

Design of Novel Conformational and Genotype-Specific Antigens for Improving Sensitivity of Immunoassays for Hepatitis C Virus-Specific Antibodies

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The current commercially licensed enzyme-linked immunosorbent assays (ELISAs) for hepatitis C virus (HCV) mainly use recombinant proteins containing linear epitopes. There is evidence, however, that conformational epitopes of HCV are more immunoreactive. Thus, we have designed an HCV antibody assay that employs a conformational protein, NS3NS4a PI (with functional protease and helicase activities), and a linear fusion protein, multiple-epitope fusion antigen 7.1 (MEFA 7.1) or MEFA 7.2. We have shown that NS3NS4a PI detects early-seroconversion conformation-sensitive antibodies better than c33c antigen. The correct conformation of NS3NS4a PI also cross-reacts with different genotype samples better than the c33c antigen. MEFA 7.1 and MEFA 7.2 incorporate all the major immunodominant and genotype-specific epitopes of HCV core, E1, E2 hypervariable region 1 (HVR1), E2 HVR1-plus-HVR2 consensus, NS3, NS4, and NS5. Since MEFA 7.1 is degraded by the active NS3NS4a PI protease, we designed a second MEFA 7.2 construct in which the six protease cleavage sites found in MEFA 7.1 were eliminated by amino acid mutation. We demonstrate here that MEFA 7.2 remains intact in the presence of NS3NS4a PI and preserves the epitopes present in MEFA 7.1. Compared to currently licensed assays, an ELISA incorporating a combination of the two antigens NS3NS4a PI and MEFA 7.1 or 7.2 demonstrates better serotype sensitivity and detects seroconversion earlier in many commercially available panels. We believe that an assay using NS3NS4a PI and MEFA 7.1 or 7.2 may have the potential to replace current HCV immunoassays for better sensitivity.

Hepatitis C virus (HCV) is the major etiologic agent for blood transfusion-associated and community-acquired non-A, non-B viral hepatitis (1, 9, 19). HCV currently affects approximately 3% of the world's population, and 70% of those individuals develop chronic HCV infection, which often progresses to liver cirrhosis and hepatocellular carcinomas (3, 19, 23). The incidence of posttransfusion HCV has steadily declined since the implementation of routine screening for HCV antibodies and HCV nucleic acid amplification testing among blood donors (21). Despite the proven utility of these assays for blood screening and for the diagnosis of HCV infection in symptomatic patients, important challenges to the improvement of immunoassay performance remain. Such challenges include detecting antibody earlier, improving the detection of HCV samples from immunosuppressed patients, and increasing assay sensitivity to detect antibodies to the different HCV genotype-specific epitopes.

HCV is an enveloped virus with a single-stranded positive-sense RNA genome of approximately 9.5 kb that encodes about 3,010 amino acids (10, 24). The HCV polyprotein is processed by host and viral proteases into several mature proteins: core protein (C), envelope glycoproteins (E1 and E2), and six nonstructural proteins (NS2, NS3, NS4a, NS4b, NS5a, NS5b) (14, 17). NS3 is a 630-amino-acid protein with three enzymatic activities: the N-terminal 180 amino acids have a

serine protease function, whereas the remaining C-terminal domains have both helicase and nucleoside triphosphatase activities (2, 18, 22). The NS3 protease is responsible for cleavages at the NS3/4a, NS4a/4b, NS4b/5a, and NS5a/5b junction sites (11, 13). NS4a is a 54-amino-acid polypeptide that acts as a cofactor of the NS3 protease and is essential for polyprotein processing (12).

The current commercially licensed enzyme-linked immunosorbent assays (ELISAs) for HCV-specific antibodies use recombinant proteins containing linear epitopes. For example, three recombinant HCV proteins from the core (c22-3), NS3 and NS4 (c200), and NS5 regions are used in the Ortho HCV Version 3.0 ELISA Test System (25). The first HCV conformational protein identified that might have played an important role in immunoreactivity in HCV-infected patients was the HCV envelope antigen E2 (5, 20). Furthermore, in earlier designs of ELISAs for HCV antibodies, we observed that a recombinant HCV NS3 protein (c33c), purified under partially denatured conditions, was much more immunoreactive to seroconversion samples than denatured c33c antigen. Thus, we believe the HCV conformational epitopes may be important for the detection of early-seroconversion patient samples.

In this study, we investigated the use of a conformational antigen, NS3NS4a PI, for detection of HCV antibodies. NS3NS4a PI, when purified under nondenaturing conditions, maintains fully functional HCV protease and helicase enzymatic activities. We found that the conformational antigen NS3NS4a PI can detect early-seroconversion antibodies and cross-react with different genotype samples with better sensi-

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tivity than the c33c antigen. To complement the NS3NS4a PI conformational antigen, we added multiple-epitope fusion antigen 7.1 (MEFA 7.1) or MEFA 7.2 for detecting different HCV genotype-specific epitopes and core specificity. The MEFA 7.1 and 7.2 proteins were designed based on our previous epitope analysis study (6, 7). These constructs incorporate all of the major immunodominant epitopes from the core, envelope, and nonstructural functional regions of the HCV genome. We report here the design, purification, and characterization of the MEFA 7.1, MEFA 7.2, and NS3NS4a PI proteins and demonstrate the utility of these new antigens in improving early HCV antibody detection.

MATERIALS AND METHODS

Samples. Hepatitis C seroconversion panels PHV 904, PHV 905, and PHV 907 to 914 were purchased from Boston Biomedica Inc. (West Bridgewater, MA); panels HCV6212 to -6214 and HCV6222 were from Impath/Bioclinal Partners (Franklin, MA); panels SC-0010, SC-0030, and SC-0040 were from North American Biologics Inc. (Boca Raton, FL). According to the product inserts, each panel consists of patient sera collected longitudinally through the “window” of HCV antigen/antibody seroconversion and through a period of HCV RNA replication prior to antigen/antibody development. These patients’ sera had the virus (i.e., they were PCR positive for HCV RNA) before clinical seroconversion occurred and had antibodies against HCV NS3 and/or core antigens detected in later bleeds. HCV genotype samples were purchased from Teragenix Corp. (Fort Lauderdale, FL). Randomly selected volunteer blood donor samples were obtained from the Community Blood Center of Kansas City (Kansas City, MO).

