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Structure and Organization of the Hepatitis C Virus Genome Isolated from Human Carriers

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Hepatitis C virus (HCV) is a major causative agent of posttransfusion non-A, non-B hepatitis, which often develops into malignant chronic diseases, including liver cirrhosis and hepatocellular carcinoma. We have cloned from human carriers overlapping cDNAs (9,416 bp) covering the entire coding region of the HCV genome. The latter encodes a 3,010-amino-acid polyprotein. In addition, there are 332 and 54 bases of 5′ and 3′ noncoding sequences, respectively. Our HCV strain has a 77% nucleic acid identity to the HCV strain cloned by workers at Chiron Corporation. The hydrophobicity profile of the putative polyprotein is similar to those of flaviviruses, but it has limited amino acid homology to polyproteins of flaviviruses and other viruses, indicating that HCV is at most distantly related to flaviviruses.

Blood-borne non-A, non-B hepatitis is a major form of posttransfusion hepatitis which frequently progresses into malignant liver diseases such as cirrhosis and cancer. In the past decade, a great deal of effort has been devoted to identifying the etiologic agent of blood-borne non-A, non-B hepatitis. Based on its physicochemical properties, the agent was suggested to be a virus (2, 3, 23), but virtually nothing was known about it until a recent breakthrough made by workers at Chiron (5, 10). They cloned cDNAs corresponding to part of the genome of a virus isolated from a chimpanzee which had been infected with the blood of a patient with posttransfusion non-A, non-B hepatitis and which subsequently developed acute non-A, non-B hepatitis. The C100-3 protein encoded by one of the cDNA clones was found to react specifically with sera from patients with blood-borne non-A, non-B hepatitis (5, 13). The close association of anti-C100-3 antibodies with chronic posttransfusion non-A, non-B hepatitis was subsequently confirmed in laboratories worldwide (12, 13, 19). This virus was thus determined to be a causative agent of non-A, non-B hepatitis and named hepatitis C virus (HCV) (13).

Houghton et al. (10) have suggested that the virus has a positive-stranded RNA genome and is related to flaviviruses. More recently, several research groups have isolated cDNAs (0.3 to 1.9 kb) spanning part of an HCV genome directly from human patients by using probes based on the Chiron sequence and have found that the HCVs isolated were closely related to Chiron's (approximately 92% amino acid homology) (12, 16, 20). Despite recent dramatic progress, however, the entire structure of HCV has not been described.

In an effort to identify an etiologic agent of posttransfusion non-A, non-B hepatitis, we have recently cloned overlapping cDNAs that span the entire coding region of HCV isolated from the plasma of Japanese carriers. Our HCV has an 85% amino acid homology to Chiron's and 90 to 98% homology to HCVs cloned by others (10, 12, 16, 20). The structure and organization of the encoded protein indicate that HCV is distantly related to flaviviruses.

Here we present the primary structure and organization of the genome of HCV isolated from human carriers. The availability of sequence information as well as cloned cDNAs spanning the entire coding region should greatly facilitate virological studies of HCV, including the development of diagnostic tools and vaccines to prevent the majority of posttransfusion non-A, non-B hepatitis cases as well as HCV-associated malignant liver diseases.

MATERIALS AND METHODS

Preparation of RNA from human plasma. Blood from potential HCV carriers, which was negative for hepatitis A or B virus antigens but had more than 35 IU of alanine aminotransferase per ml, was obtained from a regional blood bank. Combined blood samples obtained from 50 such individuals, approximately 10% of whom had detectable levels of anti-C-100 antibody, were processed to prepare plasma. By continuous-flow centrifugation through a 30% (wt/vol) sucrose cushion at 48,000 × *g* for 13 h at 4°C, viral particles were sedimented from 4.8 liters of plasma. The pellet was suspended in 50 mM Tris-HCl (pH 8.0)–1 mM EDTA and resedimented by centrifugation at 250,000 × *g* for 3 h at 4°C. RNA was prepared from the pellet by the guanidine thiocyanate-Cs-trifluoroacetic acid method as described previously (21).

Cloning of HCV cDNAs. Approximately 750 ng of the RNA was converted to double-stranded cDNA by random hexanucleotide-primed reverse transcription with the Amersham cDNA synthesis kit (Amersham International, Amersham, U.K.). The synthetic *Eco*RI adapter was ligated to the cDNA and phosphorylated with T4 kinase. About 130 ng of the cDNA with *Eco*RI adaptors attached was then ligated to 1 μg of λgt11 phage vector arms. The ligated DNA was packaged in vitro by using a commercial packaging kit, Gigapack Gold (Stratagene). The packaged phage particles yielded a total of 8×10^5 plaques, which were then screened for HCV cDNA clones by the enzyme immunoassay method

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TABLE 1. Characterization of HCV cDNA clones

Clone	Size (kb)	Immunoreaction[a] (no. positive)		Homology to human chromosomal DNA[b]
		HHS (n = 10)	NANB (n = 11)	
BK102	1.7	0	10	—
BK103	0.8	0	9	—
BK105	2.0	0	11	—
BK106	1.1	0	11	—
BK108	1.5	0	9	—
BK109	1.8	0	9	—
BK110	1.8	0	9	—
BK111	1.0	0	9	—
BK112	3.6	0	10	—
Chiron C-100		0	9	

[a] HHS, Healthy human serum; NANB, non-A, non-B hepatitis patient serum. Immunoreaction was assayed by the enzyme immunoassay method under the same conditions as for immunoscreening of HCV clones. Immunoreaction to Chiron C-100 was assayed with the Ortho HCV diagnosis kit (Ortho Diagnostic Systems, Tokyo, Japan).

