

CORE ANTIGEN PROTEIN OF HEPATITIS C VIRUS,
AND DIAGNOSTIC METHOD AND KIT USING THE SAME

The invention relates to a DNA molecule, a polypeptide expressed by the molecule and their use in diagnosis and their methods of production. More particularly, it relates to a diagnostic DNA molecule, a diagnostic protein, diagnostic antibodies and a protective polypeptide for hepatitis C virus.

Most cases of hepatitis arising from blood transfusion are viral-induced, and distinguishable from other forms of viral-associated liver diseases caused by known hepatitis viruses such as hepatitis A virus (HAV) and hepatitis B virus (HBV). The etiological agent(s) of said Non-A, Non-B hepatitis (NANBH) has long been sought by many research groups and is presently believed to be the hepatitis C virus (HCV). Post-transfusion hepatitis (PTH) occurs in approximately 10% of transfusion patients, and NANBH accounts for up to 90% of these cases. The major problem in this disease is the frequent progression to chronic liver damage (25-55%). Therefore, the demand for sensitive, specific methods for detecting HCV in contaminated blood or blood products is significant.

The hepatitis C virus (HCV) was first identified by molecular cloning and characterization of its RNA genome by Choo, et al. (Science 244: 359-362, 1989). A specific assay by using said HCV antigen, designated C100-3, synthesized by the recombinant DNA method in yeast, was also developed for detection of the antibody of HCV (Science 244: 362-364). A detailed description regarding the genome of HCV, the cDNA sequences derived therefrom and

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DATE OF RECEIPT

polypeptides derived from said HCV genome or from HCV cDNA as well as methodologies, was given in EP 0 318 216 A1 in the name of Chiron Corporation. In particular, the European patent provides a synthesized polypeptide, C100-3 containing 363 viral amino acids which can be used for the detection of HCV antibodies. Now, kits for detecting HCV antibodies on the basis of C100-3 have been commercialized by Abbott Laboratories.

As suggested in the above European patent, HCV may be a flavivirus or flavi-like virus. Generally, with respect to morphology, flavivirus contains a central nucleocapsid surrounded by a lipid bilayer. It is believed that hepatitis C virus protein is composed of structural proteins including a nucleocapsid (core) protein (C) and two glycosylated envelope proteins (E1, E2) and nonstructural proteins (NS1-5). However, the corresponding virus has not yet been isolated nor characterized. It is only confirmed that said C100-3 disclosed by Choo et al. is a protein encoded by part of the nonstructural regions 3-4 of the HCV genome.

Besides, an enzyme-linked immunosorbent assay (ELISA) was developed for serological diagnosis of hepatitis C virus (HCV) infection, by using said HCV core protein (p22) synthesized by a recombinant baculovirus by Chiba, et al. (Proc. Natl. Acad. Sci. USA 88:4641-4645, 1991). It was found that C100-3 antibody was not detected in all post-transfusion NANBH cases, probably because of the delayed response to C100-3 antigen. Such delayed response was considered to be due to the fact that the C100-3 is an HCV nonstructural protein. However, Chiba, et al. used an unglycosylated 22-kDa nucleocapsid (core) protein, and established an antibody detection system to develop a specific sensitive method for diagnosing HCV infection.

The invention discloses a DNA molecule coding for an HCV core antigen protein and provides an alternative of HCV

antibody assay.

SUMMARY OF THE INVENTION

5 Accordingly, it is an object of the present invention to provide a DNA molecule coding for a polypeptide displaying the antigenicity of a hepatitis C virus (HCV) core antigen protein. More specifically, the invention provides a DNA molecule derived from the genome of HCV.
10 The DNA molecule was cloned from the plasma of a patient with hepatitis C via a Polymerase Chain Reaction (PCR) technique.

 Portions of the DNA molecule are useful as probes to
15 diagnose the presence of virus in samples. As well, the DNA molecule of the invention is capable of producing, in an appropriate host, viral polypeptides displaying the antigenicity of an HCV core antigen protein.

20 It is another object of the present invention to provide a polypeptide displaying the antigenicity of an HCV core antigen protein. Said polypeptide was produced by an appropriate host transformed with the DNA molecule of the invention.

25 Also, it is an object of the present invention to provide a process for producing a polypeptide displaying the antigenicity of an HCV core antigen protein. The process comprises incubating host cells transformed with an
30 expression vector containing a DNA molecule of the invention.

 Still, it is another object of the present invention to provide a method for detecting HCV antibodies in samples
35 by using said HCV core antigen protein as a probe. The method may be carried out by immunoassay or Western blotting which is characterized by using said HCV core antigen as a probe, capable of binding to antibodies

directed against HCV in samples, to form an antigen-antibody complex.

5 The invention also includes a method for detecting HCV antibodies in samples by using said HCV core antigen protein combined with an HCV nonstructural protein as probes.

10 It is yet object of the present invention to provide a kit for analyzing samples for the presence of HCV antibodies in samples, comprising said HCV core-antigen protein. Specifically, a kit for immunoassay for detecting HCV antibodies comprises said HCV core antigen protein and a suitable solid phase.

15 It is another further object of the present invention to provide monoclonal and polyclonal antibodies directed against said HCV core antigen protein. Also included in the invention is the process for producing said antibodies by immunizing an animal with said HCV core antigen protein. A method for analyzing HCV in samples can be established by using the obtaining antibodies.

25 It is yet further object of the present invention to provide vaccines for prevention of HCV infection comprising an immunogenic polypeptide of the invention, or an inactivated preparation or an attenuated preparation thereof.

30 These and other objects, advantages and features of the present invention will be more fully understood and appreciated by reference to the written specification.

BRIEF DESCRIPTION OF THE DRAWINGS

35 Fig. 1A shows the DNA sequence derived from the genome of HCV nucleocapsid region (SEQ ID No: 3) and part of the envelope region in accordance with the invention.

The amino acid sequence of the polypeptide encoded therein is shown in Fig. 1B (SEQ ID No: 4).

Fig. 2 shows the structure of the expression vector pEN-2 constructed by inserting the cDNA coding for an HCV core antigen into a plasmid. The figure also shows the restriction map illustrating the significant features of the vector pEN-2.