Expression and purification of MEFA 7.1 and 7.2 polyproteins. The MEFA 7.1 and 7.2 constructs were genetically engineered for expression in *Saccharomyces cerevisiae* under the regulation of the ADH2/GAPDH hybrid promoter. The MEFA 7.1 and MEFA 7.2 antigens are chimeric polypeptides consisting of recombinant human superoxide dismutase (rhSOD), which is used to increase the level of expression of the recombinant proteins in yeast (15, 16), and different epitopes of the HCV genome (see Fig. 1B). MEFA 7.1 and MEFA 7.2 are identical except for three pairs of mutations in the helicase domain (T1428L and S1429P, N1455L and T1456P, T1657L and S1658P) and one pair of mutations in each of the 5-1-1 domains (C1711P and S1712I in 5-1-1 domain 1, C1711P and A1712I in 5-1-1 domain 2, and C1711P and S1712I in 5-1-1 domain 3). The expression cassette was created with unique restriction sites to facilitate cloning of the promoter, rhSOD, and different HCV DNA fragments into the yeast expression vector pBS24.1. This vector contains the α -factor terminator, the 2 μ m sequence for autonomous replication in yeast, and the yeast genes *leu2d* and *ura3* as selectable markers. The β -lactamase gene and the ColE1 origin of replication, required for plasmid replication in bacteria, are also present in this expression vector (7).

S. cerevisiae strain AD3 (Chiron, Emeryville, CA) was transformed with a pS.MEFA 7.1 or pS.MEFA 7.2 plasmid by using Invitrogen’s *S.c.* EasyComp transformation kit. Transformants grown on agar plates lacking uracil (Ura- agar plates) were streaked for single colonies and patched onto Leu- 8% glucose agar plates to increase the plasmid copy number. Leu- liquid starter cultures were grown for 24 h at 30°C and then diluted 1:20 in YEPD (yeast extract, Bacto peptone, 2% glucose) medium. Cells were grown for 48 h at 30°C and harvested. To test for expression of the MEFAs, aliquots of cells were lysed with glass beads in lysis buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10 mM dithiothreitol [DTT]). The insoluble fraction of each yeast lysate was analyzed by Coomassie staining of sodium dodecyl sulfate (SDS) protein gels.

Purification of MEFA 7.1 and 7.2 proteins followed the procedure described in our earlier publication (7), except that a second gel filtration was added in the final step to obtain proteins with improved purity, i.e., MEFA 7.1 and 7.2 proteins eluted from a Pharmacia Sephacryl S-400 HR column were reappplied to a Pharmacia Sephacryl S-300 HR column and then eluted and concentrated on an Amicon YM-30 membrane. MEFA 7.1 and 7.2 proteins were purified to >90% purity. The presence of MEFA 7.1 and 7.2 proteins or their fragments was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) (4 to 20% Tris-glycine gel) and Western blotting, using a monoclonal antibody directed against rhSOD, since the MEFA 7.1 and 7.2 constructs are rhSOD fusion proteins.

Expression and purification of NS3NS4a PI. NS3NS4a PI is a full-length NS3NS4a protein (amino acids 1027 to 1711) with mutations of T1428P and S1429I to remove the putative autohydrolysis site of the protease (8). A restric-

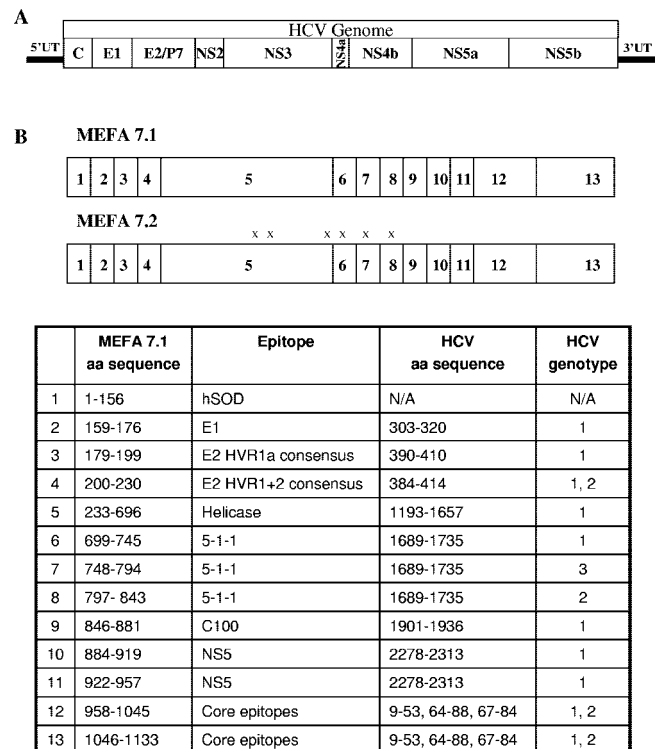


FIG. 1. (A) HCV genome-encoded structural and nonstructural proteins: core (C), envelope (E1 and E2), and nonstructural (NS2, NS3, NS4a, NS4b, NS5a, NS5b) proteins. (B) MEFA 7.1 and MEFA 7.2 epitope sequence, HCV genotype, and corresponding HCV amino acid (aa) sequence. X, six putative NS3NS4a PI protease cleavage sites in MEFA 7.1 were destroyed in MEFA 7.2 as described in Materials and Methods.

tion fragment encoding the NS3NS4a PI sequence was cloned with the ADH2/GAPDH hybrid promoter into the pBS24.1 yeast expression vector described above. Yeast transformation and expression analysis were performed under the conditions described for the MEFA 7.1 and 7.2 polyproteins.

The recombinant protein NS3NS4a PI was purified as follows. *S. cerevisiae* cells expressing the NS3NS4a PI protein were harvested and suspended in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 μ M pepstatin, 1 μ M leupeptin) and lysed in a Dyno-Mill (Willy A. Bachofen, Basel, Switzerland) or an equivalent apparatus using glass beads, at a cell/buffer/0.5-mm glass bead ratio of 1:1:1. The lysate was centrifuged at 30,100 \times g for 30 min at 4°C, and the pellet containing the insoluble protein fraction was added to the wash buffer (6 ml/g of cell pellet) and rocked at room temperature for 15 min. The wash buffer consisted of 50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl, 5 mM β -mercaptoethanol, 10% glycerol, 0.05% octyl glucoside, 1 mM EDTA, 1 mM PMSF, 0.1 μ M pepstatin, and 1 μ M leupeptin. Cell debris was removed by centrifugation at 31,000 \times g for 30 min at 4°C. The supernatant was discarded and the pellet retained.