[b] —, No hybridizing band detected.

with combined sera from patients clinically diagnosed as having chronic non-A, non-B hepatitis as described before (30). Plaque hybridization was carried out as described before (1).

Sequence analysis of cloned cDNA. cDNA inserts were subcloned into the *Eco*RI site of pUC19 and sequenced by the dideoxynucleotide method after making various deletions with exonuclease III and mung bean nuclease (9). Verification of sequences was done by resequencing both strands.

Southern blot hybridization. Human placental DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst*I, and 10 μg of each of the digests was electrophoresed on agarose gels. After blotting to nitrocellulose filters, the filters were hybridized with each HCV cDNA insert labeled with ^{32}P. As a positive control to verify the sensitivity of the detection method, 1 to 10 pg of each insert was mixed with the digests and analyzed in parallel.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the GenBank, EMBL, and DDBJ nucleotide sequence data bases under accession number M58335.

RESULTS AND DISCUSSION

Isolation of cDNA clones of the HCV genome. Blood plasma from potential non-A, non-B hepatitis virus carriers was used as the starting material for cDNA cloning. Viruses were sedimented by continuous-flow ultracentrifugation through a 30% (wt/vol) sucrose cushion. RNA was then prepared and converted into double-stranded cDNA by reverse transcription with random hexanucleotides as primers, and the double-stranded cDNA was inserted into the λgt11 phage vector. After screening of 8.5×10^5 plaques, 9 plaques were found to react specifically with combined sera from patients clinically diagnosed as having chronic posttransfusion non-A, non-B hepatitis. The positive clones contained 0.8- to 3.6-kb overlapping cDNA inserts except for clone BK106 (Table 1, Fig. 1a). None of the clones hybridized to human placental genomic DNA, as judged by extensive Southern blot hybridization analyses after various restriction enzyme digestions (Table 1). The proteins encoded by the nine clones specifically reacted with sera from 11 patients with chronic non-A, non-B hepatitis patients, 9 of whom were anti-C-100 antibody positive (Table 1).

To isolate other overlapping clones, the cDNA library was rescreened with ^{32}P-labeled fragments obtained from the 5′ end of clone BK102 (0.7 kb), the 3′ end of clone BK112 (0.7 kb), and the entire insert of clone BK106 as probes. This rescreening resulted in the isolation of an additional 59 clones (0.4 to 4.8 kb of cDNA) which cover the 9.4 kb of the HCV genome. Some representative clones are shown in Fig. 1b.

Primary structure of HCV. The nucleotide sequence and deduced amino acid sequence of HCV were determined from overlapping clones BK157, BK146, BK102, BK112, and BK166 (Fig. 2). Nucleotide identities among overlapping sequences were greater than 99.3%. The combined sequence shown in Fig. 2 is from the 5′-terminal sequence of BK157, the entire sequence of BK146, the internal region of BK102, the entire sequence of BK112, and the 3′-terminal sequence of BK166. The combined sequence is 9,416 bp in length and has a long open reading frame capable of encoding a 3,010-amino-acid protein. The 5′ and 3′ noncoding sequences are 332 and 54 bp long, respectively. The 5′ noncoding region contains several in-frame termination codons, indicating that the combined clones cover the entire coding region and, possibly, the entire genome of the HCV strain. As marked by circles, triangles, and underlines, the 5′ and 3′ noncoding regions contain several direct and inverted repeats, which might play a role in replication of the genome. At the end of the 3′ noncoding region, a stretch of 12 U residues was found. Whether or not this poly(U) stretch is the 3′ tail of the genome is unknown.

Deduced amino acid sequence of encoded protein. The RNA genomes of flaviviruses, including Japanese encephalitis virus (JEV), yellow fever virus, and dengue virus, encode one large polyprotein that is subsequently cleaved into three structural proteins, the core (C) protein, the prematrix (pre-M) protein, which is further processed into the matrix (M) protein, the envelope (E) protein, and seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. While the structural proteins form the viral particles, the nonstructural proteins are thought to be involved in replication of the viral genome. In fact, NS3 and NS5 are a helicase and an RNA-dependent RNA polymerase, respectively (11, 15, 24). The viral protein appears to be processed by at least two proteases, one of cellular origin and the other of viral origin, which recognize and cleave the polyprotein at specific sites (8, 22, 28).

