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(54) Title: HCV FUSION POLYPEPTIDES

(57) Abstract: The invention provides HCV fusion polypeptides including truncated or full- length HCV NS5 polypeptides, and a portion of the HCV NS2 polypeptide, fused to at least one other HCV epitope derived from another region of the HCV polyprotein. The fusions can be used in methods of stimulating an immune response to HCV, for example a cellular immune response to HCV, such as activating hepatitis C virus (HCV)-specific T cells, including CD4+ and CD8+ T cells. The method can be used in model systems to develop HCV-specific immunogenic compositions, as well as to immunize against HCV.

Replacement Sheet

HCV FUSION POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Ser. Nos. 60/840,162, filed August 25, 2006, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present invention relates to hepatitis C virus (HCV) polypeptides. More particularly, the invention relates to nucleic acids and proteins wherein the nucleic acids encode truncated HCV fusion proteins comprising E2, a portion of the carboxy terminus of NS2, a mutated NS3, NS4, a truncated NS5 and optionally a core polypeptide from HCV. The proteins are useful for stimulating immune responses, such as cell-mediated immune responses, for priming and/or activating HCV-specific T cells, as well as for diagnostic reagents. The invention also relates to methods of enhancing production of HCV fusion polypeptide.

BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) infection is an important health problem with approximately 1% of the world's population infected with the virus. Over 75% of acutely infected individuals eventually progress to a chronic carrier state that can result in cirrhosis, liver failure, and hepatocellular carcinoma. See, Alter *et al.* (1992) N. Engl. J. Med. 327:1899-1905; Resnick and Koff. (1993) Arch. Intem. Med. 153:1672-1677; Seeff (1995) Gastrointest. Dis. 6:20-27; Tong *et al.* (1995) N. Engl. J. Med. 332:1463-1466.

HCV was first identified and characterized as a cause of NANBH by Houghton et al. The viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. HCV has a 9.5 kb positive-sense, single-stranded RNA genome and is a member of the Flaviridae family of viruses. At least six distinct, but related genotypes of HCV, based on phylogenetic analyses, have been identified (Simmonds et al., *J. Gen. Virol.* (1993) 74:2391-2399). The virus encodes a single polyprotein having more than 3000 amino acid residues (Choo et al., *Science* (1989) 244:359-362; Choo et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455; Han et

al., *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715). The polyprotein is processed co- and post-translationally into both structural and non-structural (NS) proteins.

In particular, as shown in Figure 1, several proteins are encoded by the HCV genome. The order and nomenclature of the cleavage products of the HCV polyprotein is as follows: NH_2-C-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. Initial cleavage of the polyprotein is catalyzed by host proteases which liberate three structural proteins, the N-terminal nucleocapsid protein (termed "core") and two envelope glycoproteins, "E1" (also known as E) and "E2" (also known as E2/NS1), as well as nonstructural (NS) proteins that contain the viral enzymes. The NS regions are termed NS2, NS3, NS4 and NS5. NS2 is an integral membrane protein with proteolytic activity and, in combination with NS3, cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease serves to process the remaining polyprotein. In these reactions, NS3 liberates an NS3 cofactor (NS4a), two proteins (NS4b and NS5a), and an RNA-dependent RNA polymerase (NS5b). Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease.

Despite extensive advances in the development of pharmaceuticals against certain viruses like HIV, control of acute and chronic HCV infection has had limited success (Hoofnagle and di Bisceglie (1997) N. Engl. J. Med. 336:347-356). In particular, generation of cellular immune responses, such as strong cytotoxic T lymphocyte (CTL) responses, is thought to be important for the control and eradication of HCV infections.

Immunogenic HCV fusion proteins capable of generating cellular immune responses are described in International Application WO/2004/005473 and U.S. Patent Nos. 6,562,346; 6,514,731 and 6,428,792. Nevertheless, there remains a need in the art for additional effective methods of stimulating immune responses, such as cellular immune responses, to HCV.