To the pellet, 6 ml/g extraction buffer was added, and the solution was rocked at room temperature for 15 min. The extraction buffer consisted of 50 mM Tris (pH 8.0), 1 M NaCl, 5 mM β -mercaptoethanol, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 0.1 μ M pepstatin, and 1 μ M leupeptin. After centrifugation, the supernatant was retained, ammonium sulfate was added to a final concentration of 17.5%, and the mixture was stirred on ice for 10 min. The solution was centrifuged at 17,700 \times g for 30 min at 4°C, and the pellet was solubilized and run on a poly(U)-Sephacryl 4B column (Amersham Pharmacia) at 4°C as follows. The pellet was resuspended in poly(U) equilibration buffer (6 ml resin/gram of pellet). The equilibration buffer consisted of 25 mM HEPES, pH 8.0, 200 mM NaCl, 5 mM DTT (added fresh), 10% glycerol, and 1.2% octyl glucoside. The solution was rocked at 4°C for 15 min and centrifuged at 31,000 \times g for 30 min at 4°C. The supernatant was retained and loaded onto a poly(U) column (1 ml resin/gram of pellet). The column was washed to baseline with the equilibration

buffer, and the protein was eluted with a step elution in the following poly(U) elution buffer: 25 mM HEPES (pH 8.0), 1 M NaCl, 5 mM DTT (added fresh), 10% glycerol, 1.2% octyl glucoside. The column eluate was run on an SDS-PAGE gel, and aliquots containing NS3NS4a PI were frozen and stored at -80°C . The presence of the NS3NS4a PI protein was confirmed by Western blotting using a polyclonal antibody directed against the NS3 protease domain and a monoclonal antibody against the NS4 5-1-1 epitope. Using the procedure, the NS3NS4a PI protein was purified to $>90\%$ purity.

Monoclonal and polyclonal antibody binding assays. Monoclonal and polyclonal antibodies raised against HCV-specific recombinant core, E1, E2, NS3, NS4, and NS5 antigens were used to evaluate the antigenicity and epitope exposure of both the MEFA 7.1 or 7.2 protein and NS3NS4a PI. Purified MEFA 7.1 and 7.2 or NS3NS4a PI under various conditions was diluted to an optimal coating concentration in phosphate-buffered saline (PBS), pH 7.4, and was used to coat Costar high-binding plates (Corning Inc., Corning, NY). Antibodies against either linear epitopes or conformational epitopes were diluted appropriately and added to the plate. After incubation at 37°C for 1 h, the plate was washed and incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse or goat-anti-rabbit immunoglobulin G (IgG) for 1 h at 37°C . Finally, a developing buffer containing H_2O_2 and the substrate *o*-phenylenediamine dihydrochloride (OPD) was added to the wells. The optical densities (OD) at 492 and 620 nm were determined using a microplate reader.

NS3NS4a PI and MEFA 7.1 or 7.2 immunoassay procedure. Costar flat-bottom, high-binding plates were used for all immunoassays. The antigens (NS3NS4a PI at 0.45 $\mu\text{g}/\text{ml}$; MEFA 7.1 [or MEFA 7.2] at 0.375 $\mu\text{g}/\text{ml}$) were mixed in coating buffer (50 mM Na_3PO_4 [pH 7.0], 2 mM EDTA, and 0.1% chloroacetamide), and used to coat the plates (200 $\mu\text{l}/\text{well}$) for 16 h at room temperature. The plates were then washed with wash buffer (PBS-0.1% Tween 20) and tapped dry. Finally, 0.28 ml Ortho Post Coat Buffer (PBS [pH 7.4]-1% bovine serum albumin [BSA]-3% sucrose) was added to each well. After incubation for at least 1 h, the Post Coat Buffer was removed and the plates were dried in vacuo overnight at 2 to 8°C . The plates were used within a day or pouched with desiccants and stored at 4°C for future use.

The HCV antibody assay was conducted as follows. A 200- μl volume of specimen diluent buffer was added to the coated plates, followed by 20 μl of serum sample. The specimen diluent consisted of 1 g/liter casein, 100 mg/liter rhSOD, 1 g/liter chloroacetamide, 10 g/liter BSA, 500 mg/liter yeast extract, 0.366 g/liter EDTA, 1.162 g/liter K_2PO_4 , 5 ml/liter Tween 20, 29.22 g/liter NaCl, 1.627 g/liter Na_3PO_4 , and 1% SDS. The plates were incubated at 37°C for 1 h and then washed with wash buffer. Conjugate solution (200 μl of an HRP-conjugated mouse anti-human IgG, diluted in Ortho Conjugate diluent [Ortho-Clinical Diagnostics, Raritan, N.J.]) was added and incubated for 1 h at 37°C . After a wash, 200 μl substrate solution (1 OPD tablet/10 ml) was added. The substrate solution contains OPD and hydrogen peroxide for HRP reaction color development. This solution was incubated for 30 min at room temperature in the dark. The reaction was stopped by addition of 50 μl 4 N H_2SO_4 , and the plates were read at 492 nm, relative to absorbance at 690 nm as a control. The cutoff value was set at 0.600 plus the average signal (OD) of three negative-control sera. Samples with S/CO values (ratio of the OD of the signal to the cutoff) equal to or greater than 1.0 were considered to be positive, and those below 1.0 were considered to be negative.

Immunoreactivity of the NS3NS4a PI conformational epitope versus denatured NS3NS4a PI. The immunoreactivity of the NS3NS4a PI conformational antigen was compared to that of an NS3NS4a PI antigen that had been denatured by adding SDS to the NS3NS4a PI conformational epitope preparation to a final concentration of 2%. The denatured NS3NS4a PI, the conformational NS3NS4a PI, and the recombinant c200 antigen were used to coat microtiter plates under identical conditions. The immunoreactivity was tested against two early HCV seroconversion panels, PHV 904 and PHV 914, using the ELISA procedure described above.