Based on sequence similarities between HCV and flaviviruses, putative cleavage sites of HCV protein were tentatively assigned (Fig. 3) and are shown in the amino acid sequence (Fig. 2). It should be stressed, however, that we have no information on the processing of the HCV proteins, and these putative cleavage sites were determined solely by computer analysis. Nevertheless, when the hydrophobicity profiles of the encoded proteins were compared based on the provisional cleavage sites, a significant similarity was found between the nonstructural proteins of HCV and JEV (Fig. 4). If our putative cleavage site assignments are correct, the structural proteins of HCV are half as long as those of JEV, and the existence of pre-M in HCV is uncertain. The putative C protein consists of 114 amino acids with a molecular mass of 13,000 daltons. It has a high arginine-lysine content (23.5%), as do other flavivirus C proteins, and may be able to bind to the RNA genome. The putative E

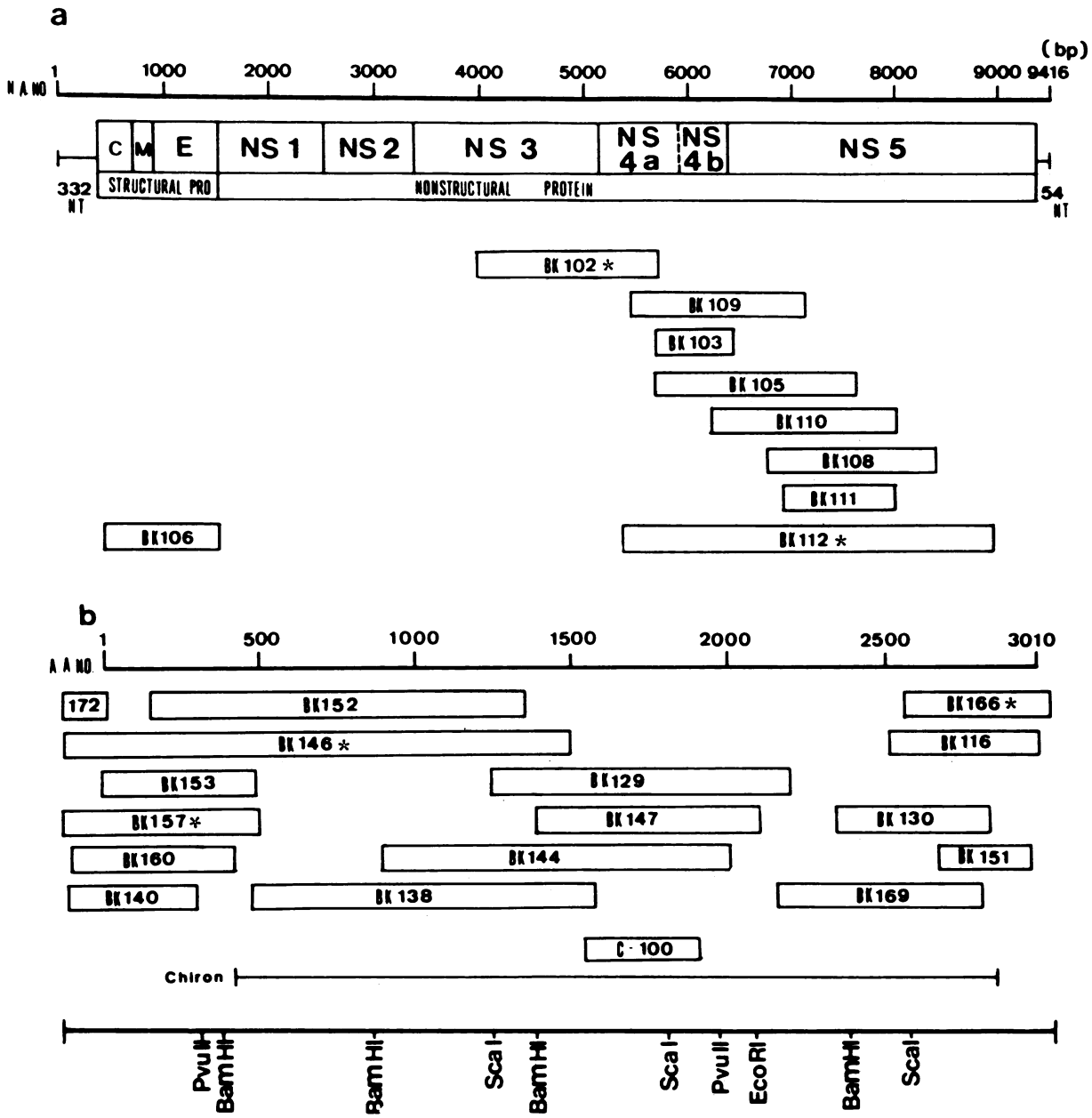

FIG. 1. HCV genome and mapping of HCV cDNA clones. (a) Mapping of nine cDNA clones initially isolated by immunoscreening. (b) Mapping of 16 representative clones isolated by plaque hybridization with the initially isolated cDNAs as probes. The predicted coding regions for structural and nonstructural proteins are shown. Asterisks indicate the clones that were used to determine the primary structure of HCV genome shown in Fig. 2. Chiron denotes the HCV sequence reported by Houghton et al. (10), and C100 is the region which was used to prepare the antigen for the Ortho HCV diagnosis kit. N.A., Nucleic acid; A.A., amino acid; NT, nucleotides.

protein is 198 amino acids long and has a molecular mass of 21,400 daltons, and its hydrophobic C-terminal half may interact with the host membrane. Potential glycosylation sites are indicated by asterisks in Fig. 2.

