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Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections

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ABSTRACT **E2/nonstructural protein 1, the putative envelope glycoprotein (gp72) of HCV, possesses an N-terminal hypervariable (E2 HV) domain from amino acids 384 to 414 of unknown significance. The high degree of amino acid sequence variation in the E2 HV domain appears to be comparable to that observed in the human immunodeficiency virus type 1 gp120 V3 domain. This observation and the observation that the HCV E2 HV domain lacks conserved secondary structure imply that, like the V3 loop of human immunodeficiency virus 1 gp120, the N-terminal E2 region may encode protective epitopes that are subject to immune selection. Antibody–epitope binding studies revealed five isolate-specific linear epitopes located in the E2 HV region. These results suggest that the E2 HV domain is a target for the human immune response and that, in addition to the three major groups of HCV, defined by nucleotide and amino acid sequence identity among HCV isolates, E2 HV-specific subgroups also exist. Analysis of the partial or complete E2 sequences of two individuals indicated that E2 HV variants can either coexist simultaneously in a single individual or that a particular variant may predominate during different episodes of disease. In the latter situation, we found one individual who developed antibodies to a subregion of the E2 HV domain (amino acids 396–407) specific to a variant that was predominant during one major episode of hepatitis but who lacked detectable antibodies to the corresponding region of a second variant that was predominant during a later episode of disease. The data suggest that the variability in the E2 HV domain may result from immune selection. The findings of this report could impact vaccine strategies and drug therapy programs designed to control and eliminate HCV.**

HCV is the major etiologic agent of transfusion-associated and community-acquired non-A, non-B hepatitis on several continents (1–3). A high rate of chronic hepatitis is associated with HCV infections (4, 5). Two patterns of HCV-associated chronic liver disease have been described in humans and chimpanzees (4). Patients with intermittent hepatitis have flares of disease characterized by alternating periods of abnormally high liver enzyme (alanine aminotransferase, ALT) levels in the blood and quiescent periods in which ALT values are normal. Other individuals have persistently elevated ALT values throughout the course of chronic liver disease. The mechanism(s) of HCV pathogenesis is unknown.

HCV has been proposed as a member of the family Flaviviridae (6), which includes the pestiviruses (hog cholera virus and bovine viral diarrhea virus) and the flaviviruses, examples of which are dengue and yellow fever virus. Two putative HCV envelope glycoproteins, E1 (gp33) and E2/nonstructural protein 1 (NS1) (gp72), have been identified from vaccinia expression (Q.-L. Choo and R. Ralston, personal communication) and *in vitro* translation studies (7, 8). Based on comparative genome alignments, HCV E1 corresponds to the envelope glycoprotein gp33 (bovine viral diarrhea virus)/gp25 (hog cholera virus) of the pestiviruses and the envelope proteins (M/E) of the flaviviruses, and E2/NS1 corresponds to the gp53 (bovine viral diarrhea virus)/gp55 (hog cholera virus) envelope glycoprotein of the pestiviruses and the NS1 of the flaviviruses (6). Since HCV has greater nucleotide and amino acid sequence homology with the pestiviruses (6, 9–11) and gp72 is not secreted into the medium by stably transfected mammalian CHO cells, R. Spaete (personal communication) proposed that gp72 more likely represents a virion envelope protein (E2) equivalent to the pestivirus gp53/55 than the secreted NS1 of the flaviviruses. Although both the pestiviral gp55/gp53 and flaviviral NS1 glycoproteins are known to elicit protective antibodies in hosts vaccinated with these proteins (13, 14), nothing is known about neutralizing or protective epitopes in the putative HCV envelope glycoproteins.

Previous studies on HCV identified a hypervariable region (E2 HV) in the N terminus of E2/NS1 based on the comparative amino acid sequence analysis of several HCV isolates (15, 16). We report here that (*i*) the E2 HV domain has characteristics typical of rapidly evolving protein domains such as the V3 loop of human immunodeficiency virus 1 (HIV-1) gp120 (for review, see ref. 17), which contain linear neutralizing epitopes, (*ii*) the E2 HV domain contains isolate-specific antibody-binding linear epitopes, (*iii*) distinct episodes of hepatitis can be associated with particular E2 HV variants that possibly result from immune selection of escape mutants, and (*iv*) individuals can be coinfected with HCV E2 HV variants. These findings suggest a mechanism for persistent chronic HCV infections and may significantly influence future strategies for vaccine development and drug therapy.

