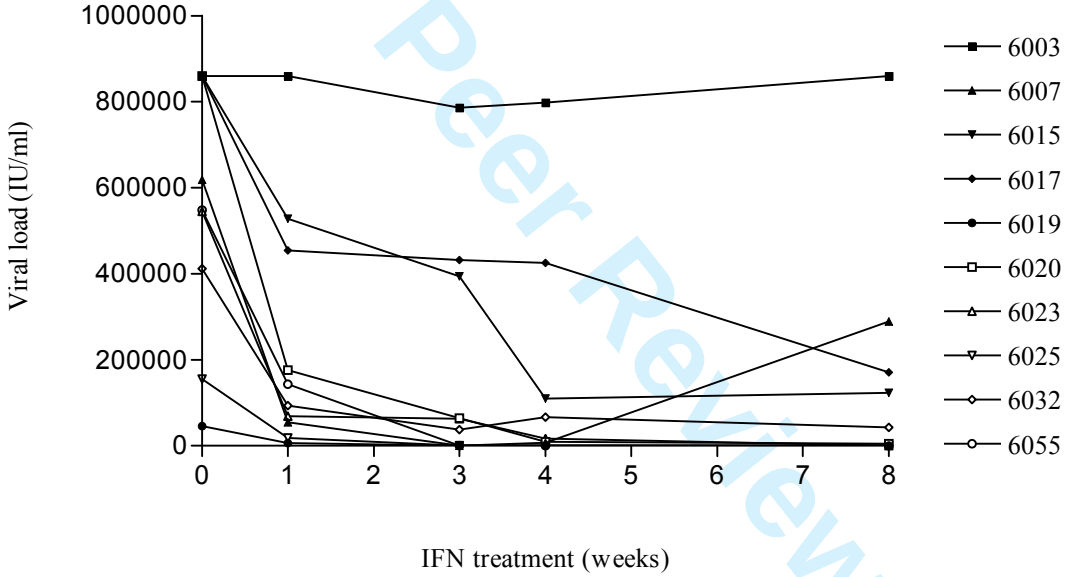
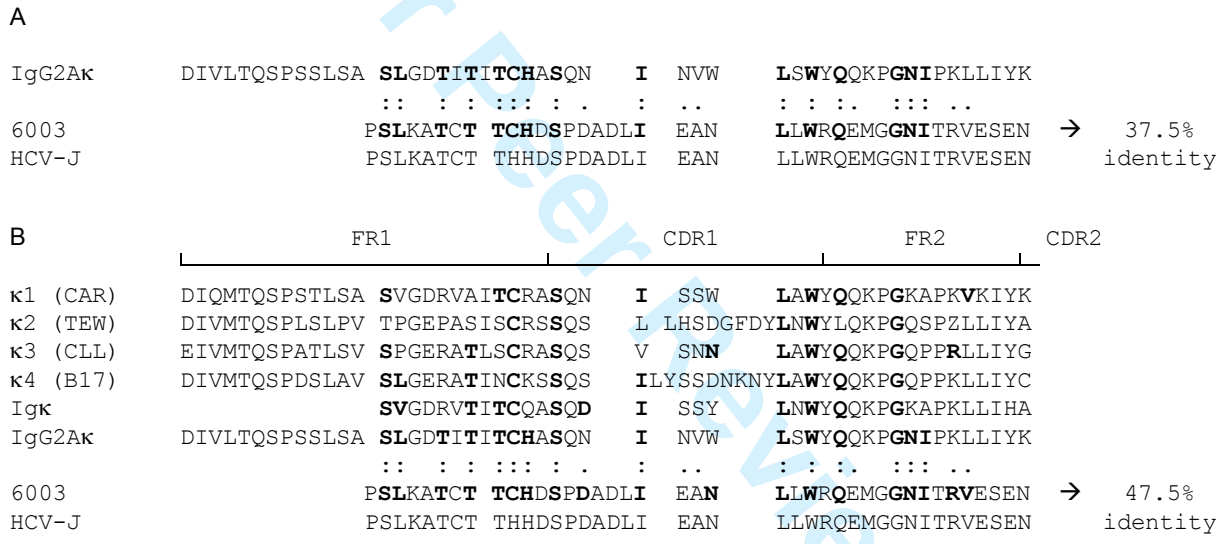


Figure 2.