Competition studies. Purified NS3NS4a PI or c200 at 0.5 μg and 1.0 μg was mixed with 20 μl of sample PHV 914-5 (an early-seroconversion bleed) in a total volume of 220 μl in PBS. The mixture was incubated for 1 h in microwells at 37°C . The mixture was then transferred to NS3NS4a PI-coated plates and incubated for 1 h at 37°C . Plates were washed and assayed as described above. Controls consisted of sample PHV 914-5 in PBS without added antigen.

Commercial immunoassay procedure. Commercial anti-HCV ELISA kits (Abbott PRISM, Ortho 3.0 SAve, and Pasteur Monolisa anti-HCV Plus, version 2) were used, and all experiments were performed according to the manufacturers' specifications.

All mouse monoclonal antibodies used were developed in cooperation with Bios Chile (Santiago, Chile). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

TABLE 1. ELISA analysis of MEFA 7.1 and MEFA 7.2 for exposed HCV epitopes

Antibody	Clone	OD with:	
		MEFA 7.1	MEFA 7.2
Mouse monoclonal			
Anti-core	5H8/H6	0.830	0.745
Anti-core	6H4/H8	0.742	0.807
Anti-5-1-1	6C10/D1	3.368	3.366
Anti-c33c	4D1-1	3.142	3.237
Anti-NS5	3E1/F1	3.510	3.477
Normal mouse serum		0.152	0.122
Rabbit polyclonal			
Anti-E1		1.649	1.223
Anti-E2 HVR1a consensus		1.784	1.783
Anti-E2 HVR1+2 consensus		1.302	1.441
Anti-c22		1.528	1.483
Anti-helicase		1.802	1.815
Anti-c100		1.795	1.811
Normal rabbit serum		0.100	0.102

RESULTS

Design and characterization of an HCV linear fusion protein and a conformational HCV antigen. We have designed three new antigens for the HCV antibody immunoassay: two linear fusion proteins, MEFA 7.1 and 7.2, and the conformational antigen NS3NS4a PI. Figure 1A illustrates the genetic organization of the HCV polyprotein with seven functional proteins: the core, the envelope (E1, E2), and nonstructural (NS2, NS3, NS4, NS5) proteins. The major linear epitopes of HCV-1 have been identified from the core, NS3, NS4, and NS5 regions (6). In constructing the MEFA 7.1 and 7.2 proteins, only the most reactive and most prominent epitopes (i.e., those that were able to detect antibodies over the greatest period throughout the course of infection) were chosen. More than one copy of immunodominant epitopes as well as genotype-specific epitope sequences are incorporated into the design of the MEFA 7.1 and 7.2 constructs (Fig. 1B). MEFA 7.2 has a sequence almost identical to that of MEFA 7.1, except that all six putative NS3 protease cleavage sites in MEFA 7.1 have been removed by amino acid mutagenesis (Fig. 1B). The epitope sequence arrangement in the MEFA 7.1 and 7.2 proteins is identical to that of MEFA 6 (7). In comparison with MEFA 6, the MEFA 7.1 and 7.2 proteins incorporate the E2 hypervariable region (HVR) and the E2 HVR1-plus-HVR2 (HVR1+2) consensus sequence to enhance the recognition of HCV antibodies against envelope proteins. In addition, the entire helicase region of NS3 is included in the MEFA 7.1 and 7.2 constructs. Thus, the MEFA 7.1 and 7.2 proteins are considerably larger than MEFA 6, with a molecular size of 120 kDa. The expression level of MEFA 7.1 or 7.2 protein in yeast, however, is similar to that of MEFA 6. Furthermore, the MEFA 7.1 and 7.2 proteins can be purified to $>90\%$ purity.

The epitopes of the MEFA 7.1 and 7.2 proteins were characterized with HCV-specific monoclonal and polyclonal antibodies (Table 1). Each of the epitope-specific antibodies tested reacted with both MEFA 7.1 and 7.2, indicating that (i) all the major epitopes on the fusion proteins are exposed and thus

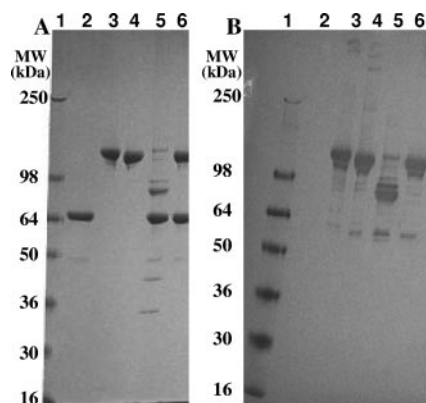


FIG. 2. Protease activity of the NS3NS4a PI antigen. MEFA 7.1, MEFA 7.2, and NS3NS4a PI were incubated alone or in combination at room temperature for 30 min. Protein gel samples were prepared and run on 4 to 20% Tris-glycine SDS-PAGE gels followed by Coomassie blue staining (A) or Western blotting (B) using an antibody against the rhSOD portion of MEFA 7.1 or 7.2. Lanes 1, molecular weight standard; lanes 2, NS3NS4a PI; lanes 3, MEFA 7.1; lanes 4, MEFA 7.2; lanes 5, MEFA 7.1 plus NS3NS4a PI; lanes 6, MEFA 7.2 plus NS3NS4a PI.

accessible for detection and (ii) the amino acid mutations in MEFA 7.2 did not distort exposure of the linear epitopes.

The other antigen used in the study is a conformational protein, NS3NS4a PI. The NS3 part of the protein is a 73-kDa protein that has dual serine protease and helicase functions. The NS4a part of the protein is a 6-kDa polypeptide and serves as a cofactor for NS3 protease activity. In order to purify full-length NS3NS4a protein, two adjacent amino acid mutations, T1428P and S1429I, were introduced into NS3NS4a PI to eliminate self-catalysis and degradation of NS3 (8). NS3NS4a PI was expressed in yeast and purified under non-denaturing conditions. The purified NS3NS4a PI had both helicase (data not shown) and protease activities. The protease activity was demonstrated by the cleavage of MEFA 7.1 (Fig. 2, lanes 5). MEFA 7.1 is a natural substrate of the protease, since it is a linear fusion protein composed of multiple epitopes from the NS3, NS4, and NS5 regions. To avoid complications associated with degradation of MEFA 7.1, we designed MEFA 7.2 with amino acid mutations intended to eliminate the cleavage sites of NS3NS4a PI protease. Both Coomassie blue-stained gels and Western blotting using a monoclonal antibody against rhSOD showed that MEFA 7.2 was not degraded when incubated with NS3NS4a PI (Fig. 2, lanes 6).