MATERIALS AND METHODS

Patient Samples and RNA Extraction. Asymptomatic HCV carriers HCT-18 and HCV-J1 and chronically infected HCV patient Th have been described (15, 18). Patient Q was diagnosed with chronic active hepatitis based on a liver biopsy and was placed on interferon α_{2b} therapy (3 million units, three times weekly) for 6 months. The ALT profile of

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Abbreviations: HCV, hepatitis C virus; HV, hypervariable; NS1, nonstructural protein 1; HIV-1, human immunodeficiency virus 1; ALT, amino alaninetransferase; nt, nucleotide(s).

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patient Q is shown in Fig. 3*A*. RNA from 0.2 ml of plasma was extracted according to the method of Chomczynski and Sacchi (19) using RNAzol B reagent (Cinna Scientific, Inc., Friendswood, TX) as indicated by the manufacturer.

cDNA and PCR (20). RNA extracted from 10 μl of plasma was reverse-transcribed in a 25-μl cDNA reaction mixture (BRL cDNA synthesis kit, 8085SB) using 100 pmol of the PCR primers described below. cDNA reaction mixtures were boiled for 5 min, quick-cooled on ice, and added to the PCR reagents with final concentrations according to the Perkin–Elmer/Cetus PCR kit (N801-0055) specifications. Thirty-five PCR cycles (94°C for 1 min, 45°C for 2 min, and 72°C for 3 min) were performed. Ten microliters of the first PCR reaction mixture was added to a second PCR reaction mixture containing nested PCR primers and was amplified for 35 cycles as indicated above. PCR products were subcloned (21) either directly into M13 vectors for DNA sequencing or into a pGEM-3 plasmid vector (Promega). Insert DNA from plasmid vectors was subcloned into M13 sequencing vectors prior to DNA sequencing (22). All consensus sequences are derived from at least four cloned inserts with the exception of the HCV-J1.2 E2 HV sequence, which was derived from two clones.

Cloning and sequencing of HCT-18 and Th have been reported (15). The nested pairs of PCR primers used to clone fragments of the E2 gene from patient Q were as follows: X(E2)14 [nucleotides (nt) 1048–1067]S/X(E2)18J(nt 1291–1269)A and X(E2)4(nt 1087–1106)S/X(E2)19J(nt 1262–1243)A; X(E2)14(above)S/J1rc12(nt 1995–1978)A and US(E2)5(nt 1641–1659)S/J1rc13(nt 1941–1921)A. The following nested pairs of PCR primers were used to clone the HCV-J1 E2 gene: X(E2)14(above)S/J(E2)rc30**(nt 2030–2011)A and E2384.5*(nt 1150–1176)S/DSCON1JBX*(nt 1989–1954)A; J1IZ-2*(nt 1596–1616)S/J(E2)rc32**(nt 2337–2317)A and J1IZ-1*(nt 1641–1659)S/J(E2)rc31**(nt 2246–2227)A. One or two asterisk(s) denote a nucleotide sequence from Takeuchi *et al.* (23) or Kato *et al.* (24), respectively. PCR primers, sense (S) or antisense (A), are given in the 5′-to-3′ orientation according to the nucleotide numbers of Choo *et al.* (6).

Synthesis of Biotinylated Peptides. The overlapping octapeptides for the E2 HV regions of three strains of HCV were synthesized on cleavable-linker derivatized polyethylene pins as described (25). A "spacer" consisting of Gly-Ser-Gly-Ser-Gly was coupled to the N terminus of each peptide. Biotin was then coupled to the N terminus. The biotinylated peptides were side-chain-deprotected and washed, and the peptide from each pin was cleaved in 200 μl of 0.1 M sodium phosphate (pH 7.2). Microtiter plates containing the cleaved peptide solutions were stored at −20°C until needed.

ELISA Testing of Biotinylated and Nonbiotinylated Peptides. ELISA testing of pin-synthesized Q1 and Q3 peptides was performed according to Geysen *et al.* (26). Biotinylated peptides used in Fig. 2 were first bound to streptavidin-coated plates and then incubated with diluted sera in the ELISA assay.

