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# Expression of Processed Core Protein of Hepatitis C Virus in Mammalian Cells

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**A structural protein of hepatitis C virus (HCV) was expressed in monkey COS cells under the control of an exogenous promoter, and a protein of 22 kDa was identified by immunoblot analysis. This protein (p22), which was produced by processing in COS cells, reacted specifically to sera of chronic hepatitis C patients, and its coding region was mapped at the most amino-terminal part of the HCV polyprotein. These results suggested that the p22 protein is the nucleocapsid (core) protein of HCV. Moreover, the assay detecting antibody to p22 was found to be useful for early diagnosis of HCV infection.**

The molecular cloning of cDNA of the hepatitis C virus (HCV) genome was a breakthrough in the research of non-A, non-B hepatitis (NANBH) afflicting one million patients per year worldwide (4, 11, 21, 22). HCV has a positive-strand RNA genome of about 10 kb (4). On the basis of the hydropathy profile of the putative HCV polyprotein and partial sequence homology at the amino acid level, HCV is considered to be distantly related to flaviviruses or pestiviruses (15, 23). The length of the putative HCV structural region was significantly shorter than those of either flaviviruses or pestiviruses. Two domains, which presumably encode nucleocapsid (core) protein and envelope protein, are proposed in the HCV structural region (23). However, no HCV-coded proteins have been identified yet, mainly because of the lack of cell culture systems supporting the replication of HCV.

The assay detecting antibody to HCV using a molecularly expressed nonstructural HCV protein, C100, has been applied for blood screening and diagnosis of chronic hepatitis (7, 12, 16). However, early diagnosis of this elusive hepatitis is particularly important because about half of acute cases become chronic and many eventually develop into hepatocellular carcinoma (1, 17). Accumulating data showed that prompt interferon treatment can prevent the disease from becoming chronic (5, 6). Here we expressed and identified the HCV core protein, p22, in mammalian cells, in which the specific processing to yield the protein takes place. The assay detecting antibody against p22 (p22 Ab) was found useful for early diagnosis of hepatitis C.

## MATERIALS AND METHODS

**Plasmid construction.** To prepare expression plasmid pSR316, an HCV cDNA fragment from nucleotide (nt) 308 (*Acc*I site) to nt 1662 (*Eco*RI site created by linker ligation) was cut out from plasmid pS7/1-216, which contains the whole structural coding region of HCV cDNA derived from Japanese healthy HCV carriers (22). The cDNA fragment, starting from 12 nt upstream of the polyprotein initiation codon, was then inserted into pcDLSRα296 (20) by using *Pst*I and *Kpn*I linkers. pSR312 was prepared from pSR316 by deleting the sequence after nt 1186 (*Pvu*II site). pSR316d46 was prepared also from pSR316 by deleting an *Apa*I fragment (from nt 441 to nt 642) by partial digestion.

**Western immunoblot analysis.** Protein samples were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel according to the method of Laemmli (13). After transfer to nitrocellulose (25), strips were immersed in 1% skimmed milk in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl), then incubated with 10- or 20-fold-diluted patient serum for 14 h at 4°C, treated with biotinylated anti-human immunoglobulin G and with peroxidase-conjugated avidine, and then developed with 4-chloro-1-naphthol.

## RESULTS AND DISCUSSION

To express the HCV structural proteins in mammalian cells, an expression plasmid, pSR316, and its derivatives were constructed (Fig. 1). pSR316 contained HCV cDNA covering the whole structural region under the control of the SRα promoter (20), a strong promoter in mammalian cells. pSR312 lacked a carboxy-terminal part of the putative envelope region, and pSR316d46 had an in-frame deletion of 67 amino acids within the core region. COS1 cells (8), plated on 50-mm-diameter dish 24 h before transfection, were transfected with 10 μg of plasmid DNA by the calcium phosphate precipitation method and then were fixed with cold methanol 48 h after transfection. Subsequently, expressed HCV protein was visualized by immunofluorescence with serum from a chronic NANBH patient with a positive C100 antibody (C100 Ab) test. Strong fluorescence was observed in the cytoplasm of about 10% of the cells transfected with either pSR316 or pSR312. Very few or no cells showed marked fluorescence when pSR316d46 or the control vector plasmid, respectively, was used for transfection (Fig. 2A and B for pSR316 and vector, respectively). The fluorescence was not detected when normal control serum was used (data not shown). These results suggested that the HCV protein detected by immunofluorescence was encoded entirely in both pSR316 and pSR312 but only partially in pSR316d46 and that the patient serum used for detection contained the