10 DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein. The DNA molecule is isolated from nucleic acid sequences present in the plasma of an HCV infected patient which includes the steps of isolating the viral particles from the patient's plasma, extracting and purifying the viral nucleic acid sequences, and then cloning the desired DNA molecule via a Polymerase Chain Reaction (PCR) technique. The primers used for cloning are

- (i) 5'-GGATCCATGAGCACAAATCCTAAACCT-3' (SEQ ID No: 1) and
- (ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3' (SEQ ID No: 2).

The cloned DNA molecule is subject to a hybridization with original HCV particles for identification. The molecule thus obtained is designated EN-80-2. The DNA sequence of the molecule EN-80-2 is given in Fig. 1A (SEQ ID No: 3). The DNA molecule is derived from the genome of HCV nucleocapsid and envelope regions and has 669bp.

Portions of the DNA molecule are useful as probes to diagnose the presence of HCV nucleic acids in samples.

The DNA molecule of the present invention is inserted into an appropriate vector to form an expression vector.

The vector can be a plasmid, bacteriophage or other DNA sequence which is able to replicate in a host cell. The preferable vector is a plasmid with the promoter lac. Referring to Fig. 2, we have shown an expression plasmid, pEN-2, constructed by a vehicle plasmid and the DNA molecule of the present invention.

An appropriate host is transformed with an expression vector containing the DNA molecule of the invention and the polypeptide displaying the antigenicity of an HCV core antigen protein can be produced in the transformed host. A process for producing the polypeptide displaying the antigenicity of an HCV core antigen has been established.

The invention also provides a polypeptide displaying the antigenicity of an HCV core antigen protein. The amino acid sequence of the polypeptide is given in Fig. 1B (SEQ ID No: 4). The polypeptide has a molecular weight of about 25,000 as measured by electrophoresis through a sodium dodecyl sulfate-polyacrylamide gel and is deduced to have about 220 amino acids. The ability of the obtained polypeptide to bind to HCV antibodies is confirmed by Western Blotting. Obviously, the polypeptide is reactive with the sera of the patients with hepatitis C but not reactive with the sera of persons without hepatitis C. This fact suggests that the polypeptide capable of detecting the presence of HCV antibodies in samples holds a potential in diagnosis of hepatitis C. Besides, the polypeptides displaying the antigenicity of an HCV core antigen, or an inactivated preparation or an attenuated preparation thereof in an immunogenically effective amount can be formulated as vaccines for prevention of HCV infection.

The method for detecting HCV antibodies in samples is included in the invention. The method is characterized by the HCV core antigen's capability of binding to HCV antibodies as a probe. The method can be carried out by

immunoassay or Western Blotting. A preferred method is solid-phase immunoassay. The solid phase such as microtiter plates, beads and semipermeable membranes can be used to carry out the immunoassay. The substances as
5 labels include enzymes, isotopes, fluorescent materials and any other materials which can be directly detected. The immunoassay is conveniently achieved by a Sandwich method. Particularly, an enzyme-linked immunosorbent assay (ELISA) was established. The ELISA comprises: 1) coating the
10 polypeptide of the present invention onto a solid phase, 2) incubating a sample suspected of containing HCV antibodies with the polypeptide coated onto said solid phase under conditions which allow the formation of an antigen-antibody complex, 3) adding an anti-antibody (such as anti-IgG)
15 conjugated with a label to be captured by the resulting antigen-antibody complex bound to the solid phase, and 4) measuring the captured label and determining whether the sample has HCV antibodies.

20 An alternative assay can be carried out by using the polypeptide of the invention combined with an HCV nonstructural protein as a probe. The assay is thought to be more specific and sensitive.

25 The invention still provides a kit for analyzing samples for the presence of HCV antibodies. The kit
- - comprises a polypeptide of the invention and an appropriate solid phase wherein preferably, said polypeptide is coated to said phase.

30 The present invention provides monoclonal and polyclonal antibodies directed against said HCV core antigen protein. The antibodies are produced by using the polypeptide of the invention as an immunogen through
35 standard procedures for preparing a hybridoma and/or conventional methods. The obtained antibodies are potential to develop a method for detecting HCV.

The following example is offered to aid in understanding of the present invention and are not to be construed as limiting the scope thereof.

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EXAMPLES

I. Cloning of an HCV cDNA

10 The plasma of the patients infected with Hepatitis C virus are collected and ultracentrifuged at 4°C and then the viral particles are obtained. Subsequently, the viral nucleic acid (RNA) is extracted and purified by using guanidine isothiocyanate and acidic phenol from the viral particles.

15

The following oligonucleotide sequences :

- (i) 5'-GGATCCATGAGCACAAATCCTAACCT-3' (SEQ ID No: 1) and
20 (ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3 (SEQ ID No: 2);

were used as primers in the subsequent cloning steps. A single-stranded antisense DNA molecule was produced using
25 primer (ii) and reverse transcriptase and then the RNA template was replaced with the corresponding DNA molecule (using primer (i)) to produce a double stranded DNA molecule. The double stranded DNA molecule was amplified by the PCR method using Taq polymerase.

30

The cloned DNA molecule is subject to a hybridization with original HCV particle for identification. The obtained molecule is designated EN-80-2. The DNA sequence of the molecule EN-80-2 is given in Fig. 1A (SEQ ID No: 3).
35 The DNA molecule is derived from the genome of HCV nucleocapsid and envelope regions and has 669bp.

II. Construction of a Plasmid Containing an HCV cDNA

The molecule EN-80-2 is treated with restriction endonucleases Bam HI and EcoRI to obtain a transposon
5 containing said HCV cDNA. The obtained transposon is inserted into a vehicle plasmid which is first cleaved with restriction endonucleases Bam HI and EcoRI to obtain an expression plasmid, designated pEN-2. The expression of the HCV cDNA is under the control of a promoter lac. The
10 structure of the expression plasmid pEN-2 and restriction map are given in Fig. 2.

III. Transformation of E. coli

15 The expression plasmid pEN-2 is incubated with E. coli BL21 (DE3) at 37°C overnight. The E. coli colonies producing HCV core antigen protein are selected by screening their expression products by Western Blotting. The selected clone, designated Clone EN-80-2, is
20 exemplified by a culture deposited in American Type Culture Collection, MD, USA, on 14 July 1993 (Access Number ATCC 55451).

IV. Production of HCV Core Antigen

25

The transformed E. coli colonies are incubated in a conditioned culture medium. The colonies are centrifuged and lysed by freezing-defrosting cycles and enzyme-digestion. The protein product is released by the lysed
30 cells and purified by chromatography. The purity of the obtained polypeptide is more than 90%.

