Acta virol. **37**: 132–142, 1993

MOLECULAR CLONING OF THE IMMUNODOMINANT REGIONS OF HEPATITIS C VIRUS TYPE III AND IV BY IMMUNOSCREENING

A. SATO[1], J. KAZAMI[1], T. ARIMA[2], H. NAKAMURA[1], T. KUNITOMO[1]

[1] Medical Devices and Diagnostic Res. Labs., Toray Industries, Inc., 3-3-7, Sonoyama, Otsu, Shiga 520; and [2]Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890, Japan

Received October 29, 1992

Summary. – We have identified the immunodominant regions of hepatitis C virus (HCV) type III and IV by immunoscreening of a lambda gt11 cDNA library, which was constructed with RNA extracted from pooled sera of patients. In addition to the nucleocapsid protein, nonstructural region 3 (NS3), and NS4 regions, we found that the region at the N terminus of NS5 in type III and IV were also immunoreactive as well as in the prototype and type II, although amino acid homologies were approximately 60 % between the prototype (or HCV type II) and HCV isolates of type III or IV. This result indicated that the region at the N terminus of NS5 may be a candidate for serotyping studies of HCV. This report presents the primary structure of immunodominant portions at the N terminus of NS5 in two HCV subtypes and a preliminary study of the immune responses to these antigens.

Key words: hepatitis C virus; subtype III, IV; cloning; immunoscreening; immunodominant region; serotyping

Introduction

HCV is the main causative agent of non-A and non-B posttransfusional hepatitis (NANBH) (Alter *et al.*, 1989). In 1989, a complementary DNA (cDNA) fragment from HCV RNA genome was isolated from a chimpanzee clinically infected with NANBH by immunoscreening with sera from patients of NANBH (Choo *et al.*, 1989).

Recently, many studies on HCV mutants using the polymerase chain reaction (PCR) techniques were reported (Garson *et al.*, 1990; Hosoda *et al.*, 1991). The 5' noncoding and putative nucleocapsid protein regions were highly conserved (Takeuchi *et al.*, 1990; Kubo *et al.*, 1989). Based on sequence variations of the PCR products, classification of HCV isolates has been proposed. Sequence analysis of amplified cDNA fragments containing a portion of NS5 region indicated that there are two major types of HCV in Japan (Enomoto *et al.*, 1990). Four types of HCV have been proposed on the basis of sequence variations in

the core protein region (Okamoto *et al.*, 1992*a*). More recently, the entire sequence of HCV type IV have been reported by using PCR amplification technique (Okamoto *et al.*, 1992*b*) and four distinct genotypes of HCV have been proposed based on the comparative sequence analysis (Kato *et al.*, 1990; Takamizawa *et al.*, 1991; Okamoto *et al.*, 1991; Okamoto *et al.* 1992*b*).

Despite numerous reports on HCV sequence variation and genotype classification, serotyping study on HCV appears to be scarce. In order to understand the distribution of antigenic regions on newly reported HCV genomes (type III and IV), a lambda gt11 library of cDNA derived from HCV-infected sera was constructed and immunoscreened. We identified the immunodominant region at the N terminal of NS5 in which amino acid homology was only 60 % between the prototype and HCV isolates of type III or type IV, suggesting the possibility of detection of type-specific antibody. Here we describe the primary structure of the immunodominant regions at the N terminal of NS5 of two HCV types, the identities among other HCV clones and a preliminary study of immune responses to these cloned antigens.

Materials and Methods

Construction of cDNA library and immunoscreening. Sera (1.8 l) from patients with non-A, non-B hepatitis, which were positive for N14 antibody (Arima *et al.*, 1990) and negative for hepatitis B virus antigens, were pooled and used for extraction of RNA by the modified AGPC method (Chomczynski *et al.*, 1987; Arima *et al.*, 1989*a, b*). Approximately 5 μg of RNA was reverse transcribed by random hexanucleotide primers (Takara Biochemicals, Japan) and a lambda gt11-cDNA library was constructed with a commercially available kit (Bethesda Research Laboratory, U. S. A.). A cDNA library of 5×10^6 plaques was immunoscreened (Arima *et al.*, 1989*a, b*). Fiftyfold diluted sera pooled from 23 patients in the chronic phase and 1000-fold diluted peroxidase-conjugated goat antihuman IgG (Cappel Corp., U. S. A.) were used as the 1st and 2nd antibodies, respectively.