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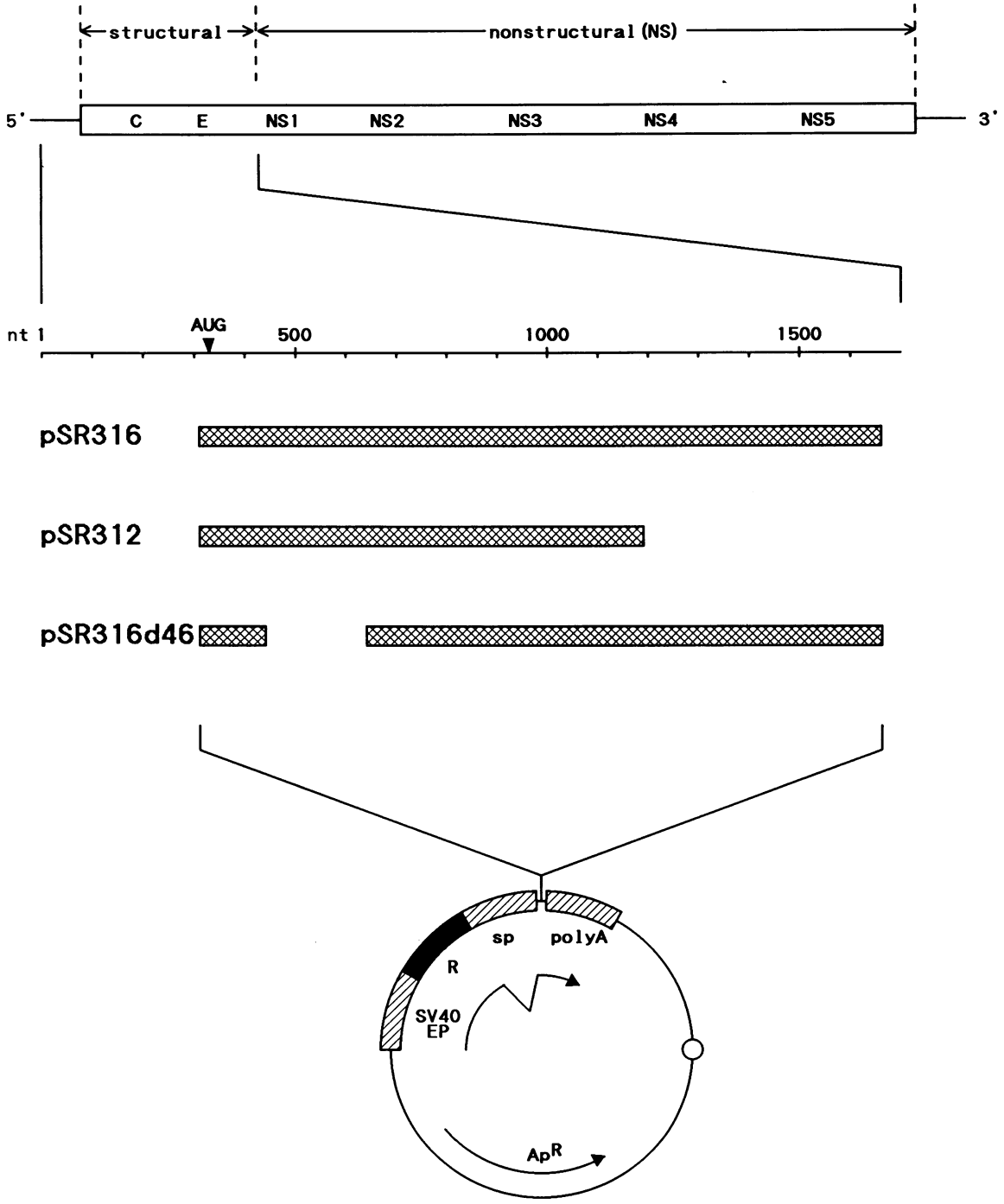



FIG. 1. Construction of plasmids expressing the HCV core protein. At the top is shown the HCV genome structure. The coding frame of the polyprotein is represented by an open box, and each protein region is shown in it. C, core; E, envelope; NS, nonstructural protein. In the middle is shown a map of the structural region. nt 1 is the 5′ end of the HCV genome. cDNA is shown as hatched boxes under the genome map. AUG, initiation codon of the polyprotein. Note that cDNA in pSR316d46 has an in-frame deletion of 201 nt. At the bottom is shown expression vector pcDLSRα296 (20). SV40 EP, simian virus 40 early promoter; R, R segment of human T-cell leukemia virus I long terminal repeat; sp, SV40 late splicing sequence; polyA, SV40 late polyadenylation signal; $Ap^R$, ampicillin resistance gene; open circle, plasmid replication origin.

