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(54) **HEPATITIS-C VIRUS TESTING**

Detektion des Hepatis-C Virus.

RECHERCHE DU VIRUS DE L'HEPATITE C

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DescriptionTechnical Field

[0001] The present invention relates to the discovery of new types of hepatitis C virus, that we have termed type 3 (HCV-3) and type 4 (HCV-4). In particular, it relates to the etiologic agent of hepatitis C virus type 3 and 4, and to polynucleotides and immunoreactive polypeptides which are useful in immunoassays for the detection of HCV-3 and HCV-4 in biological samples; and also to the use of antigenic HCV-3 and HCV-4 specific polypeptides in vaccines.

Background of the Invention

[0002] Acute viral hepatitis is a disease which may result in chronic liver damage. It is clinically diagnosed by a well-defined set of patient symptoms, including jaundice, hepatic tenderness, and an increase in the serum levels of alanine aminotransferase and aspartate aminotransferase. Serologic immunoassays are generally performed to diagnose the specific type of viral causative agent. Historically, patients presenting with symptoms of hepatitis and not otherwise infected by hepatitis A, hepatitis B, Epstein-Barr or cytomegalovirus were clinically diagnosed as having non-A, non-B hepatitis (NANBH) by default.

[0003] For many years, the agent of non-A, non-B hepatitis remained elusive. It has now been established that many cases of NANBH are caused by a distinct virus termed hepatitis C virus (HCV). European Patent Application EP-A-0318216 discloses cDNA sequences derived from HCV, polynucleotide probes and polypeptides for use in immunoassays. Further information is provided in European Application EP-A-0388232.

[0004] The HCV genome encodes a large polyprotein precursor, which contains structural and non-structural regions. The single protein is apparently cleaved into a variety of proteins after production. Most of the structural and non-structural proteins have now been identified from in vitro RNA translation and expression as recombinant proteins. The C and E regions encode for nucleocapsid structural proteins and for envelope structural proteins, respectively. At least five additional regions follow, which encode for non-structural (NS) protein of undefined function. The organisation is believed to be as follows (A. Alberti, Journal of Hepatology, 1991; 12; 279 to 282)

5' 3'
NCR: C : E1 : E2 : NS1 : NS2 : NS3 : NS4 : NS5

Certain immunoreactive proteins have been described as recombinant proteins, for example C22 (in the core region), C33 (in NS3 region), 5-1-1 and C100 (both in the NS4 region), and NS5 (NS5 region). Diagnosis of hepatitis C is still largely based on methods which detect antibodies against the product of the C-100 clone. This clone was ligated with overlapping clones to produce a larger viral antigen (C100) corresponding to part of the NS3-NS4 genomic region. C100 was then fused with the human superoxide dismutase (SOD) gene, expressed in use as a large recombinant fusion protein (C100-3) and used on solid phase to develop radio-labelled (RIA) and enzyme-linked immunosorbent assays (ELISA).

[0005] Polynucleotides useful for screening for HCV are disclosed in European Patent Specification EP-A-0398748. European Patent Specification EP-A-0414475 purports to disclose the propagation of HCV in culture cells and the production of antigens for use in diagnostics. European Patent Specification EP-A-0445423 discloses an improved immunoassay for detecting HCV antibodies.

[0006] Blood banks in the United Kingdom have recently begun routine testing of blood donors for antibodies to components of HCV. One assay involves the detection of HCV antibodies to C100-3 polypeptides. The C100-3 antibody recognises a composite polyprotein antigen within non-structural regions of the virus and is a consistent marker of HCV infection. However, in acute infections this antibody is unreliable because of the delay (typically 22 weeks) in seroconversion after exposure. Furthermore, the C100-3 antibody test lacks specificity for the hepatitis C virus.

[0007] Second generation antibody tests employ recombinant antigens or synthetic linear peptides representing structural antigens from the highly conserved core region of the virus as well as non-structural antigens. However, it is found that some second-generation ELISA tests can yield false-positive reactions. The recombinant immunoblot assay (RIBA-2) incorporating four antigens from the HCV genome, provides a method for identifying genuine anti-HCV reactivity. However, the result can be "indeterminate". The present workers have reported (The Lancet, 338; Oct. 19, 1991) varying reactivity of HCV-positive blood donors to 5-1-1, C100, C33C and C22 antigens, and compared these with the results of the direct detection of HCV RNA present in the blood samples using polymerase chain reaction (PCR) to amplify HCV polynucleotides. However, the work demonstrates that the unambiguous diagnosis of HCV infections is not yet possible.

[0008] Recently there has been discovered a second type of HCV (References 1, 2) called K2 that differs considerably in sequence from the published prototype (Reference 3) or the first type K1 sequences (References 4 and 5).

Summary of the Invention

[0009] The present invention provides a method of testing a sample for the presence of an anti-HCV antibody, which comprises contacting the sample with at least one HCV-3 or HCV-4 type-specific NS-4 antigen that comprises a sequence of amino acids from the region consisting of amino acids 1691 to 1728 inclusive, and detecting any resulting antibody-antigen complex.

[0010] The present invention is based on the discovery of a previously unknown type 3 and 4 variants of HCV, by a comparison of sequences amplified by PCR in certain regions of the HCV genome and confirmed by phylogenetic analysis. The invention has thus identified polynucleotide sequences and polypeptides which are HCV-3 and HCV-4 specific. These may be used to diagnose HCV-3 and HCV-4 infection and should thus be included in any definitive test for HCV infection.

[0011] One aspect of the invention provides a method of in vitro testing a sample for the presence of an anti-HCV antibody, which comprises contacting the sample with at least one HCV-3 or HCV-4 type-specific NS-4 antigen that comprises a sequence of amino acids from the region consisting of amino acids 1691 to 1708 inclusive or 1710 to 1728 inclusive, and detecting any resulting antibody-antigen complex.

[0012] Another aspect of the invention provides a type-specific isolated HCV-3 or HCV-4 antigen that comprises a sequence of amino acids from the region consisting of amino acids 1691 to 1708 inclusive or 1710 to 1728 inclusive.

[0013] Labelled antigenic HCV-3 or HCV-4 specific polypeptide (or mixtures thereof, from the NS4 region) is suitable for use in an immunoassay.

[0014] Antibodies to HCV-3 or HCV-4 specific antigens, particularly monoclonal antibodies, are suitable for use in therapy and diagnosis. Thus labelled antibodies may be used for in vivo diagnosis. Antibodies carrying cytotoxic agents may be used to attack HCV-3 or HCV-4 infected cells.

[0015] A vaccine may comprise immunogenic HCV-3 or HCV-4 specific polypeptide.

[0016] The HCV-3 or HCV-4 specific polynucleotide sequences may be used for identification of the HCV virus itself (usually amplified by PCR) by hybridisation techniques.

[0017] Oligonucleotides corresponding to variable regions in the NS-4 region could be used for type-specific PCR. Outer sense and inner sense primers may be used in combination with the two conserved anti-sense primers for a specific detection method for HCV types 1, 2, 3 and 4.

[0018] Immunoreactive HCV-3 or HCV-4 specific polypeptides from the NS4 region may be used to detect HCV-3 and HCV-4 antibodies in biological samples, and may also provide the-basis for immunogens for inclusion in vaccines. The term "peptide" is used herein to include epitopic peptides having the minimum number of amino acid residues for antigenicity, through oligopeptides, up to proteins. The peptide may be a recombinant peptide expressed from a transformed cell, or could be a synthetic peptide produced by chemical synthesis.

[0019] In particular, the invention allows blood donor screening by conventional assays (using HCV type 1 encoded antigens) to be supplemented with a second test that contains two oligopeptides corresponding to first and second antigenic regions found in the NS-4 sequence of HCV type 3 (positions 1691 to 1708; sequence KPALVPDKEV-LYQQYDEM and positions 1710 to 1728; sequence ECSQAAPYIEQAQVIAHQF) and two derived from the equivalent regions of HCV type 2, R(A/V)V(V/I) (A/T)PDKE(I/V)LYEAFDEM and ECAS(K/R)AALIEEGQR(M/I)AEML.

[0020] The corresponding HCV-4 antigens from substantially positions 1691 to 1708 and 1710 to 1728 may be used for HCV-4 detection.

[0021] Production and detection of the antigen-antibody immune complex may be carried out by any methods currently known in the art. For example, a labelling system such as enzyme, radioisotope, fluorescent, luminescent or chemiluminescent labels may be employed, usually attached to the antigen. Labelled anti-antibody systems may also be used. The recombinant antigen may be either used in liquid phase or absorbed onto a solid substrate.

[0022] oligopeptides corresponding to the antigenic regions of all three major types may also be used separately to serologically distinguish individuals infected with different HCV types. Such an assay could be in the format of an indirect enzyme immunoassay (EIA) that used sets of wells or beads coated with oligopeptides of the two major antigenic regions for HCV types 4, 3 and 2, and with type 1 (KPA(V/I)IPDREVLRYREFDEM and RPAV(I/V)PDREVLRYQEF-DEM and ECSQHLPYIEG(M/A)AEQF) - Minor degrees of cross-reactivity, should they exist, can be absorbed out by dilution of the test serum in a diluent that contained blocking amounts of soluble heterologous-type oligopeptides, to ensure that only antibody with type-specific antibody reactivity bound to the solid phase.

[0023] Immunogens for use in vaccine formulations may be formulated according to techniques currently known in the art, including the use of suitable adjuvant and immune-stimulation systems.

[0024] Finally, the present invention also encompasses assay kits including polypeptides which contain at least one epitope of HCV-3 or HCV-4 antigen (or antibodies thereto), as well as necessary preparative reagents, washing rea-

gents, detection reagents and signal producing reagents.

Description of the Drawings

[0025] Examples relating to the HCV3 and HCV4 NS4 antigens of the invention will now be described by way of example only.

Figures 1 and 1a give cDNA sequences obtained from PCR amplification of a region -255 to -62 of the 5' non-coding region of HCV samples from 18 blood donors and a comparison with previously published nucleotide sequences (see Table 2); sequence numbering corresponding to the prototype HCV-1 sequence (ref.4) and previous designations of type 1 or 2 being indicated;

Figure 2 is a phylogenetic analysis showing clustering of the sequences into three types viz: HCV-1, HCV-2 and HCV-3 for the 5' NCR results of Figure 1 using the maximum likelihood algorithm, shown as an unrooted tree. Numbers 1-18 in full circles correspond to blood donor sequences E-b1 through E-b18. Numbers 1 to 26 in open circles correspond to the previously published sequences identified in Table 2.;

Figure 3 is a comparison of deduced amino acid sequences in the NS-5 region of blood donors (E-b1, E-b2, E-b3, E-b7 (type 3) and E-b12 (type 2) with those previously published (Table 2). Amino acid residue numbering follows that of the HCV-1 polyprotein (4) and uses single letter amino acid codes;

Figure 4 is a phylogenetic analysis of the NS-5 region using the maximum likelihood algorithm, shown as an unrooted tree. Symbols are as described for Figure 2;

Figure 5 is a comparison of deduced amino acid sequences in the NS-3 region of blood donors (E-b1, E-b2, E-b6, E-b7 (type 3) with those previously published (Table 2). Group 1/1: amino acid sequence of f1, f3, f4, f5, h2, h3, h4 (one), i2, i3, i4, p1, p2; Group 1/2: amino acid sequence of 15; Group 1/3: amino acid sequence of h2, h3, h4 (one), h5, f2, p3, i1; Group 1/4: amino acid sequence of h1 (one); Group 1/5: amino acid sequence of h1 (one). Numbering, symbols and abbreviations are as described for Figure 3;

Figure 6 is a phylogenetic analysis of the NS-3 region using the maximum likelihood algorithm, shown as an unrooted tree. Representative nucleotide sequences of the 5 groups of type 1 sequences shown in Figure 5 coded as follows: 19 (full circle) i3; 20 (full circle) i4; 21 (full circle) h5; 22 (full circle) h3; 23 (full circle) h1. Symbols are as described for Figure 2;

Figure 7 is a comparison of deduced amino acid sequences in the core region of blood donor E-b1 (type 3) with those previously published (Table 2). Numbering, symbols and abbreviations are as described for Figure 3;

Figure 8 is a phylogenetic analysis of the core region using the maximum likelihood algorithm, shown as an unrooted tree. Symbols are as described for Figure 2.

Figure 9(a) shows nucleotide, and Figure 9(b) shows deduced amino acid sequences of HCV type 3 variants amplified from 5 Scottish blood donors (nos. 40, 38, 36, 26 and 1787) in the putative NS-4 region of HCV (nucleotides and amino acid residues numbered as in Choo et al., (1991). Nucleotide codes: G: guanine; C: cytosine; A: adenine; U: uridine; amino acid codes: A: alanine; R: arginine; N: asparagine; D: aspartic acid; C: cysteine; Q: glutamine; E: glutamic acid; G: glycine; H: histidine; I: isoleucine; L: leucine; K: lysine; M: methionine; F: phenylalanine; P: proline; S: serine; T: threonine; W: tryptophan; Y: tyrosine; V: valine; ".": sequence not determined; difference from consensus shown in bold.

Figure 10(a) shows a comparison of amino acid sequences between residues 1679 and 1768 (Choo et al., 1991) of the three major variants of HCV. T16, T42, T77, T1801, T1825: Scottish blood donors infected with HCV type 1; T351: Scottish blood donor infected with HCV type 2; T59, T940, T810: Scottish blood donors infected with HCV type 2; T40, T38, T36, T26, T1787: Scottish blood donors infected with HCV type 3; and Figure 10(b) shows the derivation of consensus sequences for HCV types 3, 2 and 1 oligopeptide series. Differences from consensus shown in bold. Amino acid codes: A: alanine; R: arginine; N: asparagine; D: aspartic acid; C: cysteine; Q: glutamine; E: glutamic acid; G: glycine; H: histidine; I: isoleucine; L: leucine; K: lysine; M: methionine; F: phenylalanine; P: proline; S: serine; T: threonine; W: tryptophan; Y: tyrosine; V: valine; ".": not determined.

Figures 11(a) to 11(c) show amino acid sequences of nonameric oligopeptides used for epitope mapping, derived from consensus HCV type 3, type 2 and type 1 sequences respectively. Amino acid codes: A: alanine; R: arginine; N: asparagine; D: aspartic acid; C: cysteine; Q: glutamine; E: glutamic acid; G: glycine; H: histidine; I: isoleucine; L: leucine; K: lysine; M: methionine; F: phenylalanine; P: proline; S: serine; T: threonine; W: tryptophan; Y: tyrosine; V: valine;

Figures 12a, 12b and 12c show antibody reactivity of three sera from blood donors infected with HCV type 3 with HCV type 3-encoded oligopeptides in the antigenic region of NS-4 (sequences 1-82 shown in Figure 11a). Antibody reactivity to oligopeptides (x-axis), recorded as optical densities in the range from -0.1 to 0.75 (and >0.75) recorded on the y-axis.

Figure 13 is a comparison of divergent HCV sequences with representative type 1, 2 and 3 sequences in variable

regions of the 5'NCR. Sequences from -255 to -246, -215 to -186, -115 to -102 and -69 to -62 identical to prototype sequence. ".": sequence identity with HCV-1; "-": gap introduced in sequences to preserve alignment; "-": sequence not determined. Origins of sequences : Eg- 1-33 : Egypt; NL-26 : Holland; HK- 1-4 : Hong Kong; IQ-48: Iraq; XX-96 : xxxxx. Figures in parentheses number each non-identical sequence.

Figure 14 is a phylogenetic analysis of the 5'NCR region using the maximum likelihood algorithm, shown as an unrooted tree. Sequences 1-17 in solid circles are numbered as in Figure 13; previously published sequences numbered as in table 1 of (992). Scottish blood donor sequences Eb-1-Eb-12 numbered 51-62 in hollow circles. For clarity, only non-identical sequences are shown in tree; e.g. Sequence 1 corresponds to those found in samples Eg-16 and Eg-29 etc. (Figure 1). Hollow squares are published sequences from Zaire; Hollow small circles are sequences from South Africa; Hollow small solid circles are sequences obtained elsewhere in the world.

Figure 15 A/B is a comparison of nucleotide (A) and amino acid (B) sequences in the core region. Symbols as for Figure 13. Single letter amino acid codes are used;

Figure 16 is a phylogenetic analysis of part of the core region using the maximum likelihood algorithm, shown as an unrooted tree. Sequences are numbered as in Figure 14; sequence 30 is that of HC-J8 (Okamoto et al. Virology 188:331 -341); and

Figure 17 shows cleavage patterns for A) HaeIII/RsaI and B) ScrFI in 5'NCR.

I) ANALYSIS OF HEPATITIS C VIRUS AND PHYLOGENETIC RELATIONSHIP OF TYPES 1, 2 and 3

Introduction

[0026] Sequence analysis of the 5' non coding region of hepatitis C virus (HCV) amplified from the plasma of individuals infected in Britain revealed the existence of three distinct groups of HCV, differing by 9-14% in nucleotide sequence. Two of the groups identified were similar to those of HCV variants previously termed type 1 and type 2, while the third group appeared to represent a novel virus type. Sequence comparisons were then made between the three virus types in other regions of the viral genome. In the NS-5 region, a high degree of nucleotide and amino acid sequence diversity was observed, with samples classified here as type "3" again forming a distinct group that was phylogenetically distinct from type 1 and type 2 variants. Type 3 sequences were similarly differentiated in the NS-3 and core regions from HCV type 1 sequences. The designation of virus types, including an observed sub-division of type 1 sequences into geographically distinct variants is discussed in relation to the new sequence data obtained in this study.

Discussion

[0027] Replication of nucleotide sequences by polymerase chain reaction (PCR) is a recently established technique. Synthetic complementary primer sequences are hybridised to single-stranded DNA on either side of a genome region to be copied. The second strand is built up under the action of a heat-stable polymerase in the region between the primers. Heating then dissociates the two-strands and the replication process starts again. The PCR technique allows tiny amounts of polynucleotide to the amplified provided that there is sufficient sequence information to synthesise the primer sequences.

[0028] The major problem associated with the use of the PCR to assess sequence variation using the PCR is the possibility that mismatches between the primers and the variant sequence will prevent amplification. We have used several strategies to overcome this problem. For initial virus detection, we used primers in the 5'NCR, which are reported to highly conserved amongst type 1 variants (4, 11, 13, 16, 23, 24, 26, 33), and between K1 and K2 (23). Sequence analysis of the blood donors allowed the identification of type 1 and type 2 variants by comparison with published sequence data. This analysis also revealed the existence of a third "type" of HCV that appeared to be as distinct from type 1 as type 2 was (Figs. 1,2; Table 3). Based on our initial tentative classification, we sought corroboration of our findings in other (coding) and more variable regions of the viral genome.

[0029] Analysis of the NS-5 region, which was based on several sequences of each of the three types (Figs. 3, 4; Table 3), confirmed the existence of 3 major groups, with type 3 sequences forming a relatively homogeneous group that was quite distinct from types 1 and 2. The proposed separation of type 1 sequences into PT and K1 "sub-types" and type 2 sequences into K2a and K2b is supported by this analysis, in which the single type 2 blood donor sequence obtained in this study appears most similar to K2b. Differentiation of HCV type 1 sequences into two groups is also clearly shown in the core (Fig.7) and NS-3 regions (Fig.5), in both cases with the type 3 sequences appearing considerably more distant.

[0030] The clustering of phylogenetically distinct groups, their mixed distributions in a single geographic area (1, 7, 23, 27, 35) and our own finding of dual or triple infections in individual haemophiliacs all strongly suggest that the three types described here are distinct viruses rather than simply representing geographical or epidemiologically clustered

variants of a single, highly variable but monophyletic group.

[0031] Our own phylogenetic analysis of the 5'NCR reveals the existence of three distinct groups. This contrasts with analyses of coding region, where there appears to be a very prominent differentiation of type 1 sequences into two "subtypes". However, unlike type 2 and 3 variants, the two subtypes are geographically distinct, one sub-type comprising sequences obtained exclusively from Japanese patients, and the other comprising predominantly USA/European sequences (Table 2). Indeed the only exception to this geographical classification is the HC-J1 sequence (26); one apparent exception (Pt-1) was obtained from a Japanese haemophiliac treated with imported factor VIII of USA origin (7,23), which is likely to have contained HCV variants corresponding to the other sub-type. There is insufficient sequence data to indicate whether the two proposed type 2 subtypes, K2a and K2b (7,23) represent geographically distinct variants.