Immunoplaque assay. To confirm the specificity and sensitivity of the candidate clones, an immunoplaque assay was performed (Maeno *et al.*, 1990).

Sequencing. Determination of the nucleotide sequences was accomplished by the dideoxy chain-termination method (Sanger *et al.*, 1977). HCV cDNA clones were subcloned into the *Eco*RI site of pUC18. Double-stranded plasmid DNA was alkali-denatured prior to use in sequence reactions (Genesis 2000 Single-stranded Sequenase™ kit, DuPont, U. S. A.). Samples were electrophoresed on 6 % polyacrylamide gels and run on a sequencing apparatus (automated DNA sequencer Genesis 2000, DuPont, U. S. A). When necessary, oligonucleotide primers were synthesized using the Applied Biosystems Synthesizer Model 380A (Applied Biosystems Japan Inc., Japan). Sequence and protein analysis were carried out by the Genetyx program (Software development Co., Japan).

Samples. A panel of 21 serum samples with clinical evidence of NANBH was used to elucidate the immune responses to the cDNA clones.

Commercial HCV ELISA. Anti-N14 and C100-3 antibodies were determined by commercial assay kits (N14: Eiken Immunochemical Co., Japan, C100-3: Ortho Diagnostics, U. S. A.).

Results and Discussion

Isolation of cDNA clones from pooled human sera

Approximately 5 μg of RNA, which was recovered from pooled human sera,

was used to construct a cDNA library. A lambda gt11 cDNA library of about 5×10^6 plaques was immunoscreened with sera collected from hepatitis C (HC) patients and healthy human carriers. Fifty-six plaques were found to react with pooled sera of HC patients in the first screening and 30 plaques of them were confirmed to be specific for sera of HC carriers in the second screening. None of the clones reacted with sera of 2 HB patients or of 2 healthy subjects confirmed by immunoplaque assay (Arima *et al.*, 1989*a, b;* Maeno *et al.*, 1990). Furthermore, all of 30 plaques obtained from a cDNA library were found to react with the stocked sera of blood donors collected in the 1970s. This result indicated that these clones may contain conserved antigenecity in their sequences (Table 1).

Mapping of HCV cDNA clones on HCV genome

The entire nucleotide sequence of 27 clones and the partial sequence of 3 clones were determined and mapped on HCV genome. We isolated 4 clones in the nucleocapsid protein (core) region, 1 clone in the NS3 region, 2 clones in the NS4 region and the rest of the clones in the NS5 region (Fig. 1). No isolates were obtained in the NS1, NS2 or the envelope (E) protein region. Similar results for HCV type II were also reported by another group (Takamizawa *et al.*, 1991). This may be because of their low antigenecity or the sequence variations especially in the E region (Okamoto *et al.*, 1990; Takeuchi *et al.*, 1990).

Analysis of the nucleotide (nt) and deduced amino acid (aa) sequence of HCV clones: Sequence comparison of HCV clones with HCV type I and II

In order to investigate the sequence variations and identities to that of HCV-1 (type I, Choo *et al.*, 1991) or HCV-J (type II, Kato *et al.*, 1990), we analyzed the entire or partial sequence of 30 clones isolated by immunoscreening. Table 2 shows the sequence identities of our representative clones to those of reported isolates at the nt and aa levels. Comparison of their sequences revealed high

Table 1. cDNA clones isolated by immunoscreening

Analysis	Positive clones/Total clones
1st screening (pooled sera of HC patients)	$56/5 \times 10^6$
2nd screening (pooled sera of healthy carriers)	30/56
1st cross-reaction test (2 HB patients)*	0/30
2nd cross-reaction test (2 healthy subjects)*	0/30

