A cDNA clone closely associated with non-A, non-B hepatitis

Mitsugu Maéno*, Kazuyoshi Kaminaka, Hiroyuki Sugimoto, Mariko Esumi, Nakanobu Hayashi, Kohei Komatsu, Kenji Abe[1], Sadayoshi Sekiguchi[2], Michitami Yano[3], Kyosuke Mizuno[4] and Toshio Shikata

Department of Pathology, Nihon University School of Medicine, 30-1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173, [1] Department of Pathology, National Institute of Health, Tokyo, [2]Hokkaido Red Cross Blood Center, Sapporo, [3]Institute for Clinical Research, Nagasaki Chuo National Hospital, Nagasaki and [4]Chemo- sero- therapeutic Research Institute, Kumamoto, Japan

Received January 16, 1990; Revised and Accepted March 28, 1990 EMBL accession no. X51587

ABSTRACT

A lambda gt11 cDNA library was constructed from RNA purified from hepatitis B viral surface antigen-negative human plasma with high alanine aminotransferase activity. A cDNA clone, designated as C8-2, was isolated by immunoscreening with mixed sera from non-A, non-B hepatitis (NANBH) carrier and convalescent chimpanzees. The recombinant protein produced by C8-2 reacted specifically with sera of patients in the chronic phase of NANBH. The sequence of C8-2, 269 bp, did not hybridized with any human or chimpanzee genomic DNA, and had no homology with those of primates and viruses. The existence of this sequence in RNA of possibly infectious plasma was shown by RNA blot hybridization and by Southern blot analysis of products amplified by the polymerase chain reaction. These results strongly suggest that C8-2 is derived from the agent of this viral hepatitis.

INTRODUCTION

Post-transfusion non-A, non-B hepatitis (NANBH) is a major clinical problems because it is thought to be the cause of up to 90% of all cases of post-transfusion hepatitis and of at least 50% of those of sporadic hepatitis (1). The transmissible agent of this hepatitis has not yet been identified, in spite of intensive studies using various conventional techniques (2−4). Thus the recent study by Choo and collaborators (5) was highly significant in that they isolated cDNA derived from viral RNA from plasma of an infected chimpanzee. They have also shown by enzyme-linked immunosorbent assay that antibody to this recombinant antigen (C100-3) appears specifically in the sera of patients with NANBH (6). These workers named this RNA virus hepatitis C virus (HCV).

Previously we constructed a cDNA library from pooled plasma of Japanese blood donors that possibly contained the infectious agent, and screened it using pooled sera from chronic carrier and convalescent chimpanzees that had been infected with the F-strain of the NANBH agent (7). In the present study, a clone of exogenous origin was isolated and shown to be derived from RNA in infectious human plasma. The antibody to the fusion protein expressed in *E. coli* was shown to be detected in patients in the chronic phase of NANBH.

MATERIALS AND METHODS

Construction of a lambda gt11 cDNA library from RNA of human plasma

Eight liters of hepatitis B viral surface antigen-free plasma from 148 Japanese blood donors whose alanine aminotransferase (ALT) values were more than 100 mIU/ml, were pooled. The viral fraction was precipitated with 3.6% (W/W) polyethylene glycol 6000 (Wako chem., Tokyo) and 500 mM NaCl, and a suspension of the precipitate was centrifuged on a stepwise sucrose gradient (5, 10, 15, and 20% (W/W)) for 12 hrs at 80000G. The resultant pellet was suspended with 8 ml of PBS (10 mM sodium phosphate buffer pH7.4, 150 mM NaCl).

The RNA was purified by the method of Chirgwin *et al.* (8) with slight modification. Five volumes of extraction buffer (4M guanidium thiocyanate, 50 mM Tris-HCl pH7.6, 10 mM ethylene diaminetetraacetic acid (EDTA), 100 mM 2-mercaptoethanol, 2% sarcosil) was added to the concentrated plasma described above, and total nucleic acid was extracted with phenol-chloroform, and precipitated with ethanol with 20 μg/ml glycogen as carrier. The RNA was further purified by DNase treatment (1.15KU/ml DNase, 50 mM Tris-HCl pH7.4, 1 mM EDTA, 10 mM $MgCl_2$) for 30 min at 37°C, and anion exchange chromatography (Qiagen pack-100, Diagen). Complementary DNA was synthesized with random primers, and cloned into lambda gt11, using a lambda gt11 cloning kit (Amersham).

