Isolation of a cDNA Clone Derived from a Blood-Borne Non-A, Non-B Viral Hepatitis Genome
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have a more stabilizing effect on the IRE-BP than direct iron starvation or administration. Finally, treatment of RD4 cells with puromycin at doses that completely inhibit protein synthesis has no effect on the induction of IRE-binding activity in response to Df (*19*).

We therefore propose that chelation of iron by Df results in the activation of the IRE-BP by leading to the reduction of an intramolecular disulfide in the IRE-BP. At least one of the now free cysteinyl residues is required for a high affinity interaction between the protein and the IRE which, in turn, is responsible for the repression of ferritin mRNA translation. In essence, alteration in cellular iron status operates a "sulfhydryl switch" by reversible oxidation or reduction of critical sulfhydryl group or groups in the IRE-BP. This hypothesis raises the question of whether such a switch could be physiologically relevant in the reducing environment of the cytosol. The major redox buffer in the cytosol is the glutathione system. The vast excess of reduced over oxidized glutathione is largely responsible for the reducing potential of the cytosol. A study on the reversible oxidation-reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase demonstrates that oxidized sulfhydryls can exist and even predominate within the cytosolic glutathione redox buffer system (*23*). Two factors can determine the redox state of a protein sulfhydryl within the cytosol. One is the ratio of reduced to oxidized glutathione, which can change significantly under physiologic conditions (*23*). The second is the oxidation equlibrium constant (K_{ox}) for a particular sulfhydryl group within a protein. Equilibrium constants for protein sulfhydryls can vary over many orders of magnitude, reflecting the effects of the local environment around the cysteinyl moiety on its K_{ox} (*24*). These local effects may reflect the stabilization or destabilization of the thiolate anion. Conformational changes that alter this local environment can therefore affect the K_{ox} of a particular cysteine sulfhydryl group. In this way allosteric effectors can perturb the K_{ox} of sulfhydryls on specific proteins and thereby alter the redox state of the protein, even in the presence of a constant cytosolic redox buffer. Our data on the IRE-BP provide an example of the utility of oxidation-reduction as a reversible covalent modification in the regulation of cellular protein function.

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17. Partial purification of the IRE-binding protein was achieved by RNA affinity chromatography. An IRE-containing RNA was transcribed in vitro by RNA polymerase T7 (*18*) with two synthetic oligodeoxynucleotides that when hybridized constituted an RNA polymerase T7 promoter and a template for the ferritin IRE (Fig. 1) plus the 3′ nucleotides $\mathrm{UCUCUCUCU}_{24}$. The reaction included 3 m*M* biotin-11–uridine triphosphate (UTP) (Bethesda Research Laboratories) and 1 m*M* UTP, in addition to [α-^{32}P]guanosine triphosphate. The RNA was purified by hybridization chromatography on poly(A) Sepharose (Pharmacia). RNA was added to a lysate of K562 cells and incubated for 30 min at room temperature before addition of 5 mg of sodium heparin (Hynson, Westcott, and Dunning) per milliliter. This sample was then added to streptavidin agarose (Bethesda Research Laboratories) and the mixture was incubated for an additional 15 min before the resin was washed five times with 20 volumes of 40 m*M* KCl, 25 m*M* tris-Cl, *p*H 8, and 1% Triton X-100. Elution of IRE-BP was accomplished with 1*M* KCl, 5 mg of sodium heparin per milliliter, 25 m*M* tris-Cl, *p*H 8, and 1% Triton X-100. Based on recovery of IRE-binding activity and the recovery of labeled K562 protein from a parallel experiment with a [^{35}S]methionine lysate, we estimate that the IRE-binding protein was purified approximately 50-fold by this procedure.
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Isolation of a cDNA Clone Derived from a Blood-Borne Non-A, Non-B Viral Hepatitis Genome

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A random-primed complementary DNA library was constructed from plasma containing the uncharacterized non-A, non-B hepatitis (NANBH) agent and screened with serum from a patient diagnosed with NANBH. A complementary DNA clone was isolated that was shown to encode an antigen associated specifically with NANBH infections. This clone is not derived from host DNA but from an RNA molecule present in NANBH infections that consists of at least 10,000 nucleotides and that is positive-stranded with respect to the encoded NANBH antigen. These data indicate that this clone is derived from the genome of the NANBH agent and are consistent with the agent being similar to the togaviridae or flaviviridae. This molecular approach should be of great value in the isolation and characterization of other unidentified infectious agents.