* Confirmatory test was carried out by immunoplaque assay

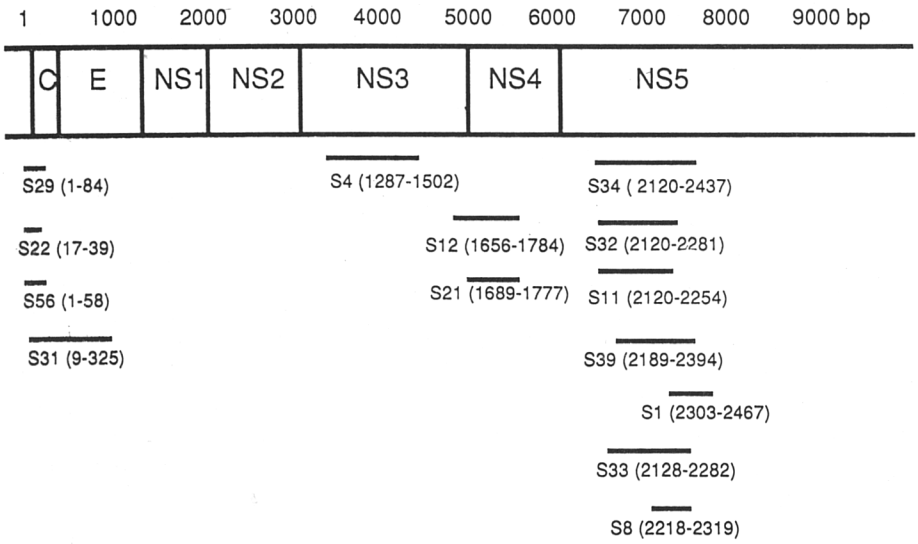

Fig. 1

Mapping of HCV cDNA clones isolated by immunoscreening

The amino acid positions of HCV cDNA clones are numbered in parentheses as compared with the sequence of HCV-1 (Choo *et al.*, 1991). Fourteen representative clones are mapped on HCV genome.

Table 2. Homologies among HCV clones (%)

Clones		Core		NS3	NS4	NS5						
		S29	S31	S4	S12	S11	S34	S32	S33	S8	S39	S1
HCV-1	nt	81.6	81.9	80.5	77.4	77.6	58.4	64.6	62.8	63.3	56.2	53.8
	aa	87.8	86.9	93.0	82.1	88.7	53.4	63.6	61.5	53.7	53.2	52.4
HCV-J	nt	82.3	93.1	89.7	88.6	90.4	58.4	62.8	62.1	61.5	56.9	57.4
	aa	90.2	96.3	94.0	93.3	96.3	57.6	66.0	65.2	57.9	56.2	51.6

