Additional Glycosylation Within a Specific Hypervariable Region of Subtype 3a of Hepatitis C Virus Protects Against Virus Neutralization

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Background. The envelope glycoprotein E2 of hepatitis C virus (HCV) contains several hypervariable regions. Interestingly, 2 regions of intragenotypic hypervariability within E2 have been described as being specific to HCV subtype 3a. Based on their amino acid position in E2, they were named HVR495 and HVR575. Here, we further investigated these regions in order to better understand their role in HCV infection.

Methods. Sequences of HCV envelope glycoproteins from Pakistani patients infected with subtype 3a were cloned and compared with other subtype 3a sequences. The entry functions and the sensitivity to antibody neutralization of selected HCV glycoprotein sequences were tested in the HCV pseudotyped particles (HCVpp) system. In addition, the cell-cultured HCV system (HCVcc) was also used to confirm some of the data obtained with the HCVpp system.

Results. We observed interesting new features within HVR495 and HVR575 for several subtype 3a isolates. Indeed, changes in glycosylation sites were observed with the appearance of a new glycosylation site within HVR495. Importantly, HCVpp and HCVcc that contained this new HVR495 glycosylation site were less sensitive to antibody neutralization.

Conclusions. We identified a new glycosylation site within the HVR495 region of HCV subtype 3a that has a protective effect against antibody neutralization.

Keywords. hepacivirus; hepatitis C virus; viral glycoproteins; neutralizing antibodies; sequence variability.

Hepatitis C virus (HCV) is a major infectious agent that has a tendency to establish a persistent infection in the human liver. Worldwide, approximately 160 million people are infected by this virus and are at risk of developing chronic inflammation of the liver, cirrhosis, and hepatocellular carcinoma [1]. In the past, antiviral therapy against HCV consisted of a bitherapy of pegylated interferon and ribavirin [2]. Recently, new direct-acting antivirals that target HCV NS3-4A protease, telaprevir and boceprevir, have been added to the therapy. This has resulted in an increase in the sustained virological response in patients infected with HCV genotype 1 of up to 70% [3]. However, this treatment remains relatively toxic and expensive, which limits its potential use in many developing countries. For such countries, the development of an efficient vaccine remains a priority. However, the development of a vaccine that is protective against HCV has proven
to be an extremely challenging task. Extensive research in this area suggests that a successful HCV vaccine will need to stimulate the production of neutralizing antibodies and potent HCV-specific T-cell responses. To this end, it is crucial to define all neutralizing determinants in HCV envelope glycoproteins and particularly conserved structural features that could be targeted to obtain cross-neutralization between different virus genotypes and to minimize the likelihood of immunological escape. In addition, it is important to understand the molecular basis of HCV neutralization resistance.

The HCV genome encodes 2 envelope glycoproteins, E1 and E2. These 2 proteins assemble as noncovalent heterodimers within infected cells and as large disulfide-linked oligomers on the surface of HCV particles [4]. Since they are present on the virion, HCV envelope glycoproteins are recognized by neutralizing antibodies, with E2 being the major target [5]. This protein is highly glycosylated with up to 11 N-glycosylation sites [6]. Interestingly, at least 5 of these glycans reduce the sensitivity of HCV to neutralization, indicating that glycans can limit the recognition of neutralizing epitopes at the surface of E2 [6, 7].

There is no high-resolution structure of HCV envelope glycoprotein E2. However, based on a comparison with other fusion proteins from viruses belonging to the same family as well as the identification of E2 disulfide bonds, a model of the E2 ectodomain, consisting of 3 separate domains, has recently been proposed [8]. Furthermore, located within the E2 ectodomain are 3 highly variable sequences: hypervariable region 1 (HVR1), HVR2, and the intergenotypic variable region (igVR) [9]. Interestingly, 2 regions of intragenotypic hypervariability within the envelope protein E2 have also been described as being specific to subtype 3a [10, 11]. Based on their amino acid position in E2, they were named HVR495 and HVR575. It is worth noting that HVR575 is located within the intergenotypic variable region igVR [9]. Importantly, longitudinal analysis of patients with acute HCV subtype 3a infection showed that positively selected mutations within HVR495 and HVR575 arose early during primary infection, suggesting an influence of the host immune response in the variability of these regions [10, 11]. Here, we further investigated these regions in order to better understand their role in HCV infection. To this aim, we analyzed E2 sequences from subtype 3a. We observed interesting new features within HVR495 and HVR575 for these isolates, with the appearance of a new glycosylation site within HVR495. Our data show that this new glycosylation site plays a major role in protection against antibody neutralization.