The polypeptide has a molecular weight of about 25,000 as measured by electrophoresis through a sodium
35 dodecyl sulfate-polyacrylamide gel.

V. Immunological Reactivity of HCV Core Antigen with HCV Antibodies by Western Blotting

The purified polypeptide is subject to an SDS electrophoresis by standard procedures. The SDS-PAGE gel is washed with deionized water at 4°C for 15 minutes and washed with Blotting Buffer (0.15M sodium phosphate buffer, pH 6.7) at 4°C for 20 minutes. The peptide map on the gel is then electroblotted onto a nitrocellulose paper under the Blotting Buffer at 1.3A for 1-1.5 hours. The paper is washed with Wash Buffer (PBS-Tween 20, pH 7.4) and blocked with Blocking Buffer (0.1M NaCl, 5mM EDTA, 50mM Tris, pH 7.2-7.4, 0.2% fetal bovine serum albumin, 0.05% Nonidet p-40, 1M urea) overnight.

The paper is reacted with the sera of the persons infected with/without hepatitis C which are first diluted with 40% NBBS/Tris-HCl (pH 7.4), 10X, at 40°C for 2 hours. After the reaction, the paper is washed with Wash Buffer three times. The paper is reacted with the anti-hIgG:HRPO conjugate (which is prepared as described hereafter) at 40°C for 2 hours. After the reaction, the paper is washed with Wash Buffer three times and then reacted with 10 ml Substrate Solution (0.01% 4-chloro-1-naphthol, 18% methanol, 0.04M Tris, pH 7.2-7.4, 0.1M NaCl and 0.01% H₂O₂) for 20 minutes. As shown in the Western blot, the polypeptide of the present invention is reactive with the sera of HCV patients but not reactive with the sera of healthy persons.

VI. ELISA for HCV Antibodies

(1) Treatment of Microtiter Plate

The microtiter plate is coated with the purified polypeptide of the invention at appropriate concentrations and blocked with a buffer containing bovine serum albumin. The treated microtiter plate is stored at 2-8°C.

(2) Preparation of Anti-hIgG:HRPO Conjugate

The purified anti-human Immunoglobulin G (anti-hIgG) is conjugated with horse radish peroxidase (HRPO) using NaIO₄ to obtain the anti-IgG:HRPO conjugate. The conjugate is purified by chromatography.

(3) Components of Reagents

- 10 (a) Wash Solution: Phosphate Buffer containing 0.9% NaCl and Thimerosal.
- (b) Anti-hIgG:HRPO Conjugate Solution: the anti-hIgG:HRPO conjugate prepared as described above
15 dissolved in Tris Buffer containing a proteineous stabilizer and antiseptics.
- (c) Sample Diluent: Tris Buffer containing a
20 proteineous stabilizer and antiseptics.
- (d) OPD Substrate Solution: o-phenylene diamine (OPD) dissolved in citrate-phosphate buffer containing H₂O₂. (If the solution becomes
25 orange, it means that the solution has been contaminated and cannot be used any more.)
- (e) Stopping Solution: 2N H₂SO₄ solution.
- (f) Positive/Negative Controls: the serum samples
30 of persons infected with/without hepatitis C diluted with phosphate buffer containing a
 proteineous stabilizer and antiseptics at an appropriate concentration.

35 (4) Procedure:

- (a) One hundred and fifty microliter (μl) of test samples diluted with Sample Diluent (1:10) and

Positive/Negative Controls are added into the wells of the treated microtiter plate. Some wells have to be retained as substrate blanks.

- 5 (b) The plate is gently mixed by shaking and incubated at 37-40°C for 1 hour.
- (c) The plate is washed with 0.3 ml of Wash Solution per well by a washer three times.
- 10 (d) One hundred μ l of anti-hIgG:HRPO Conjugate Solution is added to each well.
- (e) The plate is gently mixed by shaking and incubated at 37-40°C for 30 minutes.
- 15 (f) The plate is washed five times.
- (g) One hundred μ l of OPD Substrate Solution is added into each well and the plate is incubated at 15-30°C in the dark for 30 minutes.
- 20 (h) One hundred μ l of Stopping Solution is added into each well and gently mixed to stop the reaction.
- 25 (i) The OD value per well is measured by a spectrophotometer at 492 nm.

30 (5) Determination:

The OD_{492nm} value per well subtracts the mean of the readings of the blanks (backgrounds). The difference (PCx-NCx) between the mean of the readings of the positive controls (PCx) and that of the negative controls (NCx) is equal to or more than 0.5.

The Cut-off value (CO) is calculated by the

following formula:

$$CO = PCx \times 0.15 + NCx$$

5 When the readings of test samples are less than the CO value, the samples are considered negative (i.e. HCV antibodies cannot be detected in the samples).

10 When the readings of test samples are equal to or more than the CO value, the samples are expected to be positive; however, it is necessary to repeat the assay for
- the samples in duplicate. If the reading of either of the duplicate samples is less than the CO value, the samples are negative. If the duplicate samples are both more than
15 or equal to the Cut-off value, the samples will be positive.

 When the readings of test samples are more than NCx but less than the CO value by 20%, the samples should be
20 regarded as questionable samples and the assay has to be repeated for those samples.

 Twenty-seven samples were tested by the ELISA according to the invention. At the same time, the samples
-25 were also tested by the HCV antibody assay by using
- - structural and nonstructural proteins as probes (i.e. Abbott's kit (II)). The comparison between the test results of Abbott's kit and those of the assay of the invention is given in Table I. It is noted that the
30 results of Sample G 229 was negative by the Abbott's kit (II) but positive by the assay of the present invention. It is suggested that samples infected with HCV cannot be all detected by the Abbott's assay.