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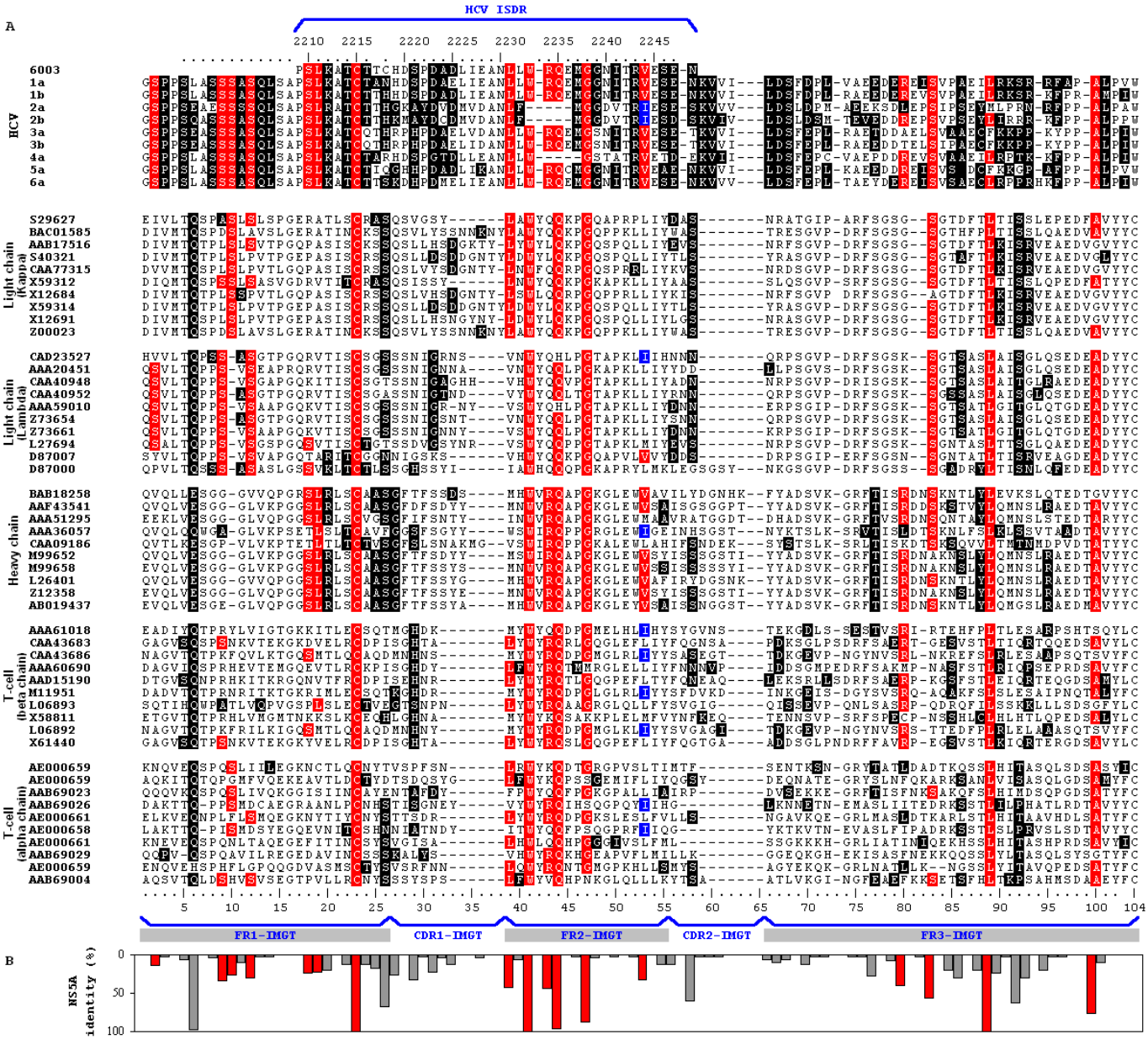