Computer-Generated Secondary Structure Predictions. The α-helix, β-sheet, and β-turn secondary structure probabilities for the N-terminal region of E2 (amino acids 384–420) were determined using an algorithm (H.M.G., unpublished data) that assigns the probabilities for each of the three secondary structural motifs to each residue. The coefficients used in the algorithm were obtained for all pair-wise combinations of residues of the structural data base (27). The prediction parameters obtained from these coefficients were fitted to the observed outcome when the algorithm was applied to the data base to obtain probabilities that a given residue would be found in one of the three defined secondary structural motifs.

RESULTS

Secondary Structure Analysis of the N-Terminal Region of the HCV E2 Polypeptide. Linear epitopes are more likely associated with less-structured regions of proteins, in particular, the ends of proteins or extended surface loops such as that observed for foot and mouth disease virus (28) and HIV-1 (29). A modified computer program that indicates the probability that an individual residue is associated with a defined secondary structural motif was applied to 15 E2 HV amino acid sequences [HCV-1 (6); Q1/Q3 (see Fig. 3); HCT-23, EC10, Th, HCT-27, HCT-18 (15); HC-J1 and HC-J4 (30); HCV-E1 (31); HCV-J1.1 (23)/HCV-J1.2 (see below); HCV-J (24); HCV-BK (32)] between residues 384 and 420. Fig. 1 shows that the region between the E2 N-terminal residue 384 and the strongly predicted highly conserved β-turn (residues 415–418) is relatively unstructured, as indicated by <50% probability of α-helix, β-sheet, or β-turn character. Lack of strongly predictive structure in the E2 HV domain is consistent with the tolerance for extensive sequence variation found between isolates and is in contrast with highly structured regions that contribute to tertiary folding of the protein. The HCV E2 HV domain appears even less structured than the V3, principal neutralizing domain of

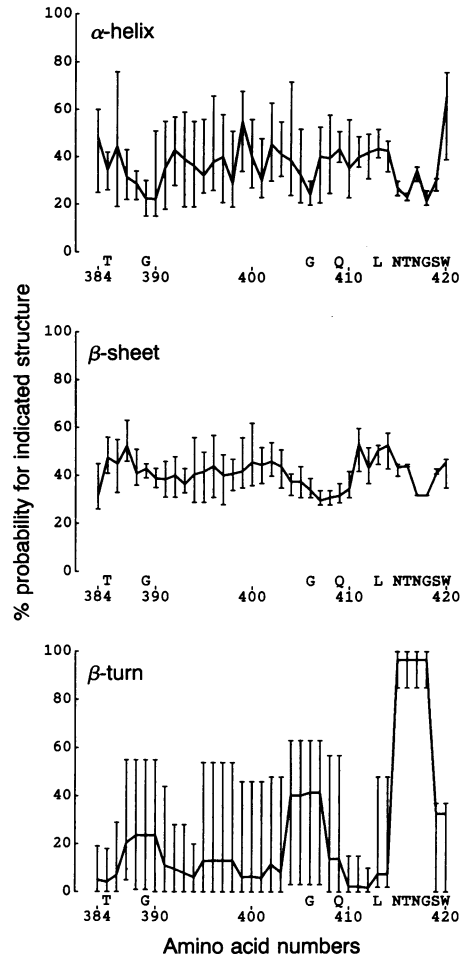

FIG. 1. Secondary structure analysis of the N terminus of the putative HCV E2 polypeptide. Percentage probabilities that a given residue from the N-terminal region of the HCV E2 protein (amino acids 384–420) will be found in an α-helix, β-sheet, or β-turn secondary structural motif. Range bars indicate the variation in the predicted probabilities at each position for 15 HCV E2 sequences described in *Results*. Amino acid numbers are according to Choo *et al.* (6). Amino acids conserved among all 15 sequences analyzed are indicated on the *x* axis in the single letter amino acid code (33).

HIV-1 gp120, reported to contain a β-strand-type-II–β-turn–β-strand–α-helix motif (34) and may have greater structural constraints on amino acid variability than the HCV E2 HV domain.