SUMMARY OF THE INVENTION

It is an object of the invention to provide reagents and methods for stimulating an immune response, such as a cellular immune response to HCV, such as priming and/or activating T cells which recognize epitopes of HCV polypeptides. It is also an object of the invention to provide compositions for the prevention and/or treatment of HCV infection. It is also an object of the invention to provide reagents and methods for use in diagnostic assays for detecting the presence of HCV in a biological sample.

Accordingly, in one embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine.

In another embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine.

In another embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine.

In yet another embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2,

amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine.

In further embodiments, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine.

In another embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine.

In another embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine.

In yet another embodiment, the invention is directed to an immunogenic composition comprising an isolated HCV fusion polypeptide consisting of, in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position

1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine.

In a separate embodiment, the invention comprises a nucleic acid encoding a HCV fusion polypeptide of the invention.

All HCV protein regions (eg, E2, NS2, NS3, NS4, NS5, core) are numbered relative to the amino acid sequence of the full-length HCV-1 polyprotein.

In additional embodiments, the HCV polypeptides present in the fusion polypeptide are derived from the same HCV isolate. In other embodiments, at least one of the HCV polypeptides present in the fusion is derived from a different isolate than at least one of the other peptides present in the fusion.

In certain embodiments, the HCV fusion polypeptides comprise an HCV core polypeptide that comprises a C-terminal truncation, such a core polypeptide that consists of the sequence of amino acids depicted at amino acid positions 1772-1892 of Figure 3A-3J.

In yet further embodiments, the invention is directed to a composition comprising a HCV fusion polypeptide according to any of the embodiments above in combination with a pharmaceutically acceptable excipient. In certain embodiments, the compositions include an immunogenic HCV polypeptide, such as an HCV E1E2 complex. The E1E2 complex can be provided separately from the fusion protein.

In additional embodiments, the invention is directed to a method of stimulating a cellular immune response in a vertebrate subject comprising administering to the subject a therapeutically effective amount of a composition as described above.

In further embodiments, the invention is directed to a recombinant vector comprising:

(a) a polynucleotide encoding one or more of the HCV fusion polypeptides as described above; and

(b) at least one control element operably linked to the polynucleotide, whereby the coding sequence can be transcribed and translated in a host cell.

In additional embodiments, the invention is directed to a host cell comprising the recombinant vector described above.

In further embodiments, the invention is directed to a method for producing an HCV fusion polypeptide, the method comprising culturing a population of host cells

as described above under conditions for producing the protein. The invention also includes a method for enhancing the recombinant expression of an HCV fusion polypeptide by positioning HCV E2 amino acid sequences at the N-terminal of the fusion polypeptide.

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These and other embodiments of the subject invention will readily occur to those of skill in the art in view of the disclosure herein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of the HCV genome, depicting the
10 various regions of the HCV polyprotein.

Figure 2 (SEQ ID NOS:3 and 4) depicts the DNA and corresponding amino acid sequence of a representative native, unmodified NS3 protease domain.

Figure 3A-3J (SEQ ID NOS:5 and 6) shows the DNA and corresponding amino acid sequence of a region of a representative modified fusion protein, with the
15 NS3 protease domain deleted from the N-terminus and including amino acids 1-121 of Core on the C-terminus.

Figures 4A and 4B show a comparison of expression levels of NS5tCore121 (amino acids 1973-2990 of NS5 and 1-121 of core) and NS5Core121 (full-length NS5, amino acids 1973-3011 of NS5 and 1-121 of core) in *S. cerevisiae* strain AD3.
20 Figure 4A shows expression levels at 25°C and Figure 4B shows expression levels at 30°C. Lane 1, standard; Lane 2, plasmid control; Lane 3, plasmid encoding NS5tCore121 (clone 6); Lane 4, plasmid encoding NS5tCore121 (clone 7); Lane 5, plasmid encoding NS5Core121 (clone 8); Lane 6, plasmid encoding NS5Core121 (clone 9); Lane 7, standard.

25 Figures 5A-5E (SEQ ID NOS:7 and 8) show the DNA and corresponding amino acid sequence of a region of a representative fusion protein that includes a C-terminally truncated NS5 polypeptide with the C-terminus of the NS5 polypeptide fused to a core polypeptide. In particular, the C-terminally truncated NS5 polypeptide includes amino acids 1973-2990 of the HCV polyprotein, numbered relative to
30 HCV-1 (see, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455), fused to a core polypeptide that includes amino acids 1-121 of the HCV polyprotein.