WITH THE DEVELOPMENT OF SPECIFIC diagnostics for the hepatitis A virus (HAV) and the hepatitis B virus (HBV) in the 1970s, it became clear that most cases of hepatitis arising from blood transfusion were not caused by infections with these or other known viral agents (*1–4*). Despite over a decade of research, the agent or agents responsible for this so-called non-A, non-B hepatitis (NANBH) remains unidentified (*5, 6*), although there is evidence that one blood-borne NANBH agent may be a small, enveloped virus that is readily transmissible to chimpanzees (*7, 8*). A major impediment to progress in studies of this virus has been that despite intensive work, conventional immunological methods have consistently failed to identify specific viral antibodies and antigens (*5, 6*). Although this failure could be interpreted in terms of a lack of viral antibody, we consid-

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ered it to be due more likely to insufficient concentrations of viral antigen in NANBH infections.

Therefore, in order to increase viral antigen concentrations, a cDNA library derived from infectious material was constructed in the bacteriophage λgt11. This vector allows the efficient expression of cDNA-encoded polypeptides and was designed originally to facilitate the isolation of cDNA clones by means of well-characterized antibodies that bind to clones synthesizing the polypeptide of interest (*9*). This library was then screened for rare clones expressing viral antigen with serum from a chronic NANBH patient as a presumed source of viral antibodies. To increase the probability of detecting viral clones, the cDNA library was derived from chimpanzee plasma containing a relatively high infectious titer (*10*). This plasma was subjected to extensive ultracentrifugation in order to ensure the pelleting of a small virus, and nucleic acid was recovered from the pellet. Since the nature of the genome was unknown, the recovered nucleic acid was completely denatured before synthesizing cDNA from both RNA and DNA with random primers of reverse transcriptase. Screening $\sim 10^6$ of the resulting recombinant λgt11 phage led to the identification of positive cDNA clone 5-1-1.

To investigate its potential viral origin, a larger overlapping clone (clone 81) was first isolated from the same library, and the cDNA was hybridized to human and chimpanzee DNA by Southern blot analyses. This cDNA did not hybridize either to control human DNA or to DNA derived from two chimpanzees with NANBH infections (Fig. 1A). In a control experiment, the single-copy interferon gene from human fibroblasts yielded clear hybridization signals under identical conditions (Fig. 1B). This proves that clones 5-1-1 and 81 are not derived from the host genome and that DNA replication intermediates related to these sequences were undetectable.

Whereas homologous DNA sequences were undetectable, total RNA extracted from infectious chimpanzee liver hybridized specifically to the cloned cDNA but not total RNA derived from control, uninfected chimpanzee livers (Fig. 2A). The abundance of homologous RNA in total liver RNA from the infected animal was estimated to be ~0.00001% (w/w). Furthermore, total nucleic acid extracted from ultracentrifuged pellets of the high-titer NANBH chimpanzee plasma hybridized to these clones, but this hybridization signal was lost after treatment with ribonuclease but not deoxyribonuclease (Fig. 2B). Hence, it appears that these clones are derived from an exogenous RNA molecule associated with NANBH infection. This RNA from infectious plasma appears to be single-stranded since only one of the strands in clone 81 cDNA could hybridize to it even though both strands hybridized with equal efficiency to the double-stranded clone (Fig. 2C). To analyze the size of the RNA homologous to these cDNA clones, we separated RNA derived from infectious chimpanzee liver by electrophoresis through a denaturing formaldehyde agarose gel, transferred it to nitrocellulose, and hybridized it with clone 81 cDNA. Although the hybridization signal obtained with total RNA was weak, there was strong hybridization to a heterogeneous population of RNA molecules that bound to oligo(dT)-cellulose (Fig. 2D). The approximate size of this RNA was estimated to be between 5,000 to 10,000 nucleotides. The observed smear may reflect degradation during preparation and the maximum size of the original RNA may be at least 10,000 nucleotides. The binding to oligo(dT)-cellulose indicates that there is either a 3′ terminal polyadenylate sequence or an A-rich tract elsewhere in the molecule.