**MATERIALS AND METHODS**

**Patients**

HCV-positive patients were enrolled for this study under approval of the Internal Review Board of the National University of Sciences and Technology, Atta ur Rhaman School of Applied Biosciences, Pakistan; a patient consent form was duly signed for each patient. HCV patients selected for this study were under interferon and ribavirin therapy and had completed 3–4 months of initial treatment. Their characteristics are presented in Supplementary Table 1. HCV RNA was quantified with a HCV RNA quantification kit (RoboGene; AJ Roboscreen) according to the manufacturer’s instructions.

**Amplification, Cloning, and Sequencing of E1 and E2 Genes**

Viral RNA was extracted from serum using an RNA extraction kit (Qiagen) according to the manufacturer’s protocol. Extracted RNA was used as the template for cDNA synthesis, and the HCV core-NS2 region was amplified with a high-fidelity Taq DNA polymerase (Roche). The sequences of primers were as follows: 5’-AAAGAATTCGCCCACATGCTAGGATGCGGGA ATACGTCTGGCC-3’ (sense) and 5’-AAAGGCGCCGCTCAC CCCAGGTAGACCTTGATTTCC-3’ (antisense). Polymerase chain reaction (PCR) products were cloned into PCRII TOPO cloning vector (Invitrogen). Selected clones were subjected to sequencing and were used to clone full-length E1E2 into pCDNA3.1 expression vector (Invitrogen). Mutations were introduced into the E2 sequence by sequential PCR steps as described [12] using a PCR system (Expand High Fidelity PLUS, Roche). Mutated clones were confirmed by sequencing.

**Sequence Analyses and Structure Predictions**

Sequence analyses were performed using the Web site tools of the European HCV database [13] and network protein sequence analysis [14], available at the Institut de Biologie et Chimie des Protéines. Multiple sequence alignments and amino acid conservations were carried out with the ClustalW program using default parameters [15]. Amino acids are numbered with respect to the polyprotein of HCV strain H77 consensus sequence that is used as a reference strain [16].

**Antibodies**

Polyclonal antibodies were purified from a pool of 20 Pakistani patients infected with HCV 3a subtype. Purified immunoglobulins from uninfected patients were used as a negative control. Monoclonal antibodies (Mabs) 3/11 (anti-E2) [17], JS81 (anti-CD81; BD-Pharmingen), R187 (anti-murine leukemia virus capsid; American Type Culture Collection [AATC]-CRL1912), and C167 (anti-scavenger receptor B1 [SRB1]) [18] were used in this work. Anti-claudin-1 (CLDN1) Mab has been previously described [19]. Anti-ApoE antibody was from EMD Millipore.