The putative NS1 protein consists of 340 amino acids and has a molecular mass of 38,000 daltons. The flavivirus NS1 has been determined to be a soluble complement-fixing antigen sometimes found on the surface of infected cells (29). Immunization with the NS1 protein effectively protects hosts from flavivirus infection (25, 26), perhaps by selective complement-induced lysis of infected cells. The NS1s of flaviviruses contain two or more potential glycosylation sites. Similarly, the NS1 of HCV has nine potential glycosylation sites, indicated by asterisks in Fig. 2.

The putative NS2 and NS4 proteins are 277 and 398 amino acids long. NS3 and NS5, which may be a helicase and an RNA-dependent RNA polymerase, respectively, have 609 and 997 amino acids. They are well conserved among flaviviruses and even in some other viruses. The sequence Gly-Asp-Asp (GDD) is found in all RNA-dependent RNA polymerases, including the NS5s of all flaviviruses, poliovirus, and tobacco mosaic virus (11, 15, 24, 27). This sequence

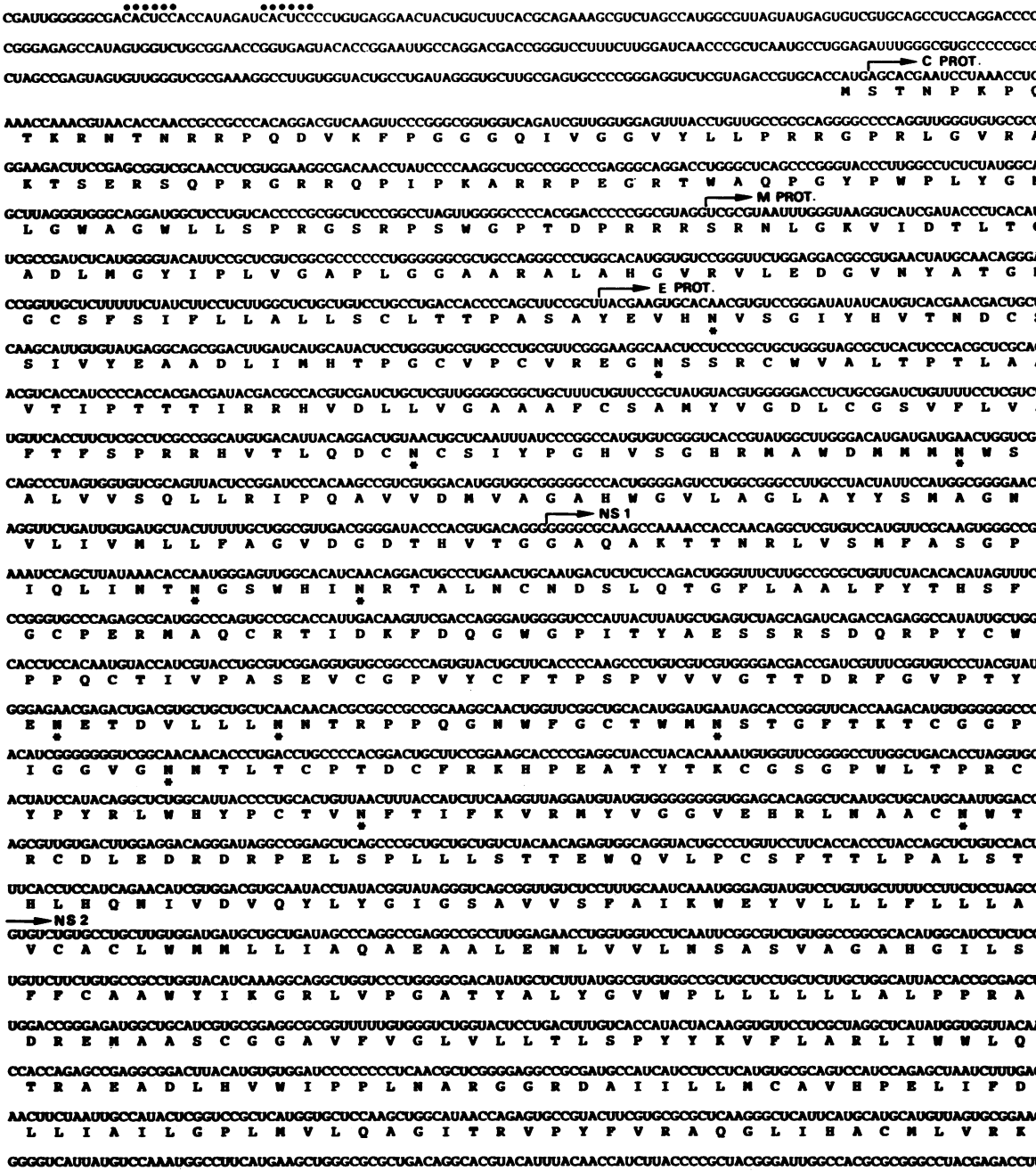

FIG. 2. Nucleotide and deduced amino acid sequences of the HCV genome. The nucleotide sequence was determined from clones BK157, BK146, BK102, BK112, and BK166 as described in the text. Nucleotides are numbered from the 5′ end, and amino acids are numbered from the first methionine in the polyprotein. Potential glycosylation sites (Asn-X-Ser/Thr) are indicated by asterisks. The sequence Gly-Asp-Asp found in all the RNA-dependent RNA polymerases of flaviviruses, poliovirus, tobacco mosaic virus, and cowpea mosaic virus (11) is boxed. Two palindromic sequences at the 3′ end are indicated by a double line. Identical direct repeats of six nucleotides at the 5′ and 3′ ends are marked by solid circles. Two additional direct repeats (CCGGCUG and GCUGGUU) at the 3′ terminus are indicated by open and solid triangles. The sequence continues on the following pages.