Immunoscreening and immunoplaque assay

A library containing 1.2×10^6 plaque forming units was immunoscreened with 10-fold diluted mixed sera from 4 chimpanzees in the convalescent phase and one chimpanzee in the carrier phase of NANBH. All these chimpanzees had been

* To whom correspondence should be addressed

inoculated with the F-strain of NANBH agent as described elsewhere (9). For confirmation of the specificity of the immunoreaction of sera against the recombinant antigen with respect to NANBH diagnosis, a phage lysate from the candidate clone was mixed in a 1:1 ratio with control phage without insertion, plated on the same agar plate, and immunoassayed with sera from normal and NANBH virus-infected chimpanzees, and from a human panel of patients (see Fig. 1).

Western blot analysis

Y1089 was infected with lambda gt11 phage containing the C8-2 insert, and lysogens were induced as described by Snyder *et al* . (10). A fusion protein was expressed in Y1089 by addition of isopropyl beta-D-thiogalactopyranoside (IPTG), and pelleted cells were solubilized with RIPA buffer (100 mM Tris-HCl pH7.5, 1% sodium deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 300 mM NaCl). The lysate was separated in 7.5% SDS-polyacrylamide gel, and transferred to nitrocellulose paper. After treatment with 5% skim milk, the paper was incubated with 20-fold diluted patient's serum. The reactive material was detected with peroxidase-conjugated anti-human IgG and 4-chloro-1-naphthol (Sigma) as substrate. For determination of molecular weight of the C8-2/lacZ fusion protein, thyroglobulin (330kd), ferritin (220kd), phosphorylase b (94kd) and albumin (67kd) were used as standard proteins to establish calibration curve.

Sequence analysis

The cDNA inserts obtained by immunoscreening were subcloned into the *EcoRI* site of pUC118 (Takara, Tokyo) and both strands of DNA were sequenced by the dideoxy nucleotide chain termination technique (11). Sequencing and protein analysis were performed by the programs supplied by DNASIS software (Hitachi, Tokyo).

Southern blot analysis and RNA dot hybridization

Human and chimpanzee genomic DNA, purified from leukocytes and liver, respectively, were digested overnight with *EcoRI* at 37°C, subjected to electrophoresis on 1% agarose gel, and transferred to a nitrocellulose membrane. The blot was hybridized for 16 hr at 42°C with ^{32}P-labeled C8-2-5 DNA probe excised from plasmid in a mixture containing 50% formamide, 5× Denhardt's solution, and 5× SSC (150 mM NaCl, 15 mM trisodium citrate) and then washed 4 times with 0.1× SSC containing 0.2% SDS at 68°C. The beta-actin gene with 1.5 kb purified probe was used as a positive control.

For detection of RNA in serum, total nucleic acid was isolated from 50 ml of pooled human plasma with high ALT activity by the same method as used for preparation of the cDNA library. Precipitated nucleic acid was resuspended in 40 μl of FA/FA buffer (6.5% formaldehyde, 50% formamide, 20 mM MOPS pH 7.0, 5 mM sodium acetate, 0.5 mM EDTA pH 8.0), spotted on a nitrocellulose paper, and hybridized with C8-2-5 DNA probe. Treatment with RNase (30 μg/ml) was performed in buffer containing 50 mM Tris-HCl (pH. 7.4), 1 mM EDTA, and 10 mM $MgCl_2$.