[0032] The genomic organisation of HCV corresponds to that of flaviviruses and pestiviruses, with a single open reading frame encoding a polyprotein that is subsequently cleaved into structural and non-structural proteins. Weak sequence homologies have been detected with several other virus groups that have positive-sense RNA genomes (19,21). Although the overall degree of sequence dissimilarity between types 1, 2 and 3 cannot be measured by comparison of the small regions of sequence analysed in this study, a rough estimate of the extent of divergence in protein coding regions is given by an examination of the divergence of the partial core sequence. This shows that the difference between HCV type 1 and type 3 core region (approximately 10% amino acid sequence divergence) is comparable to that which exists between different serotypes of the flavivirus, tick-borne encephalitis virus (14%; ref.20), but lower than that which is found between serotypes of a mosquito borne flavivirus, dengue fever virus (33%), and the West Nile (WN) subgroup (28-43% divergence). The 5'NCR sequences of the different members of WN subgroup are also considerably more diverse than those of the three types of HCV (=50% similarity; ref.5), although within each of the members e.g. Murray Valley encephalitis virus, the 5'NCR is extremely well conserved (>95% similarity; ref.5). On the basis of these analogies, we speculate that the major types of HCV represent distinct "serotypes", each capable of human infection irrespective of the immune response mounted against other HCV types.

METHODS

[0033] Samples. Plasma from 18 different blood donors (E-b1 through E-b18), that were repeatedly reactive on screening by Abbott 2nd generation enzymeimmunoassay (EIA), and confirmed or indeterminate by a recombinant immunoblot assay (RIBA; Ortho; ref 1) were the principal samples used in this study. Sequences in the NS-3 region from 5 anti-HCV positive IVDUs (abbreviated as i1 - i5 in ref. 31), 5 haemophiliacs who had received non-heat treated clotting concentrate, and who were also anti-HCV positive (h1-h5), 3 pools of 1000 donations collected in 1983 (p1-p3), and 5 separate batches of commercially available non-heat treated factor VIII (fl - f5) correspond to those described previously (31).

[0034] Primers. The primers used for cDNA synthesis and polymerase chain reaction (PCR) are listed in Table 1. They were synthesised by Oswel DNA Service, Department of Chemistry, University of Edinburgh.

[0035] RNA Extraction and PCR. HCV virions in 0.2-1.0 ml volumes of plasma were pelleted from plasma by ultracentrifugation at 100,000g for 2 hours at 4°C. RNA was extracted from the pellet as previously described (2,31). First strand cDNA was synthesized from 3ul of RNA sample at 42°C for 30 min. with 7 units of avian myeloblastosis virus reverse transcriptase (Promega) in 20ul buffer containing 50 mM Tris-HCl (pH 8.0), 5mM MgCl₂, 5 mM dithiothreitol, 50 mM KCl, 0.05 ug/ul BSA, 15% DMSO, 600 uM each of dATP, dCTP, dGTP and TTP, 1.5 uM primer and 10 U RNAsin (Promega).

[0036] PCR was performed from 1 ul of the cDNA over 25 cycles with each consisting of 25 sec. at 94°C, 35 sec. at 50°C and 2.5 min. at 68°C. The extension time for the last cycle was increased to 9.5 min. The reactions were carried out with 0.4 unit Taq polymerase (Northumbria Biologicals Ltd.) in 20 ul buffer containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 33 uM each of dATP, dCTP, dGTP and dTTP and 0.5 uM of each of the outer nested primers. One ul of the reaction mixture was then transferred to a second tube containing the same medium but with the inner pair of nested primers, and a further 25 heat cycles were carried out with the same programme. The PCR products were electrophoresed in 3% low melting point agarose gel (IBI) and the fragments were detected by ethidium bromide staining and UV illumination. For sequence analysis, single molecules of cDNA were obtained at a suitable limiting dilution at which a Poisson distribution of positive and negative results was obtained (30).

[0037] Direct Sequencing of PCR Products. The PCR products were purified by glass-milk extraction ("GeneClean"; Bio101, Inc.). One quarter of the purified products was used in sequencing reactions with T7 DNA polymerase (Sequenase; United States Biologicals) performed according to the manufacturer's instructions except that the reactions were carried out in 10% DMSO and the template DNA was heat denatured before primer annealing.

[0038] Phylogenetic Methods. The sequences were compiled by version 2.0 of the programs of Staden (32) and analysed by programs available in the University of Wisconsin Genetics Computer Group sequence analysis package, version 7.0 (6). Phylogenetic trees were inferred using two different programs available in the PHYLIP package of

Felsenstein (version 3,4 June 1991; ref.9). The program DNAML finds the tree of the highest likelihood (the maximum likelihood tree) given a particular stochastic model of molecular evolution and has been shown to perform well in simulation studies (28). In the analyses performed here the global (G) option was used as this searches a greater proportion of all possible trees. The second program used was NEIGHBOR which clusters (following the algorithm of Saitou & Nei; ref.29) a matrix of nucleotide distances previously estimated using the program DNADIST (which itself was set, using the D option, to use the same stochastic model as underlies DNAML in order to estimate distances corrected for the probabilities of multiple substitution). In all cases the maximum likelihood and neighbour joining procedures produced congruent trees and thus only the former have been presented here.

[0039] To establish the interrelationships of the major types of HCV, we have separately analysed several regions of the viral genome that differ in sequence variability and evolutionary constraint. Thus the conclusions drawn from the sequence comparisons are not subject to spurious evolutionary phenomena that may affect a particular region. However, one problem with the analysis presented here was the absence of a viral sequence that was sufficiently distantly related to HCV to serve as an out-group. Thus, although we describe the interrelationships of different sequence variants of HCV, it should be stressed that we have no means of deciding which sequence is ancestral to the others. The trees are thus drawn in the less familiar un-rooted form to indicate this.

RESULTS

[0040]

1) Analysis of the 5' non-coding region. Samples were obtained from 18 blood donors that were repeatedly reactive in the Abbott 2nd Generation enzyme immunoassay and which were confirmed or indeterminate in the Chiron 4-RIBA (E-b1 through E-b18, ref.10). HCV sequences present in stored plasma samples from each donor were amplified with primers corresponding to sites in the 5'NCR (12,25) that are well conserved between all known HCV type 1 and type 2 variants (4,11,13,16,23,24,26,33). Sequencing of the PCR product, after limiting dilution to isolate single molecules of cDNA before amplification, allowed approximately 190 bps in the centre of the region to be compared with equivalent published sequences (fig.1).

Within the sequences, constant as well as variable regions can be found. Six sequences from donors E-b13 through E-b18 closely resembled those previously described as type 1 (4,11,13,16,23,24,26,33) and others resembled type 2(23) sequences (E-b9 through E-b12). However, eight sequences (E-b1 through E-b8) were distinct from both types, and have been provisionally termed type 3. Division of the sequences into three types is supported by formal phylogenetic analysis using the maximum likelihood (fig.2) and neighbour joining algorithms (data not shown) of the blood donor sequences along with previously published sequences (identified in table 2). Sequence variability within the three groups is in each case considerably less than that which separates the types. No sequence intermediate between the three types were found. This tree shows that the provisionally identified type 3 group is equally distinct from type 1 as is type 2. Using the DNAML model, the corrected distances between sequences within each type were in each case less than 3%. Between groups, they ranged from 9% (between type 1 and type 3, and between type 1 and 2), to 14% between type 2 and type 3 (table 3).

2) Analysis of the NS-5 Region. The nucleotide sequence of the NS-5 region has been found to vary significantly between the previously described K1 and K2 variants of HCV (7). To investigate whether type 3 sequences were equally distant from the other two types in this region as well as in the 5'NCR, we compared sequences from four type 3 blood donors (E-b1, E-b2, E-b3 and E-b7) and one type 2 donor (E-b12) with previously published sequences (Fig. 3; Fig.4; table 3).

A remarkable variation was observed between sequences of the three types in this region. Again, type 3 sequences form a separate group from type 1 and type 2 in this region. However, unlike the 5'NCR, there appear to be subdivisions within the type 1 and type 2 groups. Type 1 sequences are split between those found in Japanese infected individuals (e.g. HCV-J; HCV-BK; sequence numbers 12, 13, 16-20 in table 2) and those of U.S.A. origin (HCV-1, Pt-1, H77, H90; sequence numbers 1-4; fig.4). There is also some evidence for a split between type 2 sequences, those corresponding to their previous designation as K2a (7) appearing distinct from type K2b sequences and the Scottish blood donor, E-b12.

Table 3 shows that the average nucleotide distances between the two groups of HCV type 1 sequences is 25% (indicated here as type 1a [USA] and type 1b [Japanese]), with variation of only 4-7% within each group. The nucleotide sequence divergence within the two type 1 groups is similar to that which exists between K2a and K2b (table 3). However, both of these distances are considerably less than those which exist between type 1 and type 2 sequences (52-62%), and type 3 (48-49%), and the distance between type 2 and type 3 sequences (53-60%).

3) Analysis of the NS-3 region. Amplification reactions were carried out using previously published primer sequenc-

es in the NS-3 region (37), and a pair of empirically derived inner primers (31). Although these primers amplified HCV sequences from a high proportion of anti-C-100 positive sera from haemophiliacs (31), they were less effective with sera from IVDUs (31), and with blood donor samples (3 positive out of 15 tested; data not shown). Two conserved sites in the amplified fragment were identified by sequence analysis of the NS-3 region from the haemophiliac and IVDU patients, and two new primers corresponding to these were specified (207, 208; Table 1). The combination of 288-208 (first round) and 290-207 (second round) primers successfully amplified samples from four donors infected with HCV type 3 (E-b1, E-b2, E-b6 and E-b7) but none of those infected with HCV type 2 (data not shown). This enabled a comparison of the new type with our own (31) and previously published type 1 sequences (figs. 5, 6; table 3). For clarity, only seven of the type 1 sequences obtained in this study (E-b16, E-b17, i3, i3, h5, h3 and h1) are shown in the tree. These sequences are representative of the range of variation found in this region in individuals infected in Britain; comparison of the tree previously published (31) with fig.6 shows that the former forms a very small component of the overall tree obtained once Japanese type 1 and type 3 sequences are added.

The maximum likelihood tree shows that type 1 and type 3 have diverged considerably from each other. As was found in the NS-5 region, subtypes of type 1 sequences are found in NS-3. Again, sequences of Japanese origin (HCV-J, HCV-BK and JH) are distinct from the prototype (PT) sequence, and those found in Scottish blood donors (E-b16, E-b17, pl-3), IVDUs (i1-5) and haemophiliacs (h1-5), all of which correspond to the prototype sequence (fig.5). However, the average subtype difference (23%) is lower than those that exist between HCV-I and HCV-J with the four type 3 sequences (37-43%). As reported previously (31), the majority of nucleotide substitutions that exist between type 1 sequences are silent (i.e. do not affect the encoded amino acid sequence), while numerous amino acid substitutions exist between type 1 and type 3 sequences (fig.5). The analysis of the NS-3 region includes the sequence of clone A (35) which was obtained from Japanese patients with NANB hepatitis, and which was reported to be distinct from existing HCV type 1 sequences. In Fig. 6, this sequence appears to be distinct from both HCV type 1 and type 3, with corrected sequence distances of 33-43% and 36% respectively. Although it is not possible to assign this sequence to any known group at this stage, these distances are not inconsistent with the hypothesis that it represents a type 2 sequence, or an equally distinct novel HCV type.

4) Partial Sequence of the Putative Core Region of HCV.

The region encoding the putative core protein is comparatively well conserved in its nucleotide sequence between known type 1 variants, showing nucleotide and amino acid sequence similarities of 90-98% and 98-99% respectively (11, 24). Part of the core region from the blood donor Eb1, who has type 3 sequences in other regions analysed was amplified with primers 410 and 406 and compared with previously published type 1 sequences (fig. 7, 8; table 3). This analysis confirms that the type 3 sequence was distinct from those of type 1, and again there was a prominent subdivision of type 1 sequences into Japanese (HCV-J, HCV-BK, HC-J4, JH and J7) and USA/European (HCV-1, H77, H90, GM1, GM2) sequences. As was found in NS-3, very little amino acid sequence variation is found in the core regions of type 1 sequences; almost all of the nucleotide differences between the two groups are at "silent" sites. By contrast, the type 3 sequence shows 7-8 amino acid substitutions on comparison with type 1 sequences.

TABLE 1

SEQUENCES AND SOURCES OF PRIMERS USED FOR AMPLIFICATION OF HCV GENOME.

Name	Region	Position	Sense ^b	Sequences 5'-3'	Ref.
of 5'base ^a					
209	5'NCR	8	-	ATACTCGAGGTGCACGGTCTACGAGACCT	(12)
211	5'NCR	-29	-	CACTCTCGAGCACCCCTATCAGGCAGT	(12)
939	5'NCR	-297	+	CTGTGAGGAACTACTGTCTT	(25)
940	5'NCR	-279	+	TTCACGCAGAAAGCGTCTAG	(25)
410	CORE	410	-	ATGTACCCCATGAGGTCGGC	
406	CORE	-21	+	AGGTCTCGTAGACCGTGCATCATGAGCAC	
288	NS-3	4951	-	CCGGCATGCATGTCATGATGTAT	(31)
290	NS-3	4932	-	GTATTGGTGACTGGGTGCGTC	(31)
208	NS-3	4662	+	TCTTGAATTTTGGGAGGGCGTCTT	
207	NS-3	4699	+	CATATAGATGCCCACTTCCTATC	
007	NS-4	5293	-	AACTCGAGTATCCCACTGATGAAGTTCCACAT	
220	NS-4	5278	-	CACATGTGCTTCGCCCAGAA	
<i>HCV type 3A</i>					
221	NS-4	4858	+	GGACCTACGCCCCCTTCTATA	
008	NS-4	4878	+	TCGGTTGGGGCCTGTCCAAAATG	
<i>HCV type 2:</i>					
281	NS-4	4858	+	GGTCCCACCCCTCTCCTGTA	
509	NS-4	4878	+	CCGCTTGGGTTCCGTTACCAACG	
<i>HCV type 1:</i>					
253	NS-4	4858	-	GGGCCAACACCCCTGCTATA	
196	NS-4	4878	+	CAGACTGGGCGCCGTTCAGAATG	

Table 1 cont.

242	NS-5	8304	-	GGCGGAATTCCTGGTCATAGCCTCCGTGAA (7)
555	NS-5	8227	-	CCACGACTAGATCATCTCCG
243	NS-5	7904	+	TGGGGATCCCGTATGATACCCGCTGCTTTGA (7)
554	NS-5	7935	+	CTCAACCGTCACTGAACAGGACAT

*Position of 5' base relative to HCV genomic sequence in ref. no. (4)

^bOrientation of primer sequence (+: sense; -: anti-sense)

‡ Abbreviations: A: adenine, c: cytidine, G: guanidine, T: thymidine.

¶ Separate sense primers required to enable amplification of each HCV type.

TABLE 2

SOURCE AND CITATION OF PREVIOUSLY PUBLISHED HCV SEQUENCES USED IN THIS STUDY					
No.	Type	Abbreviation	Geographical Source	Reference	Ref. No.
1	1	HCV-1	U.S.A.	Choo <i>et al.</i> , 1991	(4)
2	1	Pt-1	Japan	Nakao <i>et al.</i> , 1991	(23)
				Enomoto <i>et al.</i> , 1990	(7)
3, 4	1	H77, H90	U.S.A.	Ogata <i>et al.</i> , 1991	(24)
5, 6	1	GM-1, GM-2	Germany	Fuchs <i>et al.</i> , 1991	(11)
7	1	J1	Japan	Han <i>et al.</i> , 1991	(13)
8	1	A1	Australia	Han <i>et al.</i> , 1991	(13)
9	1	S1	S. Africa	Han <i>et al.</i> , 1991	(13)
10	1	T1	Taiwan	Han <i>et al.</i> , 1991	(13)
11	1	U18/I24	U.S.A/Italy	Han <i>et al.</i> , 1991	(13)
12	1	HCV-J	Japan	Kato <i>et al.</i> , 1990	(16)
13	1	HCV-BK	Japan	Takamizawa <i>et al.</i> , 1991	(33)
14,15	1	HC-J1, -J4	Japan	Okamoto <i>et al.</i> , 1990	(26)
16-20	1	K1, K1- 1-4	Japan	Enomoto <i>et al.</i> , 1990	(7)
21	1	JH	Japan	Kubo <i>et al.</i> , 1990	(17)
22	1	J7	Japan	Takeuchi <i>et al.</i> , 1990	(34)
23-26	2	K2a, K2a-1, K2b, K2b-1	Japan	Nakao <i>et al.</i> , 1991	(23)
				Enomoto <i>et al.</i> , 1990	(7)
27	2	Clone A	Japan	Tsukiyama-Kohara. 1991	(35)

TABLE 3

NUCLEOTIDE DISTANCES BETWEEN THE THREE HCV TYPES IN FOUR REGIONS OF THE GENOME.						
REGION	TYPES (n ^a)	1a	1b	2a	2b	3
5'NCR	1 (20)	0.0163	n/a ^b			
	2 (6)	0.0869	n/a	0.0214		
	3 (8)	0.0948	n/a	0.1331	n/a	0.0123
CORE	1a (6)	0.0358				
	1b (5)	0.0855	0.0227			
	3 (1)	0.1801	0.1511	n/d ^c	n/d	0.0000
NS-3	1a (34)	0.0699				
	1b (3)	0.2270	0.0535			
	3 (4)	0.3689	0.4279	n/d	n/d	0.0460
NS-5	1a (4)	0.0743				
	1b (7)	0.2477	0.0372			
	2a (2)	0.6092	0.6206	0.0612		
	2b (3)	0.5214	0.5732	0.2252	0.0635	
	3 (4)	0.4754	0.4890	0.5983	0.5299	0.0322

^anumber of sequences analysed^bn/a : not applicable^cn/d : not done

II). SEROLOGICAL REACTIVITY OF BLOOD DONORS INFECTED WITH THREE DIFFERENT TYPES OF HEPATITIS C VIRUS.

[0041] HCV sequences were amplified in the 5'non-coding region (5'NCR), core, NS-3 and NS-5 regions from blood donors, haemophiliacs and intravenous drug abusers.

[0042] Blood donations that were repeatedly reactive on screening with Abbott 2nd generation enzyme immunoassay (EIA) and positive or indeterminate by Ortho recombinant immunoblot assay (RIBA) were amplified by primers in the 5'NCR (reference 10). The first fourteen PCR-positive blood donations (where PCR was used to amplify and thus detect HCV RNA present in the blood) were then typed by sequence analysis of the amplified region, and compared with their serological reactivity to a range of structural and non-structural peptides in two 1st generation EIAs (Ortho HCV ELISA; Abbott HCV EIA) and two RIBA assays (Ortho RIBA and Innogenetics LIA; Table 4). The five donations containing HCV type 1 sequences were positive in both EIAs, reacted with all antigens in the Ortho RIBA assay, and were broadly reactive in the LIA. However, all but two of the sera from donors with type 2 and 3 infections were completely negative on anti-C100 EIA screening and failed to react with 5-1-1, C100 (RIBA) and NS4 (LIA).

[0043] Furthermore, some carriers of HCV type 3 variants reacted poorly with the C33 (NS-3) peptide in the Ortho RIBA, and yielded two "indeterminate" results (donor nos. 11 and 13).

[0044] Thus, current tests using Ortho RIBA and (to a lesser extent) Innogenetics LIA tests are unable to reliably detect HCV-2 and HCV-3 genotypes. For reliable testing for all HCV types, antigens from 5-1-1, C100 and NS4 for each of the three types of HCV should preferably be included in the panel of antigens.

TABLE 4

SEROLOGICAL REACTIVITY OF SERA FROM BLOOD DONORS INFECTED WITH THREE TYPES OF HEPATITIS C VIRUS													
Donor Number	HCV genotype	anti-C100		Ortho RIBA				Innogenetics LIA					
		O*	A†	5-1-1	C100	C33	C22	NS4	NS5	C1‡	C2	C3	C4
E-b13	1	+	+	3§	4	4	4	2§	3	1	2	1	1
E-b15		+	+	4	4	4	4	2	3	3	2	2	1
E-b16		+	+	4	4	4	4	2	3	2	3	3	-
E-b17		+	+	4	4	4	4	3	3	3	2	1	1
E-b18		+	+	4	4	4	4	3	-	2	1	1	-
E-b9	2	+	+	-	1	3	4	-	-	3	1	1	3
E-b10		-	-	-	-	4	4	-	3	2	2	2	-
E-b11		-	-	-	-	4	4	-	3	4	2	2	3
E-b12		-	-	-	-	4	4	-	1	3	1	2	2
E-b1	3	-	-	-	-	-	4	-	1	3	1	-	3
E-b2		-	-	-	-	4	4	-	2	1	1	1	2
E-b3		+	+	-	-	2	4	2	2	1	2	2	1
E-b5		-	-	-	-	2	4	-	-	3	1	2	3
E-b7		-	-	-	-	-	4	-	2	3	1	1	4

*Ortho HCV ELISA (Recombinant C100-3)

†Abbott HCV EIA (Hepatitis C Recombinant DNA Antigen)

‡Core oligopeptides, 1-4

§Bands scored - (negative) to 4 (strong positive) according to manufacturers instructions.