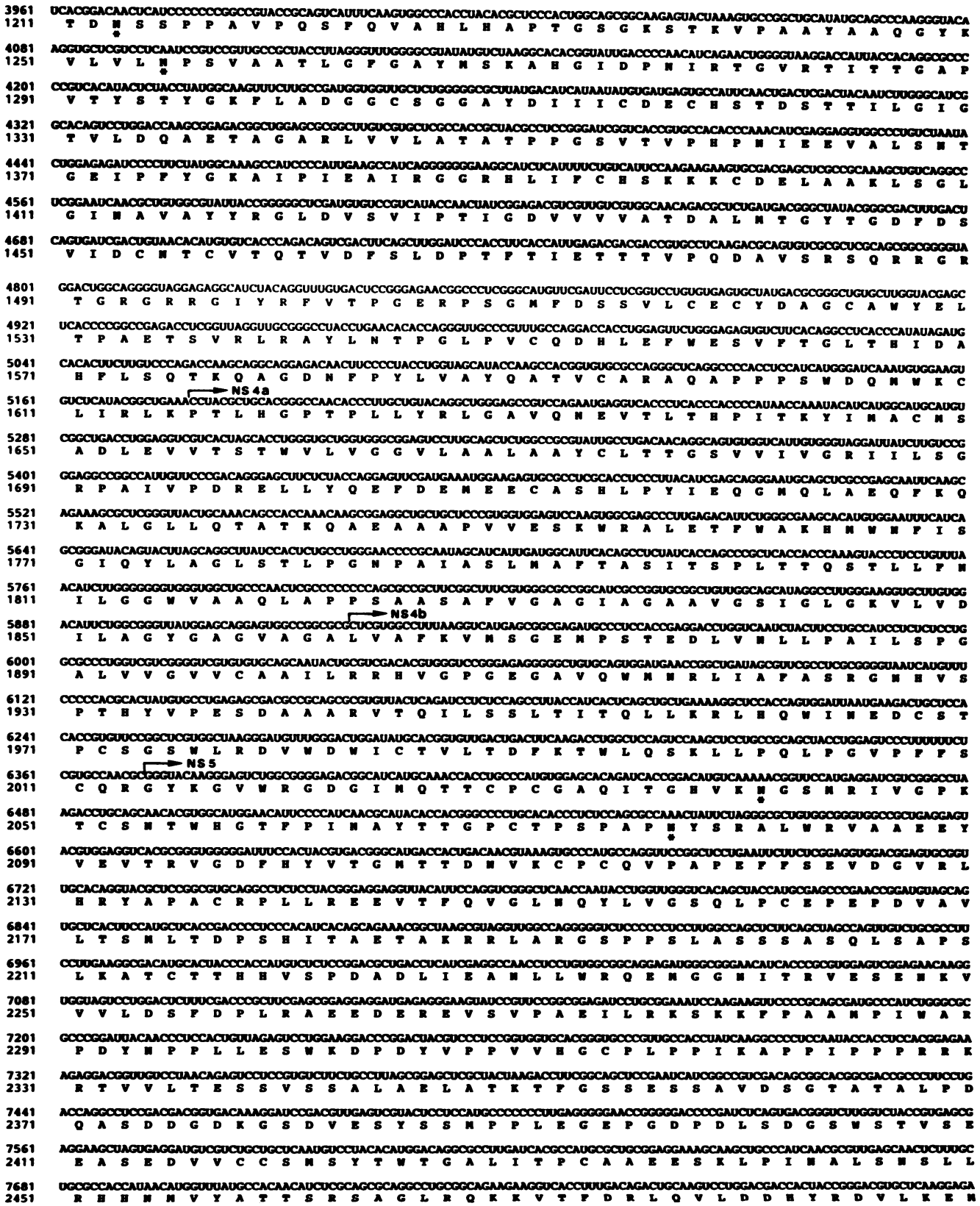

FIG. 2—*Continued.*

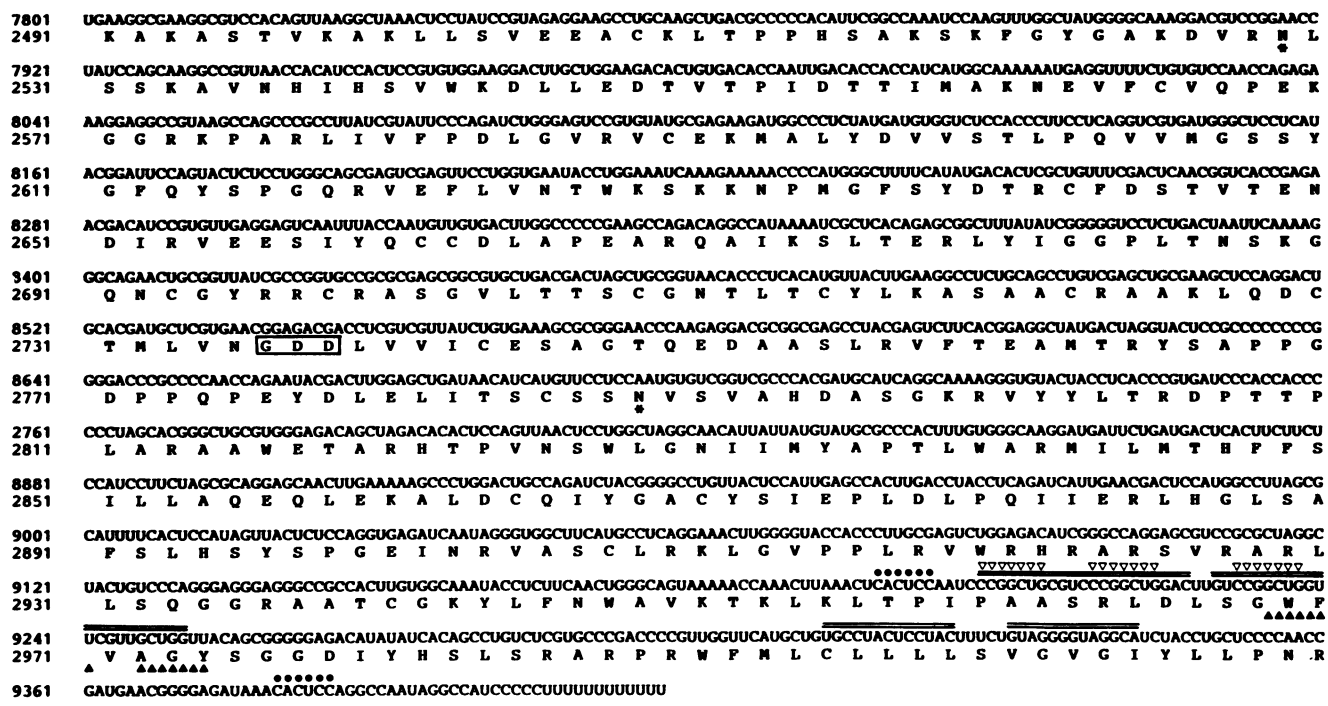

FIG. 2—*Continued.*

is also contained in the putative NS5 of HCV (amino acids 2736 to 2738 in Fig. 2).

Sequence homology among HCV cDNA clones and other viruses. Since Houghton et al. (10) first reported a partial sequence of HCV cloned from infected chimpanzees, several groups have isolated cDNAs corresponding to various parts of HCV, and significant sequence heterogeneity was apparent among the isolated cDNA clones. Kubo et al. found that there were even some sequence variations among HCV cDNA clones isolated from a blood sample from a single patient (12).