nt - nucleotide homology
aa - amino acid homology

```
          10          20          30          40          50          60
GAA TTC TTC TCT TGG GTG GAC GGG GTG CAA ATA CAC CGA TTC GTC CCC ACT CCG GGC CCC
Glu Phe Phe Ser Trp Val Asp Gly Val Gln Ile His Arg Phe Val Pro Thr Pro Gly Pro
          70          80          90         100         110         120
TTC TTT CGG GAT GAG GTA ACG TTC ACC GTA GGC CTC AAT TTC TTT GTG GTC GGC TCC CAG
Phe Phe Arg Asp Glu Val Thr Phe Thr Val Gly Leu Asn Phe Phe Val Val Gly Ser Gln
         130         140         150         160         170         180
CTC CCT TGT GAC CCT GAG CCG GAC ACC GAG GCG CTA GCC TCC ATG CTG ACA GAC CCG TCC
Leu Pro Cys Asp Pro Glu Pro Asp Thr Glu Ala Leu Ala Ser Met Leu Thr Asp Pro Ser
         190         200         210         220         230         240
CAC ATT ACT GCG GAG ACG GCA GCC AGA CGA TTG GCC AGG GGA TCT CCC CCT TCA CAG GCC
His Ile Thr Ala Glu Thr Ala Ala Arg Arg Leu Ala Arg Gly Ser Pro Pro Ser Gln Ala
         250         260         270         280         290         300
AGC TCT TCA GCG AGC CAG CTC TCC GCC CCG TCC TTG AAG GCT ACC TGC ACC ACC CAT AAG
Ser Ser Ser Ala Ser Gln Leu Ser Ala Pro Ser Leu Lys Ala Thr Cys Thr Thr His Lys
         310         320         330         340         350         360
ATG GCA TAT GAC TGT GAC ATG GTG GAT GCT AAC CTT TTC ATG GGA GGT GAT GTG ACC CGG
Met Ala Tyr Asp Cys Asp Met Val Asp Ala Asn Leu Phe Met Gly Gly Asp Val Thr Arg
         370         380         390         400         410         420
ATT GAG TCC GAC TCC AAG GTA ATC GTT CTC GAC TCC CTC GAC TCC ATG ACT GAG GTA GAG
Ile Glu Ser Asp Ser Lys Val Ile Val Leu Asp Ser Leu Asp Ser Met Thr Glu Val Glu
         430         440         450         460         470         480
GAT GAA CGT GAG CCT TCT GTA CCA TCA GAG TAC TTG ATC AGG AGG AGG AAG TTC CCA CCG
Asp Glu Arg Glu Pro Ser Val Pro Ser Glu Tyr Leu Ile Arg Arg Arg Lys Phe Pro Pro
         490         500         510         520         530         540
GCA CTA CCT CCC TGG GCC CGT CCG GAC TAT AAC CCT CCT ACG ATC GAG ATA TGG AAG AGG
Ala Leu Pro Pro Trp Ala Arg Pro Asp Tyr Asn Pro Pro Thr Ile Glu Ile Trp Lys Arg
         550         560         570         580         590         600
CCG GGC TAT GAA CCA CCT ACT GTC CTA GGC TGT GCC CTC CCC CCC ACG CCT CAA GCG CCA
Pro Gly Tyr Glu Pro Pro Thr Val Leu Gly Cys Ala Leu Pro Pro Thr Pro Gln Ala Pro
         610         620         630         640         650         660
GTG CCC CCA CCC AGG AAG CGC CGC GCC AAA GTC CTG ACT CAG GAC AAC GTG GAG GGG GTC
Val Pro Pro Pro Arg Lys Arg Arg Ala Lys Val Leu Thr Gln Asp Asn Val Glu Gly Val
         670         680         690         700         710         720
CTT AGG GAG ATG GCG GAC AAG GTG CTC AGT CCT TCC CAA GAC CAC AAT GAC TCC GGT CAC
Leu Arg Glu Met Ala Asp Lys Val Leu Ser Pro Ser Gln Asp His Asn Asp Ser Gly His
         730         740         750         760         770         780
TCC ACC GGA GCG GAC ACC GGA GGA GAC AGC TTC CAG CAG CTC TTC GAC GAG ACT GCC GCT
Ser Thr Gly Ala Asp Thr Gly Gly Asp Ser Phe Gln Gln Leu Phe Asp Glu Thr Ala Ala
         790         800         810         820         830         840
TCA GAA GCG GGA TCA CTG TCC TCC ATG CCT CCC CTT GAG GGG GAG CCG GGG GAC CCT GAC
Ser Glu Ala Gly Ser Leu Ser Ser Met Pro Pro Leu Glu Gly Glu Pro Gly Asp Pro Asp
         850         860         870         880         890         900
CTG GAG TTT GAA CCA GCG GGA TCC GGT CCC CCT TCT GAG GGG GAG TGC GAG GTC GTT GAT
Leu Glu Phe Glu Pro Ala Gly Ser Gly Pro Pro Ser Glu Gly Glu Cys Glu Val Val Asp
         910         920         930         940         950         960
TCG GAC TCT AAG TCG TGG TCC ACA GTC TCG GAT CAG GAG GAT TCT GTT ATC TGC TGC TCC
Ser Asp Ser Lys Ser Trp Ser Thr Val Ser Asp Gln Glu Asp Ser Val Ile Cys Cys Ser
         970         980         990        1000
ATG TCA TAC TCC TGG ACA GGG GCC CTC ATA ACA CCA TGT GGG GGA ATT C
Met Ser Tyr Ser Trp Thr Gly Ala Leu Ile Thr Pro Cys Gly Gly Ile
```

Fig. 2

Nucleotide sequence and deduced amino acid sequence of HCV cDNA clone S34 in the NS5 region. The deduced amino acid sequence is shown under the nucleotide sequence. Suspected *Eco*RI linker sequences are underlined.