HCV E2 HV Domain Contains Antibody Binding Epitopes. Overlapping biotinylated 8-residue peptides corresponding to and extending past the E2 HV domain (amino acids 384–416) of HCV isolates HCT-18 (Fig. 2 *A* and *D*), Th (Fig. 2 *B* and *E*), and HCV-J1 (Fig. 2 *C* and *F*) were bound to plates coated with streptavidin and incubated with plasma from either HCT-18 (Fig. 2 *A–C*) or Th (Fig. 2 *D–F*). Antibody-bound biotinylated peptides were then detected in an ELISA by reactivity with a second antibody labeled with horseradish peroxidase. The antibody binding profile of HCT-18 plasma indicates that there was one linear epitope (PKQNV, residues 407–411) when tested with peptides derived from the HCT-18 sequence (HVE-I in Fig. 2*A*), but this plasma failed to react with peptides corresponding to the E2 HV domain of two other strains, Th and HCV-J1 (Fig. 2 *B* and *C*, respectively). In contrast, antibodies in Th plasma bound to linear epitope HVE-IV (QNIQLI, residues 409–414) in the HV domain of Th (Fig. 2*E*), epitopes HVE-II and -III (MTGIVRFF, residues 396–403 and IVRFFAP, residues 399–405, respectively) in HCT-18 (Fig. 2*D*) and epitope HVE-V (LTSLFRP, residues 399–405) in HCV-J1 (Fig. 2*F*). Th, an intravenous drug user, may have been exposed to multiple strains of HCV. To validate antibody binding specificity, HCT-18 antibodies bound to biotinylated peptides containing HCT-18 amino acids 407–411 were eluted and used to block the reactivity of HCT-18 plasma with pins containing overlapping 8-residue peptides derived from the HCT-18 E2 HV domain (data not shown). The data in Fig. 2 indicate that (*i*) the E2 HV domain contains linear epitopes, (*ii*) there are multiple epitopes that map to this region, and (*iii*) epitopes HVE-I to HVE-V in Fig. 2 in the HV domain are specific to the viral isolate.

Variant E2 HV Domains Can Be Associated with Particular Flares of Hepatitis and Appear To Be Under Immune Selection. To investigate the possibility of finding HCV variants associated with the intermittent flares of hepatitis often found in chronic HCV infections, we partially sequenced the E2 gene from a patient, with chronic hepatitis (patient Q) during two episodes of hepatitis that occurred ≈2 years apart (Q1 and Q3, respectively, Fig. 3*A*). The second episode of hepatitis occurred 1.5 years after the termination of interferon treatment. The deduced amino acid sequences of the Q1 and Q3 E2 HV regions were strikingly different between amino acids 391 and 407 with seven of eight amino acid substitutions in the subregion (HVE) between amino acids 398 and 407 (Fig. 3*B*). In contrast, only one amino acid heterogeneity was observed