**Cell Culture**

Huh-7 human hepatoma cells [20] and human embryonic kidney 293T cells (ATCC number CRL-11268) were grown in Dulbecco’s modified essential medium (Invitrogen) supplemented with 10% fetal calf serum.
Figure 1. Sequence analyses of E2 of subtype 3a Pakistani patients. Multiple alignments of E2 sequences were performed with ClustalW [15]. A–D, Consensus sequences of patients (this study) are compared with consensus sequences of patients 99 and 301 [11] exhibiting an additional glycosylation site in HVR4 region. Consensus sequences were deduced from quasispecies sequences reported in Supplementary Figure 1. Sequence of the UKN3A-1.28 synthetic clone used for functional studies in this work is indicated here as genotype 3a reference sequence (accession number: AY734984). Amino acids are numbered with respect to the polyprotein of hepatitis C virus strain H77 consensus sequence used as a general reference [16] (top row). To highlight the variability at each position, amino acids identical to the UKN3A-1.28 sequence are indicated by dots. The degree of amino acid physicochemical conservation at each position relative to the UKN3A-1.28 sequence is indicated with the similarity index according to ClustalW convention (asterisk, invariant; colon, highly similar; dot, similar) [15]. Expected glycosylation sites are labeled (bottom row) and highlighted in gray. Additional and defective glycosylation sites are highlighted by a gray box and an open box, respectively. Patient sequences have been submitted to GenBank; the accession numbers are indicated in Supplementary Figure 1.
Production of Viruses
The luciferase-based HCV pseudotyped retroviral particles (HCVpp) [21] were produced as previously described [22]. The UKN3A-1.28 (AY734984) clone was used as a genotype 3a reference isolate for HCVpp experiments [23]. For experiments with cell-cultured HCV (HCVcc), a modified JFH1 virus [24], intergenotypic HCV chimeras H77/JFH1 [25], and GT3a(452)/JFH1 [26] were used.

Neutralization and CD81 Inhibition Assays
Neutralization and CD81 inhibition experiments were performed as previously described [7, 27]. Student t test was used to compare the percentages of infectivity.

RESULTS
Characterization of E2 Sequences From Pakistani Patients
To gain further information on variable regions specifically observed in subtype 3a, we sequenced several E2 clones from Pakistani patients because this subtype is highly prevalent in Pakistan. Four different clones were analyzed from each patient; their consensus sequences are presented in Figure 1 (sequence details are presented in Supplementary Figure 1). In addition, these sequences were compared with subtype 3a sequences available in the databases (Table 1 and Supplementary Figure 2). Among 476 nonredundant sequences from 71 patients available for E2, the large majority had potential glycosylation sites located at the same positions as those described for subtype 1a, except position N7, which is totally absent in subtype 3a [6]. In addition, 140 sequences also showed the absence or addition of another glycosylation site (Figure 1 and Supplementary Figures 1 and 2).

Effect of New HVR495 Glycosylation Site on the Entry Functions of HCV Envelope Proteins
To gain further information on the role of HVR495 glycosylation in E2 function, we produced HCVpp with the sequences from 2 quasispecies of patient PC (PC2 and PC3; Supplementary Figure 1). Previously described functional HCVpp from a subtype 3a (UKN3A-1.28) were used as a positive control of infectivity [23]. As shown in Figure 2, HCVpp containing the PC2 or PC3 envelope proteins were infectious. However, the level of infectivity was lower than what was observed for our positive control. This observation is not unexpected since HCVpp of genotype 3 are known to be less infectious as compared with other genotypes [23].

Table 1. Statistics of E2 Sequences of Subtype 3a Exhibiting Defective or Additional Glycosylation Sites

<table>
<thead>
<tr>
<th>Glycosylation Sites</th>
<th>Sequences</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defective sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>21</td>
<td>4.4</td>
</tr>
<tr>
<td>N2</td>
<td>7</td>
<td>1.5</td>
</tr>
<tr>
<td>N3</td>
<td>30</td>
<td>6.3</td>
</tr>
<tr>
<td>N4</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>N5</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td>N6</td>
<td>10</td>
<td>2.1</td>
</tr>
<tr>
<td>N7</td>
<td>(. . .)</td>
<td>(. . .)</td>
</tr>
<tr>
<td>N8</td>
<td>14</td>
<td>2.9</td>
</tr>
<tr>
<td>N9</td>
<td>41</td>
<td>8.6</td>
</tr>
<tr>
<td>N10</td>
<td>6</td>
<td>1.3</td>
</tr>
<tr>
<td>N11</td>
<td>6</td>
<td>1.3</td>
</tr>
<tr>
<td>Additional sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVR495</td>
<td>37</td>
<td>3</td>
</tr>
<tr>
<td>524</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>528</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>658</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Data deduced from the analysis of 476 nonredundant full-length E2 sequences of subtype 3a from 71 patients available in euHCVdb [13]. Sequence fragments exhibiting defective or additional glycosylation sites are reported in Supplementary Figure 2.
To gain more information on the potential effect of the HVR495 glycosylation site in PC isolate, we replaced the asparagine residue in HVR495 of PC2 and PC3 isolates with a proline residue, which occupies the same position in the UKN3A-1.28 clone. This mutation improved HCVpp infectivity by approximately 6 fold (Figure 2A and data not shown). To more specifically determine the effect of a glycosylation site within HVR495, we used site-directed mutagenesis to engineer a glycosylation site in this region in the context of the UKN3A-1.28 clone. As shown in Figure 2A, this single mutation reduced HCVpp infectivity by approximately 6 fold. Although there was some variation in the incorporation of the envelope glycoprotein E2 (Figure 2C), this did not correlate with the changes in infectivity. Indeed, UKN-mut495 showed a slightly higher level of incorporation into HCVpp, and infectivity was lower for this mutant (Figure 2A and C). Together, these data indicate that the selection of an additional glycosylation site within HVR495 reduces the efficiency of HCVpp entry into the hepatocyte.