homologies in the core protein, NS3, and NS4 region to that of HCV-1 or HCV-J. Judging from the higher homologies of these clones to HCV-J than HCV-1, they appear to resemble more closely HCV-J than HCV-1. On the other hand, aa identities of 6 clones obtained in the NS5 region were less than 64 % as compared with the sequence of HCV-1. The nt and deduced aa sequence of two major clones (S34 and S39) are shown in Fig. 2 and Fig. 3. These clones had some aa deletions or insertion as judged by comparison with the sequence of HCV-1 or HCV-J (Fig. 4). Such aa deletions or insertions were not reported in HCV type I or HCV type II except that type II had one aa deletion which appeared to be characteristic of this type (Kato *et al.*, 1990; Takamizawa *et al.*,

```
          10          20          30          40          50          60
GAA TTC CCG CGG GGG TCA CCC CCG TCT GAG GCA AGT TCC TCA GCG AGC CAA CTA TCG GCA
Glu Phe Pro Arg Gly Ser Pro Pro Ser Glu Ala Ser Ser Ser Ala Ser Gln Leu Ser Ala
          70          80          90          100         110         120
CCA TCG CTG CGA GCC ACC TGT ACC ACC CAC GGC AAG ACC TAC GAT GTG GAC ATG GTG GAT
Pro Ser Leu Arg Ala Thr Cys Thr Thr His Gly Lys Thr Tyr Asp Val Asp Met Val Asp
          130         140         150         160         170         180
GCT AAC CTG TTT ATG GGA GGC GAT GTG ACT CGG ATA GAA TCT GAA TCC AAA GTG GTC GTT
Ala Asn Leu Phe Met Gly Gly Asp Val Thr Arg Ile Glu Ser Glu Ser Lys Val Val Val
          190         200         210         220         230         240
CTG GAC TCC CTC GAC CCA ATG GCC GAA GAA ATG AGC GAC CTC GAG CCT TCT ATA CCA TCG
Leu Asp Ser Leu Asp Pro Met Ala Glu Glu Met Ser Asp Leu Glu Pro Ser Ile Pro Ser
          250         260         270         280         290         300
GAG TAT ATG CTC CCC AAA ACC AGG TTC CCA CCA GCC TTA CCG GCC TGG GCA CGG CCT GAC
Glu Tyr Met Leu Pro Lys Thr Arg Phe Pro Pro Ala Leu Pro Ala Trp Ala Arg Pro Asp
          310         320         330         340         350         360
TAC AAC CCA CCG TTT GTG GAA CCA TGG AGG AGA CCA GAC TAC CAA CCG CCC ACT GTT GCG
Tyr Asn Pro Pro Phe Val Glu Pro Trp Arg Arg Pro Asp Tyr Gln Pro Pro Thr Val Ala
          370         380         390         400         410         420
GGC TGT GCT CTC CCC CCC CCC AAG AAG ACC CCG ACG CCC CCC CCA AGG AGA CGC CGG ACA
Gly Cys Ala Leu Pro Pro Pro Lys Lys Thr Pro Thr Pro Pro Pro Arg Arg Arg Arg Thr
          430         440         450         460         470         480
GTG GGT CTG AGT GAG AGC ACC ATA GGA GAT GTC CTC CAA CAG ATG GCC ATT AAG ACC TTT
Val Gly Leu Ser Glu Ser Thr Ile Gly Asp Val Leu Gln Gln Met Ala Ile Lys Thr Phe
          490         500         510         520         530         540
GGC CAG CCC CCC CCA AGC GGA GAT TCA GGC CTC TCT ACG GGG GCG GAC GCC GCC GAC TCT
Gly Gln Pro Pro Pro Ser Gly Asp Ser Gly Leu Ser Thr Gly Ala Asp Ala Ala Asp Ser
          550         560         570         580         590         600
GGT AGT CGG ACG CCC CCC GAG GAG TTA GCT CTC TCG GAG ACA GGT TCT ACC TCC TCC ATG
Gly Ser Arg Thr Pro Pro Glu Glu Leu Ala Leu Ser Glu Thr Gly Ser Thr Ser Ser Met

CGG AAT TC
Arg Asn
```

Fig. 3

Nucleotide sequence and deduced amino acid sequence of HCV cDNA clone S39 in the NS5 region The deduced amino acid sequence is shown under the nucleotide sequence. Suspected *Eco*RI linker sequences are underlined.