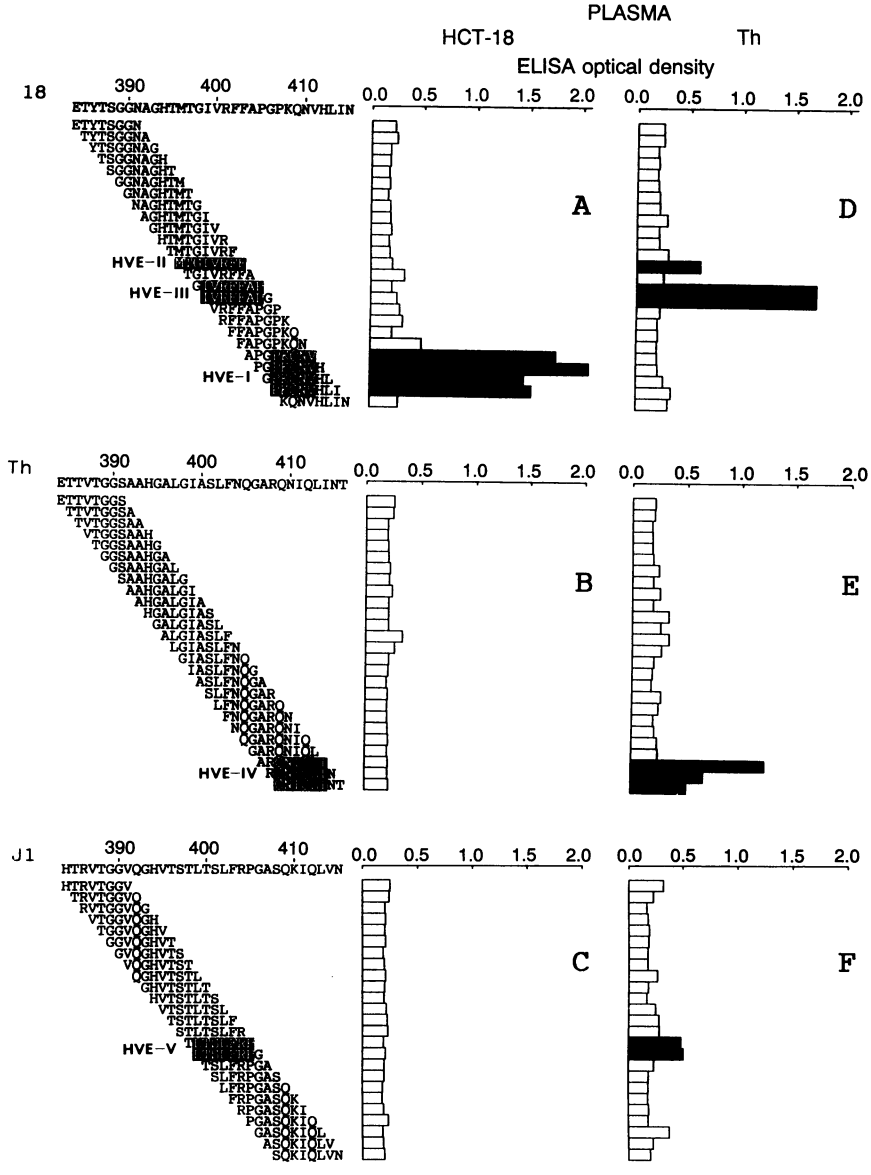

FIG. 2. Antibody-binding epitopes in the HCV E2 HV domain. Plasma from HCT-18 (*A–C*) or Th (*D–F*) was reacted with overlapping biotinylated 8-residue peptides from amino acids 384 to 416 of HCV isolates HCT-18 (designated 18) (*A* and *D*), Th (*B* and *E*), and HCV-J1 (designated J1) (*C* and *F*). HCT-18 plasma was diluted 1:200 and Th plasma was diluted 1:500. HVE-I to HVE-V represent isolate-specific antibody-binding epitopes whose amino acids are indicated in light letters in a dark background.

between amino acids 554 and 641 of the Q1 and Q3 E2 polypeptides (Fig. 3*B*).

To examine the effect of the amino acid substitutions observed in the Q1 and Q3 E2 HV domains on antibody binding, we synthesized a Q1- and Q3-specific 12-residue peptide from amino acids 396 to 407 (HVE Q1 or Q3 in Fig. 3*B*) and separately assayed the Q1 and Q3 plasmas with each peptide in an ELISA. Antibodies in the Q1 and Q3 plasma reacted with the Q1 peptide [A_{405} values of 1.158 ± 0.134 and 1.022 ± 0.123 (mean ± SD), respectively] but not with the Q3 peptide [A_{405} values of 0.691 ± 0.123 and 0.693 ± 0.036 (mean ± SD), respectively]. Statistical analysis (Student's *t* test) indicated that the binding of the Q1/Q3 plasmas to the Q1 peptide was significantly above background binding of those plasmas to a panel of 12 randomly chosen control peptides ($P < 0.001$), whereas binding of either the Q1 or Q3 plasma to the Q3 peptide was not statistically significant. The data indicate that although patient Q developed antibodies to the HCV Q1 HV domain, which were still detectable 2 years later at the Q3 time point, no detectable humoral response had developed to the Q3 E2 HV variant, which was predominant during the second episode of hepatitis.

Individuals Can Have Coexisting E2 Genes with Distinct E2 HV Domains. The partial nucleotide sequence of the E2 gene of HCV-J1 (18, 23) was determined. Two sequences, HCV-J1.1 and HCV-J1.2, were obtained from RNA extracted from a single plasma sample. Nine of 23 amino acid heterogeneities (in boldface type below) between amino acids 384 and 651 were clustered in the E2 HV domain. The deduced amino acid consensus sequences, residues 384–414, of HCV-J1.1 and HCV-J1.2 were **HTRVMGGVQGHVTSTLTSLFRP**GASQKIQLV, residues 384–414, and **NTHVTGAVQGH**GAFGLTSLFQPGASQKIQLV, residues 384–414, respectively.