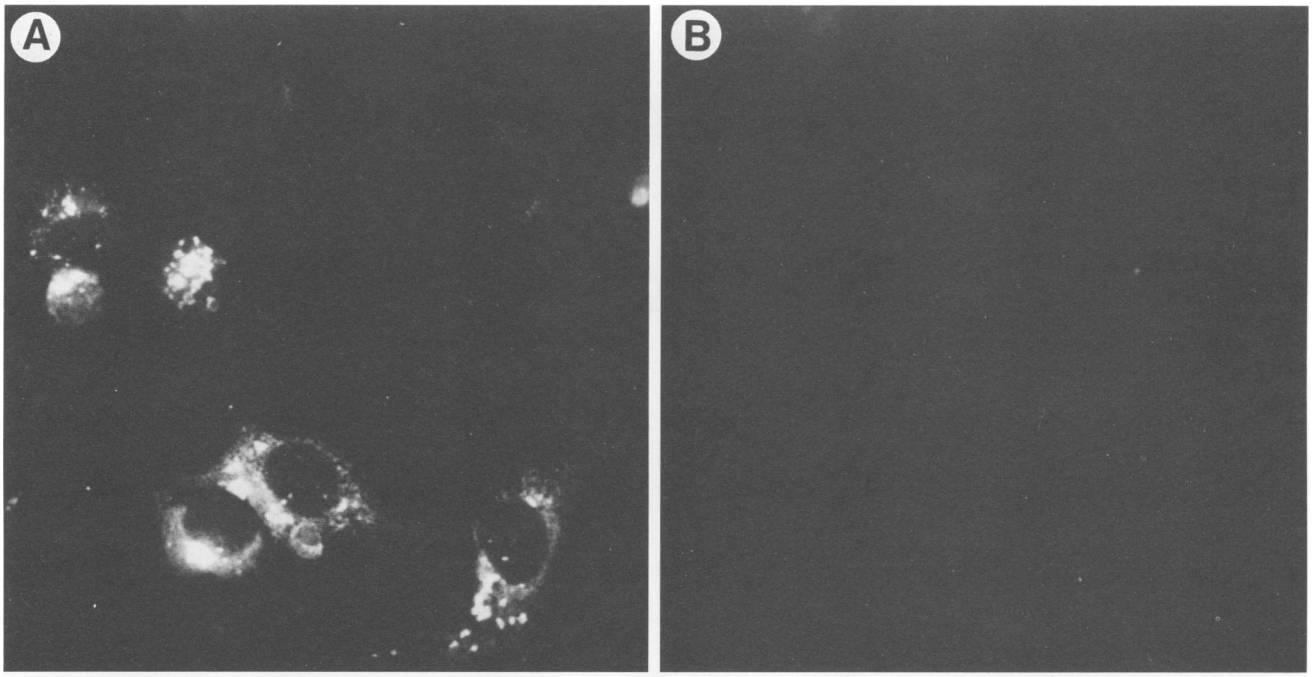



FIG. 2. Immunofluorescence of the HCV core protein expressed in COS1 cells. Cells were transfected with pSR316 (A) and vector pcDLSRα296 (B).

antibody against the expressed HCV protein. These partial mapping data are consistent with the notion that the detected protein is the HCV core protein, predicted to be the protein encoded at the most amino-terminal part of the HCV polyprotein (23).

To determine the size of the HCV core protein expressed in this system, the cell lysates obtained 48 h after transfection were analyzed by Western blot with the same patient serum (Fig. 3A). A strong band of 22 kDa was detected when either pSR316 or pSR312 was used. When pSR316d46 was used for transfection, the 22-kDa band was not observed (Fig. 3A) but, instead, a faint band of about 16 kDa was detected (data not shown). These results confirmed the specificity of the results of immunofluorescence and suggest that the 22-kDa protein is the HCV core protein and that the 16-kDa protein is a shortened form of the 22-kDa protein produced by the in-frame deletion. For some Western blot analyses, tunicamycin (20 μg/ml), which blocks N glycosylation (19, 24), was added 8 h before preparation of the cell lysates. The size of the 22-kDa protein did not change by treatment with tunicamycin (Fig. 3A). Thus, the 22-kDa protein did not appear to be N glycosylated. The size of the protein and lack of an N-glycosylation site coincide with the features of the predicted HCV core protein of about 190 amino acids (23). The 22-kDa protein is denoted p22 hereafter.