Enzymatic amplification of complementary DNA by the polymerase chain reaction

The polymerase chain reaction (PCR) from RNA template was performed as described by Gama *et al.* (12). RNA from either the serum or liver was reverse-transcribed at 37°C for 90 min in the presence of 4 KU/ml reverse transcriptase (BRL), 50mM Tris-HCl pH 8.3, 6mM $MgCl_2$, 40mM KCl, 1mM dithiothreitol, 1mM dNTPs, 1.3KU/ml RNasin, and 30mg/ml random primers. Part of the reaction mixture was then added to 50 μl of amplification mixture containing 20 U/ml *Thermus aquaticus* polymerase and 250 nM of sense (5′-CCGACCCCTCCCACATTACAGCA-3′) and antisense (3′-CGTCTGGGGAGTCATGACGGGT-5′) primers. Forty amplification cycles, consisting of denaturation at 94°C (30 sec), annealing at 55°C (30 sec), and extension at 72°C (60 sec) were carried out in a DNA Thermal Cycler (Perkin-Elmer/Cetus). Amplified DNA was separated electrophoretically in 2% agarose gel, and Southern blot analysis was performed under usual stringent condition using synthetic oligo nucleotide (5′-TAACTGACTAGCTGAAGAGCTGGCCA-3′).

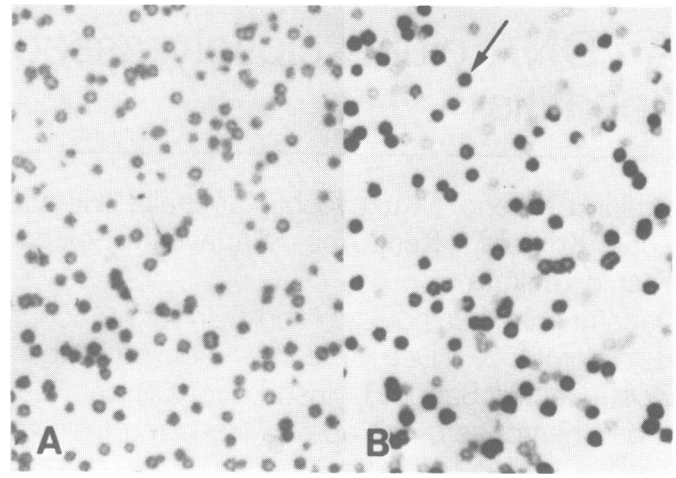

Figure 1. Detection of C8-2 antibody in chimpanzee sera by immunoplaque assay. Phage containing the C8-2 and negative control phage were mixed (1:1), and plated on agar. Replicate filters were incubated with chimpanzee serum obtained in week 0 (A) or 20 (B) during the chronic phase after inoculation of the F-strain of NANBH agent as the first antibody. Approximately half the plaques in B give a positive reaction, as shown by an arrow.

Table 1. Incidences of antibodies against C8-2 antigen in experimentally infected chimpanzees*

Diagnosis	No. positive/No. examined	(%)
NANBH, convalescent	0/7	(0)
NANBH, carrier	5/6	(83.3)
Normal	0/17	(0)

*All the chimpanzees except one (No. 59) used for detecting antibodies had been inoculated with F-strain of NANBH agent. No.59 was inoculated with serum from a patient with acute epidemic NANBH in Shimizu City, and developed chronic hepatitis (14).

RESULTS

Isolation of cDNA from human plasma RNA

A library containing 1.2×10^6 plaque forming units, which was obtained from 8 liters of possibly infectious pooled plasma, was immuno-screened with sera from chimpanzees in the convalescent and chronic carrier phases of non-A, non-B hepatitis (NANBH). One of 3 positive cDNA obtained, clone C8-2, was specifically associated with the diagnosis of NANBH: on immunoplaque assay