Figures 6A through 6F depict an exemplary cloning strategy used to generate fusion polypeptides of the present invention.

Figure 7 depicts the genetic organization of an exemplary fusion polypeptide of the present invention.

Figure 8 is a graphical representation of T cells generated in mice in response to immunization with an exemplary HCV fusion polypeptide of the invention.

Figure 9 is a graphical representation of T cells generated in mice in response to immunization with an exemplary HCV fusion polypeptide of the invention.

Figures 10A to 10D show the results of expression of exemplary HCV fusion polypeptides of the invention in yeast cells.

Figure 11 depicts the genetic organization of exemplary HCV fusion polypeptides of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, recombinant DNA techniques and immunology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *DNA Cloning*, Vols. I and II (D.N. Glover ed.); *Oligonucleotide Synthesis* (M.J. Gait ed.); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.); *Animal Cell Culture* (R.K. Freshney ed.); Perbal, B., *A Practical Guide to Molecular Cloning.*

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a polypeptide" includes a mixture of two or more polypeptides, and the like.

The following amino acid abbreviations are used throughout the text:

Alanine: Ala (A)	Arginine: Arg (R)
Asparagine: Asn (N)	Aspartic acid: Asp (D)
Cysteine: Cys (C)	Glutamine: Gln (Q)
Glutamic acid: Glu (E)	Glycine: Gly (G)
Histidine: His (H)	Isoleucine: Ile (I)
Leucine: Leu (L)	Lysine: Lys (K)

Methionine: Met (M)	Phenylalanine: Phe (F)
Proline: Pro (P)	Serine: Ser (S)
Threonine: Thr (T)	Tryptophan: Trp (W)
Tyrosine: Tyr (Y)	Valine: Val (V)

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms "polypeptide" and "protein" refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

An "HCV polypeptide" is a polypeptide, as defined above, derived from the HCV polyprotein. The polypeptide need not be physically derived from HCV, but may be synthetically or recombinantly produced. Moreover, the polypeptide may be derived from any of the various HCV strains and isolates including isolates having any of the 6 genotypes of HCV described in Simmonds et al., *J. Gen. Virol.* (1993) 74:2391-2399 (e.g., strains 1, 2, 3, 4 etc.), as well as newly identified isolates, and subtypes of these isolates, such as HCV1a, HCV1b etc. A number of conserved and variable regions are known between these strains and, in general, the amino acid sequences of epitopes derived from these regions will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, when the two sequences are aligned. Thus, for example, the term "NS5" polypeptide refers to native NS5 from any of the various HCV strains, as well as NS5 analogs, muteins and immunogenic fragments, as defined further below. The term

"HCV polypeptide" will generally be used to refer to the various individual identified and well-known HCV proteins present in the HCV polyprotein, that is core, E1, E2, p7, NS2, NS3, NS4 (including NS4a and NS4b), NS5 (including NS5a and NS5b). The term "HCV fusion polypeptide" will be used to refer to a recombinant polypeptide in which two or more of the HCV polypeptides are present in a single recombinant polypeptide molecule.

The terms "analog" and "mutein" refer to biologically active derivatives of the reference molecule, or fragments of such derivatives, that retain desired activity, such as the ability to stimulate a cell-mediated immune response, as defined below. In the case of a modified NS3, an "analog" or "mutein" refers to an NS3 molecule that lacks its native proteolytic activity. In general, the term "analog" refers to compounds having a native polypeptide sequence and structure with one or more amino acid additions, substitutions (generally conservative in nature, or in the case of modified NS3, non-conservative in nature at the active proteolytic site) and/or deletions, relative to the native molecule, so long as the modifications do not destroy immunogenic activity. The term "mutein" refers to peptides having one or more peptide mimics ("peptoids"). Preferably, the analog or mutein has at least the same immunoactivity as the native molecule. Methods for making polypeptide analogs and muteins are known in the art and are described further below.