We reasoned that the correct folding of purified NS3NS4a PI, which contributed to the observed protease and helicase activities, might also expose more conformational epitopes that could be recognized by antibodies in early HCV infection. To test this hypothesis, we used two HCV seroconversion panels, PHV 904 and PHV 914, which have been characterized as having anti-c33c (NS3 region) antibodies. Table 2 shows the comparison of the immunoreactivity of NS3NS4a PI with that of denatured NS3NS4a PI or recombinant c200 protein. NS3NS4a PI detected HCV antibodies at least two bleeds earlier than denatured NS3NS4a PI or c200 (Table 2). The c200 recombinant protein is a chimeric protein made from the NS3 and NS4 regions and rhSOD; however, it was purified under denaturing conditions. We also observed that the im-

TABLE 2. Immunoreactivity of conformational NS3NS4a PI versus that of denatured NS3NS4a PI

BBI ^a ID	S/CO ^b with:		
	NS3NS4a PI	Denatured NS3NS4a PI	c200
PHV 904-1	0.02	0.02	0.01
PHV 904-2	0.02	0.01	0.01
PHV 904-3	1.80	0.11	0.07
PHV 904-4	3.85	0.44	0.21
PHV 904-5	4.85	1.28	0.57
PHV 904-6	4.35	2.37	1.28
PHV 904-7	5.28	2.99	1.55
PHV 914-1	0.01	0.01	0.01
PHV 914-2	0.01	0.01	0.01
PHV 914-3	0.16	0.01	0.01
PHV 914-4	1.79	0.01	0.01
PHV 914-5	3.26	0.07	0.04
PHV 914-6	3.35	0.12	0.04
PHV 914-7	4.04	0.45	0.22
PHV 914-8	1.40	1.46	0.82
PHV 914-9	4.94	2.78	1.53

^a BBI, Boston Biomedica Inc.

^b An S/CO greater than or equal to 1 is positive and indicated by boldfacing. The cutoff value is calculated as described in Materials and Methods.

munoreactivity of NS3NS4a PI deteriorated after the antigen stock solution went through repeated freeze-thaw cycles or storage at room temperature. We speculated that the attenuated sensitivity might be due to loss of some of the reactive epitopes during antigen storage. To assess this, we used two kinds of monoclonal antibodies. As shown in Table 3, the binding of the NS3NS4a PI antigen to the monoclonal antibodies against conformational epitopes decreased over time, whereas its binding to an antibody against linear epitopes was unchanged. These data suggest that the conformational

TABLE 3. Conformational versus linear epitopes during NS3NS4a PI storage^a

Monoclonal antibody	Dilution	OD at the following time:			
		0 h	3 h	6 h	24 h
5B7/D7 (conformational)	1:40,000	0.481	0.216	0.093	0.029
	1:20,000	0.759	0.354	0.160	0.050
	1:10,000	1.039	0.603	0.267	0.089
	1:1,000	1.998	1.082	0.574	0.221
	1:100	1.909	1.116	0.663	0.351
1A8/H2 (conformational)	1:40,000	0.205	0.089	0.032	0.008
	1:20,000	0.323	0.149	0.058	0.022
	1:10,000	0.446	0.220	0.078	0.021
	1:1,000	0.675	0.366	0.177	0.068
	1:100	0.783	0.385	0.198	0.083
4D1-1 (linear)	1:40,000	2.868	2.800	2.840	2.936
	1:20,000	3.062	2.861	2.796	3.192
	1:10,000	3.168	3.118	2.785	3.019
	1:1,000	2.988	2.854	2.778	3.164
	1:100	3.169	2.730	2.900	2.222

^a NS3NS4a PI stock solution was stored at room temperature for 0, 3, 6, and 24 h before it was used to coat microplate wells. Mouse monoclonal antibodies against linear or conformational epitopes of NS3NS4a PI were added and incubated as described in Materials and Methods. An OD of 0.5 or greater is considered to be positive (boldfaced).

TABLE 4. Competition binding of added antigen and solid-phase NS3NS4a PI to HCV antibodies^a

OD of control ^b	Antigen added (amt ^c)	OD of PHV 914-5 + antigen	% Inhibition ^d
1.645	c200 (1.0)	1.450	12
1.687	c200 (1.0)	1.545	8
1.913	c200 (0.5)	1.557	19
1.804	c200 (0.5)	1.719	5
1.599	NS3NS4a PI (1.0)	0.054	97
1.677	NS3NS4a PI (1.0)	0.037	98
1.672	NS3NS4a PI (0.5)	0.066	96
1.524	NS3NS4a PI (0.5)	NT ^e	NT

^a Assessed by using early-seroconversion sample PHV 914-5 as described in Materials and Methods.

^b Sample PHV 914-5 alone, without added antigen.

^c In micrograms.

^d Calculated as 100 × [(OD of control) – (OD of sample with antigen added)].

^e NT, not tested.

epitopes in NS3NS4a PI are most likely responsible for the detection of early-seroconversion antibodies.

We further compared the epitope reactivity of NS3NS4a PI to that of c200 by using an inhibition assay where either NS3NS4a PI or c200 was added to an early-seroconversion sample prior to binding to solid-phase NS3NS4a PI. As shown in Table 4, prior incubation of c200 failed to inhibit binding of the antibodies to NS3NS4a PI, implying that NS3NS4a PI might be able to detect a class of antibodies different from those detected by c200.