DISCUSSION

The HCV is a unique infectious agent that is most closely related to the pesti- and flaviviruses (1, 3, 6, 9–11). Recent expression of the pestiviral hog cholera virus structural proteins in vaccinia vectors indicates that virus-neutralizing antibodies to the envelope glycoprotein gp55 are essential for the complete protection of pigs from a lethal challenge with the homologous virus (13). The NS1 of the flaviviruses has also been shown to elicit protective immunity in vaccinated subjects through a proposed mechanism whereby infected cells, displaying the NS1 epitopes on the surface, are lysed by complement-fixing antibodies that bind to the NS1 protein (14). Comparative nucleotide and amino acid sequence analyses of HCV with flavi- and pestiviruses and evidence from expression studies that indicate that the HCV E2/NS1 is most likely an envelope glycoprotein (E2) corresponding to the pestiviral gp53/55. The role of putative HCV glycoproteins, E1 and E2/NS1, with respect to virus neutralization is unknown.

A high degree of sequence variation in the surface proteins of infectious agents is thought to arise as a consequence of immune selection of escape mutants and, therefore, indicate sites containing or contributing to virus-neutralizing epitopes. Linear neutralizing epitopes have been identified for a number of RNA viruses including foot and mouth disease virus (34) and HIV-1 (17). In each of these cases the linear neutralizing epitope(s) coincide with a contiguous stretch of amino acids showing a high degree of sequence variation between isolates and flexible loop structures, as would be expected where mutation of any of the residues contributing to the linear epitope can lead to loss of binding by neutralizing antibodies without destroying important structural features of the protein conformation. The data in Fig. 1 indicate that the N terminus of the HCV E2 polypeptide lacks conserved secondary structural motifs and may be a flexible end with less structure than the HIV-1 gp120 V3 loop. The potential mechanism of HCV neutralization may have unique features since the E2 HV domain is unusual with respect to its predicted N-terminal location in the E2 polypeptide (7, 15, 16). By comparing the numbers of amino acid positions that contain sequence heterogeneities among 15 E2 HV regions (amino acids 384–414) and 15 HIV-1 V3 loop domains (amino acids 303–338), selected at random from the data base of unique HIV-1 sequences (34), the E2 HV domain appears to be at least as variable as the HIV-1 gp120 V3 region (data not shown). Thus, the evidence supports a theoretical argument suggesting that the E2 HV domain appears to have features characteristic of protein domains that contain likely sites of linear neutralizing epitopes.

To obtain direct evidence that variation in the HCV E2 HV domain might be due to selective immune pressure rather than random sequence variation, we performed antibody–epitope mapping experiments to show that the E2 HV domain was the target for the human immune response. The results shown in Fig. 2 indicate that five epitopes (HVE-I to HVE-V) mapped between amino acids 396 and 414 of the E2 HV domain. These data suggest that, in addition to having three major groups of HCV, subgroups based on differential immunologic reactivities to the E2 HV variants also exist.

Studies on patient Q revealed that the appearance of a variant E2 HV domain (Q3) was associated with the reappearance of disease in patient Q. Two closely related isolates (Q1 and Q3) with distinct E2 HV domains, containing eight amino acid heterogeneities in the HVE subregion, were extracted from plasma obtained during independent episodes of hepatitis that occurred within 2 years of each other in this

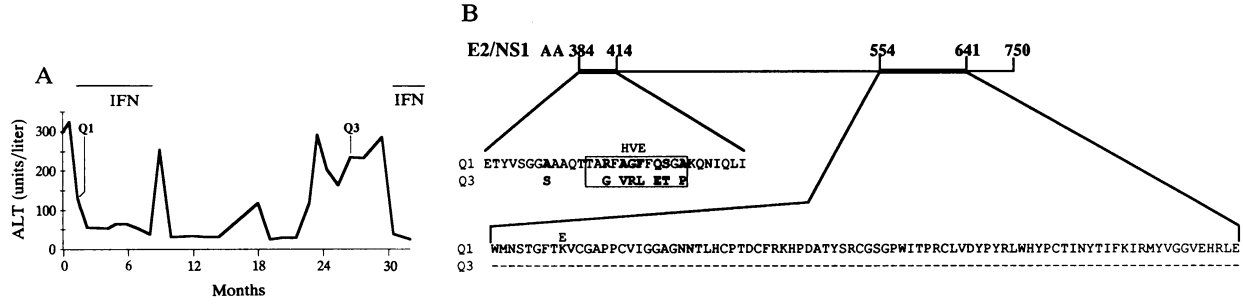

FIG. 3. ALT profile and partial amino acid sequence of the putative HCV E2 polypeptide from patient Q. The profile of ALT values (units/liter) for patient Q from June 1988 to June 1990 is shown (*A*). Q1 and Q3 denote the plasma samples from which HCV RNA was extracted. Bars indicate length of interferon (IFN) treatment. (*B*) The deduced amino acid sequences from two regions of the E2/NS1 polypeptide, amino acids 384–414 and 554–641, are given for the Q1 and Q3 isolates. The amino acid (E) above the Q1 sequence was found in one of four Q1 clones. The boxed amino acids represent the location of the HVE Q1 or Q3 12-residue peptide. Amino acid sequence differences found between Q1 and Q3 are in boldface type.

