have a more stabilizing effect on the IRE-BP than direct iron starvation or administration. Finally, treatment of RLD cells with penicillin at doses that completely inhibit protein synthesis has no effect on the induction of IRE-binding activity in response to DFO.

We therefore propose that chelation of iron by DFO 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 cysteine 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 actually 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 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 glutathiony can exist and even predominate within the cytosolic glutathione redox buffer system (23). Two factors may 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 physiological conditions (23). The second is the oxidation equilibrium constant (Kred) 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 Kred (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 Kred of a particular cysteine sulfhydryl group. In this way allosteric effectors can perturb the Kred 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.

REFERENCES AND NOTES


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 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 (3-4). Despite 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 some 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 consider

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17. Partial purification of the IRE-biding protein was achieved by RNA affinity chromatography. An IRE-containing RNA was transcribed in vitro by RNA polymerase T7 (18) with two precoate oligodeoxyribonucleotides that, when hybridized contained an RNA promoter (T7) promoter and a template for the normal IRE (F1) plus the X nucleotide. The reaction included 3 mRN2 (18-nt polynucleotide triphosphate (UTP) (RIohebra Research Laboratories) 1 mN2 UTP, in addition to [32P]guanosine triphosphate. The RNA was

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ered it to be due to more likely to insufficient concentrations of viral antigen in NANNBH infections. Therefore, in order to increase viral antigen concentrations, a cDNA library derived from infectious material was constructed in the bacteriophage Agt11. 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 NANNBH 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 titers (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 ~10<sup>6</sup> of the resulting recombinant Agt11 phage led to the identification of positive cDNA clone 51-1.

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 NANNBH 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 51-1 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 NANNBH 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 NANNBH 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 were 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 to it to nitrocellulose, and hybridized 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 51-1 indicated that the cDNA strand encoding the immunoreactive polypeptide possessed one continuous, translational open reading frame (ORF). (sequence of the genome is being completed and will be deposited in the GenBank database shortly). To investigate the relation of this polypeptide with NANNBH, this ORF was expressed in bacteria as a fusion polypeptide with human superoxide dismutase (SOD) and immuno blot analyses were performed on total bacterial lysates. The chronic NANNBH patient serum used originally to detect clone 51-1 reacted specifically with this SOD/51-1 fusion protein.
sion polypeptide (PSS), whereas there was no reaction with control lysates expressing SOD alone (Fig. 3A). Similar results were obtained with serum from 7 other NANNB patients of 11 tested, whereas serum from 10 normal donors were all negative (11). In addition, four chimpanzees experimentally infected with the NANNB agent all seroconverted to PSS 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 PSS to capture and measure reactive antibodies. Only those animals experimentally infected with the NANNB agent developed PSS 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 S-1-1 ORF is closely associated with NANNB infections. Furthermore, the A strand that hybridized with plasmoid RNA (Fig. 2C) was complementary to the strand encoding this S-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 S-1-1 and S-81 are derived from the genome of a blood-borne NANNB 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.

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Fig. 3. Immunoassay for PSS antibodies. (A) Inhibition of the chronic NANNB patient serum used to isolate clone S-1-1 (22) with bios of total human lysates (16) containing either PSS (lane 1) or control SOD (lane 2). (B) Sequential serum samples from experimentally infected chimpanzees were reacted with identical strip cut from a preparative blot of total human lysate containing PSS (16) in which congenous SOD was added as an internal control. Day 0 represents the day of virus inoculation. Infections were monitored by serum ALT measurements (international units per liter). Serum C was incubated with the same patient serum used in A. The arrow and bar indicate the position of which PSS and SOD migrate, respectively.

14, 24, 30; and four sampling days are shown. Sampling times are represented as the number of days after inoculation of virus, not done.

Clump Age Samples containing PSS (16) of which congenous SOD was added as an internal control. Day 0 represents the day of virus inoculation. Infections were monitored by serum ALT measurements (international units per liter). Serum C was incubated with the same patient serum used in A. The arrow and bar indicate the position of which PSS and SOD migrate, respectively.

Clump Age Samples containing PSS (16) of which congenous SOD was added as an internal control. Day 0 represents the day of virus inoculation. Infections were monitored by serum ALT measurements (international units per liter). Serum C was incubated with the same patient serum used in A. The arrow and bar indicate the position of which PSS and SOD migrate, respectively.

Clump Age Samples containing PSS (16) of which congenous SOD was added as an internal control. Day 0 represents the day of virus inoculation. Infections were monitored by serum ALT measurements (international units per liter). Serum C was incubated with the same patient serum used in A. The arrow and bar indicate the position of which PSS and SOD migrate, respectively.
An Assay for Circulating Antibodies to a Major Etiologic Virus of Human Non-A, Non-B Hepatitis


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 IgG 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 three of these cases and that all ten recipients seroconverted during their illnesses. In all cases, it was determined that the infected recipients had received transfusions containing HCV antibodies. A much lower frequency (15 percent) was observed in acute, resolving infections. In addition, 58 percent of NANBH patients from the United States with identifiable source of parental 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). Treated non-A, non-B hepatitis (NANBH), this entity represents greater than 90% of transfusion-associated hepatitis cases in the United States, and about 10% of transfusions have been estimated to result in NANBH (5, 6).

We recently found the occurrence of NANBH in the absence of any obvious parenthetical exposure has been well documented (7-9). Whereas acute disease is often mild, 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 clones 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 a protein 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 recombinant disulfide bond (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 (~6% 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 infected, all sera contained antibodies against recombinant C100-3. Six of these sera contained antibodies against recombinant C100-3. Six of these sera were from two control patients with alcoholic hepatitis or primary biliary cirrhosis and five non-infectious normal blood donors. These results were reproducible in quadruplicate analysis (Table 1). The only proven infectious sample was from an individual in the acute phase of post-transfusion NANBH (PT-NANBH), although many acute-phase sera from unproven infectivity was similarly negative. A blood donor implicated in transmission of NANBH but whose serum was of equivocal infectivity in chimpanzeas 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 donors and prospectively obtained recipient sera from the 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 serocconversion 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 hepatotropic viruses 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