A
HCV ISDR
2210 2215 2220 2225 2230 2235 2240 2245
HCV
6003
1a
1b
2a
2b
3a
3b
4a
5a
6a
Light chain (Kappa)
S29627
BAC01585
AAB17516
S40321
CAA77315
X59312
X12684
X59314
X12691
Z00023
Light chain (Lambda)
CAD23527
AAA20451
CAA40948
CAA40952
AAA59010
Z73654
Z73661
L27694
D87007
D87000
Heavy chain
BAB18258
AAF43541
AAA51295
AAA36057
CAA09186
M99652
M99658
L26401
Z12358
AB019437
T-cell (beta chain)
AAA61018
CAA43683
CAA43686
AAA60690
AAD15190
M11951
L06893
X58811
L06892
X61440
T-cell (alpha chain)
AE000659
AE000659
AAB69023
AAB69026
AE000661
AE000658
AE000661
AAB69029
AE000659
AAB69004
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 104
FR1-IMGT
CDR1-IMGT
FR2-IMGT
CDR2-IMGT
FR3-IMGT
B
HS5A identity (%)
0
50
100

Figure 4.

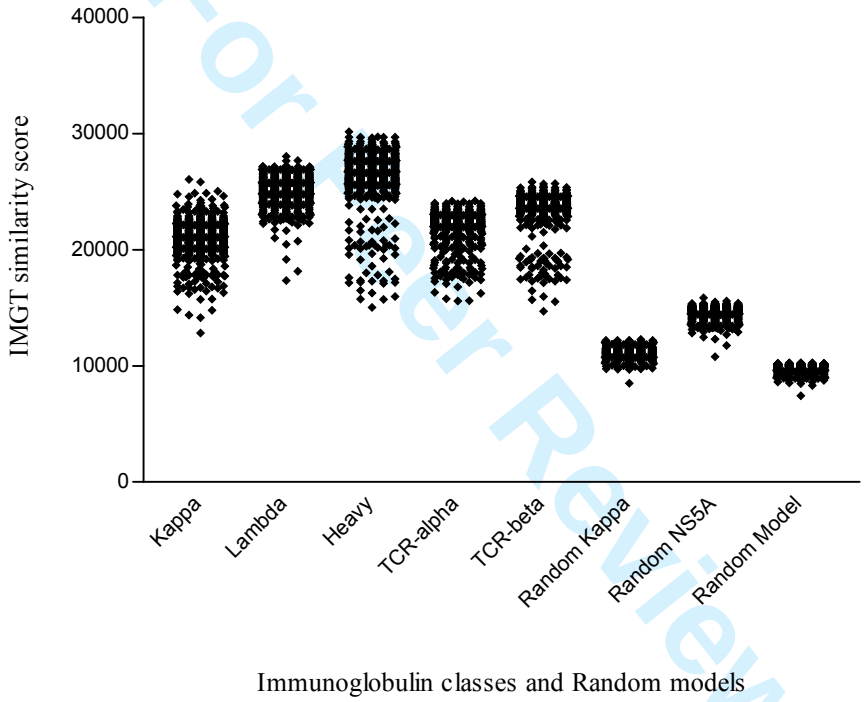


Figure 5.

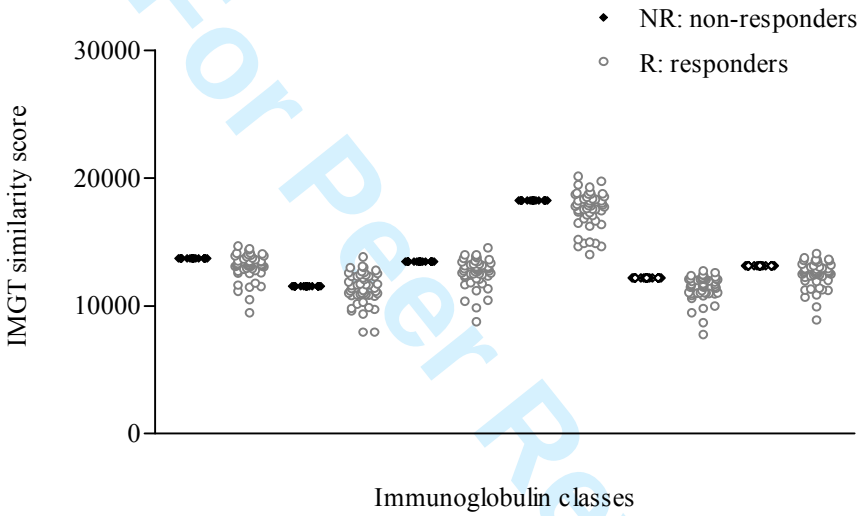


Figure 6.