**Effect of New HVR575 Mutation on the Entry Functions of HCV Envelope Proteins**

To gain additional information on the role of HVR575 in E2 function, we swapped 5 amino acids of HVR575 (DNNQA sequence) between PC2 and UKN3A-1.28 clones as well as their HVR575 mutants were used to generate HCV pseudotyped particles (HCVpp). PC2-mut575 corresponds to PC2 sequence in which the DNNQA sequence of HVR575 has been replaced by the corresponding KNESD sequence of UKN3A-1.28 isolate. UKN3A-mut575 corresponds to UKN3A-1.28 sequence in which the KNESD sequence of HVR575 has been replaced by the corresponding DNNQA sequence of PC2 isolate. Infection assays with the luciferase reporter gene were performed using target Huh-7 cells. UKN3A-del575 corresponds to UKN3A-1.28 and PC2-del575 corresponds to PC2 sequences in which the 5 amino acid segment of HVR575 has been deleted. Pseudotyped particles produced in the absence of envelope proteins (ΔE1E2) were used as a control. The results are expressed as relative light units and are reported as means ± SD. C and D Incorporation of HCV envelope proteins into HCVpp. Particles were pelleted through 30% sucrose cushions and analyzed by western blotting. HCV envelope glycoprotein E2 and the capsid protein of murine leukemia virus (MLV) were revealed with specific monoclonal antibodies (Mabs): anti-E2 (3/11) and anti-MLV capsid (R187) Mabs. Expression of mutant proteins was verified by a direct western blotting on cell lysates. Molecular mass markers are indicated on the left.
clones. We also deleted this 5–amino acid segment in both PC2 and UKN3A-1.28 clones. In addition to the change in amino acids, these mutations led to the removal of glycosylation site N9 in the UKN3A-1.28 clone when its HVR575 sequence was replaced by the equivalent sequence of PC2 as well as when this segment was deleted. On the contrary, this 5–amino acid swap in the context of PC2 isolate led to a gain in a glycosylation site at position N9. However, in contrast with the mutations in HVR495, none of these HVR575 mutations affected HCVpp infectivity (Figure 2C). It is worth noting that the mutations did not modify the level of incorporation of E2 into HCVpp (Figure 2D). Together, these data indicate that the loss of a glycosylation site at position N9 within HVR575 does not affect the efficiency of HCVpp entry into the hepatocyte.