PART III. MAPPING OF ANTIGENIC DETERMINANTS IN NS-4

Introduction

[0045] With an overall aim of improving serological screening assays, we have obtained sequence data from the antigenic region of region corresponding to c100-3 for types 2 and 3. This information was used to epitope map the region, to define additional immunoreactive peptides that could be used to improve serological anti-HCV assays.

Methods:

[0046] PCR and sequencing. Plasma samples from Scottish blood donors yielding repeatedly reactive donations on 2nd generation anti-HCV screening (Abbott or Ortho), and which were confirmed or indeterminate on confirmatory testing by RIBA (Chiron) were referred to the Department of Medical Microbiology from the Scottish National Blood Transfusion Service Microbiology Reference Laboratory. HCV RNA within the plasma samples was extracted and amplified with primers in the 5'NCR as described previously (Chan et al., 1992). HCV was typed by sequence analysis of the amplified DNA as described previously (Simmonds et al., 1990) and by RFLP analysis.

[0047] Five samples from different donors infected with HCV type 3 (nos. 40, 38, 36, 26 and 1787), four infected with type 2 (nos. 351, 59, 940 and 810) and five with type 1 infection (nos. 16, 42, 77, 1801 and 1825) were amplified with primers corresponding to sense and anti-sense sequences spanning the antigenic region of NS-4 (table 1). Nucleotide sequences obtained from the amplified DNA were compared and used to define consensus sequences for each HCV type. In-frame translation of the nucleotide sequences yielded an uninterrupted consensus amino acid sequence that was used to define a series of overlapping oligopeptides for epitope mapping.

Epitope mapping and determination of antibody specificities

[0048] Overlapping synthetic peptides were synthesised on polypropylene pins using kits commercially available from Cambridge Research Biochemicals Ltd. The principle of the addition reactions is described in refs (Geysen et al., 1984; Geysen et al., 1985). Antibody reactions were carried out on pins disrupted by sonication (30 minutes) in 1% sodium dodecyl sulphate, 0.1% 2-mercaptoethanol, 0.1M sodium dihydrogen orthophosphate. Pins were pre-coated in 1% ovalbumin, 1% bovine serum albumin, 0.1% Tween-20 in phosphate buffered saline (PBS) for one hour at room temperature. Serum or plasma was diluted 1.40 in PBS + 0.1% Tween-20 (PHST) and incubated with the blocked pins at 4°C for 18 hours. After washing in 4 changes of PBST (10 minutes at room temperature, with agitation), bound antibody was detected by incubation in a 1/20000 dilution of affinity isolated anti-human IgG, peroxidase conjugate (Sigma) for one hour at room temperature. Following washing (4 changes in PBST), pins were incubated in a 0.05% solution of azino-di-3-ethyl-benzthiazodinsulphonate in 0.1 M sodium phosphate/sodium citrate buffer (pH 4.0) containing 0.03% hydrogen peroxide for 20 minutes. Optical densities were read at 410 nm.

RESULTS

[0049] HCV RNA in plasma samples from five donors infected with HCV type 3 by sequence analysis of the 5'NCR, and by RFLP were amplified in the NS-4 region using primers listed in Table 1. Because of the high degree of sequence variability in this region, it was necessary to use separate sense primers for the amplification of different HCV types. However, the anti-sense primers were in a highly conserved region and could be used for amplification of all three types. Sequence analysis was carried out as previously described. This gave a continuous sequence from position 4911 to 5271 (numbered as in Choo et al., 1991) (Figure 5a). Little sequence variability (highlighted) was observed between the four different donors in this region.

[0050] The nucleotide sequences were used to deduce the sequence of the encoded peptide (Figure 5b). The putative protein contains mainly hydrophilic residues but no potential sites for N-linked glycosylation. Amino acid sequence variability with HCV type 3 was confined to only five residues (Figure 5b). However, this region differed considerably from the amino acid sequences of other blood donors infected with HCV types 1 and 2 (T16, 42, 77, 1801, 1825, 351, 940 and 810; Figure 6a). Sequence comparison between the major HCV types from residues 1679 to 1769 reveals three regions of considerable amino acid sequence variability. Most of the observed differences between types involve non-synonymous amino acid substitutions, particularly alternation of acidic and basic residues in the hydrophilic regions. These changes would be expected to profoundly alter the overall conformation of the protein, and its antigenicity.

[0051] The consensus amino acid sequences in this region of types 1-3 (Figure 6b) were used to define three series of 82 nonameric oligopeptides overlapping by eight of the nine residues with those before and after in the series (Figure 7a-c). These were synthesised on a 12 x 8 arrays of polypropylene pins as described in Methods. Antibody reactivity to the immobilised antigens on the pins was determined by indirect ELISA, using an overnight incubation with a 1/40

dilution of test serum overnight at 4°C, followed by washing, and detection with an anti-human IgG-peroxidase conjugate and appropriate substrate (see Methods).

[0052] Reactivity of an anti-HCV negative, PCR-negative donor, with no known risk factors for HCV infection with the three series of peptides was determined. No significant reactivity is shown with any of the HCV-encoded oligopeptides. Reactivity of sera from three donors infected with HCV type 3 to each of the oligopeptides is shown in Figures 9a-9c. All three sera reacted with peptides ranging from No. 13 (sequence KPALVPDKE; Figure 7) to No. 22 (sequence VLYQQYDEM) in the first antigenic region, although the precise peptides recognised varied slightly between individuals. All three sera reacted to varying extents with a second antigenic region, lying in the range from oligopeptides 32 to 42 (of sequence ECSQAAPYI to QAQVIAHWQ). Weaker and more variable reactivity was observed to peptides 48 to 53. Finally, significant reactivity was also observed to single oligonucleotides 2 (2 of 3 samples), 61 (2 of 3), 66 (3 of 3), 73 (3 of 3) and 80 (2 of 3).

[0053] The sequences of the major antigenic regions of HCV type 3 differ considerably from those encoded by any of the type 1 or type 2 variants. The region bounded by peptides 13 to 22 shows average homologies of 50% with HCV type 2 variants and 67% with type 1. Between peptides 32 to 42, there are homologies of 39% with type 2 and 58% with type 1 variants. Thus, although similar regions of each NS-4 sequence are antigenic, the actual epitopes differ considerably between HCV types.

Discussion

[0054] The NS-4 region of HCV type 3 shows considerable sequence divergence from other variants of HCV, that exceeds that found in the core, NS-3 or NS-5 regions previously analysed (Chan et al, 1992). The function of the protein encoded by this region of the HCV genome is unknown, and the consequences of this variability on virus replication and pathogenesis are unknown. The function of the NS-4 region in flaviviruses and pestiviruses is also poorly defined.

[0055] The degree of amino acid sequence variability, and the nature of the amino acid substitutions indicate that the major sites of antibody reactivity are also those of antigenic variability. This undoubtedly underlies the restricted cross-reactivity of HCV type 1 NS-4 encoded antigens with sera from individuals infected with different HCV types. Serological diagnosis of infection is currently based entirely on recombinant or synthetic oligopeptide sequences derived ultimately from HCV type 1 sequences (Choo et al., 1991). The serological response to infection is often very restricted in its initial stages, with antibody to only one of the recombinant antigens used for screening. Not only does this present difficulties with supplementary antibody tests, where reactivity to two HCV-encoded antigens is required for confirmation, but can lead to an increased probability of failing to detect early infection with HCV types 2 and 3.

[0056] Table 7 relates HCV typing determined by PCR to results obtained using type-specific antigens (TSA) and shows good correlation for HCV1-3 types.

TABLE 5

SEQUENCES OF NS-4 ENCODED ANTIGENS FOR (A) IMPROVED
SEROLOGICAL DIAGNOSIS. AND (B) FOR SEROLOGICAL
DISCRIMINATION OF INFECTION WITH DIFFERENT HCV TYPES

A)

Type	Region 1 (1691-1708)*	Region 2 (1710-1728)
3	KPALVPDKEVLYQQYDEM†	ECSQAAPYIEQAQVIAHQF
2‡	RVVVTPDKEILYEAFFDEM RAVIAPDKEVLYEAFFDEM	ECASKAALIEEGQORMAEML ECASRAALIEEGQRIAEML

B)

Type	Region 1 (1691-1708)	Region 2 (1710-1728)
3	KPALVPDKEVLYQQYDEM	ECSQAAPYIEQAQVIAHQF
2	RVVVTPDKEILYEAFFDEM RAVIAPDKEVLYEAFFDEM	ECASKAALIEEGQORMAEML ECASRAALIEEGQRIAEML
1	KPAIIPDREVLYREFDEM KPAVIPDREVLYREFDEM	ECSQHLPYIEGMLAEQF ECSQHLPYIEGALAEQF

* Amino acid positions numbered as in Choo *et al.*, (1991).

† Amino acid codes: A: alanine; R: arginine; N: asparagine; D: aspartic acid; C: cysteine; Q: glutamine; E: glutamic acid; G: glycine; H: histidine; I: isoleucine; L: leucine; K: lysine; M: methionine; F: phenylalanine; P: proline; S: serine; T: threonine; W: tryptophan; Y: tyrosine; V: valine.

‡ Alternative peptides. where there is variability within an HCV type..

TABLE 6

SEQUENCES OF OLIGONUCLEOTIDES SUITABLE FOR DIRECT DETECTION OF
HCV TYPE 3 IN CLINICAL SPECIMENS BY POLYMERASE CHAIN REACTION

Name	Region	Position of 5'base*	Pol.†	Sequences 5'-3'‡
007	NS-4	5293	-	AACTCGAGTATCCCACTGATGAAGTTCCACAT
220	NS-4	5278	-	CACATGTGCTTCGCCAGAA
Type 3-¶				
TS-3a	NS-4	5140	+	GCCGCCCATATATCGAACA
TS-3b	NS-4	5161	+	GCTCAGGTAATAGCCCACCA
Type 2:				
TS-2a	NS-4	5140	+	AAAGCCGCCCTCATTGAGGA
TS-2b	NS-4	5161	+	GGCAGCGGATGGCGGAGAT
Type 1:				
TS-1a	NS-4	5140	+	CACTTACCGTACATCGAGCA
TS-1b	NS-4	5161	+	GGATGATGCTCGCCGAGCA

* Position of 5'base relative to HCV genomic sequence in Choo *et al.* (1991).

† Orientation of primer sequence (+: sense; -: anti-sense)

‡ Abbreviations: A: adenine. C: cytidine. G: guanine. T: thymidine.

¶ Type-specific sense primers for amplification of HCV types 3, 2 and 1 variants.

TABLE 7

COMPARISON OF SEROLOGICAL TYPING BY HCV-TSA WITH PCR									
PCR ^a	Number tested	TYPE-SPECIFIC ANTIBODY							
		1	2	3	1+2	1+3	2+3	NTS ^b NR ^c	
1	57	63	-	-	-	1	-	3	3
2	12	-	11	-	-	-	1	1	0
3	47	1	-	45	-	2	-	4	4
Haem ^d	27	11	-	4	1	4	-	3	4

^a Genotype of HCV sequences amplified by PCR and typed by RFLP (McOmish *et al.* 1992)

^b NTS : No type-specific antibody detected

^c NR : non-reactive with NS-4 peptides

^d Samples from HCV-infected haemophiliacs, un-typed by PCR.

PART IV IDENTIFICATION OF HCV TYPE -4

Introduction

[0057] Investigations were carried out on sequence variations in the 5' non-coding region (5'NCR) of HCV samples from a variety of worldwide geographical locations (Figure 13), and also in the core region (Figures 15A and 15B). Phylogenetic analysis (Figures 14 and 16) revealed a new distinct HCV type which we refer to herein as HCV-4.

Methods

[0058] Samples. RNA was extracted from plasma samples that were repeatedly reactive on second generation screening assays for HCV, and which were either confirmed (significant reactivity with two or more antigens in the Chiron recombinant immunoblot assay; Chiron corporation, Emeryville, California, USA) or indeterminate (reactive with only one antigen) from blood donors and patients with NANBH. Most of the samples containing sequences that differed substantially from known HCV types came from Egypt (EG 1-33). Others came from Holland (NL-26), Hong Kong (HK 1-4), Iraq (IQ-48) and XX (xx-(6)).

[0059] Sequence determination. HCV sequences were reverse transcribed and amplified with primers matching conserved regions in the 5'NCR as previously described [1]. For analysis of the core region, RNA was reverse transcribed using a primer of sequence CA(T/C)GT(A/G)AGGGTATCGATGAC (5' base: xxx, numbered as in [20]). cDNA was amplified using this primer and a primer in the 5'NCR of sequence ACTGCCTGATAGGGTGCTTGCGAG (5' base: -54). The second PCR used primers of sequences AGGTCTCGTAGACCGTGCATCATG (5' base: -21) and TTGCG (G/T/C)GACCT(A/T)CGCCGGGGGTC (5'base: xxx). Amplified DNA in both regions was directly sequenced as described previously (reference 1a).

[0060] Sequence analysis. Sequences were aligned using the CLUSTAL program in the University of Wisconsin GCG package (reference 6). Phylogenetic trees were constructed by the DNAML program in the PHYLIP package of Felsenstein (version 3.4, June 1991; (reference 9), using the global option. RNA secondary structures in the 5'NCR of 4 representative HCV variants (refs) were predicted using the program FOLD. Three predictions were made from each sequence between nucleotides -341 to -1, -341 to +300, and -341 to +900 to allow for possible long range interactions. Comparison of the predicted conformations for each sequence over the different lengths showed that only relatively small scale features, such as the stem/loop analysed in the Results were at all conserved (data not shown).

[0061] All sequences reported in this part have been submitted to GenBank.

Results

[0062] Divergent 5'NCR sequences. Several sequences in the 5'NC region detected in samples of blood donors from Saudi Arabia, Holland and Hong Kong, and from NANBH patients in Iraq and xxx differed substantially from those found in Scottish blood donors and those reported elsewhere (Figure 13). Instead of showing the well characterised nucleotide substitutions that distinguish HCV types 1, 2 and 3 from each other, a new set of sequence differences were observed in the new variants that appeared to place them outside the existing system of virus classification. This can be more simply represented by reconstructing a phylogeny of the sequences and presenting the results as an evolutionary tree (Figure 14). This analysis confirms that sequences 1-10 cluster separately from the variants previously typed as 1,2 and 3. For convenience we will refer to sequences within this new group as type HCV type 4. Mean distances within type 4 and between type 4 and the other HCV types in the 5'NCR were comparable to those previously described for type 1-3. Although sequences within type 4 are relatively closely grouped, sequences 11, 12 and 13 differ considerably from any of the known types.

[0063] Using this phylogenetic tree, it can be seen that the majority of previously published 5'NCR sequences can be readily identified as types 1, 2 or 3. Furthermore, almost all of the sequences from Zaire (shown as hollow squares) cluster closely within type 4, suggesting a wider distribution in Africa. However, a further complication is that three identical sequences obtained from South African patients appeared distinct from both the type 1 and the type 4 group, and may represent yet another HCV type.

[0064] RNA from three representative type 4 variants (Eg 29, 33, 21; corresponding to 5'NCR sequences nos. 1-3) was amplified using primers in the core region of HCV polyprotein. All three sequences differed considerably at both the nucleotide and amino acid level from HCV types 1 to 3 (Figure 15A/B). Phylogenetic analysis of these sequences and those previously analysed indicated that they formed a separate, relatively homogeneous group distinct from the other types (Figure 16). Reconstructed nucleotide distances between type 4 and types 1-3 were comparable to those that exist between the three known HCV types of HCV. Although most of the nucleotide sequence differences were silent, there were between 4 and 9 amino acid differences between the new variants and other types.

PART V HCV TYPINGIntroduction

[0065] In view of the sequence variations between HCV types 1,2,3 and 4 differences in restriction enzyme cleavage sites exist, leading to different endonuclease cleavage patterns. This technique was used to identify HCV genotypes in blood samples from a variety of sources worldwide.

(A) Typing of HCV1-3METHODS

[0066] Serum Samples: Samples from blood donors in six countries, Scotland, Finland, Netherlands, Hong Kong and Australia and Japan, were available from routine 2nd Generation anti-HCV ELISA screening (Ortho or Abbott). Donor samples that were repeatedly reactive in the above tests were further investigated using a supplementary test (Ortho RIBA: Finland, Netherlands, Australia, Egypt, Abbott Matrix: Hong Kong) or samples were titred for anti-HCV by ELISA (Japan). Samples that were positive (significant reactivity with two or more HCV antigens (1+ to 4+) or indeterminate (reactivity with one antigen only) in the RIBA test or had a titre of > X 4096 by ELISA (Japan only) were tested for viral RNA by Polymerase Chain Reaction (PCR).

[0067] RNA PCR: PCR for the detection of HCV RNA was carried out as previously described by Chan et al (reference 1a) using primers in the 5'non-coding region (5'NCR) in a nested PCR, with primers 209/939 and 211/940(5) in first and second reactions respectively.

HCV TYPING

[0067] The existence of relatively conserved patterns of substitutions in the 5'NCR that are characteristic of different HCV types provide useful signature sequences for identification of HCV genotypes. Having compared large numbers of different HCV type 1, 2 and 3 sequences, we developed a method that differentiated HCV types 1-3 by restriction endonuclease cleavage of amplified DNA. However, the 19 type 4 sequences would appear as type 1 (electrophoretic types Aa and Ab), and for concurrent studies it has been necessary to modify the conditions to identify the new HCV type. All type 4 sequences showed a T-->C change at position -167 that creates a novel HinfI site that is absent in all type 1 (and type 2) sequences. In combination with ScrFI, and HaeIII/RsaI, it has now proved possible to identify the new type reliably in numerous countries in the Middle East and elsewhere.

RESULTS

[0068] The results are summarised in Table 8 for HCV types 1, 2 and 3. The Egyptian samples gave aberrant restriction patterns on the single ScrFI digest and were identified as type 4.

TABLE 8

PREVALENCE OF HCV TYPES IN DIFFERENT COUNTRIES			
COUNTRY	HCV TYPES (%)		
	HCV-1	HCV-2	HCV-3
Scotland	86(51%)	21(13%)	60(36%)
Finland	3(25%)	5(42%)	4(33%)
Netherlands	18(60%)	7(23%)	5(17%)
Hong Kong	22(63%)	0(0%)	0(0%)
Australia	13(57%)	3(13%)	7(30%)
Japan	31(77%)	9(23%)	0(0%)
Egypt	0(0%)	0(0%)	0(0%)

(B) MODIFICATION OF PCR-BASED TYPING ASSAY TO DETECT INFECTION WITH HCV TYPE 4 IN CLINICAL SPECIMENSMethods

[0069] Extraction of RNA. RNA was extracted from 100 ul aliquots of plasma of non-A, non-B patients by addition of 1 ml RNAzol solution (2M guanidinium thiocyanate, 12.5 mM sodium citrate [pH7.0], 0.25% w/v N-lauroylsarcosine, 0.05M 2-mercaptoethanol, 100mM sodium acetate [pH 4.0], 50% w/v water saturated phenol) as previously described (Chomczynski et al. 1987), and mixed until precipitate dissolved. After addition of 100 ul chloroform, each sample was spun for 5 minutes at 14000 x g and the aqueous phase re-extracted with 0.5 ml chloroform. RNA was precipitated by addition of an equal volume of isopropanol and incubation at -20°C for at least 1 hour. An RNA pellet was produced by centrifugation at 14000 x g for 15 minutes at 4°C, washed in 1ml 70% cold ethanol solution, dried and resuspended in 20 ul diethylpyrocarbonate treated distilled water. Of the 100 directly extracted samples, a total of 19 were PCR-negative (see below). Two ml volumes of the negative samples were ultracentrifuged at 200 000 x g for 2 hours and the pellet re-extracted as described above. Extraction from the larger volume of plasma yielded an additional 3 positive samples (numbers 66, 80, 85).

[0070] PCR and typing. RNA was reverse transcribed with primer 940 and cDNA amplified in a two stage nested PCR reaction with primers 940/939, followed by 209/211 as previously described (Chan et al. 1992). PCR product was radiolabelled with [³⁵S]-dATP analysed by restriction endonuclease cleavage (McOmish et al. Transfusion, 32:no.11 1992). Samples were cleaved with ScrFI and a combination of HaeIII/RsaI in two separate reactions to identify HCV types 1/4, 2, 3. Figure 17 shows endonuclease cleavage patterns. HCV types 1 and 4 were differentiated by a third reaction with HinfI (see Results). Two samples yielded restriction patterns that were different from those of the four known types of HCV and were analysed further by direct sequence analysis of the amplified DNA (Chan et al. 1992). These two samples contained 5'NCR sequences distinct from those of known HCV types and currently remain unclassified.

RESULTSModification of RFLP method to identify HCV type 4.