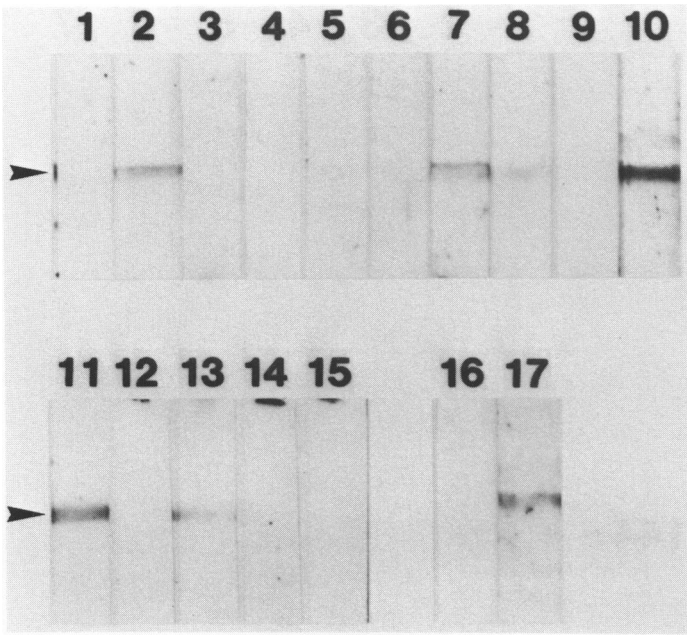

Figure 2. Detection of C8-2 antibody by Western blot analysis in human sera from patients with chronic NANBH (lanes 1–15), chronically infected chimpanzee serum, as a positive control (lane 17) and uninfected chimpanzee serum, as a negative control (lane 16). C8-2/lacZ fusion protein was separated as a band of approximately 160 kd by SDS-PAGE (arrow heads). Lanes 2, 7, 10, 11, and 13 gave a positive reaction.

as shown in Fig. 1, antibodies to C8-2 were frequently detected in the sera of chimpanzees in the carrier phase of NANBH (83.3%), but not in convalescent sera or in sera of normal chimpanzee (Table 1). In serial examinations of 4 chronic carrier chimpanzees, the antibody to C8-2 antigen was found to appear 20 weeks after inoculation in 3 chimpanzees, and 102 weeks after inoculation in one (data not shown). These results show that this antibody appears in the chronic phase, but not the acute phase of infection. The antibody did not appear in a chimpanzee infected with hepatitis B virus (HBV)(data not shown).

Close association of appearance of C8-2 antibody with NANBH in human serum

The specific incidence of C8-2 antibody in serum was also confirmed in the sera of human patients with a well-defined clinical course. As shown in Table 2, by immuno-plaque assay, the C8-2 antibody was detected in 40.0% of the sera of chronic cases and 10.0% of the sera of acute stage cases of NANBH, but was not detected in sera of patients with acute hepatitis A or B. The serum from one patient with chronic hepatitis B was positive, suggesting infection with both NANBH virus and HBV. The C8-2 antibody appeared with low frequency (1.2%) in sera of normal blood donors whose ALT values were less than 30 mU/ml. As shown in Fig. 2, Western blot analysis revealed that sera that were positive for C8-2 antibody reacted specifically with a band of 160 kd of C8-2/lacZ fusion protein. Western blotting gave almost the same results as immunoplaque assay (see Table 1), but 2 serum samples from chronic NANBH patients gave a positive reaction on Western blotting but a negative one on immunoplaque assay, and 2 gave a negative reaction on Western blotting but a positive one on immuno-plaque assay. One sample from a patient with chronic hepatitis B also gave a positive reaction only on Western blotting analysis (Table 2).

Table 2. Specific appearance of antibodies against C8-2 in human blood samples detected by immunoplaque or Western blot assay.

Diagnosis	No.positive/No.examined (%) Immunoplaque assay	Western blot assay
NANBH, acute	4/40 (10.0)	4/40 (10.0)
NANBH, chronic	12/30 (40.0)	12/30 (40.0)
HBV, acute	0/20 (0)	0/20 (0)
HBV, chronic	1/20 (5.0)	2/20 (10.0)
HAV, acute	0/20 (0)	0/20 (0)
Other liver diseases*	0/15 (0)	0/15 (0)
Normal	1/83 (1.2)	ND**

*The patients consisted of 5 with alcoholic liver injury, 5 with drug-induced liver injury, and 5 with lupoid hepatitis.
**Not done.