HVR495 Glycosylation Site Protects Against Virus Neutralization

It has been reported that several highly conserved glycans present on E2 glycoprotein protect against antibody neutralization [6, 7, 28]. We therefore wondered whether the presence of an additional glycan in HVR495, as observed in the quasispecies of patient PC, could also affect HCV sensitivity to antibody neutralization. To this aim, we used a pool of purified antibodies from patients infected with HCV of subtype 3a. The neutralization experiments were then performed for each HCVpp mutant using antibodies at a concentration that corresponded to the half maximal effective concentration (EC50), as determined on wild-type UKN3A-1.28-HCVpp. Importantly, in these conditions, HCVpp containing the envelope proteins of PC2 or PC3 isolates were not neutralized by the anti-HCV antibody, whereas the mutants lacking the HVR495 glycosylation site were neutralized as efficiently as HCVpp containing the envelope proteins of the UKN3A-1.28 clone (Figure 3A). On the contrary, the addition of a glycosylation site in HVR495 of the UKN3A-1.28 clone blocked antibody neutralization. Neutralization experiments were also performed on HVR575 mutants. As shown in Figure 3B, HCVpp containing the envelope proteins of PC2 mutated in HVR575 were more sensitive to neutralization. However, in contrast to HVR495 mutants, none of these HVR575 mutations modulated HCVpp neutralization in the context of the control UKN3A-1.28 HCVpp (Figure 3B). Together, these results indicate that HVR495 glycan contributes to the masking of important neutralizing epitopes on E2 in the context of the HCVpp system.

Analysis of HVR495 Glycosylation Site in the Context of HCVcc

To further characterize the potential role of HVR495 in protection against neutralization, we engineered a new glycosylation site in the context of the HCVcc system. We also constructed another chimeric virus in which HVR575 mutation was introduced. Unfortunately, this latter mutant was not infectious and we could not use it in our neutralization experiments. The lack of infectivity of this mutant could be due to intersequence incompatibility within the igVR region as previously reported [30].

As shown in Figure 4A, the addition of a glycosylation site in HVR495 in the JFH1 virus or in GT3a(452)/JFH1 or H77/JFH1
chimeras did not modify HCVcc infectivity. The presence of an additional glycosylation site in these mutants was confirmed by sequencing, and the change in the migration profile of E2 in the context of the GT3a(452)/JFH1 chimera was confirmed by western blotting (Figure 4B). It should be noted that the mutation did not modify the density of the major peak of the infectious particles (Figure 4C). Indeed, the EC$_{50}$ for neutralization by anti-ApoE antibodies was 3.0 ng/mL (± 0.06) for wild-type and 13.3 ng/mL (± 0.36) for mutant GT3a(452)/JFH1 (Table 2). These results indicate that HVR495 glycan masks important neutralizing epitopes on E2 in the context of the HCVcc system.

Since we previously reported that several conserved glycans reduce the accessibility of a soluble form of CD81 to its binding region on E2 [7], we also investigated whether HVR495 glycan affects the accessibility of CD81 to its binding region. To this end, we analyzed the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. As shown in Figure 5B, our results indicate that the presence of HVR495 glycan reduces the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. Indeed, the EC$_{50}$ for CD81 inhibition was 2.8 µg/mL (± 0.51) for wild-type and 8.8 µg/mL (± 0.58) for mutant GT3a(452)/JFH1, and similar results were obtained when the mutation was introduced in JFH1 and H77/JFH1 chimera (Table 2). To further determine whether the presence of HVR495 glycan affects the way HCV interacts with cellular entry factors, additional

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Figure 4. Analysis of HVR495 glycosylation site in the context of cell-cultured hepatitis C virus (HCVcc). B, Effect of HVR495 glycan on HCVcc infectivity. Huh-7 cells were electroporated with viral RNA transcribed from JFH1, GT3a(452)/JFH1 (3a/2a), or H77/JFH1 (1a/2a) chimeras containing or not containing an additional glycosylation site in HVR495 (495) at the same position as the one observed in PC quasispecies. At 72 hours post electroporation, infectivity of the supernatants was determined by titration in 50% tissue culture infective dose (TCID$_{50}$/mL). Error bars indicate standard errors of the mean values from 3 independent experiments. C, Separation of concentrated virus in iodixanol gradient. Concentrated cell-cultured HCV from wild-type GT3a(452)/JFH1 (3a/2a) or the HVR495 mutant (495-3a/2a) were separated by sedimentation through a 10%–50% iodixanol gradient. Fractions were collected from the top and analyzed for their density as well as infectivity by TCID$_{50}$.
experiments with anti-CD81, anti-SRB1, and anti-CLDN1 antibodies were performed. As shown in Figure 5 and Table 2, viruses containing HVR495 glycan were less sensitive to neutralization by anti-CD81 and anti-SRB1 antibodies but not to anti-CLDN1 antibody. These results suggest that the presence of HVR495 glycan reduces the accessibility to CD81 and SRB1 but not to