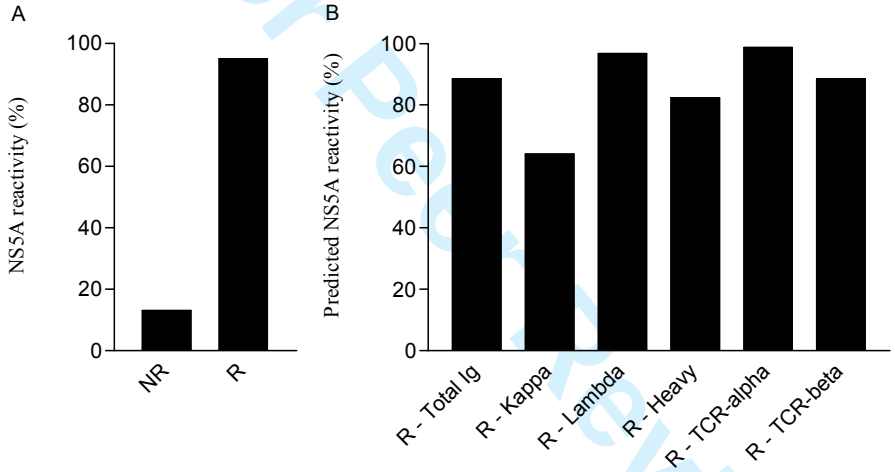


Figure 7.

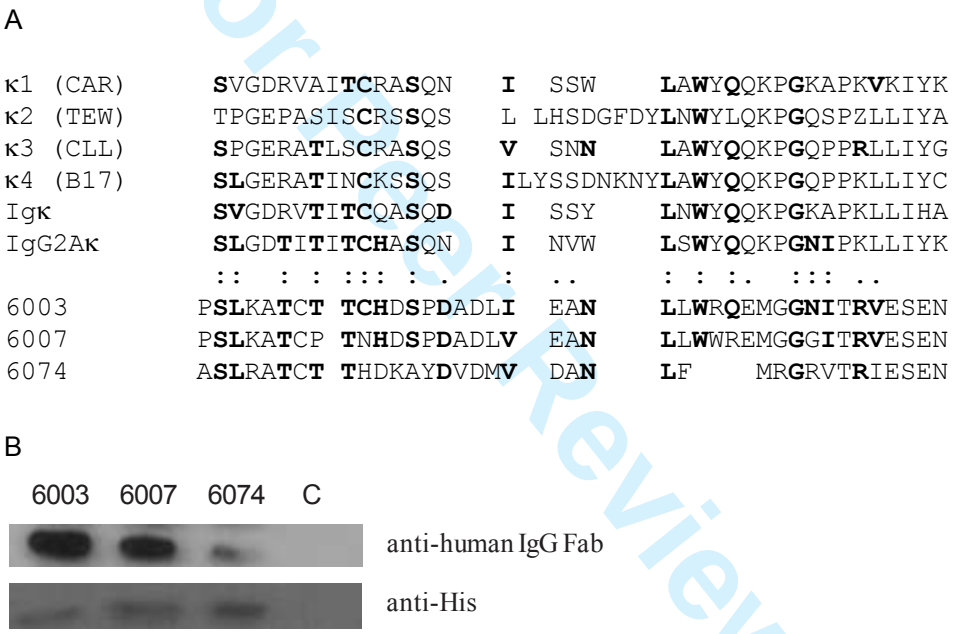


Figure 8.