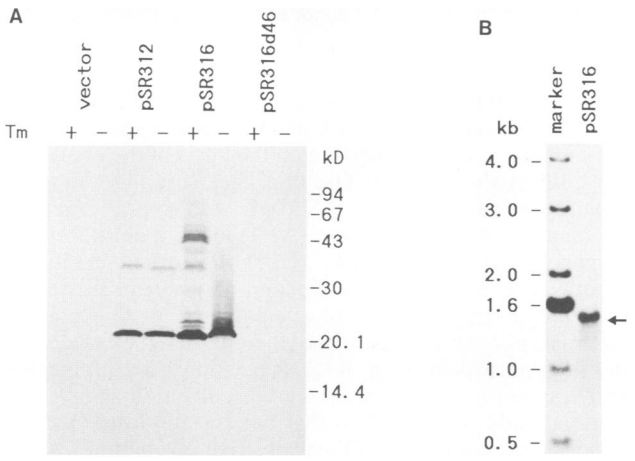



FIG. 3. HCV proteins and RNA expressed in COS1 cells. (A) Western blot analysis of expressed HCV proteins. Tm, presence and absence of tunicamycin. (B) Nuclease S1 protection experiment of cytoplasmic RNA from cells transfected with pSR316. In panel A, the bands in the lane of pSR316 without tunicamycin were somewhat broad and delayed possibly because of contamination of cellular DNA. We carried out the identical experiments twice and confirmed that the patterns in this lane were very similar to that in the Tm+ lane. In panel B, an arrow indicates the protected band of about 1.4 kb which shows that the expressed RNA covered the entire cDNA fragment inserted into pSR316.

It should be noted that HCV cDNAs inserted in pSR316 and pSR312 are capable of expressing amino-terminal parts of the viral polyprotein (441 and 289 amino acids long, respectively), both of which are much larger than the p22 protein. These proteins were actually detected as less-abundant proteins of about 50 kDa for pSR316 and 35 kDa for pSR312 (Fig. 3A). These results agreed with our previous suggestion, based on the sequencing analysis, that the HCV core protein was generated from these precursor proteins by processing, i.e., cleavage at a specific site (23). An alternative possibility, however, is that p22 is synthesized by an aberrant spliced RNA in this expression system. To examine the latter possibility, RNA from pSR316-transfected cells was analyzed by the S1 protection experiment (2, 18). Briefly, 100 μg of total cytoplasmic RNA was hybridized with 25 ng of probe DNA labeled with $^{32}$P-ATP and polynu-