[0071] Previous sequence analysis in the 5'NCR of HCV amplified from plasma of Egyptian blood donors revealed a relatively homogeneous group of novel sequence variants in both the 5'NCR and core region which were as distinct from HCV types 1, 2 and 3 as these latter types were from each other (see previous submission). This new group was designated as HCV type 4.

[0072] Comparison of cleavage patterns of type 4 sequences with those of type RFLP analysis of the previously identified type 4 sequences produced a distribution of electrophoretotypes with ScrFI and HaeIII/RsaI similar to that HCV type 1 (Table 9). Type 1 sequences yielded 9 patterns of aA/B, 35 of bA/B and 1 bC. With these enzymes alone, type 4 sequences were thus indistinguishable from type 1 (14 aA/B, 4 bA/B). However, type 1 and type 4 sequences consistently differ in the number of HinfI sites. All 18 type 4 sequences contain one or two potential cleavage sites (producing patterns band c; table 5) while none are found in any of the 45 type 1 sequences analysed (pattern a). One of the type 4 sequences was further differentiated from type 1 and other HCV types by the loss of a restriction site for RsaI, leading to a new pattern of bands designated h (44, 172, 9, 26; first column, Table 9). Finally, a single sequence, EG-28 lost two sites to produce bands of 216, 9, and 26 bps (pattern i; Table 9). This sequence was distinct from that of any of the known HCV types (including type 4) and is shown in the table in the column labelled U (unclassified)

[0073] Typing of study subjects. RNA was extracted from 100 samples of patients with NANB hepatitis and amplified with primers in the 5'NCR. Of these, 84 were PCR positive, and enabled HCV typing to be carried out by RFLP. This was initially carried out with HaeIII/RsaI and ScrFI, and allowed the identification of 10 type 2 and 10 type 3 variants (Table 10). Samples showing electrophoretic patterns aA/B or bA/B were further analysed by cleavage with hinfI, yielding 38 samples with pattern a, thus identified as type 1, 22 with pattern b and 2 with pattern c, both identified as type 4. Finally, two samples showed the unusual cleavage patterns h and i with HaeIII/RsaI and pattern b with HinfI, and were therefore directly sequenced. These two sequences were similar to each other but were unlike any of the known HCV types, and also distinct from EG-28, the other sequence showing pattern i with HaeIII/RsaI (Table 10). As they cannot be currently classified, they will be referred to as type U.

TABLE 9

PREDICTED CLEAVAGE PATTERNS OF PUBLISHED 5'NCR SEQUENCES OF HCV TYPES 1, 2, 3 AND 4 WITH
RsaI/HaeIII, ScrFI AND HinfI

Predicted cleavage pattern ^a			HCV type				
<i>HaeIII/RsaI</i>	<i>ScrFI</i>	<i>HinfI</i>	1	2	3	4	U ^b
a	A/B	a ^c	9	-	-	-	-
b	A/B	a	35	-	-	-	-
b	C	a	1	-	-	-	-
a	A/B	b ^d	-	-	-	13	-
a	A/B	c ^e	-	-	-	1	-
b	A/B	b	-	-	-	4	-
c	D	a	-	5	-	-	-
d	D	a	-	1	-	-	-
d	E	a	-	2	-	-	-
e	D	a	-	1	-	-	-
e	E	a	-	1	-	-	-
f	G	b	-	-	14	-	-
f	G	c	-	-	1	-	-
g	G	b	-	-	8	-	-
h ^f	A/B	b	-	-	-	1	-
i ^g	A/B	b	-	-	-	-	1

^a Cleavage patterns designated for *HaeIII/RsaI* and *ScrFI* as described previously (McOmish *et al.* 1992).

^b Cleavage pattern of an HCV variant of undesigned type

^c Pattern a : uncleaved by *HinfI*

^d Pattern b : DNA cleaved to generate two fragments of sizes 107 and 142 bps (in order 5'→3')

^e Pattern c : DNA cleaved to generate three fragments of 56, 51 and 142 bps

^f New cleavage pattern for *HaeIII/RsaI* designated h (bands of 44 bps, 172 bps, 9 bps, 26 bps)

^g New cleavage pattern for *HaeIII/RsaI* designated i (216 bps, 9 bps, 26 bps)

TABLE 10

IDENTIFICATION OF HCV TYPES 1-4 IN STUDY SUBJECTS BY RFLP ANALYSIS OF 5'NCR SEQUENCES WITH
RsaI/HaeIII, ScrFI AND HinfI

Observed cleavage pattern			Inferred HCV type				
<i>HaeIII/RsaI</i>	<i>ScrFI</i>	<i>HinfI</i>	1	2	3	4	U ^a
a	A/B	a	2	-	-	-	-
b	A/B	a	36	-	-	-	-
a	A/B	b	-	-	-	16	-
a	A/B	c	-	-	-	2	-
b	A/B	b	-	-	-	6	-
c	D	n.d.	-	7	-	-	-
d	E	n.d.	-	3	-	-	-
f	G	n.d.	-	-	7	-	-
g	G	n.d.	-	-	3	-	-

^a Two samples yielded unusual restriction patterns with *HaeIII/RsaI* (h, i). Sequence analysis of the 5'NCR placed them outside existing HCV classification (samples IQ-48, EG-96).

TABLE 10 (continued)

IDENTIFICATION OF HCV TYPES 1-4 IN STUDY SUBJECTS BY RFLP ANALYSIS OF 5'NCR SEQUENCES WITH <i>RsaI/HaeIII. ScrFI AND HinfI</i>							
Observed cleavage pattern			Inferred HCV type				
<i>HaeIII</i> <i>RsaI</i>	<i>ScrFI</i>	<i>HinfI</i>	1	2	3	4	U ^a
h	A/B	b	-	-	-	-	1
i	A/B	b	-	-	-	-	1
		TOTALS	38	10	10	24	2

^a Two samples yielded unusual restriction patterns with *HaeIII/RsaI* (h. i). Sequence analysis of the 5'NCR placed them outside existing HCV classification (samples IQ-48, EG-96).

PART VI Expression and Assay etc. Techniques

[0074] The present invention also provides expression vectors containing the DNA sequences as herein defined, which vectors being capable, in an appropriate host, of expressing the DNA sequence to produce the peptides as defined herein.

[0075] The expression vector normally contains control elements of DNA that effect expression of the DNA sequence in an appropriate host. These elements may vary according to the host but usually include a promoter, ribosome binding site, translational start and stop sites, and a transcriptional termination site. Examples of such vectors include plasmids and viruses. Expression vectors of the present invention encompass both extrachromosomal vectors and vectors that are integrated into the host cell's chromosome. For use in *E.coli*, the expression vector may contain the DNA sequence of the present invention optionally as a fusion linked to either the 5'- or 3'-end of the DNA sequence encoding, for example, B-galactosidase or to the 3'-end of the DNA sequence encoding, for example, the trp E gene. For use in the insect baculovirus (AcNPV) system, the DNA sequence is optionally fused to the polyhedrin coding sequence.

[0076] The present invention also provides a host cell transformed with expression vectors as herein defined.

[0077] Examples of host cells of use with the present invention include prokaryotic and eukaryotic cells, such as bacterial, yeast, mammalian and insect cells. Particular examples of such cells are *E.coli*, *S.cerevisiae*, *P.pastoris*, Chinese hamster ovary and mouse cells, and *Spodoptera frugiperda* and *Tricoplusia ni*. The choice of host cell may depend on a number of factors but, if post-translational modification of the HCV viral peptide is important, then an eukaryotic host would be preferred.

[0078] The present invention also provides a process for preparing a peptide as defined herein which comprises isolating the DNA sequence, as herein defined, from the HCV genome, or synthesising DNA sequence encoding the peptides as defined herein, or generating a DNA sequence encoding the peptide, inserting the DNA sequence into an expression vector such that it is capable, in an appropriate host, of being expressed, transforming host cells with the expression vector, culturing the transformed host cells, and isolating the peptide.

[0079] The DNA sequence encoding the peptide may be synthesised using standard procedures (Gait, *Oligonucleotide Synthesis: A Practical Approach*, 1984, Oxford, IRL Press).

[0080] The desired DNA sequence obtained as described above may be inserted into an expression vector using known and standard techniques. The expression vector is normally cut using restriction enzymes and the DNA sequence inserted using blunt-end or staggered-end ligation. The cut is usually made at a restriction site in a convenient position in the expression vector such that, once inserted, the DNA sequences are under the control of the functional elements of DNA that effect its expression.

[0081] Transformation of an host cell may be carried out using standard techniques. Some phenotypic marker is usually employed to distinguish between the transformants that have successfully taken up the expression vector and those that have not. Culturing of the transformed host cell and isolation of the peptide as required may also be carried out using standard techniques.

[0082] The peptides of the present invention may be prepared by synthetic methods or by recombinant DNA technology. The peptides are preferably synthesized using automatic synthesizers.

[0083] Antibody specific to a peptide of the present invention can be raised using the peptide. The antibody may be polyclonal or monoclonal. The antibody may be used in quality control testing of batches of the peptides; purification of a peptide or viral lysate; epitope mapping; when labelled, as a conjugate in a competitive type assay, for antibody detection; and in antigen detection assays.

[0084] Polyclonal antibody against a peptide of the present invention may be obtained by injecting a peptide, optionally coupled to a carrier to promote an immune response, into a mammalian host, such as a mouse, rat, sheep or

rabbit, and recovering the antibody thus produced. The peptide is generally administered in the form of an injectable formulation in which the peptide is admixed with a physiologically acceptable diluent. Adjuvants, such as Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA), may be included in the formulation. The formulation is normally injected into the host over a suitable period of time, plasma samples being taken at appropriate intervals for assay for anti-HCV viral antibody. When an appropriate level of activity is obtained, the host is bled. Antibody is then extracted and purified from the blood plasma using standard procedures, for example, by protein A or ion-exchange chromatography.

[0085] Monoclonal antibody against a peptide of the present invention may be obtained by fusing cells of an immortalising cell line with cells which produce antibody against the viral or topographically related peptide, and culturing the fused immortalised cell line. Typically, a non-human mammalian host, such as a mouse or rat, is inoculated with the peptide. After sufficient time has elapsed for the host to mount an antibody response, antibody producing cells, such as the splenocytes, are removed. Cells of an immortalising cell line, such as a mouse or rat myeloma cell line, are fused with the antibody producing cells and the resulting fusions screened to identify a cell line, such as a hybridoma, that secretes the desired monoclonal antibody. The fused cell line may be cultured and the monoclonal antibody purified from the culture media in a similar manner to the purification of polyclonal antibody.

[0086] Diagnostic assays based upon the present invention may be used to determine the presence or absence of HCV infection. They may also be used to monitor treatment of such infection, for example in interferon therapy.

[0087] In an assay for the diagnosis of viral infection, there are basically three distinct approaches that can be adopted involving the detection of viral nucleic acid, viral antigen or viral antibody. Viral nucleic acid is generally regarded as the best indicator of the presence of the virus itself and would identify materials likely to be infectious. However, the detection of nucleic acid is not usually as straightforward as the detection of antigens or antibodies since the level of target can be very low. Viral antigen is used as a marker for the presence of virus and as an indicator of infectivity. Depending upon the virus, the amount of antigen present in a sample can be very low and difficult to detect. Antibody detection is relatively straightforward because, in effect, the host immune system is amplifying the response to an infection by producing large amounts of circulating antibody. The nature of the antibody response can often be clinically useful, for example IgM rather than IgG class antibodies are indicative of a recent infection, or the response to a particular viral antigen may be associated with clearance of the virus. Thus the exact approach adopted for the diagnosis of a viral infection depends upon the particular circumstances and the information sought. In the case of HCV, a diagnostic assay may embody any one of these three approaches.

[0088] In an assay for the diagnosis of HCV involving detection of viral nucleic acid, the method may comprise hybridising viral RNA present in a test sample, or cDNA synthesised from such viral RNA, with a DNA sequence corresponding to the nucleotide sequences of the present invention or encoding a peptide of the invention, and screening the resulting nucleic acid hybrids to identify any HCV viral nucleic acid. The application of this method is usually restricted to a test sample of an appropriate tissue, such as a liver biopsy, in which the viral RNA is likely to be present at a high level. The DNA sequence corresponding to a nucleotide sequence of the present invention or encoding a peptide of the invention may take the form of an oligonucleotide or a cDNA sequence optionally contained within a plasmid. Screening of the nucleic acid hybrids is preferably carried out by using a labelled DNA sequence. Preferably the peptide of the present invention is part of an oligonucleotide wherein the label is situated at a sufficient distance from the peptide so that binding of the peptide to the viral nucleic acid is not interfered with by virtue of the label being too close to the binding site. One or more additional rounds of screening of one kind or another may be carried out to characterise further the hybrids and thus identify any HCV viral nucleic acid. The steps of hybridisation and screening are carried out in accordance with procedures known in the art.

[0089] The present invention also provides a test kit for the detection of HCV viral nucleic acid, which comprises

- i) a labelled oligonucleotide comprising a DNA sequence of the present invention or encoding a peptide of the present invention; and
- ii) washing solutions, reaction buffers and a substrate, if the label is an enzyme.

[0090] Advantageously, the test kit also contains a positive control sample to facilitate in the identification of viral nucleic acid.

[0091] In an assay for the diagnosis of HCV involving detection of viral antigen or antibody, the method may comprise contacting a test sample with a peptide of the present invention or a polyclonal or monoclonal antibody against the peptide and determining whether there is any antigen-antibody binding contained within the test sample. For this purpose, a test kit may be provided comprising a peptide, as defined herein, or a polyclonal or monoclonal antibody thereto and means for determining whether there is any binding with antibody or antigen respectively contained in the test sample. The test sample may be taken from any of the appropriate tissues and physiological fluids mentioned above for the detection of viral nucleic acid. If a physiological fluid is obtained, it may optionally be concentrated for any viral antigen or antibody present.

[0092] A variety of assay formats may be employed. The peptide can be used to capture selectively antibody against HCV from solution, to label selectively the antibody already captured, or both to capture and label the antibody. In addition, the peptide may be used in a variety of homogeneous assay formats in which the antibody reactive with the peptide is detected in solution with no separation of phases.

[0093] The types of assay in which the peptide is used to capture antibody from solution involve immobilization of the peptide on to a solid surface. This surface should be capable of being washed in some way. Examples of suitable surfaces include polymers or various types (moulded into microtitre wells; beads; dipsticks of various types; aspiration tips; electrodes; and optical devices), particles (for example latex; stabilized red blood cells; bacterial or fungal cells; spores; gold or other metallic or metal-containing sols; and proteinaceous colloids) with the usual size of the particle being from 0.02 to 5 microns, membranes (for example of nitrocellulose; paper; cellulose acetate; and high porosity/high surface area membranes of an organic or inorganic material).

[0094] The attachment of the peptide to the surface can be by passive adsorption from a solution of optimum composition which may include surfactants, solvents, salts and/or chaotropes; or by active chemical bonding. Active bonding may be through a variety of reactive or activatable functional groups which may be exposed on the surface (for example condensing agents; active acid esters, halides and anhydrides; amino, hydroxyl, or carboxyl groups; sulphhydryl groups; carbonyl groups; diazo groups; or unsaturated groups). Optionally, the active bonding may be through a protein (itself attached to the surface passively or through active bonding), such as albumin or casein, to which the viral peptide may be chemically bonded by any of a variety of methods. The use of a protein in this way may confer advantages because of isoelectric point, charge, hydrophilicity or other physico-chemical property. The viral peptide may also be attached to the surface (usually but not necessarily a membrane) following electrophoretic separation of a reaction mixture, such as immunoprecipitation.

[0095] After contacting (reacting) the surface bearing the peptide with a test sample, allowing time for reaction, and, where necessary, removing the excess of the sample by any of a variety of means, (such as washing, centrifugation, filtration, magnetism or capillary action) the captured antibody is detected by any means which will give a detectable signal. For example, this may be achieved by use of labelled molecule or particle as described above which will react with the captured antibody (for example protein A or protein G and the like; anti-species or anti-immunoglobulin-sub-type; rheumatoid factor; or antibody to the peptide, used in a competitive or blocking fashion), or any molecule containing an epitope contained in the peptide.

[0096] The detectable signal may be optical or radioactive or physico-chemical and may be provided directly by labelling the molecule or particle with, for example, a dye, radiolabel, electroactive species, magnetically resonant species or fluorophore, or indirectly by labelling the molecule or particle with an enzyme itself capable of giving rise to a measurable change of any sort. Alternatively the detectable signal may be obtained using, for example, agglutination, or through a diffraction or birefringent effect if the surface is in the form of particles.

[0097] Assays in which a peptide itself is used to label an already captured antibody require some form of labelling of the peptide which will allow it to be detected. The labelling may be direct by chemically or passively attaching for example a radio label, magnetic resonant species, particle or enzyme label to the peptide; or indirect by attaching any form of label to a molecule which will itself react with the peptide. The chemistry of bonding a label to the peptide can be directly through a moiety already present in the peptide, such as an amino group, or through an intermediate moiety, such as a maleimide group. Capture of the antibody may be on any of the surfaces already mentioned by any reagent including passive or activated adsorption which will result in specific antibody or immune complexes being bound. In particular, capture of the antibody could be by anti-species or anti-immunoglobulin-sub-type, by rheumatoid factor, proteins A, G and the like, or by any molecule containing an epitope contained in the peptide.

[0098] The labelled peptide may be used in a competitive binding fashion in which its binding to any specific molecule on any of the surfaces exemplified above is blocked by antigen in the sample. Alternatively, it may be used in a non-competitive fashion in which antigen in the sample is bound specifically or non-specifically to any of the surfaces above and is also bound to a specific bi- or poly-valent molecule (e.g. an antibody) with the remaining valencies being used to capture the labelled peptide.

[0099] Often in homogeneous assays the peptide and an antibody are separately labelled so that, when the antibody reacts with the recombinant peptide in free solution, the two labels interact to allow, for example, non-radiative transfer of energy captured by one label to the other label with appropriate detection of the excited second label or quenched first label (e.g. by fluorimetry, magnetic resonance or enzyme measurement). Addition of either viral peptide or antibody in a sample results in restriction of the interaction of the labelled pair and thus in a different level of signal in the detector.

[0100] A suitable assay format for detecting HCV antibody is the direct sandwich enzyme immunoassay (EIA) format. A peptide is coated onto microtitre wells. A test sample and a peptide to which an enzyme is coupled are added simultaneously. Any HCV antibody present in the test sample binds both to the peptide coating the well and to the enzyme-coupled peptide. Typically, the same peptide are used on both sides of the sandwich. After washing, bound enzyme is detected using a specific substrate involving a colour change. A test kit for use in such an EIA comprises:

- (1) a peptide, as herein defined labelled with an enzyme;
- (2) a substrate for the enzyme;
- (3) means providing a surface on which a peptide is immobilised; and
- (4) optionally, washing solutions and/or buffers.

[0101] It is also possible to use IgG/IgM antibody capture ELISA wherein an antihuman antibody is coated onto microlitre wells, a test sample is added to the well. Any IgG or IgM antibody present in the test sample will then bind to the anti-human antibody. A peptide of the present invention, which has been labelled, is added to the well and the peptide will bind to any IgG or IgM antibody which has resulted due to infection by HCV. The IgG or IgM antibody can be visualized by virtue of the label on the peptide.

[0102] It can thus be seen that the peptides of the present invention may be used for the detection of HCV infection in many formats, namely as free peptides, in assays including classic ELISA, competition ELISA, membrane bound EIA and immunoprecipitation. Peptide conjugates may be used in amplified assays and IgG/IgM antibody capture ELISA.

[0103] An assay of the present invention may be used, for example, for screening donated blood or for clinical purposes, for example, in the detection and monitoring of HCV infections. For screening purposes, the preferred assay formats are those that can be automated, in particular, the microtitre plate format and the bead format. For clinical purposes, in addition to such formats, those suitable for smaller-scale or for single use, for example, latex assays, may also be used. For confirmatory assays in screening procedures, antigens may be presented on a strip suitable for use in Western or other immunoblotting tests.

[0104] As indicated above, assays used currently to detect the presence of anti-HCV antibodies in test samples, particularly in screening donated blood, utilise antigenic peptides obtained from HIV type 1 only and, as demonstrated herein, such antigens do not reliably detect other HCV genotypes. Accordingly, it is clearly desirable to supplement testing for HIV-1 with testing for all other genotypes, for example, types 2, 3 and 4, and also any further genotypes that may be discovered.

[0105] To test for a spectrum of genotypes, there may be provided a series of assay means each comprising one or more antigenic peptides from one genotype of HCV, for example, a series of wells in a microtitre plate, or an equivalent series using the bead format. Such an assay format may be used to determine the genotype of HCV present in a sample. Alternatively, or in addition, an assay means may comprise antigenic peptides from more than one genotype, for example, a microwell or bead may be coated with peptides from more than one genotype.