As explained above, analogs generally include substitutions that are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in their side chains. Specifically, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cysteine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. For example, the polypeptide of interest may include up to about 5-10 conservative or non-conservative amino acid substitutions, or even up to about 15-25 conservative or non-conservative

amino acid substitutions, or any integer between 5-25, so long as the desired function of the molecule remains intact. One of skill in the art may readily determine regions of the molecule of interest that can tolerate change by reference to Hopp/Woods and Kyte-Doolittle plots, well known in the art.

By "C-terminally truncated NS5 polypeptide" is meant an NS5 polypeptide that comprises a full-length NS5a polypeptide and an N-terminal portion of an NS5b polypeptide, but not the entire NS5b region. Particular examples of C-terminally truncated NS5 polypeptides are provided below.

By "modified NS3" is meant an NS3 polypeptide with a modification such that protease activity of the NS3 polypeptide is disrupted, that is to say the protease activity is reduced, inhibited or absent (compared with the non-modified or wild type NS3). The modification can include one or more amino acid additions, substitutions (generally non-conservative in nature) and/or deletions, relative to the native molecule, wherein the protease activity of the NS3 polypeptide is disrupted. Methods of measuring protease activity are discussed further below.

By "fragment" is intended a polypeptide consisting of only a part of the intact full-length polypeptide sequence and structure. The fragment can include a C-terminal deletion and/or an N-terminal deletion of the native polypeptide. An "immunogenic fragment" of a particular HCV protein will generally include at least about 5-10 contiguous amino acid residues of the full-length molecule, preferably at least about 15-25 contiguous amino acid residues of the full-length molecule, and most preferably at least about 20-50 or more contiguous amino acid residues of the full-length molecule, that define an epitope, or any integer between 5 amino acids and the full-length sequence, provided that the fragment in question retains immunogenic activity, as measured by the assays described herein.

The term "epitope" as used herein refers to a sequence of at least about 3 to 5, preferably about 5 to 10 or 15, and not more than about 1,000 amino acids (or any integer therebetween), which define a sequence that by itself or as part of a larger sequence, binds to an antibody generated in response to such sequence. There is no critical upper limit to the length of the fragment, which may comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes from the HCV polyprotein. An epitope for use in the subject invention is not limited to a polypeptide having the exact sequence of the portion of the parent protein

from which it is derived. Indeed, viral genomes are in a state of constant flux and contain several variable domains which exhibit relatively high degrees of variability between isolates. Thus the term "epitope" encompasses sequences identical to the native sequence, as well as modifications to the native sequence, such as deletions, additions and substitutions (generally conservative in nature).

Regions of a given polypeptide that include an epitope can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols, supra.* Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omiga version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

For a description of various HCV epitopes, see, e.g., Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,280,927 and 6,150,087.

As used herein the term "T-cell epitope" refers to a feature of a peptide structure which is capable of inducing T-cell immunity towards the peptide structure or an associated hapten. T-cell epitopes generally comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of

MHC molecules, (Unanue et al., *Science* (1987) 236:551-557). Conversion of polypeptides to MHC class II-associated linear peptide determinants (generally between 5-14 amino acids in length) is termed "antigen processing" which is carried out by antigen presenting cells (APCs). More particularly, a T-cell epitope is defined by local features of a short peptide structure, such as primary amino acid sequence properties involving charge and hydrophobicity, and certain types of secondary structure, such as helicity, that do not depend on the folding of the entire polypeptide. Further, it is believed that short peptides capable of recognition by helper T-cells are generally amphipathic structures comprising a hydrophobic side (for interaction with the MHC molecule) and a hydrophilic side (for interacting with the T-cell receptor), (Margalit et al., *Computer Prediction of T-cell Epitopes*, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116) and further that the amphipathic structures have an α-helical configuration (see, e.g., Spouge et al., *J. Immunol.* (1987) 138:204-212; Berkower et al., *J. Immunol.* (1986) 136:2498-2503).

Hence, segments of proteins that include T-cell epitopes can be readily predicted using numerous computer programs. (See e.g., Margalit et al., *Computer Prediction of T-cell Epitopes*, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116). Such programs generally compare the amino acid sequence of a peptide to sequences known to induce a T-cell response, and search for patterns of amino acids which are believed to be required for a T-cell epitope.