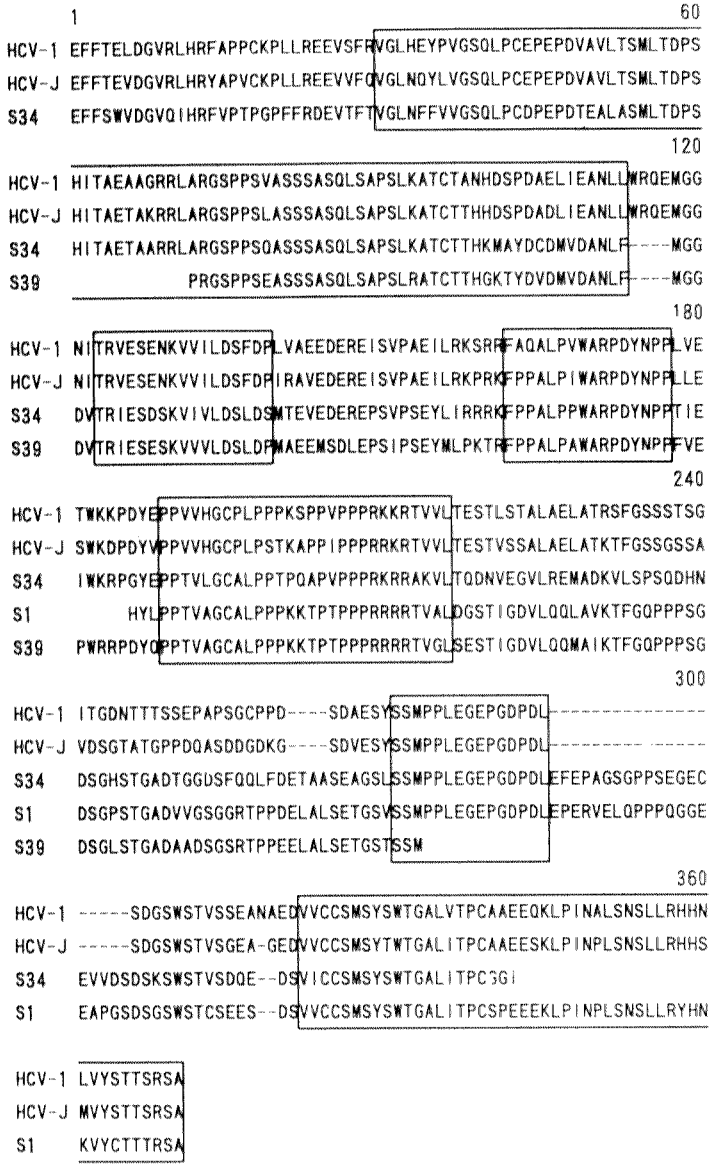

Fig. 4

Amino acid identities among HCV cDNA clones in the NS5 region

The numbers shown are percentage of identical amino acids between their sequences. [a]Amino acid identity in the NS5 region (aa 2124–2487) between HC-J6 (Okamoto *et al.*, 1991) and HC-J8 (Okamoto *et al.*, 1992*b*).

1991). Evaluating the results of sequence homologies and the patterns of aa deletions and insertions, these 6 clones should belong to other HCV subtypes. HCV was reported to tend to change its sequence and the sequence variation was found among HCV clones isolated even from a single patient (Kubo *et al.*, 1990). Moreover, our clones were isolated from a cDNA library constructed with mixed sera of HC patients. Therefore, it should be noted that whether each clone was derived from independent source or not was uncertain. Grouping of these clones revealed that they might have been derived from at least two separate patients.

Grouping of HCV clones obtained in the NS5 region and sequence comparison of them with HC-J6 (type III) and HC-J8 (type IV)

Recently, Okamoto *et al.* (1991, 1992*b*) described the isolation of 2 HCV genomes having low homology to reported isolates (HC-J6: type III, HCJ8: type IV) and proposed four distinct genotypes based on the nucleotide sequence variations. Comparison of the proteins encoded by HCV clones in the NS5 region, HC-J6 and HC-J8 was presented in Fig. 5. The data of sequence identities suggested that our HCV clones in the NS5 region could be classified into two different groups. A high degree of similarity was observed among S1, S39 and HC-J6 (more than 84.1 %) or S8, S32, S34 and HC-J8 (more than 89.2 %), indicating that S1 and S39 belong to type III and S8, S32, S33 and S34 belong to type IV, respectively. In addition to 6 clones described above, we also