The nucleotide sequence of clone 5-1-1 indicated that the cDNA strand encoding the immunoreactive polypeptide possessed one continuous, translational open reading frame (ORF) (the sequence of the genome is being completed and will be deposited in the GenBank database shortly). To investigate the relation of this polypeptide with NANBH, this ORF was expressed in bacteria as a fusion polypeptide with human superoxide dismutase (SOD) and immunoblot analyses were performed on total bacterial lysates. The chronic NANBH patient serum used originally to detect clone 5-1-1 reacts specifically with this SOD/5-1-1 fu-

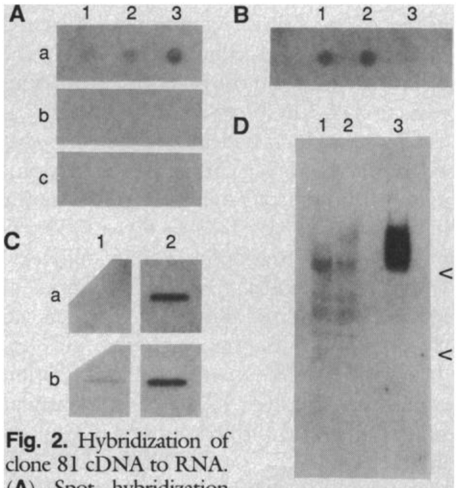

Fig. 2. Hybridization of clone 81 cDNA to RNA. (**A**) Spot hybridization (*24*) of 2, 4, or 12 μg of total liver RNA extracted (*25*) from either chronic NANBH-infected chimp 910 (a1 to a3) or from two control, uninfected animals (b1 to b3 and c1 to c3) with ^{32}P-labeled nick-translated clone 81 cDNA. (**B**) Spot hybridization of nucleic acid extracted from viral plasma pellets (*22*) before (spot 1) or after treatment with either excess deoxyribonuclease 1 (spot 2) or ribonuclease A (spot 3). Hybridization probe as in (A). (**C**) Each strand of clone 81 cDNA was subcloned into phage M13mp18 and then labeled by incubating with Klenow *Escherichia coli* DNA polymerase 1 in the presence of hybridization probe primer (New England Biolabs) and [α-^{32}P]dCTP (*23*). Each probe was then hybridized to slot blots containing either identical portions of viral RNA derived from infectious plasma (a1 and b1) or 2 pg of purified clone 81 double-stranded cDNA (a2 and b2). (**D**) Northern blot analysis (*26*) of 30 μg of total RNA (track 1), 30 μg of unbound RNA (track 2), and 20 μg of bound RNA (track 3) after chromatography on oligo(dT)-cellulose (Collaborative Research). RNA was derived from the liver of infectious chimpanzee 910. Arrows indicate the relative migration of 28*S* and 18*S* ribosomal RNA. ^{32}P-labeled nick-translated clone 81 cDNA was used as the hybridization probe.

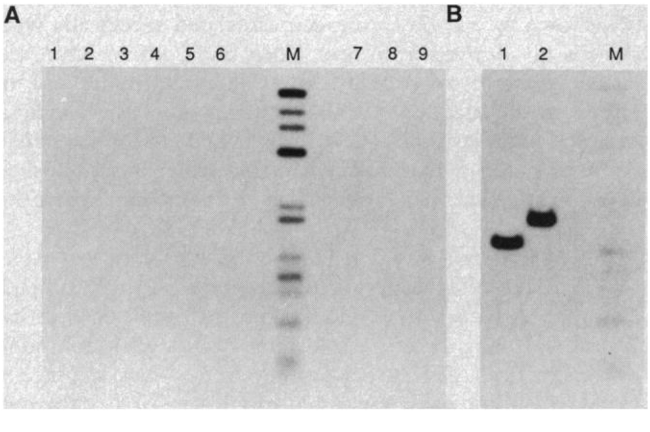

Fig. 1. Hybridization analysis of clone 81 cDNA with host DNA. (**A**) Southern blot containing 10 μg of DNA extracted either from a human placenta (lanes 7 to 9) or from proven infectious liver samples obtained from chimpanzees 1002 (lanes 1 to 3) and 910 (lanes 4 to 6) during acute or chronic infection, respectively, with the NANBH agent contaminating a human factor VIII concentrate (*10, 17*). Each DNA was digested with either Hinc II, Mbo I, or Eco RI before Southern blot analysis (*19*) with ^{32}P-labeled nick-translated clone 81 cDNA as the hybridization probe (*20*). M represents DNA standards of between 0.1 and 23.1 kbp. (**B**) Control blot of 10 μg of human placental DNA restricted with either Mbo I (lane 1) or Eco RI (lane 2) and hybridized with ^{32}P-labeled human β1-interferon cDNA (0.6 kbp) (*21*). Clone 81 was derived (*22, 23*) from an infectious plasma pool (≥10^6 chimp infectious doses per milliliter). This pool was prepared from chimpanzee 910 during chronic infection, which resulted from inoculation of chronic-phase plasma from chimpanzee 771. The latter animal was inoculated with a human factor VIII concentrate previously implicated in NANBH transmission (*10, 18*).