Immunoassay performance. (i) Seroconversion sensitivity. The c33c antigen of NS3 and the c22 core antigen are very immunogenic, and antibodies to c33c and c22 are found in early-seroconversion panels (4). We used well-characterized

commercially available c33c and c22 panels to assess seroconversion sensitivity. Microplate wells were coated with MEFA 7.1, MEFA 7.2, and NS3NS4a PI alone or in combination. ELISAs were performed as described in Materials and Methods, and the results are summarized in Table 5. For the panels tested, MEFA 7.1 alone and MEFA 7.2 alone have similar immunoreactivities. Both antigens detected c22 antibodies in seroconversion panel PHV 913 and c33c antibodies in later bleeds of PHV 904 and PHV 914 (Table 5). NS3NS4a PI, on the other hand, detected antibodies to c33c in early bleeds of PHV 904 and PHV 914 (Table 5). Thus, the combination of MEFA 7.1 or 7.2 and NS3NS4a PI detected antibodies to c22 and c33c in early-seroconversion samples. The detections were 2 and 12 days ahead of the currently licensed Ortho HCV Version 3.0 ELISA Test System and the Abbott PRISM assay for c33c and c22 antibodies, respectively. We further tested 17 commercially available HCV seroconversion panels, and the results are summarized in Table 6. For anti-c33c (NS3) type panels, the new antigens are 2 to 14 days ahead of the Ortho HCV Version 3.0 ELISA Test System and the Abbott PRISM assay in 9 out of 9 panels. For anti-c22 (core) panels, the new antigens are 2 to 5 days ahead of the Ortho HCV Version 3.0 ELISA Test System or the Abbott PRISM assay in 3 out of 8 panels, and the rest of the panels are equivalent. It should be emphasized that among the five anti-core panels showing equivalent performance between the new assay and the licensed assay, three panels had large bleed intervals between the shift from antibody negative to antibody positive: PHV909 (28 days), PHV911 (11 days), and SC-0010 (7 days) (Table 6). Due to the length of the bleed intervals, it is difficult to compare seroconversion sensitivity between the new assay and the licensed assays using these three panels.

TABLE 5. Comparison of HCV seroconversion detection

Seroconversion panel	Bleed day	S/CO ^a with:							Days ahead ^b
		MEFA 7.1	MEFA 7.2	NS3NS4a PI	MEFA 7.1 + NS3NS4a PI	MEFA 7.2 + NS3NS4a PI	Ortho HCV 3.0	Abbott PRISM	
PHV904-1	0	0.04	0.03	0.04	0.05	0.05	0.01	0.12	
PHV904-2	2	0.02	0.03	0.03	0.04	0.04	0.01	0.08	
PHV904-3	7	0.33	0.23	1.83	1.79	2.47	0.33	0.51	
PHV904-4	9	1.28	1.28	5.19	3.99	5.39	1.10	1.56	2
PHV904-5	14	2.17	2.75	5.76	5.14	5.70	3.27	3.54	
PHV904-6	21	2.43	3.29	5.81	5.24	5.78	3.92	4.45	
PHV904-7	23	2.73	3.57	6.31	6.36	6.31	4.26	4.69	
PHV914-1	0	0.02	0.02	0.02	0.03	0.03	0.00	0.06	
PHV914-2	5	0.02	0.02	0.03	0.03	0.03	0.01	0.06	
PHV914-3	9	0.03	0.02	0.07	0.11	0.14	0.01	0.06	
PHV914-4	12	0.09	0.03	1.25	1.38	2.09	0.04	0.09	12
PHV914-5	16	0.44	0.28	3.22	2.80	4.28	0.33	0.47	
PHV914-6	19	0.73	0.65	3.51	3.23	4.75	0.82	0.90	
PHV914-7	24	2.00	2.23	4.69	4.72	5.52	3.10	2.41	
PHV914-8	30	2.73	4.23	5.47	5.15	5.62	4.85	4.09	
PHV914-9	33	2.95	4.64	5.65	5.36	5.66	4.85	4.52	
PHV913-1	0	0.08	0.09	0.03	0.11	0.08	0.01	0.08	
PHV913-2	2	0.41	0.55	0.02	0.71	0.56	0.02	0.10	
PHV913-3	7	1.62	2.47	0.07	2.66	2.90	0.43	0.50	>2
PHV913-4	9	1.88	2.71	0.43	2.83	3.41	0.54	0.59	

^a An S/CO greater than or equal to 1 is positive and is indicated by boldfacing. The cutoff value is calculated as described in Materials and Methods.

^b Number of days by which detection with NS3NS4a PI in combination with MEFA 7.1 or MEFA 7.2 precedes detection by currently licensed assays.

TABLE 6. Summary of study of 17 seroconversion panels

Panel	Predominant antibody ^a	Genotype ^a	Days ahead of licensed assay ^b
PHV 904	NS3	1a	2
PHV 905	NS3	1a	7
PHV 908	NS3	3	8
PHV 914	NS3, core	2b	12
HCV 6212	NS3	Unknown	14
HCV 6213	NS3	Unknown	6
HCV 6214	NS3	Unknown	7
HCV 6222	NS3	Unknown	4
SC-0040	NS3	2b	9
PHV 913	Core	2b	>2
PHV 907	Core	1b	3
PHV 909	Core	3	0 ^c
PHV 910	Core	1b	0
PHV 911	Core	1a	0 ^c
PHV 912	Core	2b/3	0
SC-0030	Core	1a	5
SC-0010	Core	3a	0 ^c

^a Data provided by the HCV panel vendors.

^b Comparison of assay sensitivity using MEFA 7.1 in combination with NS3NS4a PI versus a currently licensed assay, the Ortho HCV Version 3.0 ELISA Test System or Abbott PRISM.

^c Bleed intervals between the shift from antibody negative to antibody positive: 28 days for PHV 909, 11 days for PHV 911, and 7 days for the SC-0010 panel.

(ii) Genotype dilution sensitivity. We also compared genotype dilution sensitivities for the new antigens versus commercially available assays. All three immunoassays were run simultaneously. As shown in Table 7, serial dilutions of all the HCV genotypes (genotypes 1 to 6) were strongly detected with MEFA 7.1 and NS3NS4a PI in the ELISA compared to HCV 3.0 or Monolisa Ver. 2 Pasteur. In a separate experiment, we compared genotype dilution sensitivities using MEFA 7.1 or NS3NS4a PI alone. It was clear that the increased genotype sensitivity resulted mainly from the conformational antigen NS3NS4a PI (Table 7).