[0106] It has been found advantageous to use more than one HCV antigen for testing, in particular, a combination comprising at least one antigenic peptide derived from the structural region of the genome and at least one antigenic peptide derived from the non-structural region, especially a combination of a core antigen and at least one antigen selected from the NS3, NS4 and NS5 regions. The wells or beads may be coated with the antigens individually. It has been found advantageous, however, to fuse two or more antigenic peptides as a single polypeptide, preferably as a recombinant fusion polypeptide. Advantages of such an approach are that the individual antigens can be combined in a fixed, predetermined ratio (usually equimolar) and that only a single polypeptide needs to be produced, purified and characterised. One or more such fusion polypeptides may be used in an assay, if desired in addition to one or more unfused peptides. It will be appreciated that there are many possible combinations of antigens in a fusion polypeptide, for example, a fusion polypeptide may comprise a desired range of antigens from one serotype only, or may comprise antigens from more than one serotype. The antigenic peptides from serotypes 2, 3 and 4 are preferably those described herein.

[0107] To obtain a polypeptide comprising multiple peptide antigens, it is preferred to fuse the individual coding sequences into a single open reading frame. The fusion should, of course, be carried out in such a manner that the antigenic activity of each component peptide is not significantly compromised by its position relative to another peptide. Particular regard should of course be had for the nature of the sequences at the actual junction between the peptides. The resulting coding sequence can be expressed, for example, as described above in relation to recombinant peptides in general. The methods by which such a fusion polypeptide can be obtained are known in the art, and the production of a recombinant fusion polypeptide comprising multiple antigens of a strain of HCV type 1 is described in GB-A-2 239 245 immunoprecipitation. Peptide conjugates may be used in amplified assays and IgG/IgM antibody capture ELISA.

[0108] The peptide of the present invention may be incorporated into a vaccine formulation for inducing immunity to HCV in man. For this purpose the peptide may be presented in association with a pharmaceutically acceptable carrier.

[0109] For use in a vaccine formulation, the peptide may optionally be presented as part of an hepatitis B core fusion particle, as described in Clarke et al (*Nature*, 1987, 330, 381-384), or a polylysine based polymer, as described in Tam (PNAS, 1988, 85, 5409-5413). Alternatively, the peptide may optionally be attached to a particulate structure, such as liposomes or ISCOMS.

[0110] Pharmaceutically acceptable carriers include liquid media suitable for use as vehicles to introduce the peptide into a patient. An example of such liquid media is saline solution. The peptide may be dissolved or suspended as a

solid in the carrier.

[0111] The vaccine formulation may also contain an adjuvant for stimulating the immune response and thereby enhancing the effect of the vaccine. Examples of adjuvants include aluminium hydroxide and aluminium phosphate.

[0112] The vaccine formulation may contain a final concentration of peptide in the range from 0.01 to 5 mg/ml, preferably from 0.03 to 2 mg/ml. The vaccine formulation may be incorporated into a sterile container, which is then sealed and stored at a low temperature, for example 4°C, or may be freeze-dried.

[0113] In order to induce immunity in man to HCV, one or more doses of the vaccine formulation may be administered. Each dose may be 0.1 to 2 ml, preferably 0.2 to 1 ml. A method for inducing immunity to HCV in man, comprises the administration of an effective amount of a vaccine formulation, as hereinbefore defined.

[0114] The present invention also provides the use of a peptide as herein defined in the preparation of a vaccine for use in the induction of immunity to HCV in man.

[0115] Vaccines of the present invention may be administered by any convenient method for the administration of vaccines including oral and parenteral (e.g. intravenous, subcutaneous or intramuscular) injection. The treatment may consist of a single dose of vaccine or a plurality of doses over a period of time.

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1-3.

SEQUENCE LISTING (FIGURE 1 and 1a)

5 **[0117]**

SEQ ID NO : 1

SEQUENCE TYPE : Nucleotide cDNA sequence

10

SEQUENCE LENGTH : 194 base pairs

STRANDEDNESS : single

15

TOPOLOGY : linear

MOLECULAR TYPE : genomic DNA from HCV types 1-3

ORIGINAL SOURCE

20

[0118]

ORGANISM : human blood samples

25

USE :

DEPOSIT :

FEATURES

30

[0119] Bases - 255 to -62 of the 5' non-coding region showing variations in cDNA sequence between HCV types 1-3.

SEQUENCE LISTING (FIGURE 3)

35 **[0120]**

SEQ ID NO : 2

SEQUENCE TYPE : deduced peptide sequence

40

SEQUENCE LENGTH : 85 amino acids

STRANDEDNESS : single

45

TOPOLOGY : linear

MOLECULAR TYPE : HCV peptide

ORIGINAL SOURCE

50

[0121]

ORGANISM : human blood samples

55

USE :

DEPOSIT :

FEATURES

[0122] Amino acids 2648 to 2732 of NS-5 region showing variations in peptide sequence between HCV types 1-3.

5 SEQUENCE LISTING (FIGURE 5)

[0123]

SEQ ID NO : 3

10

SEQUENCE TYPE : deduced peptide sequence

SEQUENCE LENGTH : 57 amino acids

15

STRANDEDNESS : single

TOPOLOGY : linear

MOLECULAR TYPE : HCV peptide

20

ORIGINAL SOURCE

[0124]

ORGANISM : human blood samples

USE :

DEPOSIT :

30

FEATURES

[0125] Amino acids 1577 to 1633 of NS-3 region showing variations in peptide sequence between HCV types 1-3.

35 SEQUENCE LISTING (FIGURE 7)

[0126]

SEQ ID NO : 4

40

SEQUENCE TYPE : deduced peptide sequence

SEQUENCE LENGTH : 124 amino acids

45

STRANDEDNESS : single

TOPOLOGY : linear

MOLECULAR TYPE : HCV peptide

50

ORIGINAL SOURCE

[0127]

ORGANISM : human blood samples

USE :

55

DEPOSIT :

FEATURES

5 **[0128]** Amino acids 5 to 128 of core region showing variations in peptide sequence between HCV types 1-3.

SEQUENCE LISTING (FIGURE 9a)

[0129]

10

SEQ ID NO : 5

SEQUENCE TYPE : Nucleotide sequence

15

SEQUENCE LENGTH : 367 base pairs

STRANDEDNESS : single

TOPOLOGY : linear

20

MOLECULAR TYPE : HCV genomic DNA

ORIGINAL SOURCE

25

[0130]

ORGANISM : human blood samples

USE :

30

DEPOSIT :

FEATURES

35

[0131] Bases 4911 to 5277 of NS-4 region of HCV-3 showing individual variations and consensus sequence.

SEQUENCE LISTING (FIGURE 9b)

[0132]

40

SEQ ID NO : 6

SEQUENCE TYPE : deduced peptide sequence

45

SEQUENCE LENGTH : 128 amino acids

STRANDEDNESS : single

TOPOLOGY : linear

50

MOLECULAR TYPE : HCV genomic DNA

ORIGINAL SOURCE

55

[0133]

ORGANISM : human blood samples

USE :

DEPOSIT :

5 FEATURES

[0134] Amino acids 1638 to 1765 of NS-4 region of HCV-3 showing individual variations and consensus sequence.

10 SEQUENCE LISTING (FIGURE 10a and 10b)

[0135]

SEQ ID NO : 7

15 SEQUENCE TYPE : peptide sequence

SEQUENCE LENGTH : 90 amino acids

20 STRANDEDNESS : single

TOPOLOGY : linear

MOLECULAR TYPE : HCV peptide

25 ORIGINAL SOURCE

[0136]

30 ORGANISM : human blood samples

USE :

DEPOSIT :

35 FEATURES

[0137] Amino acids 1679 to 1768 of NS-4 region of HCV1-3 showing individual variations and consensus sequence.

40 SEQUENCE LISTING (FIGURE 11a and 11c)

[0138]

SEQ ID NO : 8

45 SEQUENCE TYPE : Nonameric peptide sequences

SEQUENCE LENGTH : 9 amino acids each

50 STRANDEDNESS : single

TOPOLOGY : linear

MOLECULAR TYPE : nonameric HCV peptides

55

ORIGINAL SOURCE

[0139]

5 ORGANISM : synthetic

USE : epitope mapping

10 DEPOSIT :

FEATURES

[0140] Nonapeptides corresponding to NS4 region of HCV1-3 used for epitope mapping.

15 SEQUENCE LISTING (FIGURE 13)

[0141]

20 SEQ ID NO : 9

SEQUENCE TYPE : Nucleotide cDNA sequences

SEQUENCE LENGTH : 30, 70 and 32 base pairs

25 STRANDEDNESS : single

TOPOLOGY : linear

30 MOLECULAR TYPE : genomic DNA from HCV types 1 to 4

ORIGINAL SOURCE

[0142]

35 ORGANISM : human blood samples

USE :

40 DEPOSIT :

FEATURES

[0143] Bases -245 to -216; -185 to -116; and -101 to -70 in the 5'NCR region of HCV types 1-4.

45 SEQUENCE LISTING (FIGURE 15A and 15B)

[0144]

50 SEQ ID NO : 10

SEQUENCE TYPE : Nucleotide with derived protein sequence

SEQUENCE LENGTH : 240 base pairs

55 STRANDEDNESS : single

TOPOLOGY : linear

MOLECULAR TYPE : genomic DNA

ORIGINAL SOURCE

[0145]

ORGANISM : human blood samples

USE :

DEPOSIT :

FEATURES

[0146] Base pairs 23 - 262 and amino acids 5 to 89 of the core region of HCV types 1 - 4.

Claims

1. A method of in vitro testing a sample for the presence of an anti-HCV antibody, which comprises contacting the sample with at least one HCV-3 or HCV-4 type-specific NS-4 antigen that comprises a sequence of amino acids from the region consisting of amino acids 1691 to 1708 inclusive or 1710 to 1728 inclusive, and detecting any resulting antibody-antigen complex.
2. A method as claimed in claim 1, wherein the HCV antigen is HCV-3 type-specific.
3. A method as claimed in claim 1 or claim 2, wherein the sample is contacted with at least one type-specific HCV NS-4 antigen from each of two or more HCV types including said at least one HCV-3 or HCV-4 type-specific antigens.
4. A method as claimed in claim 1 or claim 2, wherein the sample is contacted separately with each member of a series of type-specific HCV NS-4 antigens including said at least one HCV-3 or HCV-4 antigen, each member of the series comprising type-specific HCV NS-4 antigen(s) from a different HCV type.
5. A method as claimed in any one of claims 1 to 4, wherein the sample is also contacted with a blocking amount of heterologous-type HCV oligopeptide antigens.
6. A method as claimed in any one of claims 1 to 5, wherein the HCV NS-4 antigen is or comprises an oligopeptide consisting of amino acids 1691 to 1708 or amino acids 1710 to 1728.
7. A method as claimed in any one of claims 3 to 5, wherein a type-specific HCV-1 NS-4 antigen is selected from the following oligopeptides:

**KPA (V/I) IPDREVLYREFDEM, RPAV (I/V) PDREVLYQEFDEM,
ECSQHLPYIEG (M/A) AEQF.**

8. A method as claimed in any one of claims 3 to 5 wherein a type-specific HCV-2 NS-4 antigen is selected from the following oligopeptides:

**R (A/V) V (V/I) (A/T) PDKE (I/V) LYEAFDEM,
ECAS (K/R) AALIEEGQR (M/I) AEML.**

9. A method as claimed in any one of claims 1 to 5, wherein a type-specific HCV-3 NS-4 antigen is selected from the following oligopeptides:

KPALVPDKEVLYQQYDEM, ECSQAAPYIEQAQVIAHQF.

10. A method as claimed in any one of claims 3 to 5, wherein the sample is contacted with HCV-1, HCV-2 and HCV-3 antigens selected from the oligopeptides defined in claims 7 to 9.

11. A method as claimed in claim 3 or any one of claims 5 to 10 when dependent on claim 3, wherein the antigen(s) from each HCV type are immobilised on a common solid substrate.

12. A method as claimed in claim 4 or any one of claims 5 to 10 when dependent on claim 4, wherein the antigen(s) from each HCV type is (are) immobilised on a separate solid substrate or at a separate location on a common solid substrate.

13. A method as claimed in any one of claims 1 to 12, wherein the sample is contacted with one or more other HCV antigens in addition to the type-specific HCV NS-4 antigen(s).

14. A method as claimed in claim 13, wherein the other HCV antigens are antigens used in blood screening.

15. A method of in vitro HCV antibody typing in a test sample comprising the steps of:

- (a) contacting the sample with a plurality of HCV antigens wherein at least one of said antigens is an HCV-3 or HCV-4 type-specific antigen that comprises a sequence of amino acids from the region consisting of amino acids 1691 to 1708 inclusive or 1710 to 1728 inclusive;
- (b) detecting any resulting antigen-antibody complexes; and
- (c) determining the HCV type in the test sample.

16. A type-specific isolated HCV-3 or HCV-4 antigen that comprises a sequence of amino acids from the region consisting of amino acids 1691 to 1708 inclusive or 1710 to 1728 inclusive.

17. A solid substrate on which is immobilised at least one type-specific antigen as defined in claim 16.

18. A solid substrate as claimed in claim 17, on which is immobilised the HCV antigens from all the HCV types used.

19. A series of solid substrates or a series of locations on a common solid substrate, on which substrate or location respectively is immobilised one or more type specific antigens as defined in claim 16, the HCV NS-4 antigen(s) immobilised at each substrate or location being from a different HCV type.

20. A solid substrate as claimed in claim 17 or 18, on which is also immobilised one or more other HCV antigens.

21. A solid substrate as claimed in claim 20, wherein the other HCV antigens are HCV antigens used for blood screening.

22. A kit comprising a solid substrate or series of solid substrates as claimed in any one of claims 17 to 21 and a series of blocking solutions, each blocking solution comprising a blocking amount of heterologous-type oligopeptide antigens.

Patentansprüche

1. Verfahren zum in-vitro-Testen einer Probe auf Vorhandensein eines Anti-HCV-Antikörpers, welches Verfahren umfasst: Kontaktieren der Probe mit mindestens einem HCV-3- oder HCV-4-Typ-spezifischen NS-4-Antigen, das eine Sequenz von Aminosäuren aus dem Bereich aufweist, bestehend aus Aminosäuren 1691 bis 1708 einschließlich oder 1710 bis 1728 einschließlich; und Detektieren eines etwaigen resultierenden Antikörper-Antigenkomplexes.

2. Verfahren nach Anspruch 1, bei welchem das HCV-Antigen HCV-3-Typspezifisch ist.
3. Verfahren nach Anspruch 1 oder 2, bei welchem die Probe mit mindestens einem Typ-spezifischen HCV-NS-4-Antigen von jeweils zwei oder mehreren HCV-Typen kontaktiert wird, einschließlich mindestens einem der HCV-3- oder HCV-4-Typspezifischen Antigene.
4. Verfahren nach Anspruch 1 oder 2, bei welchem die Probe separat mit jedem Vertreter einer Reihe von Typ-spezifischen HCV-NS-4-Antigenen kontaktiert wird, einschließlich mindestens ein HCV-3- oder HCV-4-Antigen, wobei jeder Vertreter der Reihe Typ-spezifisches/Typ-spezifische HCV-NS-4-Antigen(e) von einem anderen HCV-Typ aufweist.
5. Verfahren nach einem der Ansprüche 1 bis 4, bei welchem die Probe ebenfalls mit einer blockierenden Menge vom heterologen Typ von HCV-Oligopeptid-Antigenen kontaktiert wird.
6. Verfahren nach einem der Ansprüche 1 bis 5, bei welchem das HCV-NS-4-Antigen ein Oligopeptid ist oder aufweist, bestehend aus Aminosäuren 1691 bis 1708 oder Aminosäuren 1710 bis 1728.
7. Verfahren nach einem der Ansprüche 3 bis 5, bei welchem ein Typ-spezifisches HCV-1 eines 4-Antigen ausgewählt ist aus den folgenden Oligopeptiden:

KPA (V/I) IPDREVLRYREFDEM, RPAV (I/V) PDREVLRYQEFDEM,
ECSQHLPYIEG (M/A) AEQF.

8. Verfahren nach einem der Ansprüche 3 bis 5, bei welchem ein Typ-spezifisches HCV-2-NS-4-Antigen ausgewählt ist aus den folgenden Oligopeptiden:

R (A/V) V (V/I) (A/T) PDKE (I/V) LYEAFDEM, ECAS (K/R) AALIEEGQR
(M/I) AEML.

9. Verfahren nach einem der Ansprüche 1 bis 5, bei welchem ein Typ-spezifisches HCV-3-NS-4-Antigen ausgewählt ist aus den folgenden Oligopeptiden:

KPALVPDKEVLRYQQYDEM, ECSQAAPYIEQAQVIAHQF.

10. Verfahren nach einem der Ansprüche 3 bis 5, bei welchem die Probe kontaktiert wird mit HCV-1-, HCV-2- und HCV-3-Antigenen, ausgewählt aus den in Anspruch 7 bis 9 festgelegten Oligopeptiden.
11. Verfahren nach Anspruch 3 oder einem der Ansprüche 5 bis 10, sofern abhängig von Anspruch 3, bei welchem das/die Antigen(e) von jedem HCV-Typ auf einem gemeinsamen festen Substrat immobilisiert sind.
12. Verfahren nach Anspruch 4 oder einem der Ansprüche 5 bis 10, sofern abhängig von Anspruch 4, bei welchem das/die Antigen(e) von jedem HCV-Typ immobilisiert ist/sind auf einem separaten festen Substrat oder an einer separaten Stelle auf einem gemeinsamen festen Substrat.
13. Verfahren nach einem der Ansprüche 1 bis 12, bei welchem die Probe kontaktiert wird mit einem oder mehreren anderen HCV-Antigenen zusätzlich zu dem/den Typ-spezifischen HCV-NS-4-Antigen(en).
14. Verfahren nach Anspruch 13, bei welchem die anderen HCV-Antigene Antigene sind, die beim Blut-Screening verwendet werden.
15. Verfahren zum in-vitro-HCV-Antikörper-Typisieren in einer Testprobe, umfassend die Schritte:

(a) Kontaktieren der Probe mit einer Mehrzahl von HCV-Antigenen, wobei mindestens eines der Antigene ein HCV-3- oder HCV-4-Typ-spezifisches Antigen ist, das eine Sequenz von Aminosäuren aus dem Bereich aufweist, bestehend aus Aminosäuren 1691 bis 1708 einschließlich 1710 bis 1728 einschließlich;

(b) Detektieren etwaiger resultierender Antigen-Antikörper-Komplexe; und

(c) Bestimmen des HCV-Typs in der Testprobe.

16. Typ-spezifisches, isoliertes HCV-3- oder HCV-4-Antigen, das eine Sequenz von Aminosäuren aus dem Bereich aufweist, bestehend aus Aminosäuren 1691 bis 1708 einschließlich oder 1710 bis 1728 einschließlich.

17. Festes Substrat, auf dem mindestens ein Typ-spezifisches Antigen nach der Festlegung in Anspruch 16 immobilisiert ist.

18. Festes Substrat nach Anspruch 17, auf dem die HCV-Antigene von allen verwendeten HCV-Typen immobilisiert sind.

19. Reihe fester Substrate oder Reihe von Lokalisierungen auf einem gemeinsamen festen Substrat, wobei auf dem Substrat bzw. der Lokalisierung ein oder mehrere Typspezifische Antigene immobilisiert sind, wie in Anspruch 16 festgelegt wurde, wobei das/die HCV-NS-4-Antigen(e), das/die auf dem jeweiligen Substrat oder der Lokalisierung immobilisiert ist/sind von einem anderen HCV-Typ stammen.

20. Festes Substrat nach Anspruch 17 oder 18, auf dem ebenfalls ein oder mehrere andere HCV-Antigene immobilisiert sind.

21. Festes Substrat nach Anspruch 20, wobei die anderen HCV-Antigene HCV-Antigene sind, die für das Blut-Screening verwendet werden.

22. Satz, aufweisend ein festes Substrat oder eine Reihe von festen Substraten nach einem der Ansprüche 17 bis 21 und eine Reihe von Lösungen zum Blockieren, wobei jede Lösung eine blockierende Menge an heterologem Typ von Oligopeptid-Antigenen aufweist.

Revendications

1. Procédé d'essai *in vitro* d'un échantillon quant à la présence d'un anticorps anti-HCV, qui comprend la mise en contact de l'échantillon avec au moins un antigène NS-4 spécifique au type HCV-3 ou HCV-4 qui comprend une séquence d'acides aminés de la région constituée par les acides aminés 1691 à 1708 inclusivement ou 1710 à 1728 inclusivement, et la détection de tout complexe anticorps-antigène résultant.

2. Procédé selon la revendication 1, dans lequel l'antigène de HCV est spécifique au type HCV-3.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel l'échantillon est mis en contact avec au moins un antigène NS-4 spécifique à un type de HCV parmi chacun de deux ou plusieurs types de HCV incluant ledit au moins un antigène spécifique au type HCV-3 ou HCV-4.