Table I

	Sample No.	OD _{492nm}	Results	References Abbott's kit (II)
5				
	TSGH 56	> 2.0	positive	positive
	TSGH 57	> 2.0	positive	positive
	G 23	1.469	positive	positive
10	G 30	> 2.0	positive	positive
	G 32	> 2.0	positive	positive
	G 49	> 2.0	positive	positive
	G 56	> 2.0	positive	positive
	G 58	> 2.0	positive	positive
15	G 114	1.559	positive	positive
	G 128	> 2.0	positive	positive
	G 186	> 2.0	positive	positive
	G 208	> 2.0	positive	positive
	G 214	> 2.0	positive	positive
20	G 231	> 2.0	positive	positive
	G 250	> 2.0	positive	positive
	Y 1	> 2.0	positive	positive
	USB 9	> 2.0	positive	positive
	USB 19	> 2.0	positive	positive
25	USB 20	> 2.0	positive	positive
	USB 23	0.952	positive	positive
	USB 27	0.753	positive	positive
	G 11	0.147	negative	negative
	G 12	0.077	negative	negative
30	G 13	0.061	negative	negative
	G 14	0.116	negative	negative
	G 15	0.139	negative	negative
	G 229	0.517	positive	negative
35				

VII. Detection of HCV Antibodies by HCV Core Antigen Protein Combined with HCV Nonstructural Protein

40 The method is analogous to the ELISA as described above while HCV core antigen protein of the invention combined with an HCV nonstructural protein (9:1) is replaced for the HCV core antigen protein to be coated onto the plate.

45

Twenty-four samples were tested by the above-mentioned method. At the same time, the samples were also tested by Abbott's kit (II). The results are given in Table II. It is suggested that the results of the Abbott's

assay is the same of the above-mentioned method.

Table II

5	Sample No.	OD _{492nm}	Results	References Abbott's kit (II)
10	TSGH 56	> 2.0	positive	positive
	TSGH 57	> 2.0	positive	positive
	G 23	> 2.0	positive	positive
	G 26	> 2.0	positive	positive
	G 30	> 2.0	positive	positive
	G 32	> 2.0	positive	positive
15	G 49	> 2.0	positive	positive
-	G 56	> 2.0	positive	positive
	G 58	> 2.0	positive	positive
	G 114	> 2.0	positive	positive
	G 128	> 2.0	positive	positive
20	G 186	> 2.0	positive	positive
	G 214	> 2.0	positive	positive
	G 231	> 2.0	positive	positive
	G 250	> 2.0	positive	positive
	Y 1	> 2.0	positive	positive
25	USB 9	> 2.0	positive	positive
	USB 19	> 2.0	positive	positive
	USB 20	> 2.0	positive	positive
	USB 23	> 2.0	positive	positive
	USB 27	> 2.0	positive	positive
30	G 92	0.038	negative	negative
	G 93	0.056	negative	negative
	G 94	0.071	negative	negative

35

VIII. Preparation of Antibodies against HCV

The antibodies against HCV core antigen protein can be produced by immunizing an animal with the core antigen protein according to the invention.

According to the standard procedure for producing monoclonal antibodies, a BAL-B/C mouse is immunized with the purified core antigen protein as described in Example II mixed with an adjuvant; and then the spleen cells are fused with mouse myeloma cells (FO cell line) using polyethylene glycol to form hybridoma cells. The desired clone producing monoclonal antibodies can be obtained by screening the titer of the antibodies produced by the

hybridoma clones so prepared.

5 A diagnostic kit for detecting the presence of HCV in
a sample, or a kit for determining if the HCV cells are
successfully cultured can be developed by using the
obtained antibodies associated with EIA and sandwich
methods.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: EVERNEW BIOTECH INC.
- (B) STREET: 15F, 320, SEC. 4, CHUNG-HSIAO E. ROAD
- (C) CITY: TAIPEI
- (E) COUNTRY: TAIWAN, R.O.C.
- (F) POSTAL CODE (ZIP): NONE

10

- (ii) TITLE OF INVENTION: CORE ANTIGEN PROTEIN OF HEPATITIS C VIRUS,
AND DIAGNOSTIC METHOD AND KIT USING THE SAME

15

- (iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

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(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 07/963483
- (B) FILING DATE: 16-OCT-1992

25

(2) INFORMATION FOR SEQ ID NO: 1:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

40

- (iii) ANTI-SENSE: NO

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

45

GGATCCATGA GCACAAATCC TAAACCT

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATTCGGTG TGCATGATCA TGTCCGC

27

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 669 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGAGCACAA ATCCTAAACC TCAAAGAAAA ACCAAACGTA ACACCAACCG CCGCCCACAG 60

GACGTCAAGT TCCCGGGCGG TGGTCAGATC GTTGGTGGAG TTTACCTGTT GCCGCGCAGG 120

GGCCCCAGGT TGGGTGTGCG CGCGACTAGG AAGACTTCCG AGCGGTCGCA ACCTCGTGGA 180

AGGCGACAAC CTATCCCCAA GGCTCGCCGG CCCGAGGGCA GGACCTGGGC TCAGCCGGGG 240

TACCCTTGGC CCCTCTATGG CAATGAGGGT CTGGGGTGGG CAGGATGGCT CCTGTCACCC 300
 CGAGGCTCTC GGCCTAGTTG GGGCCCCACG GACCCCCGGC GTAGGTCGCG TAATCTGGGT 360
 5 AAGGTCATCG ATACCCTCAC AGGTGGCTTC GCCGACCTCA TGGGGTACAT TCCGCTCGTC 420
 AGCGCCCCAC TAGGAGGCGC TGCCAGGGCC CTGGGCCATG GCGTCCGGGT TCTGGAGGAC 480
 GGC GTGA ACT ATGCAACAGG GAATCTGCCC GGTGCTCTT TCTCTATCTT CCTCTTAGCT 540
 10 TTGCTGTCTT GTTTGACCAT CCCAGCTTCC GCTTACGAGG TGCGCAACGT GTCCGGGATA 600
 TACCATGTTA CGAACGATTG CTCCA ACTCA AGTATCGTGT ATGAGGCAGC GGACATGATC 660
 15 ATGCACACC 669

(2) INFORMATION FOR SEQ ID NO: 4:

20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 223 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

30 (v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

35 Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn
 1 5 10 15
 Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly
 40 20 25 30
 Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala
 35 40 45
 45 Thr Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro
 50 55 60