	S34	S32	S33	S8	S39	S1	S11	HC-J6	HC-J8
S34		96.1%	96.0%	93.6%	61.8%	57.2%	67.7%	68.4%	93.1%
S32			98.6%	94.5%	75.3%	—	69.2%	83.9%	96.8%
S33				94.9%	74.2%	—	67.2%	80.8%	95.4%
S8					67.7%	—	56.3%	68.8%	89.2%
S39						84.1%	65.1%	91.9%	63.3%
S1							—	85.8%	63.7%
S11								70.7%	68.4%
HC-J6									71.4%*
HC-J8									

Fig. 5

Multiple amino acid sequence alignment of HCV clones

The amino acid sequences of S1, S34, S39 (this study), HCV-1 (Choo *et al.*, 1991) and HCV-J (Kato *et al.*, 1990) have been aligned by introducing gaps as indicated by dashes. Gaps have been introduced to maximize homology. Numbering indicates position in alignment. Highly conserved regions are boxed.

obtained a cDNA clone (S11) which showed 96.3 % aa identity to HCV-J (Fig. 5) and included no aa deletions or insertions. This clone must be a member of HCV type II.

A multiple sequence alignment was prepared to compare the deduced aa sequences of 21, S34, S39, HCV-1 and HCV-J (Fig. 4). There are several regions which show extensive aa identities among these clones: aa 30–113; aa 122–138; aa 162–177; aa 189–216; aa 271–285; aa 322–370. These highly conserved regions may imply a biological significance. It should be stressed, however, that we have nearly no information on the function of NS5 proteins except that NS5 codes for RNA-dependent RNA polymerase similar to that of the related flaviviruses. Hydropathy profiles showed no significant difference among HCV-1, S34 and S39 (data not shown), indicating that a possible evolutionary conservation occurred without changing the functional structure of the peptide.

Appearance of antibodies to viral cDNA clones in the NS5 region

We found that the region at N terminus of NS5 of HCV type III and type IV were immunogenic despite low aa identities with other types. This result suggested that this antigenic region may be a candidate for serological studies of possible type-specific antibodies. To investigate the appearance of antibodies in sera of the patients, we selected three clones having low homologies to other subtypes (S1, S39 and S34) and performed a preliminary experiment to detect antibodies reacting with them in sera from HC patients by immunoplaque assay (Arima *et al.*, 1989*a b*; Maeno *et al.*, 1990). Table 3 shows the results of this test with serum panel constructed by authors. Among 19 clinically well-defined HC chronic patients, 11 (57.9 %) were positive for the antibody to S34 (anti-S34) and 9 (47.4 %) were positive for the antibodies to S1 (anti-S1) or S39 (anti-S39). No anti-S34, anti-S1 and anti-S39 were observed in sera of 5 HB patients and 4 healthy persons, indicating that the presence of antibodies is closely associated

Table 3. Appearance of antibodies to HCV cDNA clones

	HC patients																				
	Chronic																			Acute	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
N14	+	+	+	+	+	+	-	-	+	-	+	+	-	+	-	+	+	+	+	-	+
C100	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	-	-
S34	+	+	+	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+	-	-	-
S1	-	+	+	-	+	-	-	-	+	+	+	+	-	+	-	-	-	+	-	-	+
S39	-	+	+	-	+	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-	+

with the infection of HCV (data not shown). Differences in the immune responses were observed between individuals. It was interesting that anti-S1 and anti-S39 were detectable in the serum of a patient who showed an acute clinical course, although no anti-S34 was observed in it. This result may imply the detection of possible type-specific antibodies.

As we have shown in this study, low homologies to each HCV type in the NS5 region were expected to result in the low immunological cross-reactivity among each type. Serological studies of type-specific antibodies would be possible if the immunodominant epitope was identified in the region described here. To confirm this assumption, further analyses to investigate whether the detection of a type-specific antibody may differentiate between HCV isolates, using synthetis peptides or recombinant proteins, are necessary. The information on HCV isolates presented here will help to perform further studies on development of type-specific serodiagnosis.

Acknowledgements. We thanks Dr. E. Maeda for the assay of HCV antibody. Toray Research Center Inc., for DNA sequencing and Dr. S. Kobayashi for critical review of the manuscript and helpful discussion.