4. Procédé selon la revendication 1 ou la revendication 2, dans lequel l'échantillon est mis en contact séparément avec chaque élément d'une série d'antigènes NS-4 spécifiques à un type de HCV incluant ledit au moins un antigène de HCV-3 ou HCV-4, chaque élément de la série comprenant un (des) antigène(s) NS-4 spécifique(s) à un type de HCV, provenant d'un type de HCV différent.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel l'échantillon est aussi mis en contact avec une quantité bloquante d'antigènes oligopeptidiques de HCV de type hétérologue.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel l'antigène NS-4 de HCV est ou comprend un oligopeptide constitué par les acides aminés 1691 à 1708 ou les acides aminés 1710 à 1728.

7. Procédé selon l'une quelconque des revendications 3 à 5, dans lequel un antigène NS-4 spécifique au type HCV-

1 est choisi parmi les oligopeptides suivants:

**KPA (V/I) IPDREVLYREFDEM, RPAV (I/V) PDREVLYQEFDEM,
ECSQHLPYIEG (M/A) AEQF.**

8. Procédé selon l'une quelconque des revendications 3 à 5, dans lequel un antigène NS-4 spécifique au type HCV-2 est choisi parmi les oligopeptides suivants:

**R (A/V) V (V/I) (A/T) PDKE (I/V) LYEAFDEM, ECAS (K/R) AALIEEGQR
(M/I) AEML.**

9. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel un antigène NS-4 spécifique au type HCV-3 est choisi parmi les oligopeptides suivants:

KPALVPDKEVLYQQYDEM, ECSQAAPYIEQAQVIAHQF.

10. Procédé selon l'une quelconque des revendications 3 à 5, dans lequel l'échantillon est mis en contact avec des antigènes de HCV-1, HCV-2 et HCV-3 choisis parmi les oligopeptides définis dans les revendications 7 à 9.

11. Procédé selon la revendication 3 ou l'une quelconque des revendications 5 à 10 lorsqu'elles dépendent de la revendication 3, dans lequel le(s) antigène(s) de chaque type de HCV est (sont) immobilisé(s) sur un substrat solide commun.

12. Procédé selon la revendication 4 ou l'une quelconque des revendications 5 à 10 lorsqu'elles dépendent de la revendication 4, dans lequel le(s) antigène(s) de chaque type de HCV est (sont) immobilisé(s) sur un substrat solide séparé ou à un emplacement séparé sur un substrat solide commun.

13. Procédé selon l'une quelconque des revendications 1 à 12, dans lequel l'échantillon est mis en contact avec un ou plusieurs autres antigènes de HCV en plus du (des) antigène(s) NS-4 spécifique(s) à un type de HCV.

14. Procédé selon la revendication 13, dans lequel les autres antigènes de HCV sont des antigènes utilisés dans un dépistage sanguin.

15. Procédé de typage d'anticorps anti-HCV *in vitro* dans un échantillon d'essai, comprenant les étapes consistant:

(a) à mettre en contact l'échantillon avec plusieurs antigènes de HCV dans lesquels au moins l'un desdits antigènes est un antigène spécifique au type HCV-3 ou HCV-4 qui comprend une séquence d'acides aminés de la région constitué par les acides aminés 1691 à 1708 inclusivement ou 1710 à 1728 inclusivement;

(b) à détecter tous complexes antigène-anticorps résultants; et

(c) à déterminer le type de HCV dans l'échantillon d'essai.

16. Antigène de HCV-3 ou HCV-4 isolé spécifique à un type, qui comprend une séquence d'acides aminés de la région constituée par les acides aminés 1691 à 1708 inclusivement ou 1710 à 1728 inclusivement.

17. Substrat solide sur lequel est immobilisé au moins un antigène spécifique à un type tel que défini dans la revendication 16.

18. Substrat solide selon la revendication 17, sur lequel sont immobilisés les antigènes de HCV de tous les types de HCV utilisés.

19. Série de substrats solides ou série d'emplacements sur un substrat solide commun, substrat ou emplacement sur

lequel est (sont) immobilisé(s) respectivement un ou plusieurs antigènes spécifiques à un type tels que définis dans la revendication 16, le(s) antigène(s) NS-4 de HCV immobilisé(s) à chaque substrat ou emplacement étant d'un type de HCV différent.

5 **20.** Substrat solide selon la revendication 17 ou 18, sur lequel est aussi immobilisé un ou plusieurs autres antigènes de HCV.

10 **21.** Substrat solide selon la revendication 20, dans lequel les autres antigènes de HCV sont des antigènes de HCV utilisés pour un dépistage sanguin.

15 **22.** Nécessaire comprenant un substrat solide ou une série de substrats solides tels que revendiqués dans l'une quelconque des revendications 17 à 21 et une série de solutions bloquantes, chaque solution bloquante comprenant une quantité bloquante d'antigènes oligopeptidiques de type hétérologue.

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FIGURE 1

	-255	-235	-215	-195	-175	-156
E-b1	GGCGTTAGTA	CGAGTGTCTG	GCACGCTCCA	GGACGCGCG	TCTGCGGAGC	CGGTGAATAC
E-b2
E-b3
E-b4
E-b5
E-b6
E-b7
E-b8
2	K2a	T.....A.
E-b9
E-b10
E-b11
E-b12
2	K2b-1	T.....A.
E-b13
E-b14
E-b15
E-b16
E-b17
E-b18
1	HCV-1
1	PC-1
1	H77
1	H90
1	GM1
1	GM2
1	J1
1	A1
1	B1
1	T1
1	U18/124
1	HCV-J
1	HCV-BK
1	HC-J1
1	HC-J4

FIGURE 1a

	-155	-135	-115	-95	-75	-62
E-b1	CGGCTCTTT	CTGGAGCAA	CCGCTCAAT	ACCCAGAAAT	TTGGCGTGC	CCCCGAGAA
E-b2
E-b3
E-b4
E-b5
E-b6
E-b7
E-b8
K2a	T.....TA..	...A..T..	G..G.TC..A..
E-b9	T.....TA..	...A..T..	G..G.CC..A..
E-b10	T.....TA..	...A..T..	G..G.CC..A..
E-b11	T.....TA..	...A..T..	GT..G.TC..A..
E-b12	T.....TA..	...A..T..	GT..G.TC..A..
K2b-1	T.....TA..	...A..T..	GT..G.TC..A..
E-b13T..	G.TG..G..A..
E-b14T..	G.TG..G..A..
E-b15T..	G.TG..G..A..
E-b16TT..	G.TG..G..A..
E-b17TT..	G.TG..G..A..
E-b18T..	G.TG..G..A..
HCV-1T..	G.TG..G..A..
PC-1TA..	G.TG..G..A..
H77TA..	G.TG..G..A..
H90TA..	G.TG..G..A..
GM1TA..	G.TG..G..A..
GM2TA..	G.TG..G..A..
J1TA..	G.TG..G..A..
A1TA..	G.TG..G..A..
S1TA..	G.TG..G..A..
T1TA..	G.TG..G..A..
U18/124TA..	G.TG..G..A..
HCV-JT..	G.TG..G..A..
HCV-BKT..	G.TG..G..A..
HC-J1TA..	G.TG..G..A..
HC-J4T..	G.TG..G..A..

FIGURE 2

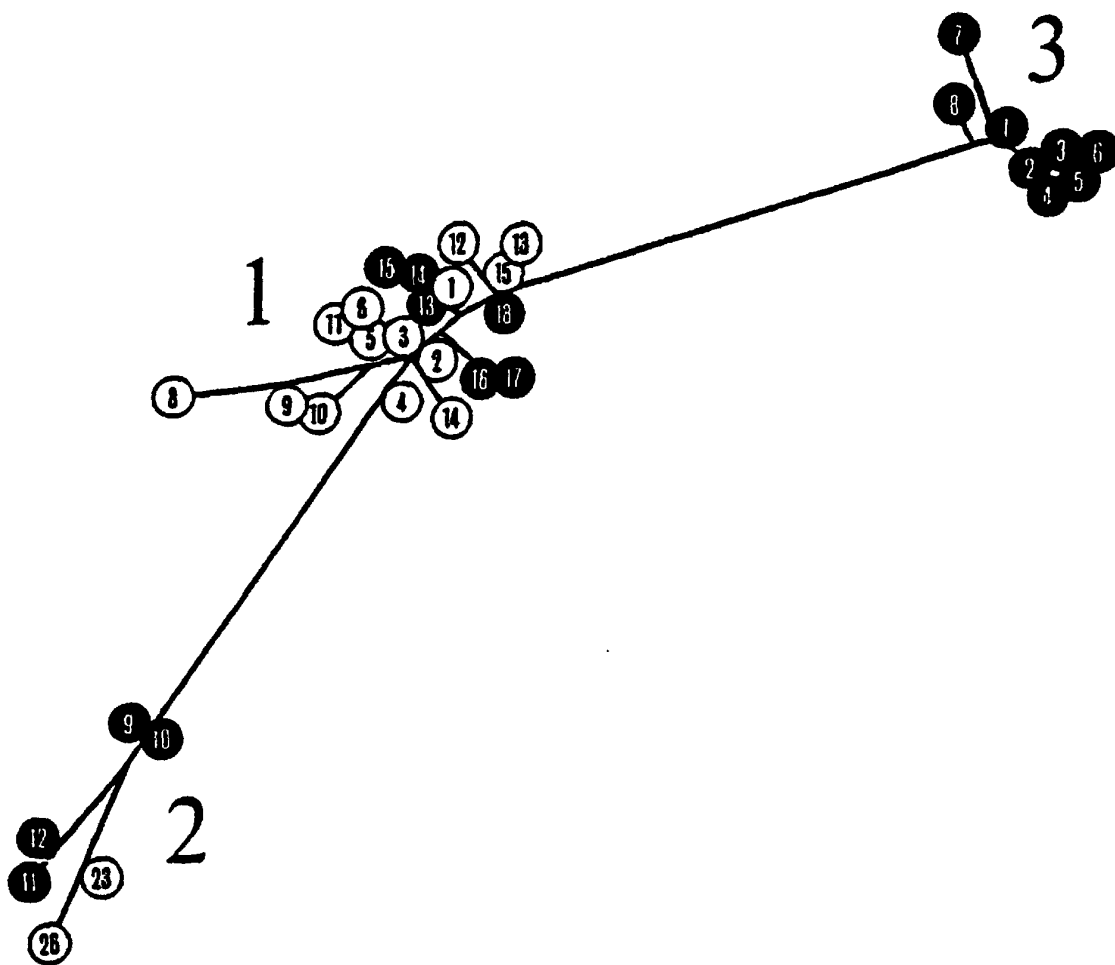


FIGURE 3

	2648	2668	2688	2708	2732				
	VTEQDIRVME	MITOCCHLEP	EARKVISSLT	ERLYCOOPNF	MSKGAOCOTR	RCRASGVLPFT	SFGNTITCYI	KATAACEAAG	LRNPD
E-b1									
E-b2									
E-b3									
E-b7									
K2a	...R...T...	S...RA.S.PE	...HIA.H...	...V...V...	...QT...	...T...	...M...V...	...L...K...	IVA.S
K2a-1	...R...T...	S...A.S.PE	...TA.H...	...V...V...	...QT...	...T...	...M...V...	...L...K...	IVA.T
E-b12	..	S...A.S.PQ	...T.H...	...V...T...	...QS...	...PT...	...M...M...	...L...K...	IVD.V
K2b	...R...T...	S...A.S.PQ	...TA.H...	...V...T...	...QS...	...PT...	...M...M...	...L...K...	IVD.I
K2b-1	...R...T...	S...A.S.PE	...T.H...	...V...T...	...QS...	...PT...	...M...M...	...L...K...	IVD.I
HCV-1	...S...T...	A...D.D.	Q.VA.K...	...V...LT	...R.EN...	...T...	...C...L...	...R...R...	QDCT
Pt-1	...S...T...	A...D.D.	Q.VA.K...	...V...LT	...R.EN...	...T...	...C...L...	...R...R...	QDCT
H77	...S...T...	A...D.D.	Q.VA.K...	...V...LT	...R.EN...	...T...	...C...L...	...R...R...	QDCT
H90	...S...T...	A...D.D.	Q.VA.K...	...V...LT	...R.EN...	...T...	...C...L...	...R...R...	QDCT
HCV-J	...N...T...	S...D.A.	...QA.R...	...V...LT	...ON...	...T...	...C...L...L	...R...K	QDCT
HCV-BK	...N...T...	S...D.A.	...QA.R...	...I...LT	...ON...	...T...	...C...L...L	...R...K	QDCT
K1	...N...	S...S.D.A.	...QA.R...	...I...LT	...ON...	...T...	...C...L...L	...R...K	QDCT
K1-1	...N...	S...D.A.	...QA.R...	...I...LT	...ON...	...T...	...C...L...L	...R...K	QDCT
K1-2	...N...	S...D.A.	...QA.R...	...I...LT	...ON...	...T...	...C...L...L	...R...K	QDCT
K1-3	...N...	S...D.A.	...QA.R...	...I...LT	...ON...	...T...	...C...L...L	...R...K	QDCT
K1-4	...N...	S...D.A.	...QA.R...	...I...LT	...ON...	...T...	...C...L...L	...R...K	QDCT

FIGURE 4

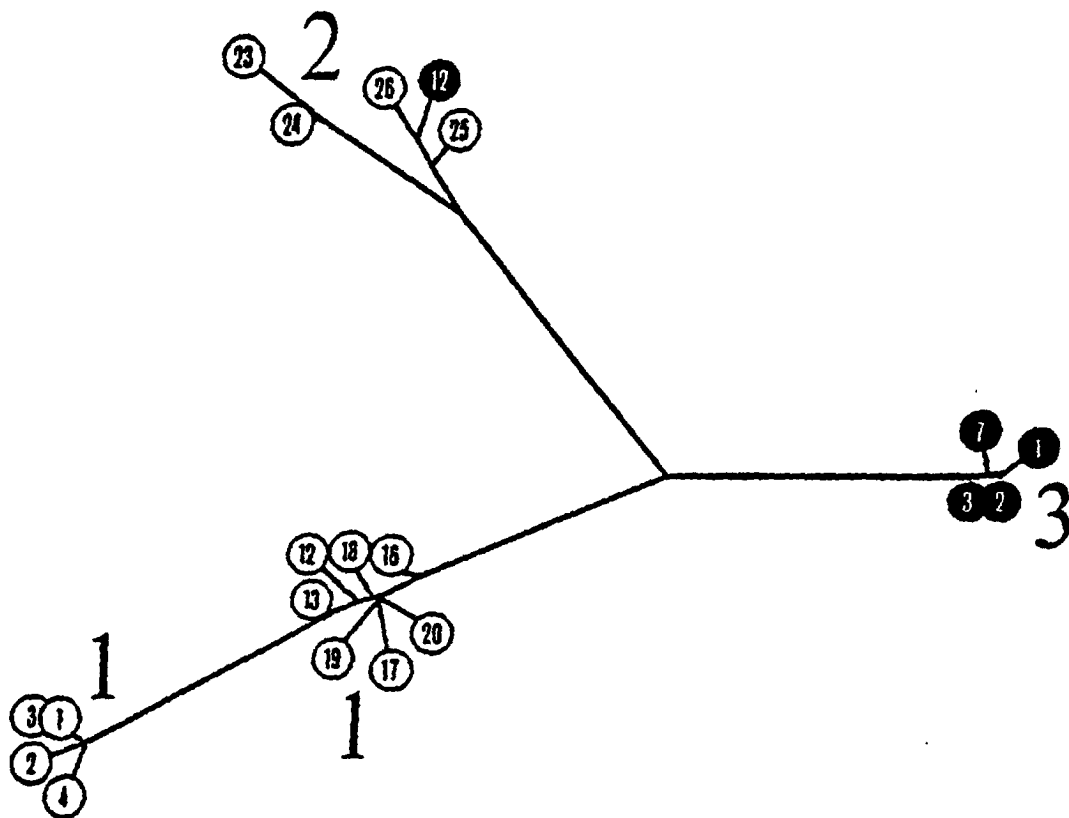


FIGURE 5

	1577	1597	1617	1633
E-b1	KQGLNFBYL	TAIQATVCAR	AQALPPSNDR	TWKCLVRLKP
E-b2P.....
E-b6P.....
E-b7	A.....	...P.....
?	Clone A	K.P.....
E-b16
E-b17	V.....	...P.....
Group	1/1	V.....	...P.....
	1/2	V.....	...P.....
	1/3	V.....	...P.....
	1/4	V.....	...P.....
	1/4	V.....	...P.....
	1/4	V.....	...P.....
HCV-1P.....
H77	V.....	...P.....
H90	V.....	...P.....
HCV-J	V.....	...P.....
HCV-BK	V.....	...P.....
JH	V.....	...P.....

FIGURE 6

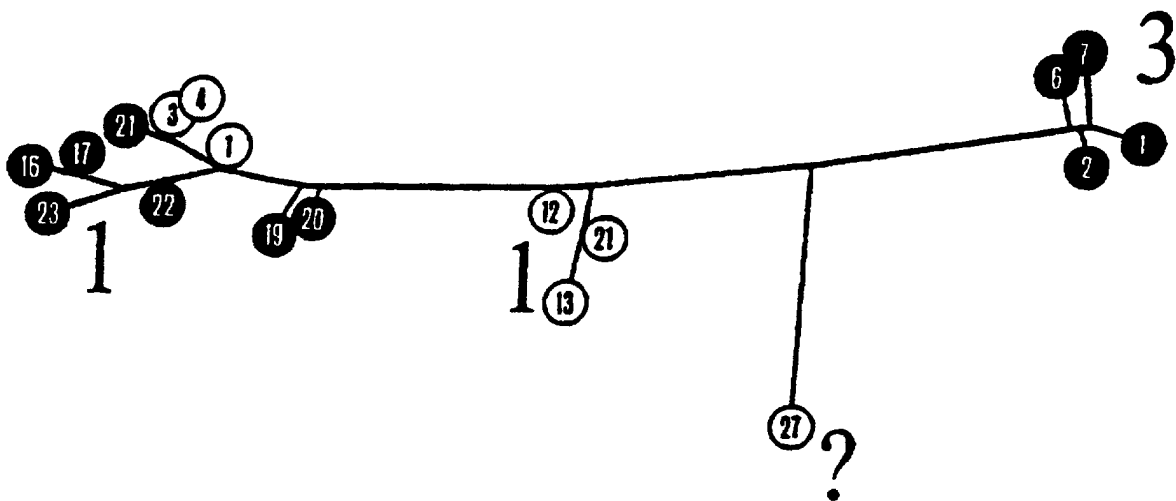


FIGURE 7

	5	25	45	64		
	AKPQRKTKRN	TIRRPQDVKE	PGGGQIVGGV	YVLPRRGPERL	GVCATRTKTSB	RSQPRGRRQP
E-b1						
HCV-1	P...K.N...	N.....	L.....	R.....
H77	P.....	N.....	L.....	R.....
H90	P.....	N.....	L.....	R.....
GM1	N.....	L.....	R.P.....
GM2	N.....	L.....	R.....
HCV-J	P.....	N.....	L.....	R.....
HCV-BK	P.....	N.....	L.....	R.P.....
HC-J1	P.....	N.....	L.....	R.....
HC-J4	P.....	N.....	L.....	R.....	W.....
JH	P.....	N.....	L.....	R.....
J7	P.....	N.....	L.....	R.....

	5	85	105	128			
	IPKARRSEGR	SNAQPGYPWP	LYGNEGCGWA	GWLLSPRGSR	PBWGPNDPRR	RSRNLGRVID	TLTW
E-b1							
HCV-1P...	T.....	T.....	C.....
H77P...	T.....	T.....	C.....
H90P...	T.....	M.....	T.....	C.....
GM1P...	T.....	T.....	C.....
GM2L...	T.....	T.....	C.....
HCV-JP...	T.....	M.....	T.....	C.....
HCV-BKP...	T.....	L.....	T.....	C.....
HC-J1	V.P...	T.....	T.....	C.....
HC-J4P...	A.....	L.....	T.....	C.....
JHP...	T.....	L.....	T.....	C.....
J7P...	T.....	L.....	C.....