	Ile	Pro	Lys	Ala	Arg	Arg	Pro	Glu	Gly	Arg	Thr	Trp	Ala	Gln	Pro	Gly	
	65						70				75					80	
5	Tyr	Pro	Trp	Pro	Leu	Tyr	Gly	Asn	Glu	Gly	Leu	Gly	Trp	Ala	Gly	Trp	
					85					90					95		
	Leu	Leu	Ser	Pro	Arg	Gly	Ser	Arg	Pro	Ser	Trp	Gly	Pro	Thr	Asp	Pro	
				100					105					110			
10	Arg	Arg	Arg	Ser	Arg	Asn	Leu	Gly	Lys	Val	Ile	Asp	Thr	Leu	Thr	Gly	
				115				120					125				
	Gly	Phe	Ala	Asp	Leu	Met	Gly	Tyr	Ile	Pro	Leu	Val	Ser	Ala	Pro	Leu	
15				130			135					140					
	Gly	Gly	Ala	Ala	Arg	Ala	Leu	Gly	His	Gly	Val	Arg	Val	Leu	Glu	Asp	
	145					150					155				160		
	Gly	Val	Asn	Tyr	Ala	Thr	Gly	Asn	Leu	Pro	Gly	Cys	Ser	Phe	Ser	Ile	
20					165					170					175		
	Phe	Leu	Leu	Ala	Leu	Leu	Ser	Cys	Leu	Thr	Ile	Pro	Ala	Ser	Ala	Tyr	
				180					185					190			
25	Glu	Val	Arg	Asn	Val	Ser	Gly	Ile	Tyr	His	Val	Thr	Asn	Asp	Cys	Ser	
				195				200					205				
	Asn	Ser	Ser	Ile	Val	Tyr	Glu	Ala	Ala	Asp	Met	Ile	Met	His	Thr		
30				210				215				220					



CLAIMS

1. A DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein which is derived from the genome of nucleocapsid and part of envelope regions of an HCV, and degenerate sequences thereof.
2. A DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein comprising the DNA sequence as given in Fig. 1A (SEQ.ID NO:3), and degenerate sequences thereof.
3. A DNA molecule as claimed in claim 1 or claim 2 coding for an antigenically significant subfragment or variant of a polypeptide as defined in claim 1 or claim 2.
4. A DNA molecule according to any one of claims 1-3 which is cloned from the plasma of a patient infected with HCV.
5. Probes for Hepatitis C virus (HCV) nucleic acid which are derived from the DNA molecule according to any one of claims 1 to 4.
6. A DNA molecule according to any one of claims 1-3 capable of expressing a polypeptide displaying the antigenicity of an HCV core antigen protein in an appropriate host.
7. A process for cloning a DNA molecule according to any one of claims 1-3 which comprises isolating RNAs from hepatitis C patients' sera, and cloning said DNA molecule via a Polymerase Chain Reaction (PCR) technique.
8. A process according to claim 7 wherein the following sequences are used as primers:

(i) 5'-GGATCCATGAGCACAAATCCTAAACCT-3' (SEQ.ID NO:1) and





(ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3' (SEQ. ID NO:2).

9. A polypeptide displaying the antigenicity of an HCV core antigen protein, which is encoded by the genome of nucleocapsid and part of envelope regions of hepatitis C virus, and derivatives thereof.

10. A polypeptide displaying the antigenicity of an HCV core antigen having the amino acid sequence as given in Fig. 1B (SEQ.ID NO:4), and derivatives thereof.

11. A polypeptide which comprises an antigenically significant subfragment or variant of a polypeptide as claimed in claim 9 or 10.

12. A polypeptide according to claim 9 or 10 having a molecular weight of about 25,000.

13. A polypeptide according to any one of claims 9-11 produced by an appropriate host transformed with the DNA molecule as defined in any one of claims 1-3.

14. A process for producing a polypeptide displaying the antigenicity of an HCV core antigen protein by the expression of a DNA molecule as defined in any one of Claims 1-3.

15. A plasmid for expression of a polypeptide displaying the antigenicity of an HCV core antigen protein, designated pEN-2.

16. E. Coli transformed to produce a polypeptide displaying the antigenicity of an HCV core antigen protein, designated Clone EN-80-2 and deposited under Accession Number ATCC 55451.

17. A method for detecting HCV antibodies in samples, characterized by using a polypeptide as defined in any one of claims 9-11 as a probe, capable of binding to antibodies



directed against an HCV antigen in samples to form an antigen-antibody complex.

18. A method according to claim 17, characterized by
5 using a polypeptide as defined in any one of claims 9-11
combined with an HCV nonstructural protein as probes.

19. A kit for analyzing samples for the presence of HCV
antibodies in samples, comprising a polypeptide as defined
10 in any one of claims 9-11.

20. Antibodies or antigen binding fragments thereof
directed against an HCV core antigen protein which are
elicited by using a polypeptide as defined in any one of
15 claims 9-11 as an immunogen.

21. A process for producing antibodies directed against
HCV core antigen comprising immunizing an animal with a
polypeptide as defined in any one of claims 9-11.

22. A method for analyzing HCV in samples characterized
by using antibodies as defined in claim 20 as a probe.

23. Vaccines for prevention of HCV infection comprising
25 an immunogenic polypeptide as defined in any one of claims
9-11, or an inactivated preparation or an attenuated
preparation thereof.

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CORE ANTIGEN PROTEIN OF HEPATITIS C VIRUS, DIAGNOSTIC
METHOD AND KIT USING THE SAME

ABSTRACT

5

The invention relates to DNA molecules, polypeptides expressed by them and their use in diagnosis and their methods of production. More particularly, it relates to a diagnostic DNA molecule, a diagnostic protein, diagnostic
10 antibodies and protective antigen and antibodies for hepatitis C virus (HCV). The DNA molecule disclosed herein is characterized by the DNA molecule derived from the genome of an HCV, and coding for a polypeptide having the antigenicity of an HCV core antigen protein. The
15 polypeptide may be used in the detection of HCV.

Nucleotide Sequence

5'-ATGAGCACAAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGCCGCCACAG 60
GACGTCAAGTTCCCGGGCGGTGGTCAGATCGTTGGTGGAGTTTACCTGTTGCCGCGCAGG 120
GGCCCCAGGTTGGGTGTGCGCGCGACTAGGAAGACTTCCGAGCGGTGCAACCTCGTGGA 180
AGGCGACAACCTATCCCCAAGGCTCGCCGGCCCCGAGGGCAGGACCTGGGCTCAGCCGGGG 240
TACCCTTGGCCCCCTCTATGGCAATGAGGGTCTGGGGTGGGCAGGATGGCTCCTGTCACCC 300
CGAGGCTCTCGGCCTAGTTGGGGCCCCACGGACCCCCGGCGTAGGTCGCGTAATCTGGGT 360
AAGGTCATCGATACCCTCACAGGTGGCTTCGCCGACCTCATGGGGTACATTCCGCTCGTC 420
AGCGCCCCACTAGGAGGCGCTGCCAGGGCCCTGGGCCATGGCGTCCGGGTTCTGGAGGAC 480
GGCGTGAACCTATGCAACAGGGAATCTGCCCGGTTGCTCTTTCTCTATCTTCCTCTTAGCT 540
TTGCTGTCTTGTTTGACCATCCCAGCTTCCGCTTACGAGGTGCGCAACGTGTCCGGGATA 600
TACCATGTTACGAACGATTGCTCCAACCTCAAGTATCGTGTATGAGGCAGCGGACATGATC 660
ATGCACACC-3' 669