sion polypeptide (PS5), whereas there was no reaction with control lysates expressing SOD alone (Fig. 3A). Similar results were obtained with serum from 7 other NANBH patients of 11 tested, whereas serum from 10 normal donors were all negative (*11*). In addition, four chimpanzees experimentally infected with the NANBH agent all seroconverted to PS5 antibody after acute infection, whereas seven animals infected with either HAV or HBV showed no such seroconversion (see Fig. 3B for representative examples). Sera from these animals were also assayed with a radioimmunoassay containing purified PS5 to capture and measure reactive antibodies. Only those animals experimentally infected with the NANBH agent developed PS5 antibodies (Table 1). These data along with results from a large study of well-characterized patients (*12*) demonstrate that the polypeptide encoded by the clone 5-1-1 ORF is closely associated with NANBH infections. Furthermore, the cDNA strand that hybridized with plasma-derived RNA (Fig. 2C) was complementary to the strand encoding this 5-1-1 ORF, indicating therefore that this RNA is positive-stranded with respect to translation of this apparent viral antigen.

Thus, our data indicate that clones 5-1-1 and 81 are derived from the genome of a blood-borne NANBH virus that we now term the hepatitis C virus (HCV). Previous filtration studies have indicated that this virus is less than 80 nm in diameter and from its proven sensitivity to organic solvents, it would appear to possess an envelope made up of essential lipid (*7, 8*). These observations led to the suggestion that the agent may be togavirus-like (*13*). Our present data showing that the virus contains a positive-stranded RNA molecule of at least 10,000 nucleotides is consistent with it being related to the togaviridae or flaviviridae. The latter used to represent a genus in the togaviridae family but were recently elevated to their own family (*14*). The cDNA clones reported here were obtained in the absence of prior knowledge concerning the virus, the viral genome, and the presence of circulating viral antibodies. As such, this represents cloning without prior characterization of the infectious agent. This approach should be relevant to studies of other diseases in which an unknown infectious agent (viral or otherwise) might be involved.

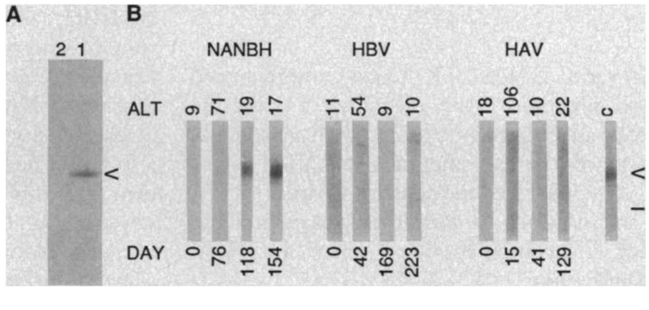

Fig. 3. Immunoblot assay for PS5 antibodies. (**A**) Incubation of the chronic NANBH patient serum used to isolate clone 5-1-1 (*22*) with blots of total bacterial lysates (*16*) containing either PS5 (lane 1) or control SOD (lane 2). (**B**) Sequential serum samples from experimentally infected chimpanzees were reacted with identical strips cut from a preparative blot of total lysate containing PS5 (*16*) to which exogenous SOD was added as an internal control. Day 0 represents the day of virus inoculation. Infections were monitored by serum ALT concentrations (international units per liter). Strip C was incubated with the same patient serum used in (A). The arrow and bar indicate the positions to which PS5 and SOD migrate, respectively.

Table 1. Incidence of PS5 antibodies in experimentally infected chimpanzees. Radioimmunoassays were performed as described (*15, 16*) on four serial serum samples obtained from each animal beginning with a sample obtained immediately prior to intravenous administration of virus (day 0). The mean of quadruplicate assays at each time point is shown (counts per minute). Values above 990 cpm (mean of uninfected controls plus three standard deviations) are considered positive. Animals 1, 7, and 8 were the same as used in Fig. 3. Animals 1 to 4 represent the third, second, fifth, and third chimpanzee passages, respectively, of the human factor VIII–derived NANBH agent (*17, 18*). The serum alanine aminotransferase (ALT) levels on the four sampling days are shown. Sampling times are represented as the number of days after inoculation of virus. nd, not done.