(iii) Specificity. A total of 500 screened human plasma samples selected from volunteer blood donor specimens were tested. Initial testing showed 1 out of 500 to be reactive. The reactive sample, however, could not be confirmed by either a RIBA 3.0 SIA (Chiron Corp.) or a Gen-Probe TMA (nucleic acid) assay. It was found later that the false positive resulted from reduction of BSA levels by DTT in the coating buffer. When the 500 normal sera were retested in the absence of DTT, none showed reactivity. Omitting DTT in the coating buffer had little impact on antigen stability: both antigens coated with DTT and those without DTT were stable for 16 h at room temperature. We also compared interference panels using NS3NS4a PI and MEFA 7.1 versus Ortho HCV 3.0. For a panel of alcoholics' and autoimmune patients' liver samples, those that tested positive by use of NS3NS4a PI and MEFA 7.1 were confirmed using RIBA HCV 3.0 SIA. For a panel of lipemic, bilirubin, cholesterol, and triglyceride samples, those with positive or negative results using NS3NS4a PI and MEFA 7.1 were confirmed as positive or negative, respectively, by the Ortho HCV Version 3.0 ELISA Test System.

In summary, the prototype HCV antibody assay employing NS3NS4a PI and MEFA 7.1 or 7.2 achieved greater sensitivity both in early-seroconversion detection and in detection of samples of different genotypes. The assay specificity matched that of the current assay. After NS3NS4a PI and MEFA 7.1 were used for coating plates and dried in vacuo, they were remarkably stable: we have obtained similar assay results using either freshly coated plates or plates that have been stored for more than 2 years.

DISCUSSION

The primary goal in the development of an immunoassay is to achieve the greatest possible sensitivity and specificity. Here we report a rational design of three novel recombinant antigens, MEFA 7.1, MEFA 7.2, and NS3NS4a PI, and demon-

TABLE 7. Comparison of HCV genotype dilution sensitivities

Genotype	Dilution	OD with:				
		NS3NS4a PI	MEFA 7.1	MEFA 7.1 + NS3NS4a PI	Ortho HCV 3.0	Monolisa Ver 2 Pasteur
1b	1:5,000	1.929	1.396	2.074	0.393	0.218
	1:10,000	1.506	0.826	1.699	0.159	0.084
	1:20,000	0.382	0.355	0.403	0.045	0.028
2a/c	1:5,000	0.868	0.717	0.917	0.136	0.782
	1:10,000	0.386	0.312	0.395	0.049	0.286
3a	1:5,000	1.879	0.964	1.622	0.218	0.353
	1:10,000	0.676	0.432	0.873	0.067	0.164
4a	1:5,000	1.392	0.824	1.752	0.193	0.181
	1:10,000	1.169	0.265	0.717	0.069	0.076
5a	1:5,000	2.889	1.763	2.744	0.827	0.988
	1:10,000	1.317	1.036	1.587	0.316	0.395
	1:20,000	0.715	0.416	0.726	0.097	0.120
6	1:1,000	2.978	2.455	3.224	2.863	ND ^a
	1:10,000	2.841	0.984	1.192	0.380	ND

^a ND, not determined.

strate their clinical utility in improving early detection of HCV infection. Previously, we have shown that MEFA 6, a single multiple-epitope fusion protein, is capable of detecting HCV at a two- to fourfold-greater dilution sensitivity than the c25 chimeric antigen made from the NS3, NS4, and core regions (7). MEFA 7.1 and 7.2 proteins were designed to further improve on MEFA 6 in overall sensitivity and in detecting genotype-specific antibodies. We added the following in the MEFA 7.1 and 7.2 constructs: (i) an E2 HVR1a cassette, because it contains linear neutralizing epitopes of E2 antigen (5, 20); (ii) an E2 HVR1+2 consensus cassette, because it can cross-react with genotype 1, 2, and 3 samples (5, 20); (iii) full-length helicase, because it is more reactive than the c33c region; and (iv) more core epitopes that are specific to HCV genotypes 1 and 2 (Fig. 1). As shown in Table 1, all the epitopes in the MEFA 7.1 or 7.2 protein are detected by epitope-specific antibodies. In seroconversion panels tested in this study, the MEFA 7.1 or 7.2 antigen core immunoreactivity is better than (Table 5) or equivalent to those of currently licensed assays. Although MEFA 7.1 has multiple NS3NS4a PI protease cleavage sites and is thus degraded to several fragments during coating (Fig. 2), the immunoreactivity of the fusion protein is unaffected (Tables 5 and 7). The improved MEFA 7.2 is intact in the presence of NS3NS4a PI (Fig. 2), and the epitope exposure and immunoreactivity are very similar to those of MEFA 7.1 (Tables 1, 5 and 7). These data suggest that the increased sensitivity of detection for early-seroconversion samples is derived from multiple epitopes in the MEFA 7.1 or 7.2 protein; the overall structure of the MEFA fusion protein itself, either intact as in MEFA 7.2 or degraded as in MEFA 7.1, has little effect on early-seroconversion detection.

NS3NS4a PI, reported here, might be considered the first of a new class of diagnostic antigens for detecting HCV antibodies. The antigen complex expressed in yeast and purified under non-denaturing conditions retains both protease and helicase functions, which implies that the protein has the correct conformation. We believe that the conformational epitopes and correct folding of NS3NS4a PI are effective in detecting early-seroconversion or low-affinity antibodies in HCV infection (Table 5). This is supported by the fact that denaturation of the NS3NS4a PI complex reduces its utility as an antigen in detecting early-seroconversion samples (Table 2). The loss of conformational epitopes (Table 3) apparently correlates with reduced sensitivity in early-seroconversion detection. Interestingly, excess c200 antigen added to an early-seroconversion sample competes poorly with NS3NS4a PI for binding of HCV antibody (Table 4). This suggests that NS3NS4a PI may detect a different class of antibodies in early HCV infection that are sensitive to the conformation of the epitopes.

The conformational NS3NS4a PI antigen detects early-seroconversion samples and genotype-specific antibodies with greater sensitivity than current assays (Table 6). One possible explanation is that NS3NS4a PI contains helicase that is highly conserved and thus may contain common epitopes that can cross-react with all genotype-specific antibodies. At present, it is unclear whether the helicase domain or the protease domain or both are required to present the conformation or epitopes for recognizing early HCV antibodies as well as detecting genotype-specific antibodies. Studies are under way to further delineate this antigen complex.

The assay described here represents the first prototype assay using a conformational NS3NS4a PI antigen as well as a linear fusion protein, MEFA 7.1 or 7.2, with all the immunodominant and genotype-specific epitopes from the HCV genome. The two antigens complement each other: MEFA 7.1 and 7.2 proteins detect core antibodies, whereas NS3NS4a PI detects c33c type antibodies. The new HCV antibody assay employing NS3NS4a PI and MEFA 7.1 or 7.2 is more sensitive than currently marketed assays and has the potential to replace current HCV immunoassays to provide improved performance.