FIGURE 8

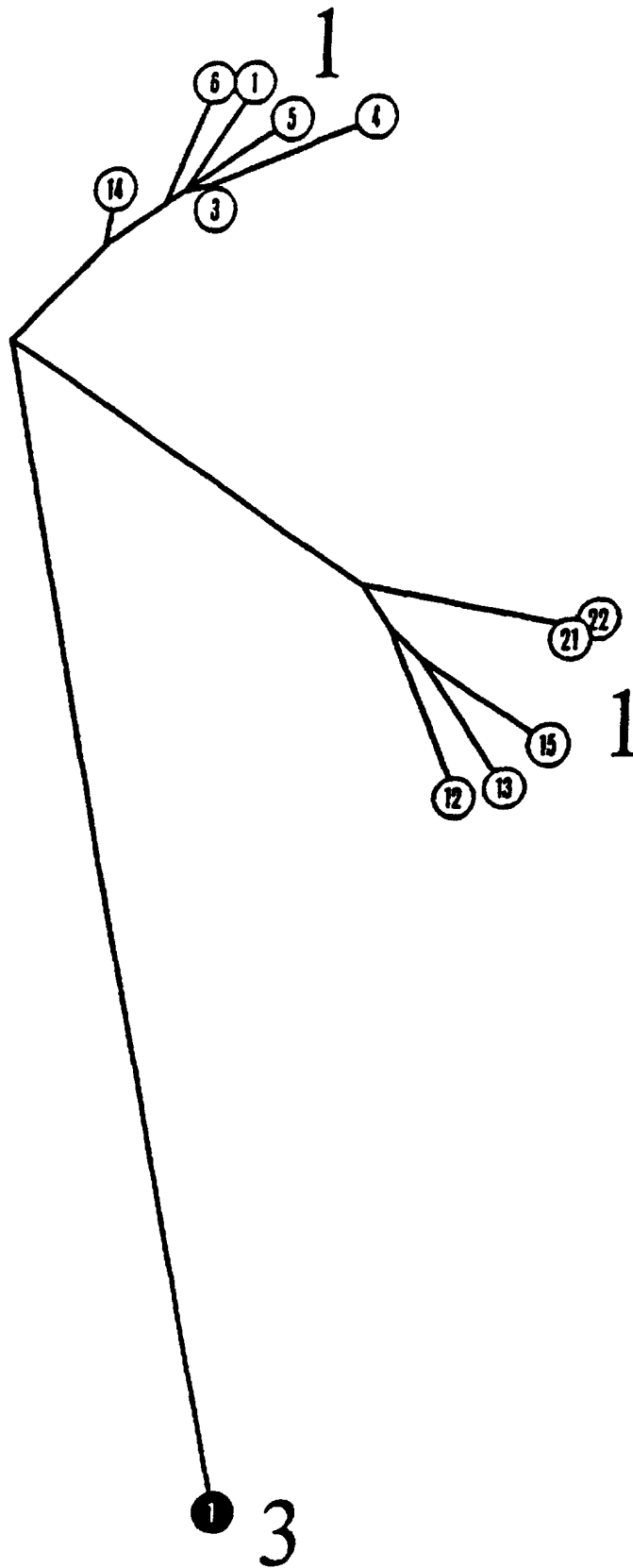


FIGURE 9a

NUCLEOTIDE SEQUENCES

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4911                                     4970
▼
T0040 GACACACCCU GUCACAAAUA ACAUCAUGGC AUGCAUGUCA GCUGAUCUGG AAGUAACCAC
T0038 ??CACACCCC GUCACAAAUA ACAUCAUGGC AUGCAUGUCA GCUGAUCUGG AAGUAACCAC
T0036 ??CACACCCC AUCACAAAUA ACAUCAUGGC AUGCAUGUCA GCUGAUCUGG AAGUAACCAC
T0026 ???CACCCC AUGCGGAAUA ACCUCAUGGC AUGCAUGUCA GCUGAUCUGG AAGUAACCAC
T1787 ???CACCCC AUCACAAAUA ACGUCAUGGC AUGCAUGUCA GCUGAUCUGG AAGUAACCAC
-----
Cons  GACACACCCU GUCACAAAUA ACAUCAUGGC AUGCAUGUCA GCUGAUCUGG AAGUAACCAC

4971                                     5030
▼
T0040 CAGCACCUGG GUGUUGCUUG GAGGGGUCCU CGCGGCCCUA GCGGCCUACU GCUUGUCAGU
T0038 CAGCACCUGG GUGUUGCUUG GAGGGGUCCU CGCGGCCCUA GCGGCCUACU GCUUGUCAGU
T0036 CAGCACCUGG GUGUUGCUUG GAGGGGUCCU CGCGGCCCUA GCGGCCUACU GCUUGUCAGU
T0026 CAGCACCUGG GUGUUGCUUG GAGGAGUCCU CGCUGCCCUA GCGGCCUACU GCUUGUCAGU
T1787 CAGCACCUGG GUGUUGCUUG GAGGGGUCCU CGCGGCCCUA GCGGCCUACU GCUUGUCAGU
-----
Cons  CAGCACCUGG GUGUUGCUUG GAGGGGUCCU CGCGGCCCUA GCGGCCUACU GCUUGUCAGU

5031                                     5090
▼
T0040 CGGCUGCGUU GUGAUUGUGG GUCAUAUUGA GCUGGGGGGC AAGCCGGCA AUCGUUCCAGA
T0038 CGGCUGCGUU GUGAUUGUGG GCCAUUUGA GCUGGGGGGC AAGCCGGCA CUCGUUCCAGA
T0036 CGGCUGCGUU GUGAUUGUGG GCCAUUUGA GCUGGGGGGC AAGCCGGCA CUCGUUCCAGA
T0026 CGGCUGCGUU GUGAUUGUGG GUCAUAUUGA GCUGGGGGGC AAGCCAGCA CUCGUUCCAGA
T1787 CGGCUGCGUU GUGAUUGUGG GUCAUAUUGA GCUGGGGGGC AAGCCGGCA CUCGUUCCAGA
-----
Cons  CGGCUGCGUU GUGAUUGUGG GUCAUAUUGA GCUGGGGGGC AAGCCGGCA AUCGUUCCAGA

5091                                     5150
▼
T0040 CAAAGAGGUG UUGUAUCAAC AAUACGAUGA GAUGGAGGAG UGCUCGCA GCUGCCCAUA
T0038 CAAAGAGGUG UUGUAUCAAC AAUACGAUGA GAUGGAGGAG UGCUCGCA GCCGCCCAUA
T0036 CAAAGAGGUG UUGUAUCAAC AAUACGAUGA GAUGGAGGAG UGCUCGCA GCCGCCCAUA
T0026 CAAAGAGGUG UUGUAUCAAC AAUACGAUGA GAUGGAGGAG UGCUCGCA GCCGCCCAUA
T1787 CAAAGAGGUG UUGUAUCAAC AAUACGAUGA GAUGGAGGAG UGCUCGCA GCCGCCCAUA
-----
Cons  CAAAGAGGUG UUGUAUCAAC AAUACGAUGA GAUGGAGGAG UGCUCGCA GCUGCCCAUA

5151                                     5210
▼
T0040 UAUCGAACAA GCUCAGGUGA UAGCCCACCA GUUCAAGGAG AAAGUCCU GGAUUGCUGCA
T0038 UAUCGAACAA GCUCAGUAA UAGCCCACCA GUUCAAGGAG AAAGUCCU GGAUUGCUGCA
T0036 UAUCGAACAA GCUCAGGUA UAGCCCACCA GUUCAAGGAG AAAGUCCU GGAUUGCUGCA
T0026 UAUCGAACAA GCUCAGGUA UAGCCCACCA GUUCAAGGAG AAAGUCCU GGAUUGCUGCA
T1787 UAUCGAACAA GCUCAGGUA UAGCCCACCA GUUCAAGGAG AAAGUCCU GGAUUGCUGCA
-----
Cons  UAUCGAACAA GCUCAGGUGA UAGCCCACCA GUUCAAGGAG AAAGUCCU GGAUUGCUGCA

5211                                     5270
▼
T0040 GCGAGCCACC CAACAACAAG CUGUUAUUGA GCCAUAGUA GCUACCAAC UGGCAAAAGCU
T0038 GCGAGCCACC CAACAACAAG CUGUUAUUGA GCCAUAGUA GCUACCAAC UGGCAAAAGCU
T0036 GCGAGCCACC CAACAACAAG CUGUUAUUGA GCCAUAGUA GCUACCAAC UGGCAAAA??
T0026 GCGAGCCACC CAACAACAAG CUGUCAUUGA GCCAUAGUA GCUACCAAC UGGCAAAA??
T1787 GCGAGCCACC CAACAACAGG CUGUCAUUGA GCCAUAGUA GCUACCAAC UGGCAAAAGCU
-----
Cons  GCGAGCCACC CAACAACAAG CUGUUAUUGA GCCAUAGUA GCUACCAAC UGGCAAAAGCU

5271
▼
T0040 UGAGACC
T0038 UGAGGCC
T0036 ???????
T0026 ???????
T1787 UGAGGCU
-----
Cons  UGAGACC

```


FIGURE 9b

DEDUCED AMINO ACID SEQUENCES

	1638												1787
	▼												▼
T0040	THPVT	KYIMA	CMSAD	LEVTT	STWVL	LGGVL	AALAA	YCLSV	GCVVI	VGHIE			
T0038	.HPVT	KYIMA	CMSAD	LEVTT	STWVL	LGGVL	AALAA	YCLSV	GCVVI	VGHIE			
T0036	.HPIT	KYIMA	CMSAD	LEVTT	STWVL	LGGVL	AALAA	YCLSV	GCVVI	VGHIE			
T0026	.HPIA	KYLMA	CMSAD	LEVTT	STWVL	LGGVL	AALAA	YCLSV	GCVVI	VGHIE			
T1787	.HPIT	KYVMA	CMSAD	LEVTT	STWVL	LGGVL	AALAA	YCLSV	GCVVI	VGHIE			
	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----			
Cons	THPVT	KYIMA	CMSAD	LEVTT	STWVL	LGGVL	AALAA	YCLSV	GCVVI	VGHIE			

	1688												1737
	▼												▼
T0040	LGGKP	ALVPD	KEVLY	QQYDE	MEECS	QAAPY	IEQAO	VIAHQ	FKEKV	LGLLQ			
T0038	LGGKP	ALVPD	KEVLY	QQYDE	MEECS	QAAPY	IEQAO	VIAHQ	FKEKV	LGLLQ			
T0036	LGGKP	ALVPD	KEVLY	QQYDE	MEECS	QAAPY	IEQAO	VIAHQ	FKEKV	LGLLQ			
T0026	LGGKP	ALVPD	KEVLY	QQYDE	MEECS	QAAPY	IEQAO	VIAHQ	FKEKV	LGLLQ			
T1787	LGGKP	ALVPD	KEVLY	QQYDE	MEECS	QAAPY	IEQAO	VIAHQ	FKEKV	LGLLQ			
	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----			
Cons	LGGKP	ALVPD	KEVLY	QQYDE	MEECS	QAAPY	IEQAO	VIAHQ	FKEKV	LGLLQ			

	1738						1765
	▼						▼
T0040	RATQQ	QAVIE	PIVAT	NWQKL	ETFWH	KHM	
T0038	RATQQ	QAVIE	PIVAT	NWQKL	EAFWH	KHM	
T0036	RATQQ	QAVIE	PIVAT	NWQ..	
T0026	RATQQ	QAVIE	PIVAT	NWQ..	
T1787	RATQQ	QAVIE	PIVAT	NWQKL	EAFWH	KHM	
	-----	-----	-----	-----	-----	---	
Cons	RATQQ	QAVIE	PIVAT	NWQKL	EAFWH	KHM	

FIGURE 10a

SEQUENCE COMPARISONS WITH TYPE 3

TYPE 3

T0040	CVVIVGHIELGGKPAIVPDKVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRA	TTQQAQVIEPIVATNWQKLETFW	HHKM...
T0038	CVVIVGHIELGGKPAIVPDKVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRA	TTQQAQVIEPIVATNWQKLEAF	FWHHKM...
T0036	CVVIVGHIELGGKPAIVPDKVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRA	TTQQAQVIEPIVATNWQKLEAF	FWHHKM...
T0026	CVVIVGHIELGGKPAIVPDKVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRA	TTQQAQVIEPIVATNWQKLEAF	FWHHKM...
T1787	CVVIVGHIELGGKPAIVPDKVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRA	TTQQAQVIEPIVATNWQKLEAF	FWHHKM...

TYPE 2

T0351	...II..L.INQRAVIA	PPDKVLYEAFDEMEECSSRAALIEGQRIAEMLRSKI	QGLLQQAASKQADIQPAVQASWP	KVEQFW...
T0059	CISIIIGRLHLNDRVVVTPDK	KEILYEAFDEMEECSSKAALIEGQRMALSKISKI	QGLLQQAATROAQDIPVQSSWP	KLEQFWAKHM...
T0940	CVSIIIGRLHL..RVV...	DK...YE.....AALIEGQRMALSKISKI	QGLLQQAATROAQDIPVQSSWP	KLEQFW...
T0810	CISIIIGRLHLNDRVVVAPDK	KEILYEAFDEMEECSSKAALIEGQRMALSKISKI	QGLLQQAATROAQDIPVQSSWP	KLEQFW...

TYPE 1

T0016	CVVIVGRIVLSGKPAIIPDRE.....PYIEQGMMLAEQFKQKALGLLQTA	SRQAEVIA	PA...QTNWQRL	ET...
T0042	CVVIVGRIVLSGKPAIIPDRE	VLYREFDEMEECSSQHLPYIEQGMMLAEQFKQKALGLLQTA	SRQAEVIA	PAVQTNWQRL
T0077	CVVIVGRIVLSGKPAIIPDRE	VLYREFDEMEECSSQHLPYIEQGMMLAEQFKQKALGLLQTA	SRQAEVIA	PAVQTNWQRL
T1801	CVVIVGRIVLSGKPAIIPDRE	VLYREFDEMEECSSQHLPYIEQGMMLAEQFKQKALGLLQTA	SRQAEVIA	PAVQTNWQRL
T1825	CVVIVGRIDLSGKPAIIPDRE	VLYREFDEMEECSSQHLPYIEQGMMLAEQFKQKALGLLQTA	SRQAEVIA	PAVQTNWQRL

FIGURE 10b

DERIVATION OF PEPTIDE SEQUENCES

	TYPE 3
T0040	CVVIVGHIELGGKPAIVDPKEVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRTQQQAVIEPIVATNWQKLETFWHKHM
T0038	CVVIVGHIELGGKPAIVDPKEVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRTQQQAVIEPIVATNWQKLETFWHKHM
T0036	CVVIVGHIELGGKPAIVDPKEVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRTQQQAVIEPIVATNWQKLETFWHKHM
T0026	CVVIVGHIELGGKPAIVDPKEVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRTQQQAVIEPIVATNWQKLETFWHKHM
T1787	CVVIVGHIELGGKPAIVDPKEVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRTQQQAVIEPIVATNWQKLETFWHKHM
Peptd	CVVIVGHIELGGKPAIVDPKEVLYQQYDEMEECSSQAAPYIEQAQVIAHQFKEKVLGLLQRTQQQAVIEPIVATNWQKLETFWHKHM
	TYPE 2
T0351	...II..L.INQRAVIAPDKEVLYEAFDEMEECASNAALIEEGQRIAEMLRSKIQGLLQQAASKQAQDIQPAVQASWPVKVEQFW
T0059	CISIIIGRLHLNDRVVVTPDKIELLYEAFDEMEECASKAALIEEGQRMALSKLQGLLQQAATRAQDIQPVVQSSWPVKLEQFWAKHMMW
T0940	CVSIIIGRLHL..RVV...DK...YE.....AALIEEGQRMALSKLQGLLQQAATRAQDIQPVVQSSWPVKLEQFW
T0810	CISIIIGRLHLNDRVVVAPDKIELLYEAFDEMEECASKAALIEEGQRMALSKLQGLLQQAATRAQDIQPAVQSSWPVKLEQFW
Peptd	CISIIIGRLHLNDRVVVTPDKIELLYEAFDEMEECASKAALIEEGQRMALSKLQGLLQQAATRAQDIQPVVQSSWPVKLEQFWAKHMMW
	TYPE 1
T0016	CVVIVGRIVLSGKPAIIPDRE.....PYIEQGMMLAEQFKQKALGLLQTASRQAEVIAP..QTNWQRLETF
T0042	CVVIVGRIVLSGKPAIIPDREVLVREFDEMEECSSQHLPIYIEQGMMLAEQFKQKALGLLQTASRQAEVIAPAVQTNWQRLETF
T0077	CVVIVGRIVLSGKPAIIPDREVLVREFDEMEECSSQHLPIYIEQGMMLAEQFKQKALGLLQTASRQAEVIAPAVQTNWQRLETF
T1801	CVVIVGRIVLSGKPAIIPDREVLVREFDEMEECSSQHLPIYIEQGMMLAEQFKQKALGLLQTASRQAEVIAPAVQTNWQRLETF
T1825	CVVIVGRIDLSGKPAIIPDREVLVREFDEMEECSSQHLPIYIEQGMMLAEQFKQKALGLLQTASRQAEVITPVVQTNWQKLEAFWAK
Peptd	CVVIVGRIVLSGKPAIIPDREVLVREFDEMEECSSQHLPIYIEQGMMLAEQFKQKALGLLQTASRQAEVIAPAVQTNWQKLEAFWAKHMMW

FIGURE 11a

TYPE 3

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 1 CVVIVGHIK
P 2 VVIVGHIK
P 3 VIVGHIK
P 4 IVGHIK
P 5 VGHILGK
P 6 GHILGK
P 7 HILGK
P 8 IELGKFP
P 9 ELGKFP
P 10 LGKFPALV

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 11 GKPALVFD
P 12 KPALVFDK
P 13 KPALVFDK
P 14 KPALVFDK
P 15 KPALVFDK
P 16 KPALVFDK
P 17 KPALVFDK
P 18 KPALVFDK
P 19 KPALVFDK
P 20 KPALVFDK

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 21 EVLTQCTD
P 22 VLTQCTD
P 23 LTQCTD
P 24 TQCTD
P 25 QCTD
P 26 CTD
P 27 TD
P 28 D
P 29 S
P 30 C

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 31 ECQAAPFT
P 32 CQAAPFT
P 33 QAAPFT
P 34 AAPFT
P 35 APFT
P 36 PFT
P 37 FT
P 38 T
P 39 S
P 40 C

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 41 NOLGVIAN
P 42 OGVIAN
P 43 VGVIAN
P 44 IGVIAN
P 45 VGVIAN
P 46 IGVIAN
P 47 VGVIAN
P 48 IGVIAN
P 49 VGVIAN
P 50 IGVIAN

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 51 KKEVLLGL
P 52 KEVLLGL
P 53 EVLLGL
P 54 VLLGL
P 55 LLGL
P 56 LGL
P 57 GL
P 58 L
P 59 S
P 60 C

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 61 ATQCCAVIE
P 62 TQCCAVIE
P 63 QCCAVIE
P 64 CCVIE
P 65 CVIE
P 66 VIE
P 67 IE
P 68 S
P 69 C
P 70 C

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 71 IVATWOKL
P 72 VATWOKL
P 73 ATWOKL
P 74 TWOKL
P 75 WOKL
P 76 OKL
P 77 KLP
P 78 LKLP
P 79 LKLP
P 80 LKLP

HCY-3 CVVIVGHIKLGKFPALVFDREVLTQCTDREKSCQAAPFTIEQAGVIANOFKKEVLLGLGRATQCCAVIEP IVATWOKLSAPFAKIDNSQF

P 81 APFAKIDNSQF
P 82 PFAKIDNSQF

FIGURE 11b

TYPE 3

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 1 CISTIGRLH
P 2 CISTIGRLH
P 3 CISTIGRLH
P 4 CISTIGRLH
P 5 CISTIGRLH
P 6 CISTIGRLH
P 7 CISTIGRLH
P 8 CISTIGRLH
P 9 CISTIGRLH
P 10 CISTIGRLH

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 11 NDVVVTFD
P 12 NDVVVTFD
P 13 NDVVVTFD
P 14 NDVVVTFD
P 15 NDVVVTFD
P 16 NDVVVTFD
P 17 NDVVVTFD
P 18 NDVVVTFD
P 19 NDVVVTFD
P 20 NDVVVTFD

ECV-1 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 21 ILYEAFD
P 22 ILYEAFD
P 23 ILYEAFD
P 24 ILYEAFD
P 25 ILYEAFD
P 26 ILYEAFD
P 27 ILYEAFD
P 28 ILYEAFD
P 29 ILYEAFD
P 30 ILYEAFD

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 31 ECASKAL
P 32 ECASKAL
P 33 ECASKAL
P 34 ECASKAL
P 35 ECASKAL
P 36 ECASKAL
P 37 ECASKAL
P 38 ECASKAL
P 39 ECASKAL
P 40 ECASKAL

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 41 ECOMHAG
P 42 ECOMHAG
P 43 ECOMHAG
P 44 ECOMHAG
P 45 ECOMHAG
P 46 ECOMHAG
P 47 ECOMHAG
P 48 ECOMHAG
P 49 ECOMHAG
P 50 ECOMHAG

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 51 ESKIQGL
P 52 ESKIQGL
P 53 ESKIQGL
P 54 ESKIQGL
P 55 ESKIQGL
P 56 ESKIQGL
P 57 ESKIQGL
P 58 ESKIQGL
P 59 ESKIQGL
P 60 ESKIQGL

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 61 ATROQDIO
P 62 ATROQDIO
P 63 ATROQDIO
P 64 ATROQDIO
P 65 ATROQDIO
P 66 ATROQDIO
P 67 ATROQDIO
P 68 ATROQDIO
P 69 ATROQDIO
P 70 ATROQDIO

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 71 VQSSWFK
P 72 VQSSWFK
P 73 VQSSWFK
P 74 VQSSWFK
P 75 VQSSWFK
P 76 VQSSWFK
P 77 VQSSWFK
P 78 VQSSWFK
P 79 VQSSWFK
P 80 VQSSWFK

ECV-2 CISTIGRLHLNDVVVTFDKILYEAFDDEKSCASKALIEGOMHAGLSEKIQGLLOQATQAGDIQFVVQSSWFKLEQFWAKHDSW

P 81 CFWAKHDS
P 82 CFWAKHDS

FIGURE 11c

TYPE 1