FIG. 1A

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Amino Acid Sequence

NH ₂ -MetSerThrAsnProLysProGlnArgLysThrLysArgAsnThr	15
AsnArgArgProGlnAspValLysPheProGlyGlyGlyGlnIle	30
ValGlyGlyValTyrLeuLeuProArgArgGlyProArgLeuGly	45
ValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly	60
ArgArgGlnProIleProLysAlaArgArgProGluGlyArgThr	75
TrpAlaGlnProGlyTyrProTrpProLeuTyrGlyAsnGluGly	90
LeuGlyTrpAlaGlyTrpLeuLeuSerProArgGlySerArgPro	105
SerTrpGlyProThrAspProArgArgArgSerArgAsnLeuGly	120
LysValIleAspThrLeuThrGlyGlyPheAlaAspLeuMetGly	135
TyrIleProLeuValSerAlaProLeuGlyGlyAlaAlaArgAla	150
LeuGlyHisGlyValArgValLeuGluAspGlyValAsnTyrAla	165
ThrGlyAsnLeuProGlyCysSerPheSerIlePheLeuLeuAla	180
LeuLeuSerCysLeuThrIleProAlaSerAlaTyrGluValArg	195
AsnValSerGlyIleTyrHisValThrAsnAspCysSerAsnSer	210
SerIleValTyrGluAlaAlaAspMetIleMetHisThr-COOH	223

FIG. 1B

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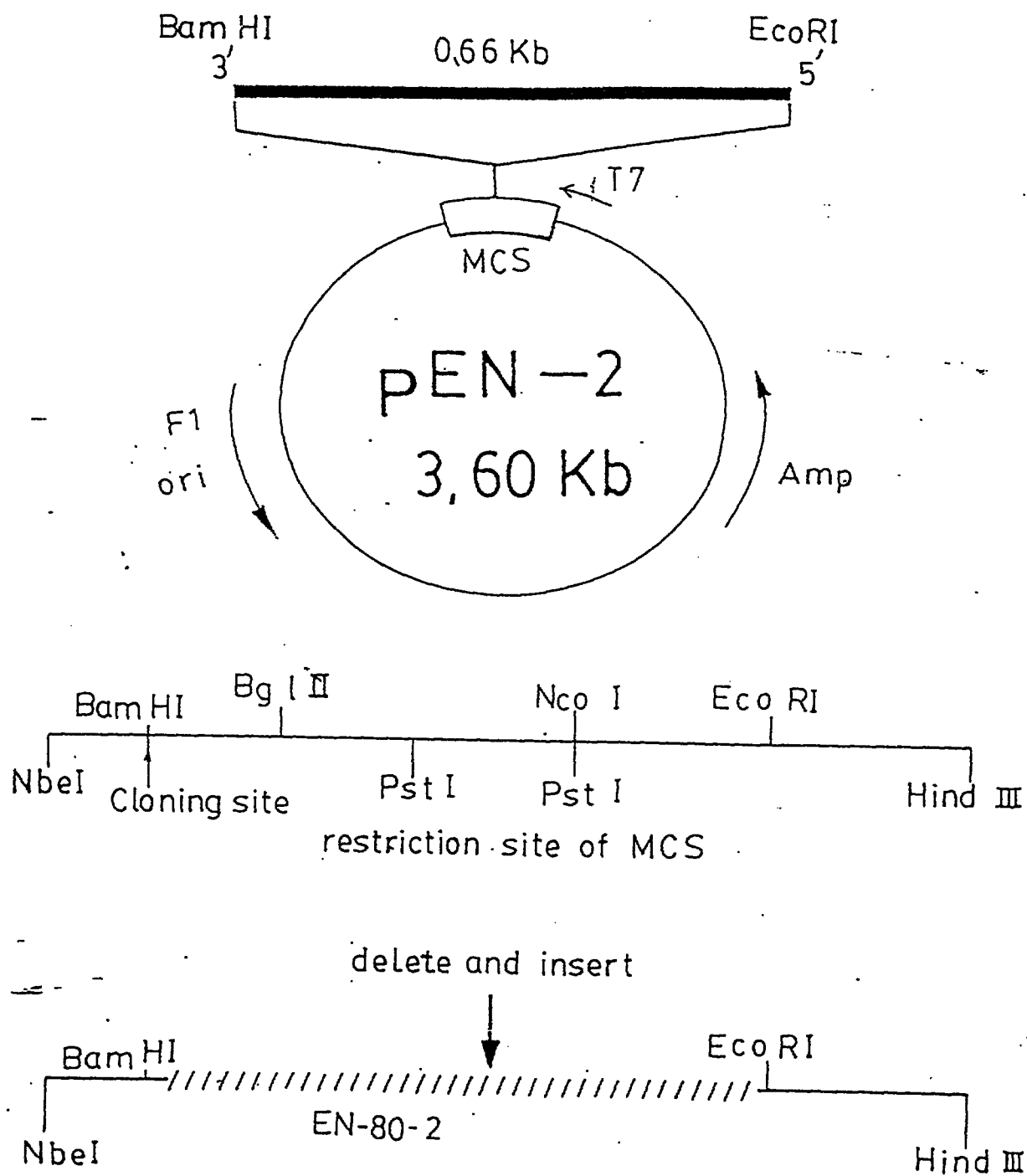