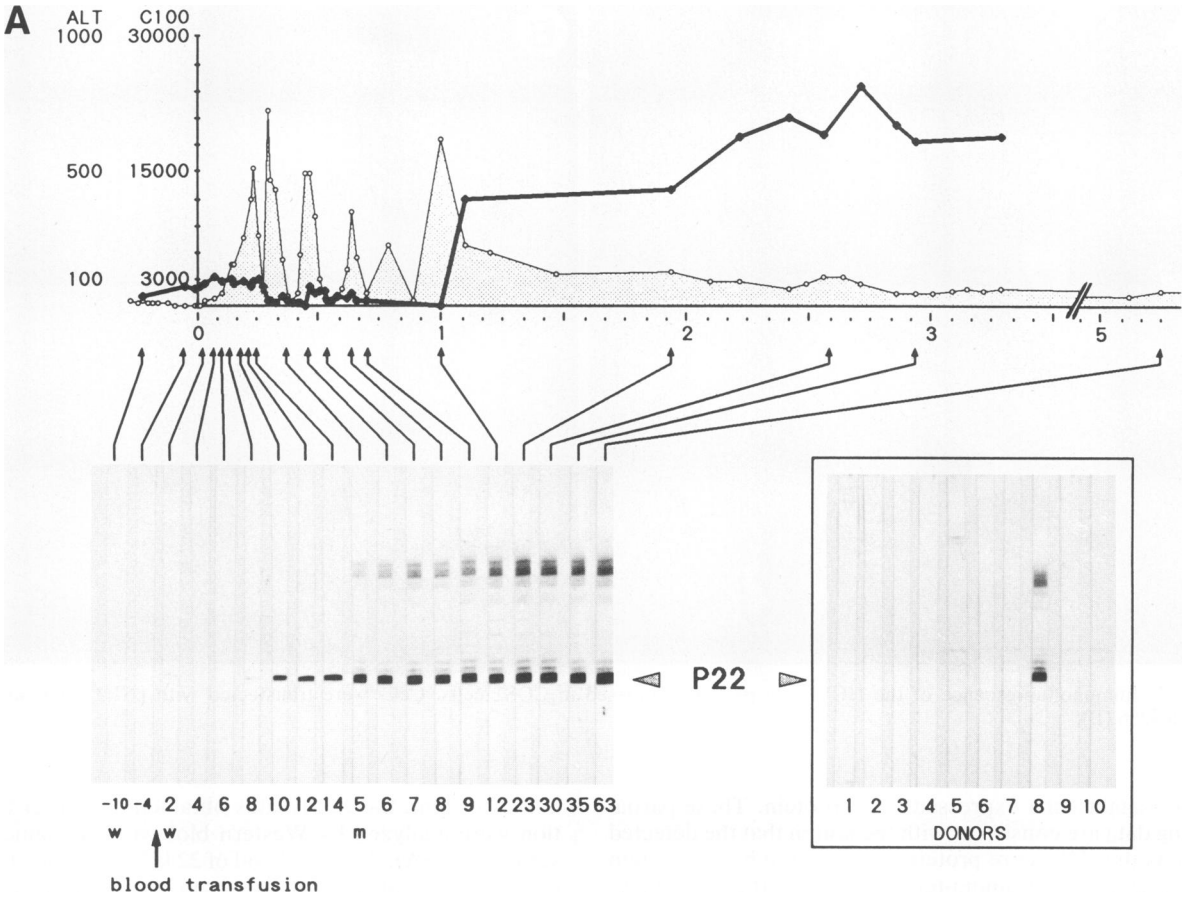


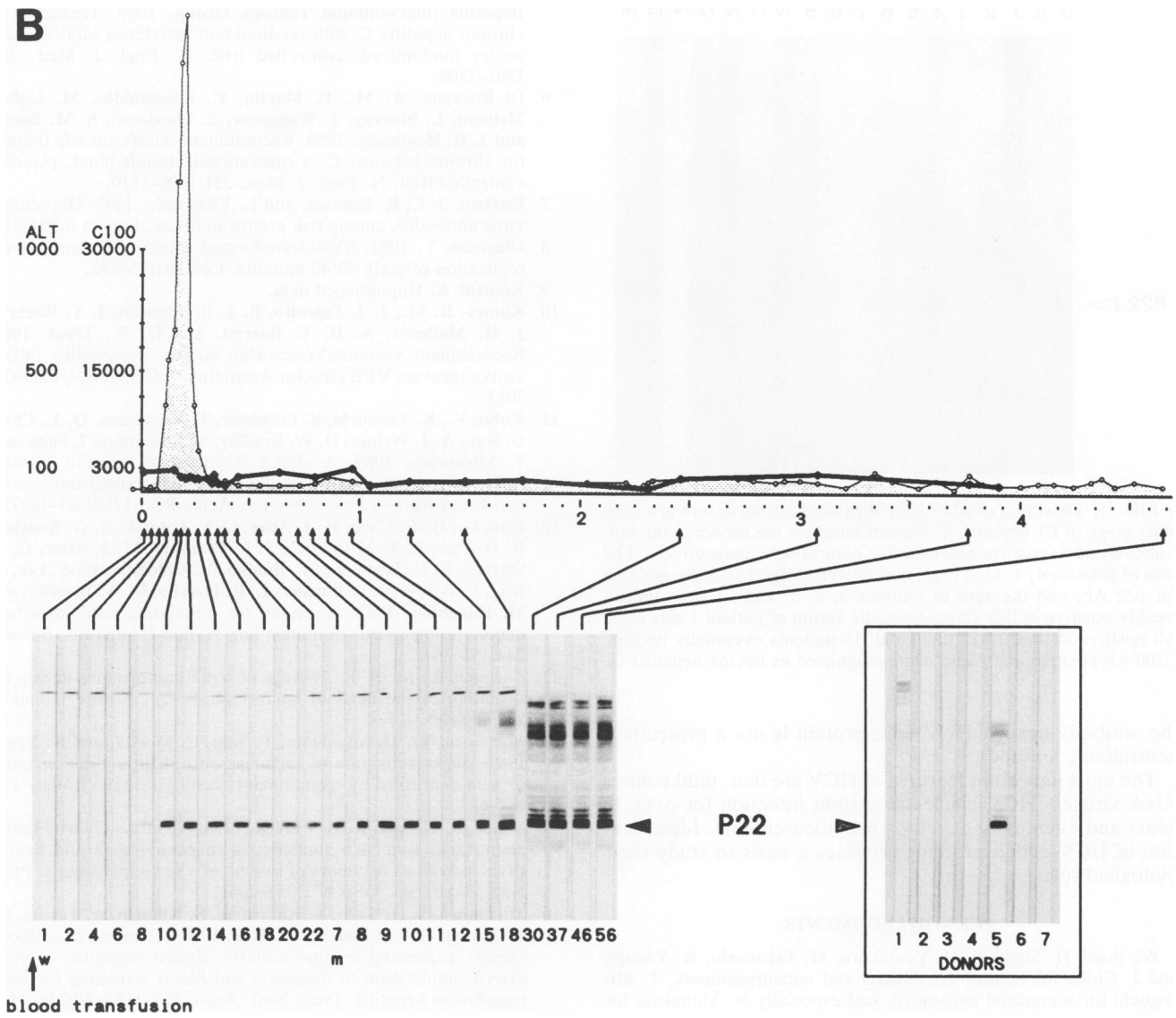

FIG. 4. Western blot analysis detecting p22 Ab in sera from PT NANBH patients. The upper portions of the panels show profiles of the ALT level (open circles) and C100 Ab level (filled circles). ALT levels are shown in international units, and C100 Ab levels are indicated in counts per minutes of radioimmunoassay. The normal levels of ALT and C100 Ab are below 35 and 4,000, respectively. The horizontal axis shows the time scale (numbers represent years after blood transfusion). The lower portions of the panels show the results of Western blot analysis. (Left) The period after blood transfusion is shown at the bottom (w, week; m, month); (right) donor number is shown at the bottom.

cleotide kinase at the 5′ ends of *Asp*718 (Boehringer)-linearized pSR316. *Asp*718 is an isochisomer of *Kpn*I and cuts at the junction between the 3′ end of HCV cDNA and the polyadenylation signal in the vector. No band was detected except for the expected band of 1.42 kb spanning both the full-length cDNA inserted in pSR316 and the 40-nt sequence after the splicing acceptor site in the vector (Fig. 3B), suggesting that p22 was generated by processing.

To investigate whether the sera of hepatitis C patients contain p22 Ab, patient sera were examined by Western blot analysis. The p22 Ab was detected in 89% (58 of 65; data from the Western blot are not shown) of sera from chronic NANBH patients, slightly more often than the positivity of C100 Ab (Ortho Diagnostics) (82% [53 of 65] in