Chimp	Agent	Sampling times	ALT	Counts per minute
1	NANBH	0, 76, 118, 154	9, 71, 19, 17	250, 306, 5664, 8301
2	NANBH	0, 21, 73, 138	5, 52, 13, 13	294, 398, 2133, 8632
3	NANBH	0, 43, 53, 159	8, 205, 14, 6	152, 349, 392, 3738
4	NANBH	0, 55, 83, 140	11, 132, 7, 7	349, 267, 392, 2397
5	HBV	0, 359, 450	12, nd, 6	804, 660, 656
6	HBV	0, 115, 205, 240	9, 126, 9, 13	618, 606, 514, 790
7	HBV	0, 42, 169, 223	11, 54, 9, 10	454, 221, 272, 198
8	HAV	0, 15, 41, 129	18, 106, 10, 22	256, 597, 266, 295
9	HAV	0, 22, 115, 139	7, 83, 5, 10	218, 176, 214, 341
10	HAV	0, 26, 74, 205	15, 130, 8, 5	162, 219, 554, 284
11	HAV	0, 25, 40, 268	4, 147, 18, 5	333, 453, 419, 358

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15. PS5 was purified from the insoluble pellet of cleared [D. B. Clewell and D. R. Helinski, *Biochemistry* **9**, 4428 (1970)] bacterial lysates (*16*) by dissolving in 6*M* urea and successive chromatography on Q-Sepharose and S-Sepharose columns (Pharmacia). Microtiter plate wells (Dynatech Immulon 2) were then coated with 250 ng of PS5 (>80% purity) and incubated with 100 μl of serum (diluted 1:100) for 1 hour at 37°C. Bound antibody was detected by a second incubation with ^{125}I-labeled sheep anti-human immunoglobulin (Amersham) and each well was counted in a gamma counter.
16. Clone 5-1-1 cDNA was subcloned into bacterial plasmid pSODcf1 [K. S. Steimer *et al.*, *J. Virol.* **58**, 9 (1986)] in order to synthesize PS5 in which the COOH-terminus represents the polypeptide encoded by the clone 5-1-1 ORF. Subclones in the opposite orientation served as control in which SOD was synthesized but not the 5-1-1 ORF-encoded polypeptide. Immunoblots of total bacterial lysates were performed [A. J. Weiner *et al.*, *J. Virol.* **62**, 594 (1988)] with ^{125}I-labeled sheep antibody to human immunoglobulin (Amersham) to detect bound antibody.
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22. Plasma was first diluted 1:5 in 50 m*M* tris-HCl, *p*H 8.0, 1 m*M* EDTA, 100 m*M* NaCl before clearing (10,000*g* for 20 min, at 20°C) and then ultracentrifuging the supernatant (104,000*g* for 5 hours at 20°C in a Beckmann SW28 rotor). Nucleic acid was extracted [D. W. Rowe *et al.*, *Biochemistry* **17**, 1581 (1978)] from the crude viral pellet and denatured with 10 m*M* CH_3HgOH prior to synthesizing cDNA (from both DNA and RNA) with random primers of reverse transcriptase (*23*). After cloning into λgt11, the resulting cDNA library was immunoscreened [T. V. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning: A Practical Approach*, D. Glover Ed. (IRL Press, Oxford, UK, 1985), vol. I, pp. 49–78] with a 10^{-2} dilution of serum from a patient that had elevated serum ALT levels for more than 6 months (up to 1000 international units per liter) in the absence of serologic markers of HAV and HBV infection [immunoglobulin M (IgM) antibody to HAV, hepatitis B surface antigen (HBsAg), antibody to hepatitis B core antigen, and antibody to HBsAg]. Positive plaque 5-1-1 was isolated and the 155-bp cDNA insert was used (T. V. Huynh *et al.*, *ibid.*) as a hybridization probe to the same library to isolate clone 81, which contains a 353-bp cDNA consisting of the 5-1-1 cDNA plus additional flanking sequence.
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An Assay for Circulating Antibodies to a Major Etiologic Virus of Human Non-A, Non-B Hepatitis