FIG. 2



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Nucleotide Sequence

5'-ATGAGCACAAATCCTAAACCTCAAAGAAAAACCAAACGTAACACCAACCGCCGCCACAG 60
 GACGTCAAGTTCCCGGGCGGTGGTCAGATCGTTGGTGGAGTTTACCTGTTGCCGCGCAGG 120
 GGCCCCAGGTTGGGTGTGCGCGCGACTAGGAAGACTTCCGAGCGGTCGCAACCTCGTGGA 180
 AGGCGACAACCTATCCCCAAGGCTCGCCGGCCCCGAGGGCAGGACCTGGGCTCAGCCGGGG 240
 TACCCTTGGCCCCCTCTATGGCAATGAGGGTCTGGGGTGGGCAGGATGGCTCCTGTCACCC 300
 CGAGGCTCTCGGCCTAGTTGGGGCCCCACGGACCCCCGGCGTAGGTCGCGTAATCTGGGT 360
 AAGGTCATCGATACCCTCACAGGTGGCTTCGCCGACCTCATGGGGTACATTCCGCTCGTC 420
 AGCGCCCCACTAGGAGGCGCTGCCAGGGCCCTGGGCCATGGCGTCCGGGTTCTGGAGGAC 480
 GGCGTGAACCTATGCAACAGGGAATCTGCCCGGTTGCTCTTTCTCTATCTTCCTCTTAGCT 540
 TTGCTGTCTTGTTTGACCATCCCAGCTTCCGCTTACGAGGTGCGCAACGTGTCCGGGATA 600
 TACCATGTTACGAACGATTGCTCCAACCTCAAGTATCGTGTATGAGGCAGCGGACATGATC 660
 ATGCACACC-3' 669

FIG. 1A

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☐	Claim(s) : paged	to paged
☒	Drawing(s) : paged 1/2	to paged 3/2
☐	Abstract : paged	to paged
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Amino Acid Sequence

NH ₂ -MetSerThrAsnProLysProGlnArgLysThrLysArgAsnThr	15
AsnArgArgProGlnAspValLysPheProGlyGlyGlyGlnIle	30
ValGlyGlyValTyrLeuLeuProArgArgGlyProArgLeuGly	45
ValArgAlaThrArgLysThrSerGluArgSerGlnProArgGly	60
ArgArgGlnProIleProLysAlaArgArgProGluGlyArgThr	75
TrpAlaGlnProGlyTyrProTrpProLeuTyrGlyAsnGluGly	90
LeuGlyTrpAlaGlyTrpLeuLeuSerProArgGlySerArgPro	105
SerTrpGlyProThrAspProArgArgArgSerArgAsnLeuGly	120
LysValIleAspThrLeuThrGlyGlyPheAlaAspLeuMetGly	135
TyrIleProLeuValSerAlaProLeuGlyGlyAlaAlaArgAla	150
LeuGlyHisGlyValArgValLeuGluAspGlyValAsnTyrAla	165
ThrGlyAsnLeuProGlyCysSerPheSerIlePheLeuLeuAla	180
LeuLeuSerCysLeuThrIleProAlaSerAlaTyrGluValArg	195
AsnValSerGlyIleTyrHisValThrAsnAspCysSerAsnSer	210
SerIleValTyrGluAlaAlaAspMetIleMetHisThr-COOH	223

FIG. 1B

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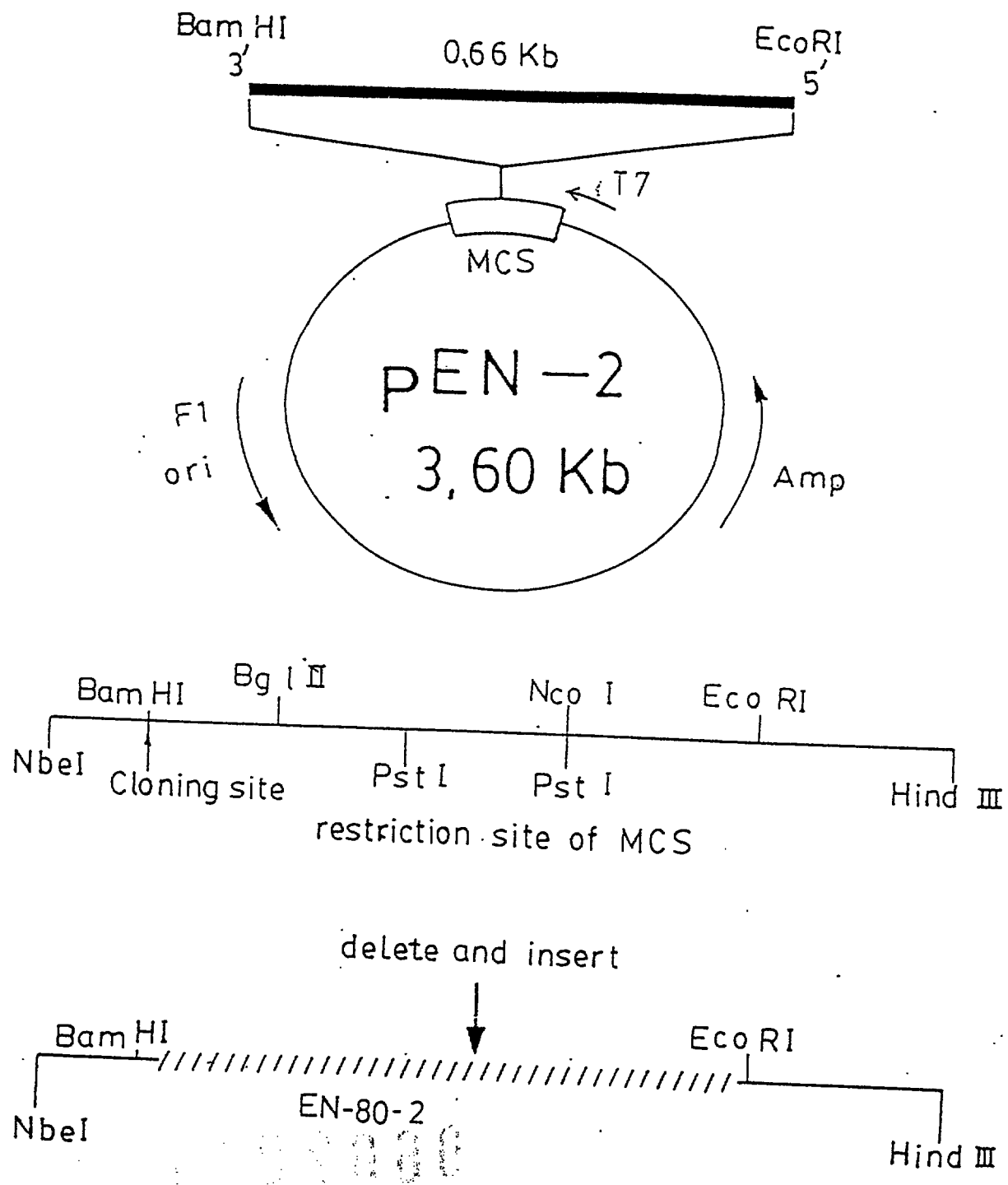