An "immunological response" to an HCV antigen (including both polypeptide and polynucleotides encoding polypeptides that are expressed *in vivo*) or composition is the development in a subject of a humoral and/or a cellular immune response to molecules present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a "cellular immune response" is one mediated by T lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T cells ("CTLs"). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Both CD8+

and CD4+ T cells are capable of killing HCV-infected cells. Another aspect of cellular immunity involves an antigen-specific response by helper T cells. Helper T cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of antiviral cytokines, chemokines and other such molecules produced by activated T cells and/or other white blood cells, including those derived from CD4+ and CD8+ T cells, including, but not limited to IFN-γ and TNF-α.

A composition or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376; and the examples below.

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T cells. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T cells and/or γδ T cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection (i.e., prophylactic) or alleviation of symptoms (i.e., therapeutic) to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

By "equivalent antigenic determinant" is meant an antigenic determinant from different sub-species or strains of HCV, such as from strains 1, 2, 3, etc., of HCV which antigenic determinants are not necessarily identical due to sequence variation,

but which occur in equivalent positions in the HCV sequence in question. In general the amino acid sequences of equivalent antigenic determinants will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, usually more than 40%, such as more than 60%, and even more than 80-90% homology, when the two sequences are aligned.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence may be located 3' to the coding sequence.

A "nucleic acid" molecule or "polynucleotide" can include both double- and single-stranded sequences and refers to, but is not limited to, cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral (e.g. DNA viruses and retroviruses) or procaryotic DNA, and especially synthetic DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their desired function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper transcription factors, etc., are present. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence, as can transcribed introns, and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, viral, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. In general, the gene of interest is cloned and then

expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

A "control element" refers to a polynucleotide sequence which aids in the expression of a coding sequence to which it is linked. The term includes promoters, transcription termination sequences, upstream regulatory domains, polyadenylation signals, untranslated regions, including 5'-UTRs and 3'-UTRs and when appropriate, leader sequences and enhancers, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A "promoter" as used herein is a DNA regulatory region capable of binding RNA polymerase in a host cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

"Expression cassette" or "expression construct" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette includes control elements, as described above, such as a promoter which is operably linked to (so as to direct transcription of) the sequence(s) or gene(s) of interest, and often includes a polyadenylation sequence as well. Within certain embodiments of the invention, the expression cassette described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (e.g., a M13 origin of replication), at least one multiple cloning site, and a "mammalian" origin of replication (e.g., a SV40 or adenovirus origin of replication).

"Transformation," as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, transformation by direct uptake, transfection, infection, and the like. For particular methods of transfection, see further below. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, an episome, or alternatively, may be integrated into the host genome.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macromolecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

The term "purified" as used herein preferably means at least 75% by weight, more preferably at least 85% by weight, more preferably still at least 95% by weight, and most preferably at least 98% by weight, of biological macromolecules of the same type are present.

"Homology" refers to the percent identity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 50% , preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98%, or more, sequence identity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length

of the shorter sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman *Advances in Appl. Math.* 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent identity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

Another method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be readily found at the NCBI internet site.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size

determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra.*

By "nucleic acid immunization" is meant the introduction of a nucleic acid molecule encoding one or more selected immunogens into a host cell, for the *in vivo* expression of the immunogen or immunogens. The nucleic acid molecule can be introduced directly into the recipient subject, such as by injection, inhalation, oral, intranasal and mucosal administration, or the like, or can be introduced *ex vivo*, into cells which have been removed from the host. In the latter case, the transformed cells are reintroduced into the subject where an immune response can be mounted against the antigen encoded by the nucleic acid molecule.