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REFERENCES

- Alter, H. J., R. H. Purcell, J. W., Shih, J. C. Melpolder, M. Houghton, Q.-L. Choo, and G. Kuo. 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N. Engl. J. Med.* **321**:1494-1500.
- Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen. 1993. Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. *J. Virol.* **67**:3835-3844.
- Bruix, J., X. Calvet, J. Costa, M. Ventura, M. Bruguera, R. Castillo, J. M. Barrera, G. Erquilla, J. M. Sanchez-Tapias, M. Vall, C. Bru, and J. Rodes. 1989. Prevalence of antibodies to hepatitis virus in Spanish patients with hepatocellular carcinoma and hepatic cirrhosis. *Lancet* **ii**:1004-1006.
- Chien, D. Y., Q.-L. Choo, A. Tabrizi, C. Kuo, J. McFarland, K. Berger, C. Lee, J. R. Shuster, T. Nguyen, D. L. Moyer, M. Tong, S. Furuta, M. Omata, G. Tegtmeyer, H. Alter, E. Schiff, M. Jeffers, M. Houghton, and G. Kuo. 1992. Diagnosis of hepatitis C virus (HCV) infection using an immunodominant chimeric polyprotein to capture circulating antibodies: reevaluation of the role of HCV in liver disease. *Proc. Natl. Acad. Sci. USA* **89**:10011-10015.
- Chien, D. Y., Q.-L. Choo, R. Ralston, R. Spaete, M. Tong, M. Houghton, and G. Kuo. 1993. Persistence of HCV despite antibodies to both putative envelope glycoproteins. *Lancet* **342**:933.
- Chien, D. Y., J. McFarland, A. Tabrizi, C. Kuo, M. Houghton, and G. Kuo. 1994. Distinct subtypes of hepatitis C virus defined by antibodies directed to putative core, NS4 and NS5 region polypeptides, p. 320-324. *In* K. Nishioka, H. Suzuki, S. Mishiro, and T. Oda (ed.), *Viral hepatitis and liver disease*. Springer-Verlag, Tokyo, Japan.
- Chien, D. Y., P. Arcangel, A. Medina-Selby, D. Coit, M. Baumeister, M., S. Nguyen, C. George-Nascimento, A. Gyenes, G. Kuo, and P. Valenzuela. 1999. Use of a novel hepatitis C virus (HCV) major-epitope chimeric polypeptide for diagnosis of HCV infection. *J. Clin. Microbiol.* **37**:1393-1397.
- Chien, D. Y., M. J. Selby, K. Shoemaker, and R. L. Warne. August 2002. Protease regulator screening assay. U.S. patent 6,436,666.
- Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359-362.
- Choo, Q.-L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, and P. J. Barr. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **88**:2451-2455.
- Eckart, M. R., M. Selby, F. Masiarz, C. Lee, K. Berger, K. Crawford, C. Kuo, G. Kuo, M. Houghton, and Q.-L. Choo. 1993. The hepatitis C virus encodes a serine protease involved in processing of the putative nonstructural proteins from the viral polyprotein precursor. *Biochem. Biophys. Res. Commun.* **192**:399-406.
- Failla, C., L. Tomei, and R. De Francesco. 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* **68**:3753-3760.
- Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**:2832-2843.
- Grakoui, A., C. Wychowski, C. Lin, S. M. Feinstone, and C. M. Rice. 1993. Expression and identification of hepatitis C virus polyprotein cleavage products. *J. Virol.* **67**:1385-1395.
- Hallewell, R. A., F. R. Masiarz, R. C. Najarian, J. P. Puma, M. R. Quiroga, A. Randolph, R. Sanchez-Pescador, C. J. Scandella, B. Smith, K. S. Steimer, et al. 1985. Human Cu/Zn superoxide dismutase cDNA: isolation of clones

- synthesising high levels of active or inactive enzyme from an expression library. *Nucleic Acids Res.* **13**:2017–2034.
16. **Hallewell, R. A., I. Laria, A. Tabrizi, G. Carlin, E. D. Getzoff, J. A. Tainer, L. S. Cousens, and G. T. Mullenbach.** 1989. Genetically engineered polymers of human CuZn superoxide dismutase. *Biochemistry and serum half-lives.* *J. Biol. Chem.* **264**:5260–5268.
 17. **Hijikata, M., H. Mizushima, Y. Tanji, Y. Komoda, Y. Hirowatari, T. Akagi, N. Kato, K. Kimura, and K. Shimotohno.** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. *Proc. Natl. Acad. Sci. USA* **90**:10773–10777.
 18. **Kim, D. W., Y. Gwack, J. H. Han, and J. Choe.** 1995. C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem. Biophys. Res. Commun.* **215**:160–166.
 19. **Kuo, G., Q.-L. Choo, H. J. Alter, G. I. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W. S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human chronic non-A, non-B hepatitis. *Science* **244**:362–364.
 20. **Lok, A. S., D. Y. Chien, Q.-L. Choo, T. M. Chan, E. K. Chiu, I. K. Cheng, M. Houghton, and G. Kuo.** 1993. Antibody response to core, envelope and nonstructural hepatitis C virus antigens: comparison of immunocompetent and immunosuppressed patients. *Hepatology* **18**:497–502.
 21. **Pillonel, J., S. Laperche, C. Saura, J. C. Desenclos, and A. M. Courouce.** 2002. Trends in residual risk of transfusion-transmitted viral infections in France between 1992 and 2000. *Transfusion* **42**:980–988.
 22. **Preugschat, F., D. R. Averett, B. E. Clarke, and D. J. Porter.** 1996. A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. *J. Biol. Chem.* **271**:24449–24457.
 23. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, Q.-L. Choo, M. Houghton, and G. Kuo.** 1990. Hepatitis C virus infection is associated with the development of hepato-cellular carcinoma. *Proc. Natl. Acad. Sci. USA* **87**:6547–6549.
 24. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* **65**:1105–1113.
 25. **Uyttendaele, S., H. Claeys, W. Mertens, H. Verhaert, and C. Vermeylen.** 1994. Evaluation of third-generation screening and confirmatory assays for HCV antibodies. *Vox Sang.* **66**:122–129.