patient (Fig. 3*A*). Patient Q developed an immune response to the Q1 E2 HV peptide associated with the first episode but failed to develop detectable antibodies to the corresponding peptide from the E2 HV domain of the Q3 isolate, which was predominant during the second episode of hepatitis. Treatment with interferon may have accelerated the *in vivo* selection process of Q3 by first eliminating viral replication of Q1 and then either (*i*) permitting an existing variant, Q3, to gain a competitive advantage over Q1 after replication recommenced in the absence of a protective immune response to the Q3 variant or (*ii*) by introducing variants, to which the host had not been previously exposed, through the rapid accumulation of mutations in the predominant HCV genome during rapid replication after interferon treatment was terminated. In either case, the lack of a detectable humoral immune response to the Q3 peptide and the coincident occurrence of a second episode of disease, most likely caused by the Q3 variant, suggests that the E2 HV domain may vary under the pressure of immune selection. Consistent with our findings, which indicate that epitope(s) between amino acids 396 and 407 appear to be under the greatest selective pressure in patient Q, Ogata *et al.* (35) found that the maximal number of clustered amino acid changes between two isolates, obtained 13 years apart, from a patient with a chronic HCV infection mapped to exactly the same location as the Q1/Q3 epitope(s). HCV may utilize antigenic variation and immune selection as a general strategy to generate escape mutants, as is known to occur for the lentivirus equine infectious anemia virus (12), which may account for the high levels of chronicity observed in HCV infections. Similar molecular and immunological analyses of a greater number of individuals will be necessary to confirm that our findings represent a widespread phenomenon in HCV-infected individuals.

Cloning the E2 HV region of group II isolate HCV-J1 revealed that some individuals can be coinfected with closely related viruses with variant E2 HV domains. Nine of 23 amino acid heterogeneities between two clones of HCV-J1 mapped to the E2 HV domain. Interestingly, only one of seven cloned sequences had a single amino acid heterogeneity in the group II-specific variable domain HVR2, described by Hijikata *et al.* (16) (data not shown), indicating that the amino acids in the HVR2 domain appear to mutate at a lower frequency in group II isolates than do those in the E2 HV domain. Subgroups of related HV domains may be discovered when extensive sequence data on HCV isolates is accumulated.

This study provides theoretical evidence that the N-terminal E2 HV domain of the HCV E2/NS1 glycoprotein may contain linear neutralizing B-cell epitopes. We cannot exclude the possibility that immune selection of T-cell epitopes may also be a contributing factor in the high degree of sequence variation observed in the E2 HV domain. Unfortunately, since there is no tissue culture system available for HCV infectivity and neutralization assays, whether the E2 HV domain elicits neutralizing antibodies awaits further advances in the field. Cross-challenge experiments in chimpanzees or direct immunization of animals with peptides may be difficult to interpret if inocula contain multiple E2 HV variants as seen in HCV-J1. Although direct evidence for the involvement of the HCV E2 domain in virus neutralization remains to be established, the possible occurrence of variant neutralizing epitopes in this region is of importance in vaccine design and drug therapy programs for HCV.