this work; 78% in the work of Kuo et al. [12] and Miyamura et al. [16]). No p22 Ab-positive sera were found in 21 apparently healthy Japanese subjects. These results suggest that the p22 Ab is specific to hepatitis C patients and that the p22 Ab assay is, at least, similarly as useful as the C100 Ab assay for diagnosis of chronic hepatitis C.

To test the time course of p22 Ab development in hepatitis C patients during HCV infection, we retrospectively examined the presence of p22 Ab by Western blot analysis in the two series of posttransfusion (PT) NANBH patient sera (16) (Fig. 4). C100 Ab was measured by radioimmunoassay as described previously (12, 16), and the patient serum samples used in this study were chosen on the basis of the analyses of the C100 Ab assay (16). The first case (Fig. 4A) showed a typical course of chronic NANBH, with multiple peaks of the alanine-amino transferase (ALT) level, a marker of liver damage caused by hepatitis. C100 Ab turned positive 13 months after the blood transfusion, and hence, this patient was first diagnosed as having hepatitis C at this point. In contrast, p22 Ab was clearly detected after 8 weeks, within the first ALT peak (Fig. 4A), suggesting its usefulness for early diagnosis. We then assayed donor blood samples that were actually transfused to that patient and found that one donor blood sample (no. 8) was positive for both p22 Ab and C100 Ab. Although the p22 Ab was detected in the serum obtained at the second week, it may have been due to passive transfer from the p22 Ab-positive donor blood.