To look for sequence variations among our cDNA clones, we sequenced BK106, -110, -109, -147, -138, -116, -152, and -160 in addition to BK157, -146, -102, -112, and -166, which were used to determine the primary structure. Comparison of overlapping sequences revealed that these clones fall into two groups. BK152 and BK160 belong to one group and the rest belong to another (Fig. 5). Within each group, the

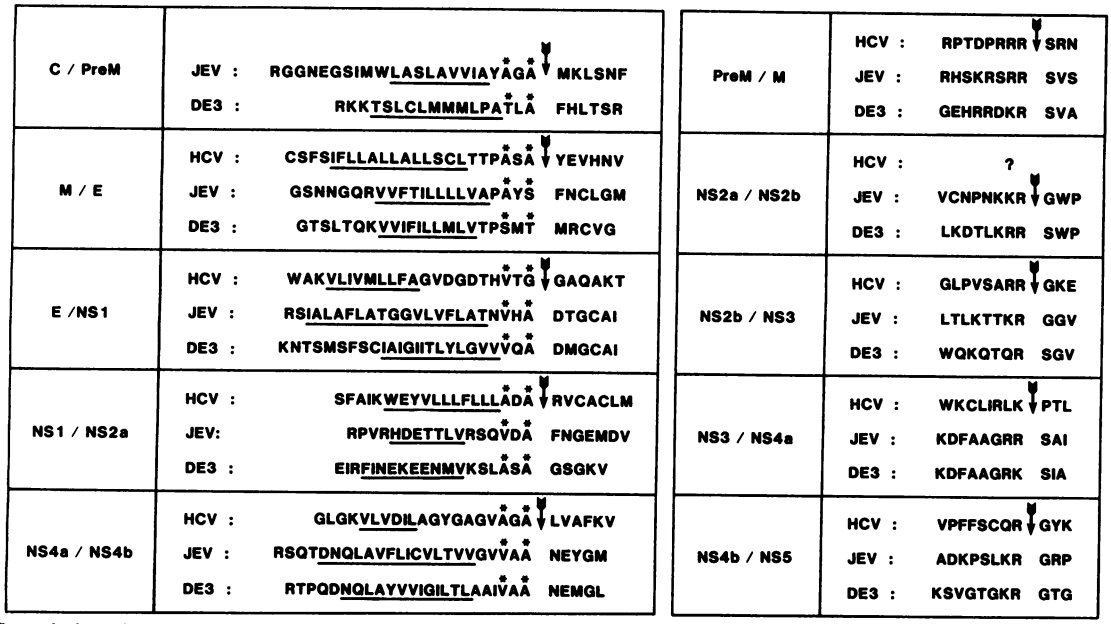

FIG. 3. Speculative cleavage sites of the HCV polyprotein compared with JEV and dengue type 3 virus (DE3). Symbols: *, signalase recognition site; underline, L-rich region; arrow, cleavage site; ?, unable to identify the putative cleavage site.