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1. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989) *Science* **244**, 359–362.
2. Kuo, G. *et al.* (1989) *Science* **244**, 362–364.
3. Choo, Q.-L., Weiner, A. J., Overby, L. R., Kuo, G., Houghton, M. & Bradley, D. W. (1990) *Brit. Med. Bull.* **46**, 423–441.
4. Dienstag, J. L. & Alter, H. J. (1986) in *Seminars in Liver Disease* (Thieme, New York) Vol. 6, pp. 67–81.
5. Alter, H. J. (1985) in *Infection, Immunity and Blood Transfusion* (Liss, New York), pp. 47–61.
6. Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G. & Houghton, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2451–2455.
7. Houghton, M., Weiner, A., Han, J., Kuo, G. & Choo, Q.-L. (1991) *Hepatology* **14**, 381–388.
8. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M. & Shimotohno, K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5547–5551.
9. Takeuchi, K., Kubo, Y., Boonmar, S., Watanabe, Y., Katayama, T., Choo, Q.-L., Kuo, G., Houghton, M., Saito, I. & Miyamura, T. (1990) *J. Gen. Virol.* **71**, 3027–3033.
10. Han, J. H., Shyamala, V., Richman, K. H., Brauer, M. J., Irvine, B., Urdea, M. S., Tekamp-Olson, P., Kuo, G., Choo, Q.-L. & Houghton, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1711–1715.
11. Miller, R. H. & Purcell, R. H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2057–2061.
12. Montelaro, R. C., Parekh, B., Orrego, A. & Issel, C. J. (1984) *J. Biol. Chem.* **259**, 10539–10544.
13. Rümenapf, T., Stark, R., Meyers, G. & Thiel, H.-J. (1991) *J. Virol.* **65**, 589–597.
14. Schlesinger, J. J., Brandriss, M. W., Cropp, C. B. & Monath, T. P. (1986) *J. Virol.* **60**, 1153–1155.
15. Weiner, A. J., Brauer, M. J., Rosenblatt, J., Richman, K. H., Tung, J., Crawford, K., Bonino, F., Saracco, G., Choo, Q.-L., Houghton, M. & Han, J. H. (1991) *Virology* **180**, 842–848.
16. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S. & Shimotohno, K. (1991) *Biochem. Biophys. Res. Commun.* **175**, 220–228.
17. Goudsmit, J., Kuiken, C. L. & Nara, P. L. (1989) *AIDS* **3**, 119–123.
18. Kubo, Y., Takeuchi, K., Boonmar, S., Katayama, T., Choo, Q.-L., Kuo, G., Weiner, A. J., Bradley, D. W., Houghton, M., Saito, I. & Miyamura, T. (1989) *Nucleic Acids Res.* **17**, 10367–10372.
19. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
20. Erlich, H. A., Gelfand, D. & Sninsky, J. J. (1991) *Science* **252**, 1643–1651.
21. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
22. Messing, J. (1983) *Methods Enzymol.* **101**, 20.
23. Takeuchi, K., Boonmar, S., Kubo, Y., Katayama, T., Harada, H., Ohbayashi, A., Choo, Q.-L., Kuo, G., Houghton, M., Saito, I. & Miyamura, T. (1990) *Gene* **91**, 287–291.
24. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. & Shimotohno, K. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9524–9528.
25. Maeji, N. J., Bray, A. M. & Geysen, H. M. (1990) *J. Immunol. Methods* **134**, 23–33.
26. Geysen, H. M., Meloen, R. H. & Barteling, S. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3998–4002.
27. Levitt, M. & Greer, J. (1977) *J. Mol. Biol.* **114**, 181–293.
28. Bittle, J. L., Houghten, R. A., Alexander, H., Shinnick, T. M., Sutcliffe, J. G. & Lerner, R. A. (1982) *Nature (London)* **298**, 30–33.
29. Modrow, S., Hahn, B. H., Shaw, G. M., Gallo, R. C., Wong-Staal, F. & Wolf, H. (1987) *J. Virol.* **61**, 570–578.
30. Okamoto, H., Okada, S., Sugiyama, Y., Yotsumoto, S., Tanaka, T., Yoshizawa, H., Tsuda, F., Miyakawa, Y. & Mayumi, M. (1991) *Jpn. J. Exp. Med.* **60**, 167–177.
31. Kremsdorf, D., Porchon, C., Kim, J. P., Reyes, G. R. & Bréchot, C. (1991) *J. Gen. Virol.* **72**, 2557–2561.
32. Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. & Okayama, H. (1991) *J. Virol.* **65**, 1105–1113.
33. Lehninger, A. L. (1975) in *Biochemistry* (Worth, New York), p. 79.
34. LaRosa, G. J., Davide, J. P., Weinhold, K., Waterbury, J. A., Profy, A. T., Lewis, J. A., Langlois, A. J., Dreesman, G. R., Boswell, R. N., Shadduck, P., Holley, H., Karplus, M., Bolognesi, D. P., Matthews, T. J., Emini, E. A. & Putney, S. D. (1990) *Science* **249**, 932–935, and correction (1991) **250**, 811.
35. Ogata, N., Alter, H. J., Miller, R. H. & Purcell, R. H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3392–3396.