```

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 1      CVVIVGRIV
P 2      VVIVGRIVL
P 3      VIVGRIVLS
P 4      IVGRIVLSG
P 5      VGRIVLSGK
P 6      GRIVLSGKP
P 7      RIVLSGKPA
P 8      IVLSGKPAI
P 9      VLSGKPAII
P 10     LSGKPAIIP

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 11      SGRPAIIPD
P 12      GKPPIIPDR
P 13      KPAIIPDRS
P 14      PAIIPDRSV
P 15      AIIPDRSVL
P 16      IIPDRSVLT
P 17      IPDRSVLTR
P 18      PDRSVLTRY
P 19      DRSVLTRYF
P 20      RSVLTRYFD

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 21      SVLTRYFDN
P 22      VLYTRYFDN
P 23      LYTRYFDNM
P 24      YTRYFDNME
P 25      TRYFDNMEC
P 26      RYFDNMECS
P 27      FYDNMECSO
P 28      DNMECSORL
P 29      NMECSORL
P 30      MECSORL

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 31      EECORLFTY
P 32      EECORLFTI
P 33      EECORLFTY
P 34      EECORLFTY
P 35      EECORLFTY
P 36      EECORLFTY
P 37      EECORLFTY
P 38      EECORLFTY
P 39      EECORLFTY
P 40      EECORLFTY

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 41      EECORLFTY
P 42      EECORLFTY
P 43      EECORLFTY
P 44      EECORLFTY
P 45      EECORLFTY
P 46      EECORLFTY
P 47      EECORLFTY
P 48      EECORLFTY
P 49      EECORLFTY
P 50      EECORLFTY

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 51      EECORLFTY
P 52      EECORLFTY
P 53      EECORLFTY
P 54      EECORLFTY
P 55      EECORLFTY
P 56      EECORLFTY
P 57      EECORLFTY
P 58      EECORLFTY
P 59      EECORLFTY
P 60      EECORLFTY

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 61      EECORLFTY
P 62      EECORLFTY
P 63      EECORLFTY
P 64      EECORLFTY
P 65      EECORLFTY
P 66      EECORLFTY
P 67      EECORLFTY
P 68      EECORLFTY
P 69      EECORLFTY
P 70      EECORLFTY

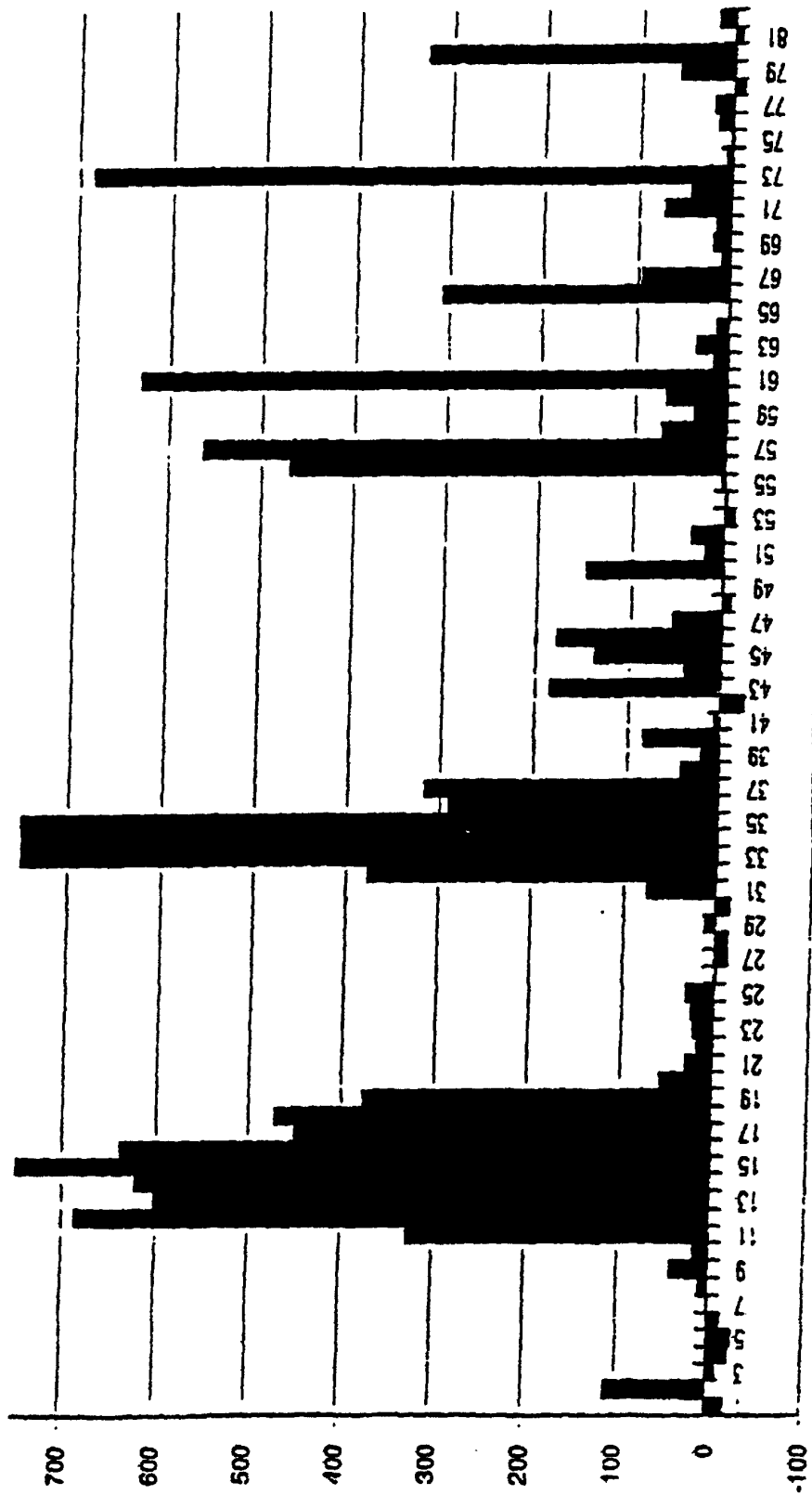
HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 71      EECORLFTY
P 72      EECORLFTY
P 73      EECORLFTY
P 74      EECORLFTY
P 75      EECORLFTY
P 76      EECORLFTY
P 77      EECORLFTY
P 78      EECORLFTY
P 79      EECORLFTY
P 80      EECORLFTY

HCV-1  CVVIVGRIVLSGKPAIIPDRVLYTRFDNMECSORLFTYIQGGGLASQFQKALGLLOTASQASVIAPAVQTNWQRLAFAWAKHNSQF
P 81      EECORLFTY
P 82      EECORLFTY

```



FIGURE 12b



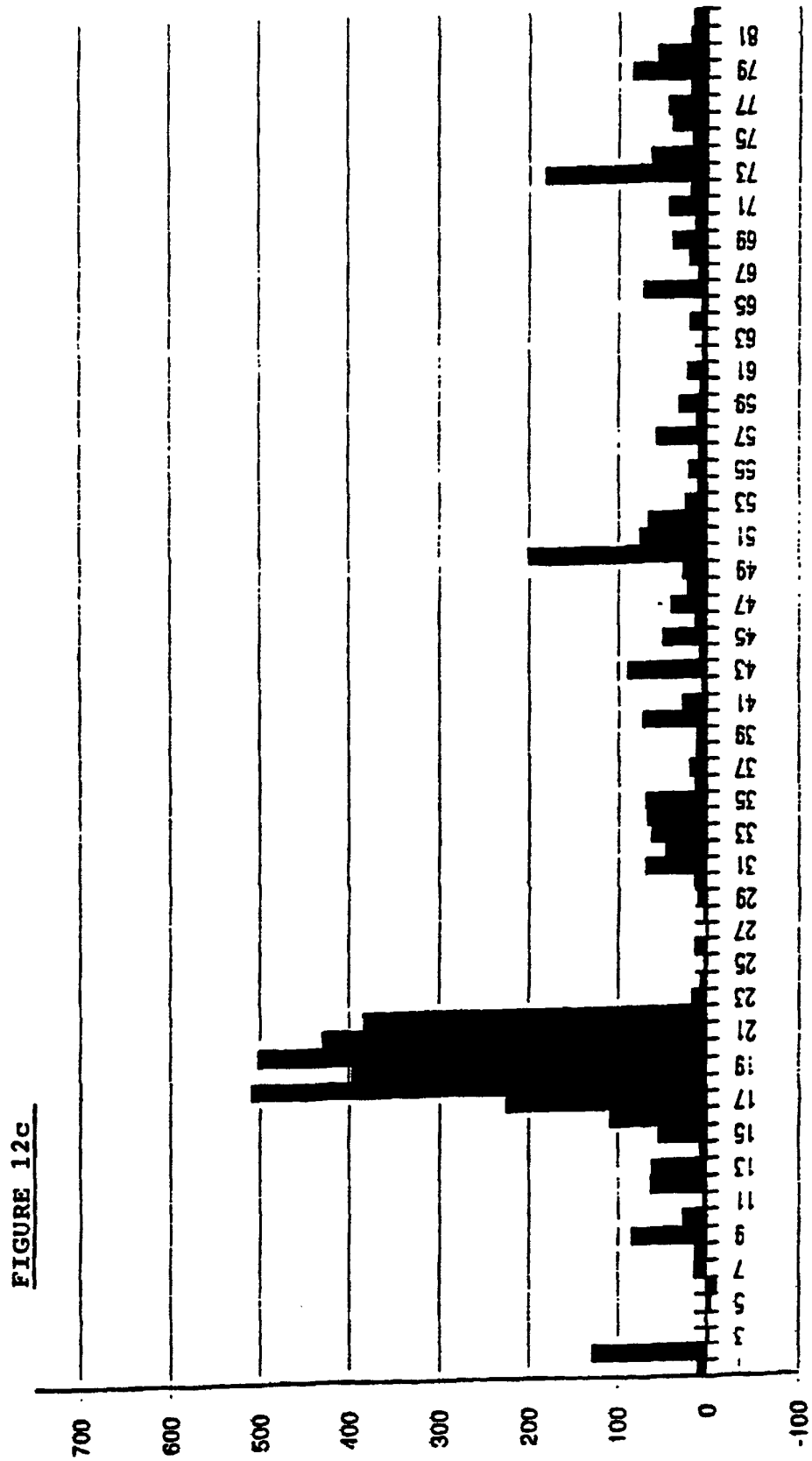


FIGURE 13

[illegible]

FIGURE 14

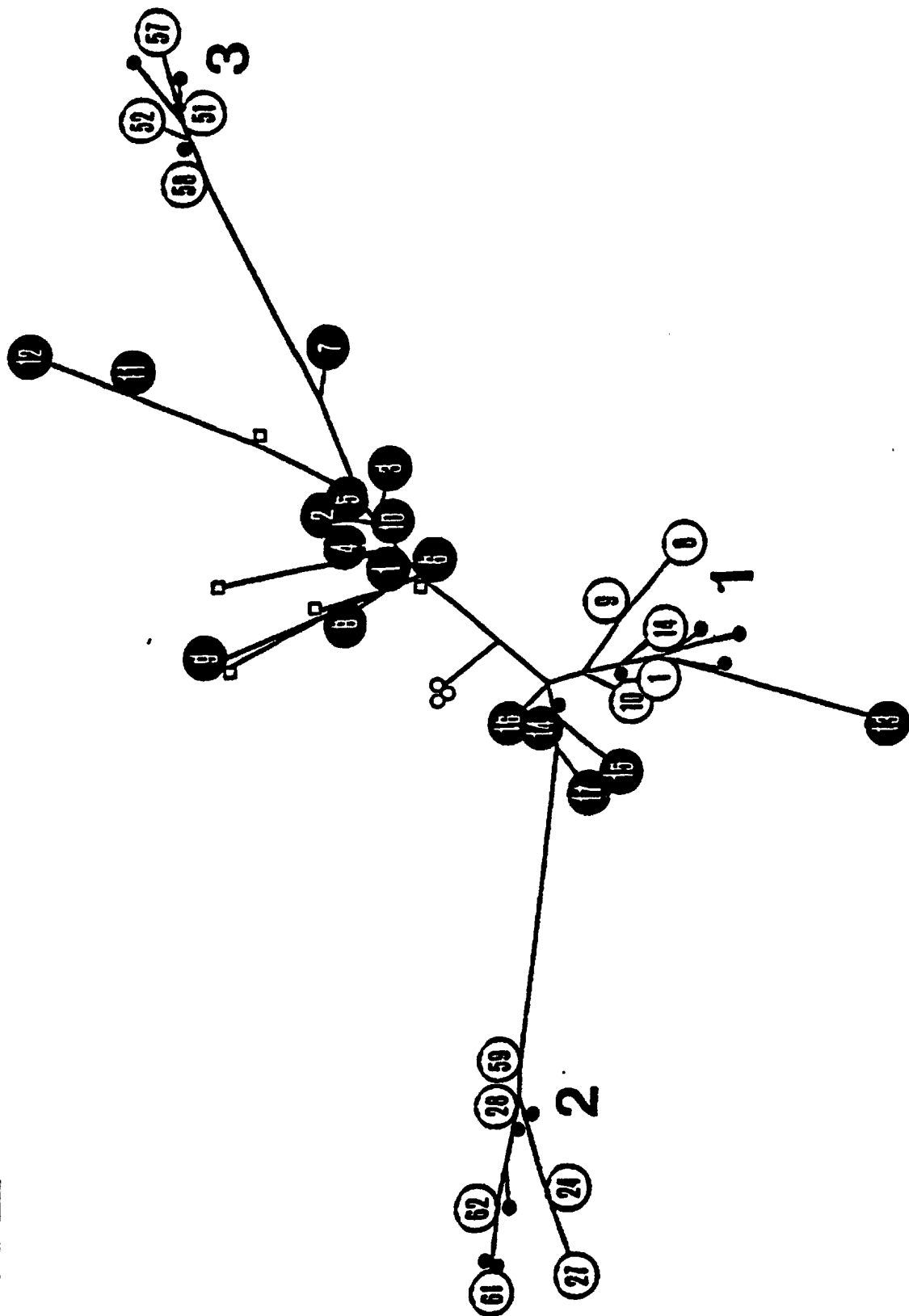


FIGURE 15a

	23	43	63	83	102
1a	HCV-1	AAACCAACG	TACACCAAC	CGTCCGCCAC	AGGACGTCAA
1b	HCV-J	...C...C...	...C...
2a	HC-J6	...C...A	A...	...T...	...C...
2b	HC-J8	...C...A	A...	...T...	...C...
3a	Eb-1	...C...A	A...A...G...
	Eg-29 (1)	...C...
	Eg-33 (2)
	Eg-21 (3)
	103	123	143	163	182
1a	HCV-1	TTGCCGCGCA	GGGGCCCTAG	ATTGGGTGTG	CGCGGCGTGG
1b	HCV-JC...
2a	HC-J6C...
2b	HC-J8C...
3a	Eb-1AC...
	Eg-29 (1)C...
	Eg-33 (2)C...
	Eg-21 (3)CC...
	183	203	223	243	262
1a	HCV-1	GCCTATCCCC	AAAGCTCGTC	GGCCCGAGGG	CAGGACCTGG
1b	HCV-J	A...	...C...
2a	HC-J6	...C...T	...A...G	...CT...ACT...	...AAT...
2b	HC-J8	...C...G	...A...G	...CT...ACC...	...AAT...
3a	Eb-29G...	...AG...A...	...
	Eg-29 (1)G...
	Eg-33 (2)G...
	Eg-21 (3)G...

FIGURE 15b

	5	25	45	65	85					
1a	HCV-1	PKPKKKKRN	TNRRPQDKF	POGQIVGGV	YLLPRQPRLL	GVRATKKTSE	RSQPRGRQP	IPKARRPEQR	TWAQPGYMP	LYGNE
1b	HCV-J	...R.T...
2a	HC-J6	...R.T...D..ST.K	S.GK.
2b	HC-J8	...R.T...D..ST.K	S.GK.
3a	Eb-1	A...R.T...	.I.....V.....	.C.....S...	S.....
	Eg-29, 33	.R.....	...M...G...S...	S.....
	Eg-21T...S...	S...P...

FIGURE 16

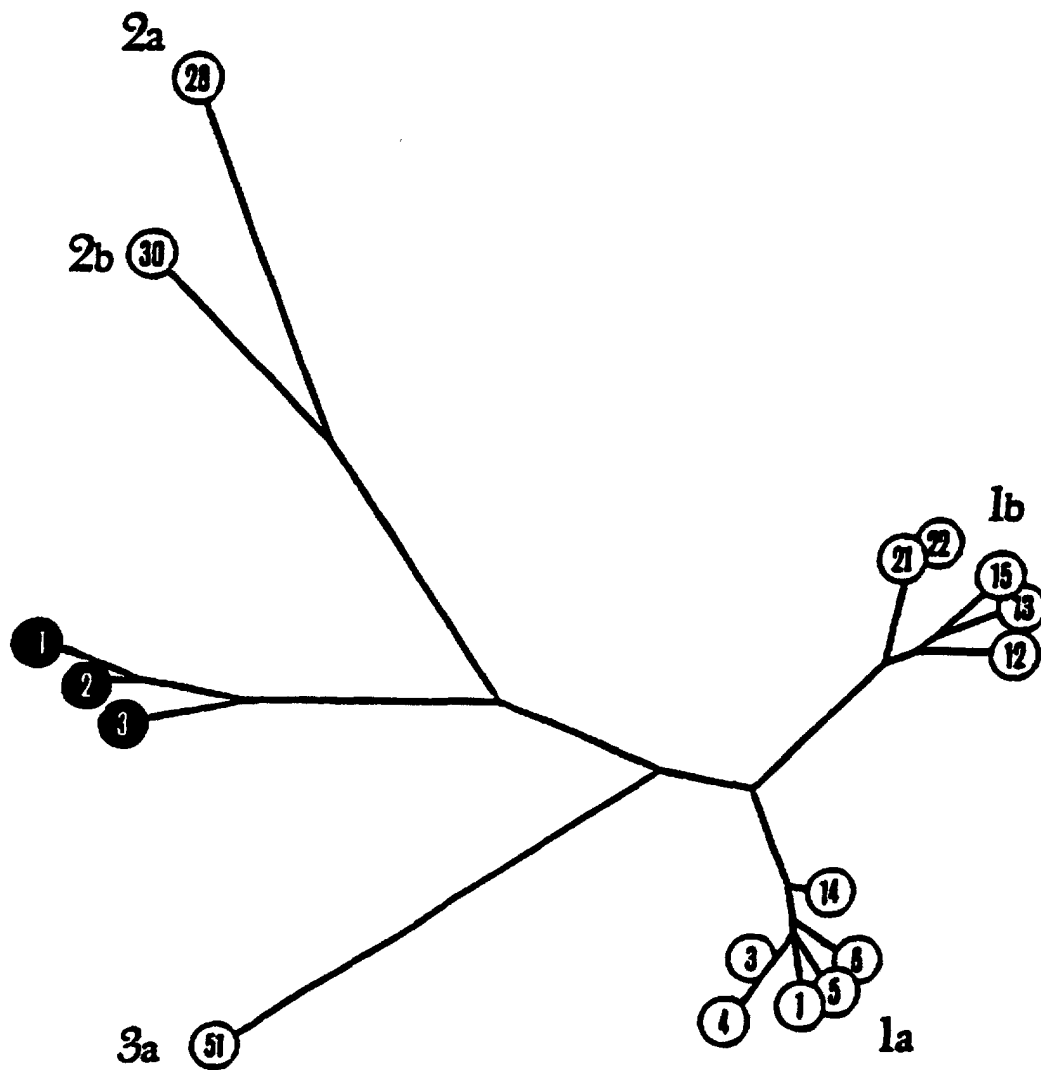


FIGURE 17