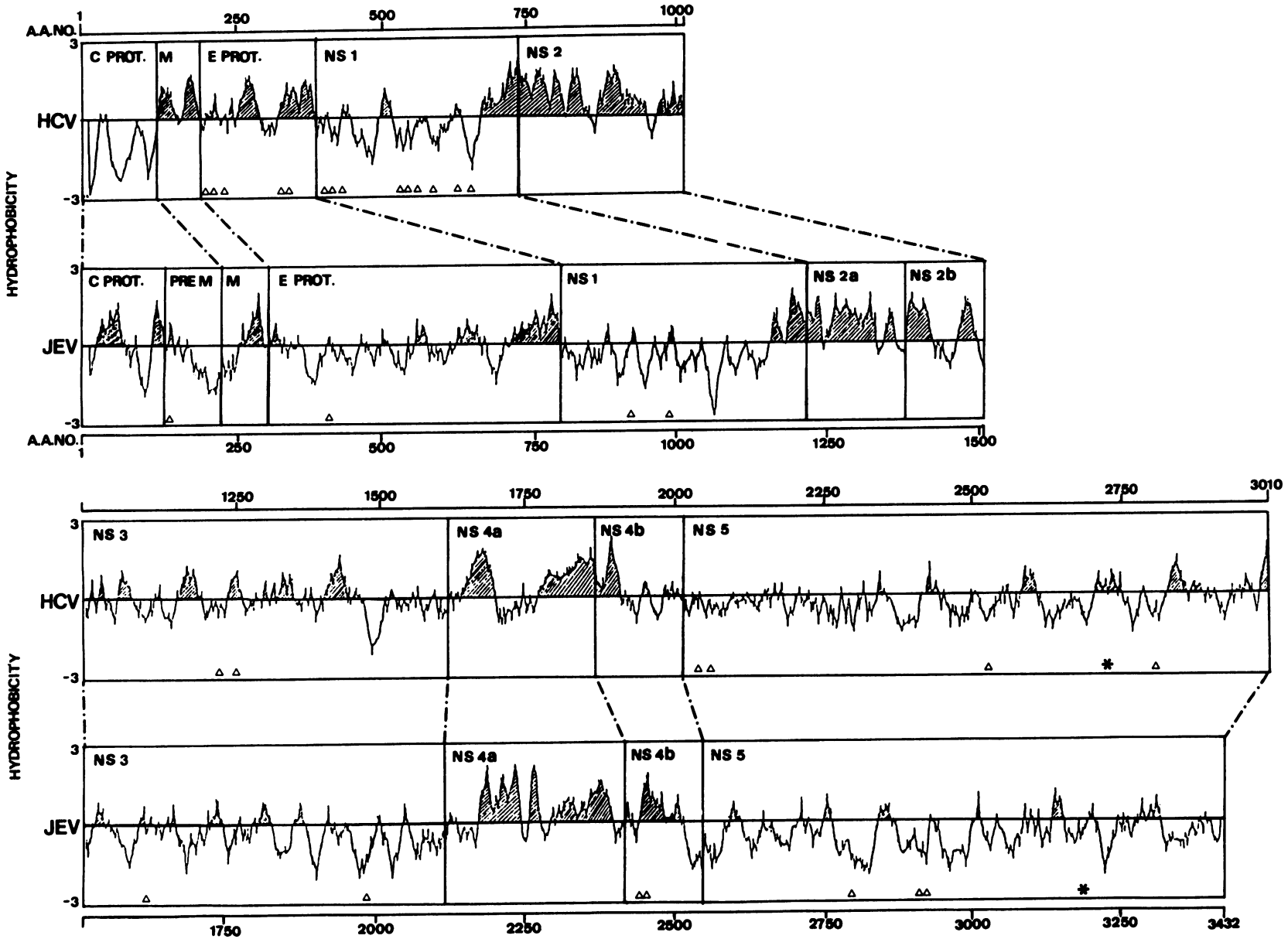

FIG. 4. Hydrophobicity plot of putative polyprotein. The average hydrophobicity index with a search length of 20 amino acids was obtained with the program of Kyte and Doolittle (14). The putative cleavage sites of predicted structural and nonstructural proteins are indicated by vertical lines. Hydrophilic regions are indicated by open peaks, and hydrophobic regions are shown by hatched peaks. Open triangles and asterisks indicate potential glycosylation sites and the sequence specific to the RNA replicase of RNA viruses shown in Fig. 2, respectively. The region of amino acid sequence homology between HCV and JEV is marked with a dotted line.