The second case (Fig. 4B) showed another typical course of chronic NANBH, having only one peak of ALT elevation but maintaining an abnormal ALT value thereafter (16). The C100 Ab test was consistently negative over 4 years, and all of the donor blood samples were also negative for C100 Ab. Therefore, this patient had not yet been diagnosed as having hepatitis C but was, rather, suspected of having unidentified




PT hepatitis. However, p22 Ab was detected in the serum at 8 weeks after transfusion, within the first ALT peak. Furthermore, one of the donor blood samples (no. 5) was found positive only by the p22 Ab assay, showing that, at least in this particular case, the p22 Ab assay identified an implicated donor blood which could not be previously identified by the C100 Ab assay.

To confirm the usefulness of the p22 Ab assay for early diagnosis of hepatitis C, p22 Ab was analyzed by Western blot in the serum samples of 16 PT hepatitis C patients in their early acute stages. The sera tested here were collected at the earliest point at which surgeons suspected hepatitis because of the first significant rise of the ALT level. All of these 16 cases were retrospectively confirmed to be hepatitis C by C100 assay. C100 Ab was detected in 6% of the serum samples (1 of 16) at such early stages, while p22 Ab was detected strongly in 38% (6 of 16) and at least 4 other serum samples were judged as weakly positive (Fig. 5). The results suggested that PT hepatitis C can often be diagnosed at the time at which the surgeon suspects PT hepatitis by using this Western blot test.

The only HCV-specific diagnostic test to date is antibody detection against C100 (12), a recombinant yeast-derived protein containing a part of HCV nonstructural 4 (NS4) protein. However, usually the C100 Ab is detectable 3 to 6 months after the onset of NANBH (16), and hence, it is not adequate for early diagnosis. Here we showed that p22 Ab is often detectable at around the first ALT peak. Recently we developed an enzyme-linked immunosorbent assay (ELISA) to detect p22 Ab (3). The usefulness of the p22 Ab assay for early diagnosis was further confirmed by using the ELISA (9). This feature may be clinically important because physicians could start interferon treatment at the very early stage of hepatitis C. Because implicated donors of PT NANBH were able to be identified by detection of p22 Ab, the detection system also appeared to be useful for the screening of donor bloods for transfusion. The results also suggest that

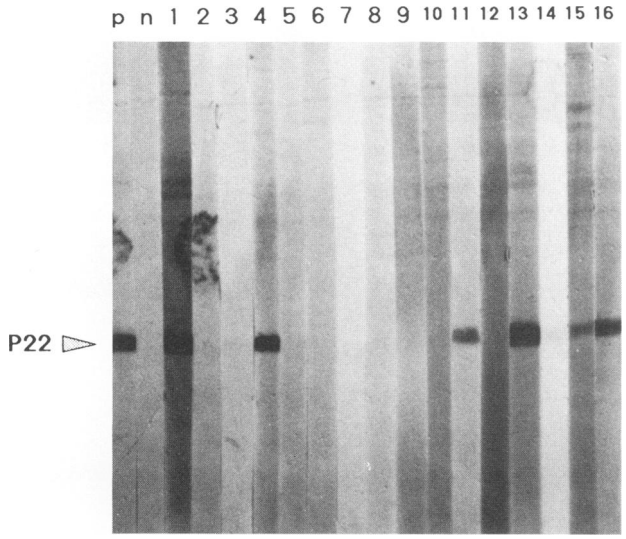



FIG. 5. Detection of p22 Ab by Western blotting in sera at a very early stage of PT hepatitis C. Patient numbers are shown at the top. Lanes p and n, positive and negative control sera, respectively. The sera of patients 1, 4, 11, 13, 15, and 16 were judged strongly positive for p22 Ab, and the sera of patients 3, 8, 9, and 14 were judged weakly positive at this stage. Only the serum of patient 1 was C100 Ab positive at this stage, though all 16 patients eventually became C100 Ab positive and hence were diagnosed as having hepatitis C.

the antibody against HCV core protein is not a protective, neutralizing antibody.

The most important features of HCV are that, unlike other RNA viruses, HCV causes persistent infection for over 30 years and eventually develops hepatocarcinoma. Identification of HCV-coded proteins provides a basis to study their biological roles.

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