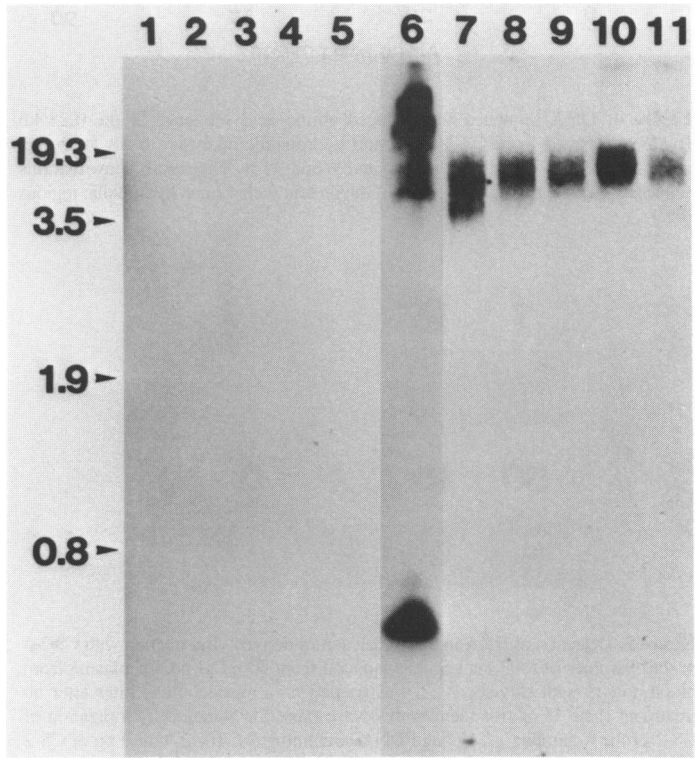

Figure 3. Southern blot analysis of liver DNA from 3 chimpanzees (lanes 1–3, 7–9), leukocyte DNA from 2 humans (lanes 4–5, 10–11), and lambda C8-2 phage DNA (lane 6). Samples of 30 μg of genomic DNA or 2 μg of phage DNA were digested with *EcoRI*, and then separated by electrophoresis in agarose gel, transferred to a nitrocellulose filter, and hybridized with C8-2 (lanes 1–6) or beta-actin (lanes 7–11) probe.

As further controls for other liver diseases, we tested for this antibody in sera of patients with clear diagnoses of alcoholic liver injury, lupoid hepatitis, and drug-induced liver injury (5 samples of each). No antibody was detected in any of these 15 samples by either immunoplaque or Western blot assay, indicating that the presence of C8-2 antibody was restricted to NANBH.

Characterization and sequencing of C8-2 clone

To prove that this cDNA clone was not of endogenous origin, we excised the DNA from the plasmid subclone, C8-2-5, and used it as a probe in Southern blot analysis of chimpanzee and human genomic DNA (Fig. 3). The control beta-actin gene probe hybridized with all samples of *EcoRI* digests of genomic DNA,

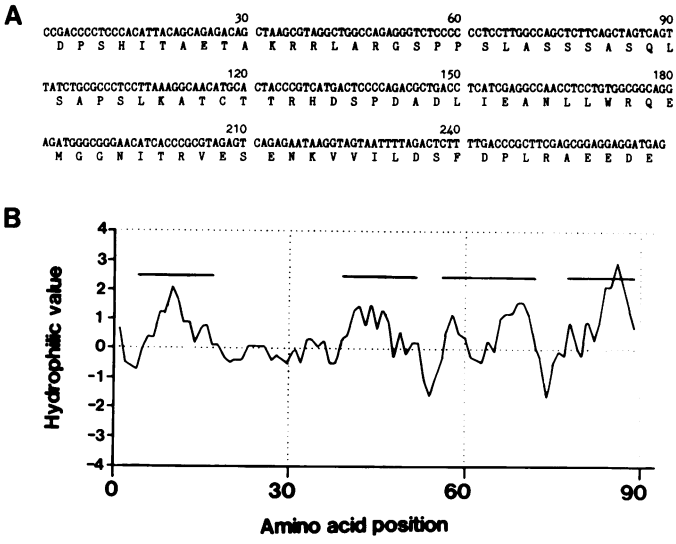

Figure 4. DNA sequence and deduced amino acid sequence of the 0.28 kb fragment derived from C8-2-5 (A) and hydropathic analysis of this 89 amino acid sequence (B) according to Hoop and Woods (13). The results show that this sequence encodes a hydrophilic part of the protein with 4 main hydrophilic regions (bar).