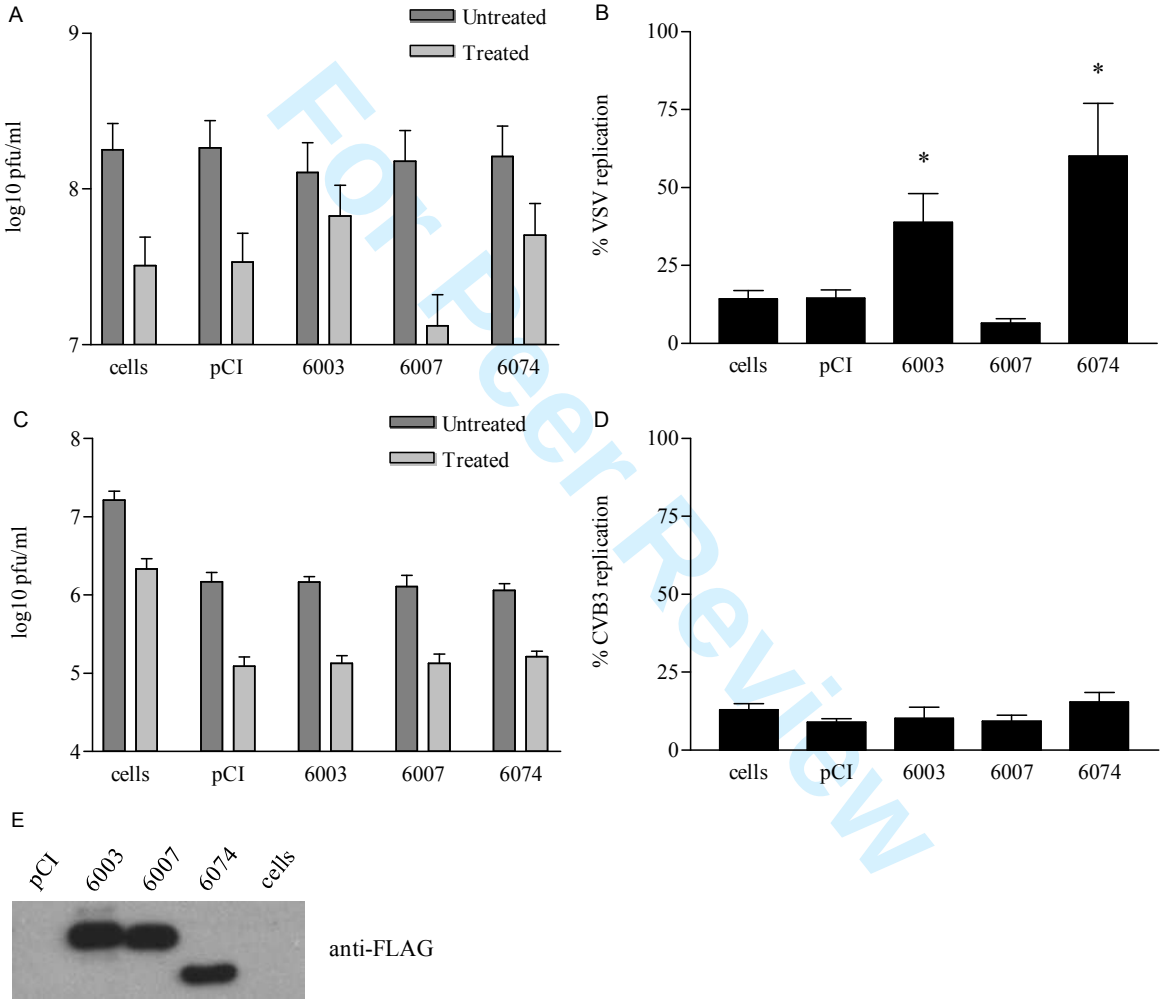


Figure 9.