As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

By "vertebrate subject" is meant any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The invention described herein is intended for use in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

II. Modes of Carrying out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the

purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

The present invention pertains to HCV fusion polypeptides that comprise amino acids 1018 to 1026 of HCV NS2, full-length HCV NS5a polypeptide and a portion of an HCV NS5b polypeptide with a C-terminal truncation. The invention also relates to polynucleotides encoding the same. In particular, the HCV fusion polypeptides of the invention include, in order from the amino terminal to the carboxy terminal, amino acids 1018 to 1026 of HCV NS2, amino acids 1027 to 1657 of HCV NS3, amino acids 1658 to 1972 of HCV NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core. The HCV fusion polypeptide can additionally include amino acids 384 to 715 of HCV E2 at the amino terminal preceding the NS2 sequence, and/or amino acids 2991 to 3011of HCV NS5 immediately following the NS5 2990 amino acid. The HCV fusion polypeptides of the present invention can be used to stimulate immunological responses, such as a humoral and/or cellular immune response, for example to activate HCV-specific T cells, *i.e.*, T cells which recognize epitopes of these polypeptides and/or to elicit the production of helper T cells and/or to stimulate the production of antiviral cytokines, chemokines, and the like. Activation of HCV-specific T cells by such fusion proteins provides both *in vitro* and *in vivo* model systems for the development of HCV vaccines, particularly for identifying HCV polypeptide epitopes associated with a response. The HCV fusion polypeptides can also be used to generate an immune response against HCV in a mammal, for example a CTL response, and/or to prime CD8+ and CD4+ T cells to produce antiviral agents, for either therapeutic or prophylactic purposes.

The HCV fusion polypeptides are therefore useful for treating and/or preventing HCV infection. The HCV fusion polypeptides can be used alone or in combination with one or more bacterial or viral immunogens. The combinations may include multiple immunogens from the same pathogen, multiple immunogens from different pathogens or multiple immunogens from the same and from different pathogens. Thus, bacterial, viral, and/or other immunogens may be included in the

same composition as the HCV fusion polypeptides, or may be administered to the same subject separately.

Moreover, the HCV fusion polypeptides of the present invention can also be used as diagnostic reagents to detect HCV infection in a biological sample.

In order to further an understanding of the invention, a more detailed discussion is provided below regarding HCV fusion polypeptides for use in the subject compositions, as well as production of the HCV fusion polypeptides, compositions comprising the same and methods of using the HCV fusion polypeptides.

HCV Fusion Polypeptides

The genomes of HCV strains contain a single open reading frame of approximately 9,000 to 12,000 nucleotides, which is transcribed into a polyprotein. As shown in Figure 1 and Table 1, an HCV polyprotein, upon cleavage, produces at least ten distinct products, in the order of NH_2-Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. The core polypeptide occurs at positions 1-191, numbered relative to HCV-1 (see, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455, for the HCV-1 genome). This polypeptide is further processed to produce an HCV polypeptide with approximately amino acids 1-173. The envelope polypeptides, E1 and E2, occur at about positions 192-383 and 384-746, respectively. The P7 domain is found at about positions 747-809. NS2 is an integral membrane protein with proteolytic activity and is found at about positions 810-1026 of the polyprotein. NS2, in combination with NS3, (found at about positions 1027-1657), cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease, found at about positions 1027-1207, serves to process the remaining polyprotein. The helicase activity is found at about positions 1193-1657. NS3 liberates an NS3 cofactor (NS4a, found about positions 1658-1711), two proteins (NS4b found at about positions 1712-1972, and NS5a found at about positions 1973-2420), and an RNA-dependent RNA polymerase (NS5b found at about positions 2421-3011). Completion of

polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease.

Table 1

Domain	Approximate Boundaries*
C (core)	1-191
E1	192-383
E2	384-746
P7	747-809
NS2	810-1026
NS3	1027-1657
NS4a	1658-1711
NS4b	1712-1972
NS5a	1973-2420
NS5b	2421-3011

*Numbered relative to HCV-1. See, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455.