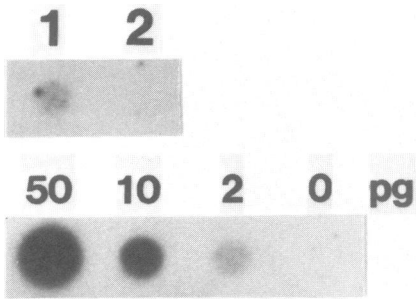

Figure 5. Detection of RNA in the human serum-derived viral fraction with C8-2-5 probe. Samples of total nucleic acid isolated from 50 ml of pooled plasma from blood donors with elevated ALT was spotted on a nitrocellulose filter after no treatment (lane 1) or treatment with RNase (lane 2). Standard hybridization of C8-2 probe to lambda gt11 phage DNA containing 50, 10, 2, and 0 pg of C8-2 DNA was shown in lower.

but the C8-2 insert DNA probe did not hybridize with any chimpanzee or human DNA. These results demonstrate that this C8-2 clone was not derived from host genomic DNA, but was of exogenous origin.

We then sequenced 0.28kb of an exogenous fragment in plasmid C8-2-5. Figure 4A shows the nucleotide sequence of 269 base pairs and its deduced amino acid sequence. This region had a high G/C content (58.7%), and 4 main hydrophilic regions, as shown by hydropathic analysis according to Hopp and Woods (13) (Fig. 4B). Homology search using DNASIS software (Hitachi, Tokyo) showed no significant homology (less than 50%) with any nucleotide sequence of primates or viruses filed in GenBank (June, 1989) or EMBL (May, 1989).

Detection of specific RNA in human plasma

Purified nucleic acid derived from 50 ml of pooled plasma from human blood donors whose ALT levels were more than 100 mU/ml were spotted on a nitrocellulose paper, and hybridized with C8-2 specific probe. A signal was detected with total nucleic

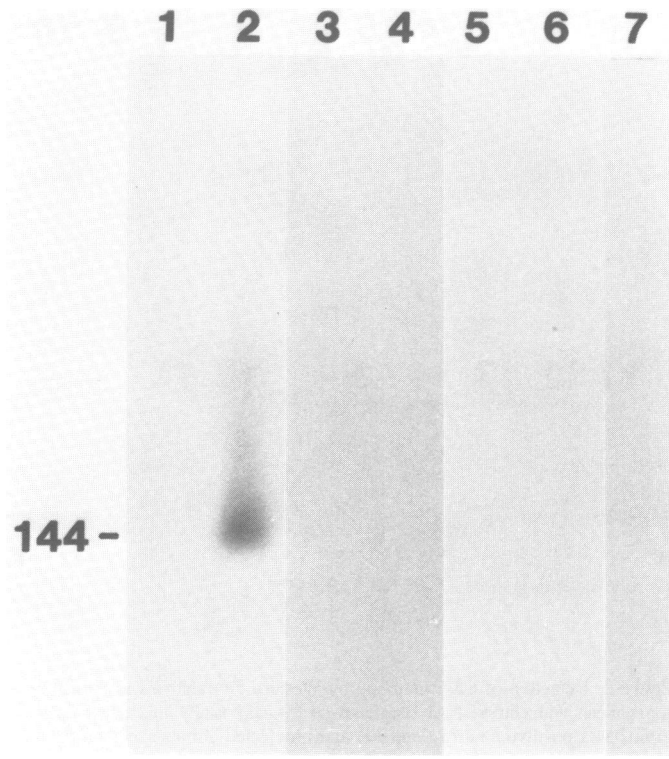

Figure 6. Southern blot analysis of products amplified by the polymerase chain reaction. Total nucleic acid from pooled plasma of chimpanzees during the chronic carrier phase (lane 1), pooled human plasma with elevated ALT (lane 2), and human plasma of two healthy individuals (lanes 3 and 4), were reverse-transcribed, amplified, and analyzed by Southern blotting as described in the Materials and Methods. Total RNA purified from the livers of two chimpanzee in the acute phase (lanes 5 and 6) was specifically amplified in the same manner. The samples subjected to electrophoresis were obtained from 15 μl of plasma, or 200 ng of liver RNA.

acid from plasma with an elevated ALT level (Fig. 5). This signal disappeared after treatment with RNase, indicating that it was derived from RNA in the serum.