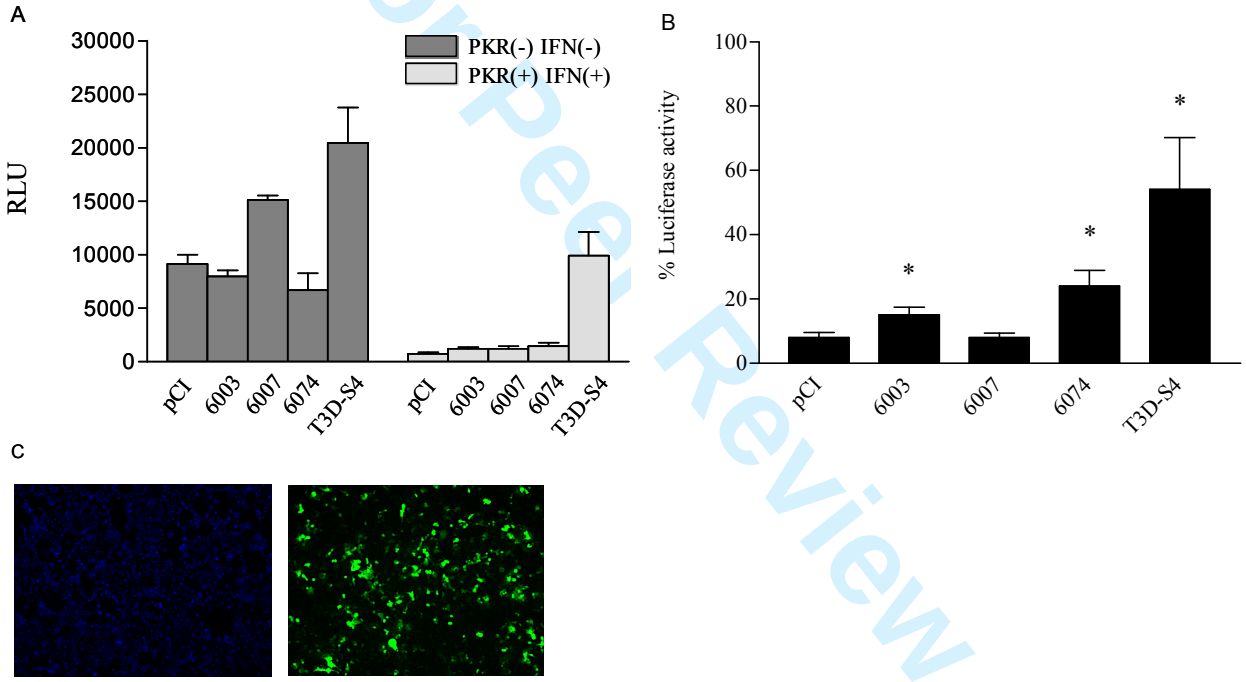
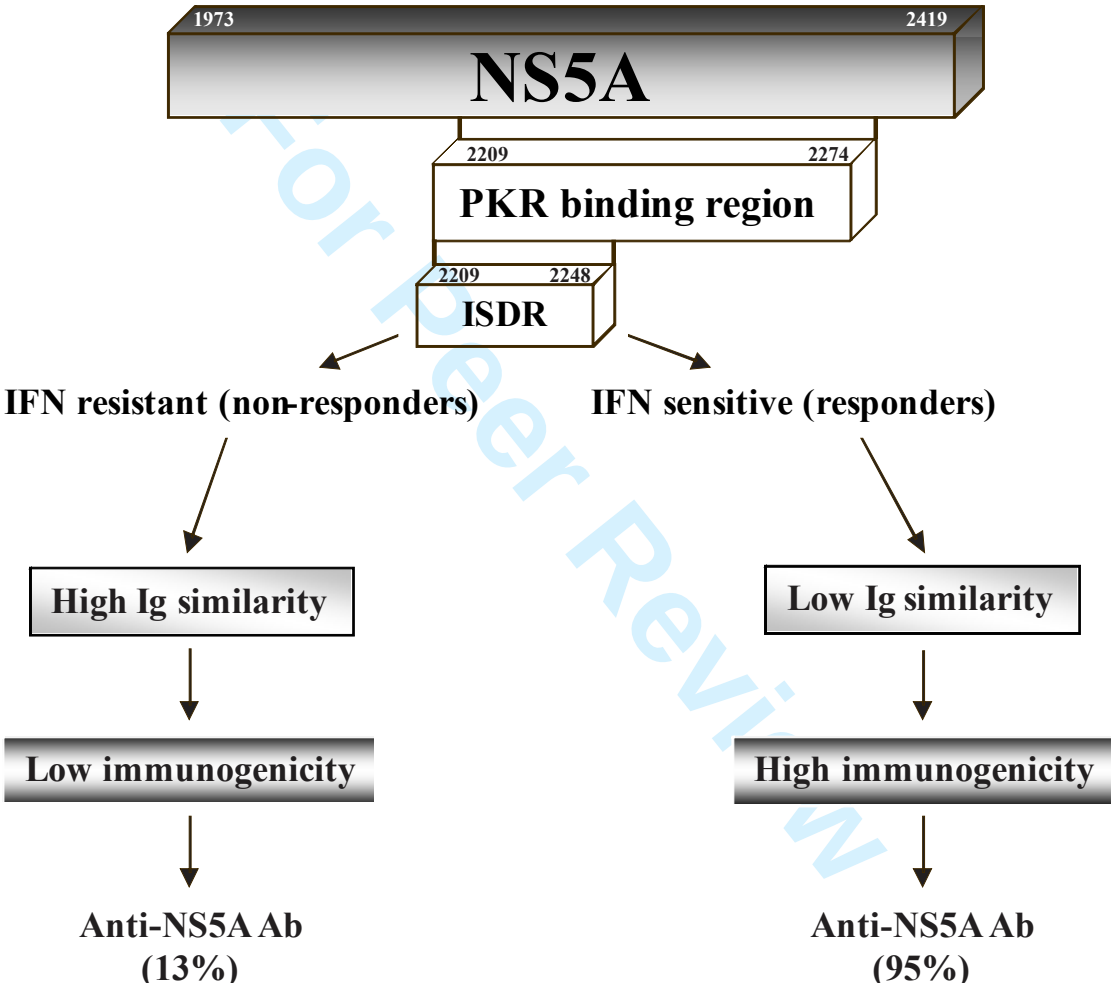