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A specific assay has been developed for a blood-borne non-A, non-B hepatitis (NANBH) virus in which a polypeptide synthesized in recombinant yeast clones of the hepatitis C virus (HCV) is used to capture circulating viral antibodies. HCV antibodies were detected in six of seven human sera that were shown previously to transmit NANBH to chimpanzees. Assays of ten blood transfusions in the United States that resulted in chronic NANBH revealed that there was at least one positive blood donor in nine of these cases and that all ten recipients seroconverted during their illnesses. About 80 percent of chronic, post-transfusion NANBH (PT-NANBH) patients from Italy and Japan had circulating HCV antibody; a much lower frequency (15 percent) was observed in acute, resolving infections. In addition, 58 percent of NANBH patients from the United States with no identifiable source of parenteral exposure to the virus were also positive for HCV antibody. These data indicate that HCV is a major cause of NANBH throughout the world.

VIRAL HEPATITIS COMMONLY OCCURS in the absence of serologic markers for such known hepatotropic agents as hepatitis A virus (HAV), hepatitis B virus (HBV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) (*1–4*). Termed non-A, non-B hepatitis (NANBH), this entity represents greater than 90% of transfusion-associated hepatitis cases in the United States, and up to 10% of transfusions have been estimated to result in NANBH (*5, 6*). More recently, the frequent occurrence of NANBH in the absence of any obvious parenteral exposure has been well documented (*7–9*). Whereas acute disease is often subclinical, at least half of NANBH infections result in chronic hepatitis, which may result in cirrhosis in approximately 20% of cases (*10*). A potential association with hepatocellular carcinoma has also been proposed (*11*). Because of the frequency and severity of NANBH, there is an urgent need to develop a direct diagnostic test for the causative agent or agents. We have recently cloned the genome of a NANBH agent (*12*), designated the hepatitis C virus (HCV), and now report the development and use of a recombinant-based assay for HCV antibodies.

Three overlapping clones were isolated by means of the cDNA in HCV clone 5-1-1, which was used as a hybridization probe to the original cDNA library (*12*). These clones have one common open reading frame (ORF) extending throughout them that encodes part of a viral antigen associated with NANBH (*12*). This continuous ORF was reconstructed from these clones and then expressed in yeast (*13*) as a fusion polypeptide with human superoxide dismutase (SOD), which facilitates the efficient expression of foreign proteins in yeast and bacteria (*13–15*). In this way, a SOD/HCV polypeptide (C100-3) containing 363 viral amino acids was synthesized at high levels (~4% total protein) in recombinant yeast. After solubilization and purification, C100-3 was used to coat the wells of microtiter plates so that circulating HCV antibodies in blood samples could be captured and measured. Detection of bound antibody was achieved with a radioactive second antibody.

Initially, to test the specificity and sensitivity of this assay, sera of known NANBH infectivity was assayed in a blind fashion (Table 1). This panel of well-pedigreed and well-characterized samples has been accepted widely as a crucial test of the validity of putative specific assays for NANBH (*16*). Of seven NANBH serum samples shown to be infectious in chimpanzees, all but one gave very high signals in the assay as compared to the results obtained with sera from two control patients with alcoholic hepatitis or primary biliary cirrhosis and five noninfectious normal blood donors. These results were reproducible in quadruplicate analysis (Table 1). The only proven infectious sample that was negative in the assay was obtained from an individual in the acute phase of post-transfusion NANBH (PT-NANBH), although another acute-phase serum of unproven infectivity was similarly negative. A blood donor implicated in transmission of NANBH but whose serum was of equivocal infectivity in chimpanzees was also found negative in this assay. Thus, the data from this panel of sera indicates a high sensitivity and specificity of the antibody assay for blood-borne NANBH. No other assay evaluated by this panel has achieved this degree of specificity and sensitivity (*16*).

Next, we assayed matched blood donor and prospectively obtained recipient sera from ten well-characterized cases of chronic PT-NANBH in the United States. The results of the HCV antibody assays of sequential samples taken at 3-month intervals from each recipient during the development of NANBH and in stored samples from the corresponding donors are shown (Table 2). Each of the ten recipients seroconverted against HCV during the course of disease, although seroconversion in case 4 was marginal and not apparent until 12 months after transfusion. In contrast, seroconversion against HCV was not observed in prospectively studied individuals infected with other viral hepatitis agents. Antibody seroconversion was generally detectable within 6 months of transfusion. The prolonged interval to antibody development may explain the observed absence of HCV antibodies in the acute-phase samples assayed in Table 1.

With one exception, significant levels of HCV antibody were detected in at least one

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