The concentration of the C8-2 sequence in serum RNA of patients was estimated to be as low as 40 fg/ml from the results of dot hybridization shown in Fig. 5. Therefore, for its detection we developed a gene amplification system by the polymerase chain reaction (PCR) with specific primers as described in the Materials and Methods. As shown in Fig. 6, a specific signal was observed by Southern blot analysis in pooled human plasma with high ALT values, but not in 2 samples of normal plasma. The signal was also not detected in 2 samples of liver from chimpanzees in the acute phase, or a sample of serum from chimpanzee in the chronic phase of NANBH after infection with the F-strain of agent. Thus the C8-2 sequence was detected in the sera of Japanese patients, but interestingly, nor in sera from chimpanzees infected with the F-strain of NANBH agent.

DISCUSSION

In the present work, by screening a lambda gt11 expression library derived from RNA of human sera suspected of being infectious, we isolated a cDNA clone that was closely associated with NANBH. This method may be better than others for screening for a gene derived from a rare infectious agent such as NANBH virus because specific antibody against the agent

might appear in large amount in the serum, even if the amount of agent is very low. Furthermore, with this method it is possible to speculate on the function of the protein encoded by the isolated cDNA clone, because the recombinant protein expressed in *E. coli* must contain one or more epitopes that are specifically recognized by antibodies in the patient's serum.

Although our results showed that the C8-2 clone was closely associated with NANBH, we cannot conclude from this finding only that this clone was derived from this hepatitis agent. But from several other findings we suggested that this clone was in fact derived from the agent of hepatitis. First antibody against C8-2 protein was detected in 40% of the sera of patients with chronic NANBH, but in only one of the patients with other forms of viral hepatitis examined and in none with alcohol liver injury, lupoid hepatitis, or drug-induced liver injury (Table 2). Second the nucleotide sequence showed no homology with those of known primate and viral nucleotides, or with human or chimpanzee genomic DNA as judged by Southern blot analysis (Fig. 3). Third amplified product blotting of nucleic acids from pooled plasma of blood donors with elevated ALT activity hybridized with the C8-2 probe, whereas nucleic acid from the plasma of normal donors did not (Fig. 6), and this sequence in the plasma was an RNA-derivative (Fig. 5). Thus the RNA sequence corresponding to that of C8-2 was present only in human plasma with elevated ALT activity.

The recent findings by Choo and collaborators (4, 5) on the specific appearance of antibody to C100-3 recombinant antigen in NANBH patients are relevant to ours. The main difference between these two studies is that they found that more than 70% of patients with chronic NANBH were positive for C100-3, whereas we found that only 40% were positive for C8-2. This difference might be partly due to low sensitivity of immunoplaque assay for detecting antibody. But it was probably mainly due to a difference in the appearance of these two antibodies against distinct epitopes. One reason for this conclusion is that the C8-2 antibody was present in a high percentage (83.3%) of chronic carrier chimpanzees, whereas a lower percentage would be expected if the difference in findings were mainly due to a difference between the sensitivities of these two assays. Furthermore, in a preliminary study we found that C100-3 and C8-2 antibodies were detected at almost the same ratio (18.2% and 16.7%, respectively), in the sera from 148 voluntary blood donors whose ALT levels were more than 100 mU/ml, suggesting similar sensitivities of these two assays. Interestingly, 4 cases gave positive reactions for C8-2 but not for C100-3 antibodies, and 7 cases gave positive reactions for C100-3 but not for C8-2 antibodies. These findings suggest that our clone and clone 81 of Choo *et al.* are derived from different viruses or different regions of a common viral gene.

In recent reports Arima *et al.* (15, 16) described two cDNA clones isolated from the plasma of human patients, one associated with the acute and chronic phases of NANBH, and the other with only the chronic phase. The nucleotide sequence of C8-2 was different from those of their two clones, indicating that these sequences are also derived from a different virus or different regions of a common viral gene.