HCV fusion polypeptides of the invention include a C-terminally truncated NS5 polypeptide (also referred to herein as "NS5t" or "NS5tr"). In particular, the C-terminally truncated NS5 polypeptide comprises a full-length NS5a polypeptide and an N-terminal portion of an NS5b polypeptide. The C-terminally truncated polypeptide can be truncated at any position between amino acid 2500 and the C-terminus, numbered relative to the full-length HCV-1 polyprotein, such as after amino acid 2505... 2550... 2600... 2650... 2700... 2750... 2800... 2850... 2900... 2950... 2960... 2970... 2975... 2980... 2985... 2990... 2995... 3000, etc, numbered relative to the full-length HCV-1 sequence. It is readily apparent that the molecule can be truncated at any amino acid between 2500 and 3010, numbered relative to the full-length HCV-1 sequence. One particularly preferred NS5 polypeptide is truncated at the amino acid corresponding to the amino acid immediately following amino acid 2990, numbered relative to the full-length HCV-1 polyprotein, and comprises an amino acid sequence corresponding to amino acids 1973-2990, numbered relative to the full-length HCV-1 polyprotein. The sequence for such a construct is shown at amino acid positions 1-1018 of SEQ ID NO:8 (labeled as amino acids 1973-2990 in Figures 5A-5E). The fusions of the invention optionally have an N-terminal methionine for expression.

The C-terminally truncated NS5 polypeptides can be used alone, in compositions described below, or in combination with one or more other HCV immunogenic polypeptides derived from any of the various domains of the HCV polyprotein. The additional HCV polypeptides can be provided separately or in the fusion. In fact, the fusion can include all the regions of the HCV polyprotein. These polypeptides may be derived from the same HCV isolate as the NS5 polypeptide, or from different strains and isolates including isolates having any of the various HCV genotypes, to provide increased protection against a broad range of HCV genotypes. Additionally, polypeptides can be selected based on the particular viral clades endemic in specific geographic regions where vaccine compositions containing the fusions will be used. It is readily apparent that the subject fusions provide an effective means of treating HCV infection in a wide variety of contexts.

Thus, NS5t can be included in a fusion polypeptide comprising any combination of NS5t with one or more immunogenic HCV polypeptide from other domains in the HCV polyprotein, i.e., an NS5t combined with an E1, E2, p7, NS2, NS3, NS4, and/or a core polypeptide. Preferably, the NS5t is combined with portions of the NS2, NS3, NS4, and core polypeptides, and optionally E2, in a HCV fusion polypeptide. These regions need not be in the order in which they occur naturally. Moreover, each of these regions can be derived from the same or a different HCV isolate. The various HCV polypeptides present in the various fusions described herein can either be full-length polypeptides or portions thereof.

The portions of the HCV polypeptides making up the fusion polypeptide each generally comprise at least one epitope, which is recognized by a T cell receptor on an activated T cell, such as 2152-HEYPVGSQL-2160 (SEQ ID NO:1) and/or 2224-AELIEANLLWRQEMG-2238 (SEQ ID NO:2). Epitopes can be identified by several methods. For example, the individual polypeptides or fusion proteins comprising any combination of the above, can be isolated by, e.g., immunoaffinity purification using a monoclonal antibody for the polypeptide or protein. The isolated protein sequence can then be screened by preparing a series of short peptides by proteolytic cleavage of the purified protein, which together span the entire protein sequence. By starting with, for example, 100-mer polypeptides, each polypeptide can be tested for the presence of epitopes recognized by a T-cell receptor on an

HCV-activated T cell, progressively smaller and overlapping fragments can then be tested from an identified 100-mer to map the epitope of interest.

Epitopes recognized by a T-cell receptor on an HCV-activated T cell can be identified by, for example, a ^{51}Cr release assay or by a lymphoproliferation assay (see the examples). In a ^{51}Cr release assay, target cells can be constructed that display the epitope of interest by cloning a polynucleotide encoding the epitope into an expression vector and transforming the expression vector into the target cells. HCV-specific $CD8^+$ T cells will lyse target cells displaying, for example, one or more epitopes from one or more regions of the HCV polyprotein found in the fusion, and will not lyse cells that do not display such an epitope. In a lymphoproliferation assay, HCV-activated $CD4^+$ T cells will proliferate when cultured with, for example, one or more epitopes from one or more regions of the HCV polyprotein found in the fusion, but not in the absence of an HCV epitopic peptide.