Clone	BK157	BK160	BK146	BK106	BK152	BK138	BK102	BK147	BK112	BK109	BK110	BK116	BK166
BK157													
BK160	92.7% (1553)												
BK146	99.7% (1923)	92.5% (1552)											
BK106	99.7% (1148)	91.8% (1143)	99.5% (1148)										
BK152	86.5% (920)	97.3% (716)	88.9% (3446)	89.1% (622)									
BK138	99.0% (208)		97.8% (3078)		89.7% (2353)								
BK102			100.0% (849)		90.1% (487)	98.6% (677)							
BK147			100.0% (392)			98.5% (677)	99.5% (1269)						
BK112							99.3% (415)	99.6% (1394)					
BK109							98.5% (334)	98.3% (1313)	98.2% (1770)				
BK110								99.4% (494)	99.6% (1791)	98.2% (958)			
BK116									99.4% (1016)		100.0% (82)		
BK166									98.6% (846)			98.8% (1314)	

FIG. 5. Nucleic acid identity among the HCV cDNA clones. cDNA clones were compared pairwise for nucleic acid sequence identity. The numbers shown are percentage of identical nucleic acids between the sequences. The numbers in parentheses are the number of nucleic acids compared.

sequence identity at the amino acid as well as the nucleic acid level was greater than 97%, while the sequence identity between groups was 87 to 93% (data for amino acid sequence comparison not shown). This suggests that these cDNA clones might have been derived from two separate carriers.

Our clones have 92% identity at both the nucleic acid and amino acid levels to the HCV sequences (0.6, 0.26, and 1.86 kb) independently isolated by others (12, 16, 20), while they have 77 and 81% nucleic acid and 86 and 82% amino acid identities, to Chiron's HCV (7.3 kb) and Okamoto's HC-J1 (1.86 kb) sequences, respectively (10, 20) (Table 2). Sequence variations among the different isolates indicate that HCV tends to change its sequence rather than having numerous subtypes. Nevertheless, production of defective viruses appears to be infrequent. Among the 13 overlapping cDNA clones sequenced (Fig. 5), only BK112 had a one-base deletion in the coding region. The rest of the clones did not have any base changes that caused an interruption of their coding capacity. Whether the sequence variations are merely a product of an unreliable RNA replicase or a reflection of the virus's ability to fight the host defense system or adapt itself to diverse environments must await further investigation.

The Chiron group has suggested that HCV is a flavivirus. The similarity in the hydrophobicity profiles of the polyproteins of our HCV and JEV appears to support their claim. However, the sequence homology comparison indicates that HCV is distantly related to flaviviruses and equally distantly related to pestiviruses and some plant viruses, as Miller and Purcell reported (18). As shown in Table 2, there was no

TABLE 2. Homology among HCV clones

Viruses compared[a]	Nucleic acid homology (%)		Amino acid homology[b] (%)								
	Total	5′ non-coding	C	M	E	NS1	NS2	NS3	NS4	NS5	Total
BK and Chiron	77.1	—	—	—	—	83.5	76.3	91.8	86.9	84.3	85.6
BK and HC-J4	92.5	99.7	97.4	97.4	94.0	—	—	—	—	—	94.9
BK and HC-J1	81.4	98.8	96.5	98.7	76.6	—	—	—	—	—	86.4
BK and HCV-J	91.2	—	—	—	—	—	—	97.6	—	—	97.6
BK and JEV	<30	<30	<10	<10	<10	<10	<10	20.3	<10	17.7	<10
JEV and WNV	71.0	61.5	63.3	80.0	78.2	76.2	58.4	80.0	70.0	81.8	76.2
YFV and JEV	59.2	33.7	26.0	39.0	43.7	44.0	32.5	49.8	36.3	60.0	46.1
BK and BVDV	<30	<30	*	*	*	*	*	23.2	*	18.6	*
BK and HOG	<30	<30	*	*	*	*	*	23.6	*	17.3	*
BK and TVM	<30	<30	*	*	*	*	*	18.9	*	<10	*

[a] BK, Our HCV; HCJ1 and HCJJ4, HCV clones (20); HCV-J, HCV clone (12); WNV, West Nile virus (4); BVDV, bovine viral diarrhea virus (6); HOG, hog cholera virus (17); TVM, tobacco vein mottling virus (7).

[b] —, Sequence not available; *, <10%, but unable to compare each protein because of structural divergence.

significant amino acid homology between the structural proteins or the nonstructural proteins NS1, NS2, and NS4 of our HCV strain and JEV. A detectable homology was found only in NS3 and NS5. This is in sharp contrast to the existence of significant amino acid homology throughout the polyprotein among flaviviruses.

Judging from the amino acid homology in the NS3 and NS5 regions among HCV, JEV, and hog cholera virus, HCV appears to be equally distant from flaviviruses and pestiviruses. These results imply that HCV should be classified as a member of a new virus family that recently diverged from flaviviruses. The sequence information and the cloned cDNAs presented here should greatly facilitate virological studies of HCV as well as the development of diagnostic tools and vaccines to prevent most cases of posttransfusion non-A, non-B hepatitis and HCV-associated cirrhosis and liver cancer.

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