As shown in Fig. 5, little, if any of the specific RNA corresponding to the C8-2 sequence was present in infectious plasma. Moreover, the C8-2 probe did not hybridize with total liver RNA from the two chimpanzees in the acute phase of NANBH (data not shown). Therefore, it was necessary to develop a system for amplifying the complementary sequence in patient's sera. Interestingly using a PCR system we found that this amplification based on the Japanese cDNA clone, C8-2, did not function in detection of the F-strain genome of the NANBH virus (Fig. 6). This suggests that the C8-2 gene and F-strain of virus have somewhat different base sequences but share the same epitope encoded in the C8-2 region. This suggestion is supported by the the fact that a very high proportion (83.3%) of chimpanzees in the chronic phase after infection with the F-strain gave positive reactions for antibodies to C8-2 antigen.

In this study we isolated a cDNA clone derived from infectious human plasma. This clone was shown to be closely associated with chronic NANBH, and might encode a different viral epitope from that described by Choo *et al.* (4). This finding should provide fundamental information on the whole gene of this virus and the functions of individual viral antigens.

ACKNOWLEDGMENT

We wish to thank Mrs. Aya Kasuya for her helpful technical assistance.

REFERENCES

1. Dienstag,J.L. (1983) Gastroenterology, **85**, 743–768.
2. Shimizu,Y.K., Feinstone,S.M., Purcell,R.H. Alter,H.J. and London,W.T. (1979) Science, **205**, 197–200.
3. Shimizu,Y.K., Oomura,M., Abe,K., Uno,M., Yamada,E., Ono,Y. and Shikata,T. (1985) Proc. Natl. Acad. Sci. USA, **82**, 2138–2142.
4. Prince,A.M., Huima,T., Williams,B.A.A., Bardina,L. and Brotman,B. (1984) Lancet, **2**, 1071–1075.
5. Choo,Q.L., Kuo,G., Weiner,A.J., Overby,L.R., Bradley,D.W. and Houghton,M. (1989) Science, **244**, 359–362.
6. Kuo,G., Choo,Q.L., Alter,H.J., Grinick,G.L., Redeker,G., Purcell,R.H., Miyamura,T., Dienstag,J.L., Alter,M.J., Stevens,C.E., Tegtmeier,G.E., Bonino,F., Colombo,M., Lee,W.S., Kuo,C., Berger,K., Shuster,J.R., Overby,L.R., Bradley,D.W. and Houghton,M. (1989) Science, **244**, 362–364.
7. Maéno,M., Kaminaka,K, Sugimoto,H., Esumi,M., Hoyashi,N, Abe,K. Sekiguchi,S., Yano,M. and Shikata,T. (1990) In 'Nihon University International Symposium on Non-A, Non-B Hepatitis and Blood Borne Infectious Diseases', Elsevier, Amsterdam, in press.
8. Abe,K., Uchino,I, Morozumi,E., Kurata,T. and Shikata,T. (1988) Acta Hepatol. Jap., **29**, 705–706.
9. Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) Biochemistry, **18**, 5294–5299.
10. Snyder,M., Elledge,S., Sweetser,D., Young,R.A. and Davis,W. (1987) Methods Enzymol., **154**, 107–128.
11. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA, **74**, 5463–5467.
12. Gama,R.E., Hughes,P.J., Bruce,C.B. and Stanway,G. (1988) Nucleic Acids Res., **16**, 9346.
13. Hopp,T.P. and Woods,K.R. (1981) Proc. Natl. Acad. Sci. USA, **78**, 3824–3828.
14. Abe,K. and Shikata,T. (1984) Acta Hepatol. Jap, **25**, 308–321.
15. Arima,T., Nagashima,H., Murakami,S., Kaji,C., Fujita,J., Shimomura,H. and Tsuji,T. (1989) Gastroenterol. Jap., **24**, 540–544.
16. Arima,T., Takamizawa,A., Mori,C., Murakami,S., Kaji,C. and Fujita,J. (1989) Gastroenterol. Jap., **24**, 545–548.