Figure 10.



TABLES

TABLE I - Amino acid sequence alignment[a] (amino acid positions 2209 to 2248 - ISDR) of NS5A protein of HCV-1b isolates obtained from 10 patients prior to IFN treatment; ISDR of HCV-2a isolate also included.

SAMPLES	AMINO ACID SEQUENCE	VIRAL LOAD (copies/ml) week[d]		
HCV-J[b](1b)	PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN	0	1	8
6003 (10 clones)	---------C------------------------------	860000	860000	860000
6007 (8 clones)	-------P-N--------V------WR----G--------	619000	54800	289000
6007 (1 clone)	-------P-S--------V------WR----A--------			
6015 (9 clones)	---------R------------------------------	860000	528000	123000
6015 (1 clone)	---------R------------------T-----------			
6017 (5 clones)	----------------V------------------------	860000	454000	171000
6017 (1 clone)	----------------V----------R------------			
6019 (10 clones)	---------R------------------------------	45800	6570	599
6020 (6 clones)	---------R------------------V-----------			
6020 (2 clones)	----------R---L---------W--K-----------	860000	176000	4970
6020 (1 clone)	----------R---L---------W--K---V-------			
6020 (1 clone)	--------AR----------------W--K----------			
6023 (5 clones)	----------N----------------L-----------			
6023 (2 clones)	---------------------------L-----------			
6023 (1 clone)	---R-------N----------------R-L----------	545000	68800	599
6023 (1 clone)	---R-------N----------------L-----------			
6023 (1 clone)	----------NP----------------L-----------			
6025 (9 clones)	--	155000	18200	599
6025 (1 clone)	------------------------C---------------			
6032 (5 clones)	--	412000	93200	43100
6032 (5 clones)	---------R------------------------------			
6055 (10 clones)	---------R------------------------------	548000	143000	599
HCV-J6[c](2a)	PSLRATCTTHGKAYDVDMVDANLF MGGDVTRIESES			
6074 (9 clones)	A---------------------- -R-R---------	187000	599	599

[a] Amino acid sequences were deduced from nucleotide sequences (HCV-1b and HCV-2a genotype) obtained by sequencing of 6-10 clones for each sample

[b] HCV-J prototype 1b sequence

[c] HCV-J6 prototype 2a sequence

[d] Week 0 corresponds to viral load before IFN treatment; week 1 and 8 correspond to viral load 1 and 8 weeks after the first administration of IFN therapy, respectively