References

Alter, H. J., Purcell, R. H., Shin, J. W., Melpolder, J. C., Houghton, M., Choo, Q.-L., and Kuo, G. (1989): Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *New Engl. J. Med.* **321**, 1494–1500.

Arima, T., Nagashima, H., Murakami, S., Kaji, C., Fujita, J., Shimomura, H., and Tsuji, T. (1989*a*): Cloning of a cDNA associated with acute and chronic hepatitis C infection generated from patients serum RNA. *Gasstroenterol. Jpn.* **24**, 540–544.

Arima, T., Takamizawa, A., Mori, C., Murakami, S., Kaji, C., and Fujita, J. (1989*b*): A lambda gt11-cDNA clone specific for chronic hepatitis C generated from pooled serum presumably infected by hepatitis C. *Gastroenterol. Jpn.* **24**, 545–548.

Arima, T., Mori, C., Takamizawa, A., Shimomura, H., and Tsuji, T. (1990): A cDNA encoding a peptide highly specific for hepatitis C infection. *Gastroenterol. Jpn.* **25**, 218–222.

Chomczynski, P., and Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradly, D. W., and Houghton, M. (1989): Isolation of a cDNA clone derived from a blood-borne non-A, non-B hepatitis genome. *Science* **244**, 359–362.

Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991): Genetic organization and diversity of the hepatitis C virus. *Proc. natn. Acad. Sci. U. S. A.* **88**, 2451–2455.

Enomoto, N., Takeda, A., Nakao, T., and Date, T. (1990): There are two major types of hepatitis C virus in Japan. *Biochem. Biophys. Res. Commun.* **170**, 1021–1025.

Garson, J. A., Tedder, R. S., Briggs, M., Tuke, P., Glazebrook, J. A., Trute, A., Parker, D., Barbara, J. A. J., Contreras, M., and Aloysius, S. (1990): Detection of hepatitis C viral sequences in blood donations by nested polymerase chain reaction and prediction of infectivity. *Lancet* **335**, 1419–1422.

Hosoda, K., Yokosuka, O., Omata, M., Kato, N., and Ohto, M. (1991): Detection and partial sequencing of hepatitis C virus RNA in the liver. *Gastroenterology* **101**, 766–711.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotono, K. (1990): Molecular cloning of the human hepatitis C virus genome from Japanese patients with

non-A, non-B hepatitis. *Proc. natn. Acad. Sci. U. S. A.*, **87**, 9524–9528.

Kubo, Y., Takeuchi, K., Boomar, S., Katayama, T., Choo, Q.-L., Kuo, G., Weiner, A. J., Bradly, D. W., Houghton, M., Saito, I., and Miyamura, T. (1989): A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. *Nucleic Acids Res.* **17**, 10367–10372.

Maeno, M., Kaminaka, K., Sugimoto, H., Esumi, M., Hayashi, N., Kamatsu, K., Abe, K., Sekiguchi, S., Yano, M., Mizuno, K., and Shikata, T. (1990): A cDNA clone closely associated with non-A, non-B hepatitis. *Nucleic Acids Res.* **18**, 2685–2689.

Okamoto, H., Okada, S., Sugiyama, Y., Kurai, K., Iizuka, H., Machida, A., Miyakawa, Y., and Mayumi, M. (1991): Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J. gen. Virol.* **72**, 2697–2704.

Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Mikawa, Y., and Mayumi, M. (1992*a*): Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J. gen. Virol.* **73**, 673–679.

Okamoto, H., Kurai, K., Okada, S., Yamamoto, K., Lizuka, H., Tanaka, T., Fakuda S., Tsuda, F., and Mishiro, S. (1992*b*): Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. *Virology* **188**, 331–341.

Sanger, F., Nicklen, S., and Coulson, A. R., (1977): DNA sequencing with chain-terminating inhibitors. *Proc. natn. Acad. Sci. U. S. A.* **74**, 5463–5467.

Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I., and Okayama, H. (1991): Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* **65**, 1105–113.

Takeuchi, K., Kubo, Y., Boomar, S., Watanabe, Y., Katayama, T., Choo, Q.-L., Kuo, G., Houghton, M., Saito, I., and Miyamura, T. (1990): The putative nucleocapsid and envelope protein genes of hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpanzee and healthy human carriers. *J. gen. Virol.* **71**, 3027–3033.