### Table 2. Effect of HVR495 Glycan on Sensitivity to Antibody Neutralization and Receptor Usage (EC50 Values)

<table>
<thead>
<tr>
<th>Reagents</th>
<th>3a/2a</th>
<th>495-3a/2a</th>
<th>JFH1</th>
<th>495-JFH1</th>
<th>1a/2a</th>
<th>495-1a/2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble CD81</td>
<td>2.8 ± 0.51</td>
<td>8.8 ± 0.58**</td>
<td>2.2 ± 0.20</td>
<td>16.3 ± 1.47**</td>
<td>0.7 ± 0.07</td>
<td>3.7 ± 0.60*</td>
</tr>
<tr>
<td>Anti-CD81</td>
<td>0.6 ± 0.05</td>
<td>3.7 ± 0.61*</td>
<td>0.6 ± 0.03</td>
<td>2.3 ± 0.10**</td>
<td>0.7 ± 0.10</td>
<td>4.4 ± 0.55***</td>
</tr>
<tr>
<td>Anti-SRB1</td>
<td>0.4 ± 0.03</td>
<td>3.0 ± 0.26***</td>
<td>1.1 ± 0.08</td>
<td>5.9 ± 0.73*</td>
<td>0.6 ± 0.06</td>
<td>2.6 ± 0.36*</td>
</tr>
<tr>
<td>Anti-claudin-1</td>
<td>0.31 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>0.4 ± 0.02</td>
<td>0.4 ± 0.04</td>
<td>0.3 ± 0.01</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>Anti-3a Abs</td>
<td>3.0 ± 0.06</td>
<td>13.3 ± 0.36***</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-ApoEAb</td>
<td>0.00078 ± 0.00004</td>
<td>0.00085 ± 0.00004</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

EC50 values are expressed in μg/ml except for anti-ApoE Ab for which the values are expressed as dilution factors.

*P < .05, **P < .01, and ***P < .001.

Figure 5. Effect of HVR495 glycan on cell-cultured hepatitis C virus (HCVcc) sensitivity to antibody neutralization and receptor usage. Inhibition experiments were performed by incubating mutant (495-2a/3a) or wild-type GT3a(452)/JFH1(2a/3a) with various concentrations of anti-HCV antibodies (3aHCV+Abs) (A), CD81 large extracellular loop (LEL) fused to GST (GST-CD81-LEL) (B), anti-CD81 Mab JS81 (C), anti-SRB1 Mab C167 (D), or anti-CLDN1 Mab (E). At 72 hours post infection, infected cells were quantified by immunofluorescence. Values are the combined data from 2 independent experiments; error bars represent standard errors of the means.
The presence of a glycan in HVR575 does not affect virus entry and does not protect against antibody neutralization. It is worth noting that the appearance of a glycosylation site in HVR495 might be associated with the disappearance of a glycosylation site within HVR575 located in igVR. The presence of the E2N9 glycan in igVR is highly conserved in all HCV genotypes, suggesting that it might play a role in HCV infection. However, this glycan does not affect HCV entry in subtypes 1a and 2a and it does not reduce HCV sensitivity to antibody neutralization in these same genotypes [6, 7, 27, 28]. In the context of patient PC, it is possible that the disappearance of E2N9 glycan is linked to the appearance of a new glycosylation site in HVR495. However, this could not be demonstrated in our experimental setting since restoring E2N9 glycan within HVR575 did not affect virus entry. This is in line with the lack of effect of E2N9 mutation in subtypes 1a and 2a [6, 7, 27, 28]. However, we cannot exclude that this potential glycosylation shift is important for maintaining some in vivo function(s) of this glycan.

In conclusion, our functional characterization of regions of hypervariability within E2 in HCV subtype 3a provides new information on the high level of plasticity of HCV envelope glycoprotein E2. Such data are important for the future design of HCV vaccines in countries that cannot afford the high cost of the current antiviral treatments.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References