FIG. 2



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CLAIMS

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☒ Claim(s) : paged 21 to paged 21
☐ Drawing(s) : paged to paged
☐ Abstract : paged to paged

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1. A DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein, the polypeptide having the amino-acid sequence given in Figure 1B (SEQ.ID NO: 4).
2. A DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein comprising the DNA sequence as given in Fig. 1A (SEQ.ID NO: 3).
3. A DNA molecule as claimed in claim 1 or claim 2 coding for an antigenically significant subfragment or variant of a polypeptide as defined in claim 1 or claim 2.
4. A DNA molecule according to any one of claims 1-3 which is cloned from the plasma of a patient infected with HCV.
5. Probes for Hepatitis C virus (HCV) nucleic acid which are derived from the DNA molecule according to any one of claims 1 to 4.
6. An expression vector comprising a DNA molecule according to any one of claims 1-3.
7. A process for cloning a DNA molecule according to any one of claims 1-3 which comprises isolating RNAs from hepatitis C patients' sera, and cloning said DNA molecule via a Polymerase Chain Reaction (PCR) technique.
8. A process according to claim 7 wherein the following sequences are used as primers.
 - (i) 5'-GGATCCATGAGCACAAATCCTAAACCT-3' (SEQ.ID NO:1) and
 - (ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3' (SEQ.ID NO:2).
9. A polypeptide displaying the antigenicity of an HCV core antigen having the amino acid sequence as given in Fig. 1B (SEQ.ID NO:4).



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10. A polypeptide which comprises an antigenically significant subfragment or variant of a polypeptide as claimed in claim 9.
11. A polypeptide according to claim 9 having a molecular weight of about 25,000.
12. A polypeptide according to any one of claims 9-11 produced by an expression vector as claimed in claim 6.
13. A process for producing a polypeptide displaying the antigenicity of an HCV core antigen protein by the expression of a DNA molecule as defined in any one of claims 1-3.
14. A plasmid for expression of a polypeptide displaying the antigenicity of an HCV core antigen protein, designated pEN-2 as deposited in clone EN-80-2 under Accession Number ATCC 55451.
15. E. Coli transformed to produce a polypeptide displaying the antigenicity of an HCV core antigen protein, designated Clone EN-80-2 and deposited under Accession Number ATCC 55451.
16. A method for detecting HCV antibodies in samples, characterized by using a polypeptide as defined in any one of Claims 9-11 as a probe, capable of binding to antibodies directed against an HCV antigen in samples to form an antigen-antibody complex.
17. A method according to claim 16, characterized by using a polypeptide as defined in any one of claims 9-11 combined with an HCV nonstructural protein as probes.
18. A kit for analyzing samples for the presence of HCV antibodies in samples, comprising a polypeptide and defined in any one of claims 9-11.



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19. Antibodies or antigen binding fragments thereof directed against an HCV core antigen protein which are elicited by using a polypeptide as defined in any one of claims 9-11 as an immunogen.
20. A process for producing antibodies directed against HCV core antigen comprising immunizing an animal with a polypeptide as defined in any one of claims 9-11.
21. A method for analyzing HCV in samples characterized by using antibodies as defined in claim 19 as a probe.
22. Vaccines for prevention of HCV infection comprising an immunogenic polypeptide as defined in any one of claims 9-11.



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☐ Drawing(s) : paged to paged
☐ Abstract : paged to paged

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CLAIMS

1. A DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein, the polypeptide having the amino-acid sequence given in Figure 1B (SEQ.ID NO: 4).
2. A DNA molecule coding for a polypeptide displaying the antigenicity of an HCV core antigen protein comprising the DNA sequence as given in Fig. 1A (SEQ.ID NO: 3).
3. A DNA molecule according to any one of claims 1-2 which is cloned from the plasma of a patient infected with HCV.
4. Probes for Hepatitis C virus (HCV) nucleic acid which are derived from the DNA molecule according to any one of claims 1 to 3.
5. An expression vector comprising a DNA molecule accordingly to any one of claims 1-2.
6. A process for cloning a DNA molecule according to any one of claims 1-2 which comprises isolating RNAs from hepatitis C patients' sera, and cloning said DNA molecule via a Polymerase Chain Reaction (PCR) technique.
7. A process according to claim 6 wherein the following sequences are used as primers.
 - (i) 5'-GGATCCATGAGCACAAATCCTAACCT-3' (SEQ.ID. NO: 1) and
 - (ii) 5'-GAATTCGGTGTGCATGATCATGTCCGC-3' (SEQ.ID NO: 2).
8. A polypeptide displaying the antigenicity of an HCV core antigen having the amino acid sequence as given in Fig. 1B (SEQ.ID NO: 4).
9. A polypeptide according to claim 8 having a molecular weight of about 25,000.
10. A polypeptide according to any one of claims 8-9 produced by an expression vector as claimed in claim 5.



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11. A process for producing a polypeptide displaying the antigenicity of an HCV core antigen protein by the expression of a DNA molecule as defined in any one of claims 1-2.
12. A plasmid for expression of a polypeptide displaying the antigenicity of an HCV core antigen protein, designated pEN-2 as deposited in clone EN-80-2 under Accession Number ATCC 55451.
13. E. Coli transformed to produce a polypeptide displaying the antigenicity of an HCV core antigen protein, designed Clone EN-80-2 and deposited under Accession Number ATCC 55451.
14. A method for detecting HCV antibodies in samples, characterized by using a polypeptide as defined in any one of Claims 8-9 as a probe, capable of binding to antibodies directed against an HCV antigen in samples to form an antigen-antibody complex.
15. A method according to claim 14 characterized by using a polypeptide as defined in any one of claims 8-9 combined with an HCV nonstructural protein as probes.
16. A kit for analyzing samples for the presence of HCV antibodies in samples, comprising a polypeptide and defined in any one of claims 8-9.
17. Antibodies or antigen binding fragments thereof directed against an HCV core antigen protein which are elicited by using a polypeptide as defined in any one of claims 8-9 as an immunogen.
18. A process for producing antibodies directed against HCV core antigen comprising immunizing an animal with a polypeptide as defined in any one of claims 8-9.
19. A method for analyzing HCV in samples characterized by using antibodies as defined in claim 17 as a probe.
20. Vaccines for prevention of HCV infection comprising an immunogenic polypeptide as defined in any one of claims 8-9.



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