The various HCV polypeptides can occur in any order in the fusion polypeptide. If desired, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more of one or more of the HCV polypeptides may occur in the HCV fusion polypeptide. Multiple viral strains of HCV occur, and HCV polypeptides of any of these strains can be used in the fusion polypeptide.

Nucleic acid and amino acid sequences of a number of HCV strains and isolates, including nucleic acid and amino acid sequences of the various regions of the HCV polyprotein, including Core, NS2, p7, E1, E2, NS3, NS4, NS5a, NS5b genes and polypeptides have been determined. For example, isolate HCV J1.1 is described in Kubo *et al.* (1989) Japan. Nucl. Acids Res. 17:10367-10372; Takeuchi *et al.*(1990) Gene 91:287-291; Takeuchi *et al.* (1990) J. Gen. Virol. 71:3027-3033; and Takeuchi *et al.* (1990) Nucl. Acids Res. 18:4626. The complete coding sequences of two independent isolates, HCV-J and BK, are described by Kato *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87:9524-9528 and Takamizawa *et al.*, (1991) J. Virol. 65:1105-1113 respectively.

Publications that describe HCV-1 isolates include Choo *et al.* (1990) Brit. Med. Bull. 46:423-441; Choo *et al.* (1991) Proc. Natl. Acad. Sci. USA 88:2451-2455 and Han *et al.* (1991) Proc. Natl. Acad. Sci. USA 88:1711-1715. HCV isolates HC-J1 and HC-J4 are described in Okamoto *et al.* (1991) Japan J. Exp. Med. 60:167-177.

HCV isolates HCT 18~, HCT 23, Th, HCT 27, EC1 and EC10 are described in Weiner *et al.* (1991) Virol. 180:842-848. HCV isolates Pt-1, HCV-K1 and HCV-K2 are described in Enomoto *et al.* (1990) Biochem. Biophys. Res. Commun. 170:1021-1025. HCV isolates A, C, D & E are described in Tsukiyama-Kohara *et al.* (1991) Virus Genes 5:243-254.

As explained above, each of the components of an HCV fusion polypeptide can be obtained from the same HCV strain or isolate or from different HCV strains or isolates. For example, the NS5 polypeptide can be derived from a first strain of HCV, and the other HCV polypeptides present can be derived from a second strain of HCV. Alternatively, one or more of the other HCV polypeptides, for example NS2, NS3, NS4, Core, p7, E1 and/or E2, if present, can be derived from a first strain of HCV, and the remaining HCV polypeptides can be derived from a second strain of HCV. Additionally, each or the HCV polypeptides present can be derived from different HCV strains.

For a description of various HCV epitopes from the HCV regions for use in the subject fusions, see, e.g., Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365; Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,280,927 and 6,150,087.

For example, fusions can comprise the C-terminally truncated NS5 polypeptide and an NS3 polypeptide. The NS3 polypeptide can be modified to inhibit or reduce protease activity, such that further cleavage of the fusion is inhibited (also referred to herein as "NS3*"). The NS3 polypeptide can be modified by deletion of all or a portion of the NS3 protease domain. Alternatively, proteolytic activity can be inhibited by substitutions of amino acids within active regions of the protease domain. Finally, additions of amino acids to active regions of the domain, such that the catalytic site is modified, will also serve to inhibit proteolytic activity.

As explained above, the protease activity is found at about amino acid positions 1027-1207, numbered relative to the full-length HCV-1 polyprotein (see, Choo et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455), positions 2-182 of Figure 2. The structure of the NS3 protease and active site are known. See, e.g., De Francesco et al., *Antivir. Ther.* (1998) 3:99-109; Koch et al., *Biochemistry* (2001) 40:631-640. Thus, deletions or modifications to the native sequence will typically

occur at or near the active site of the molecule. Particularly, it is desirable to modify or make deletions to one or more amino acids occurring at positions 1- or 2-182, preferably 1- or 2-170, or 1- or 2-155 of Figure 2. Preferred modifications are to the catalytic triad at the active site of the protease, i.e., H, D and/or S residues, in order to inactivate the protease. These residues occur at positions 1083, 1107 and 1165, respectively, numbered relative to the full-length HCV polyprotein (positions 58, 80 and 140, respectively, of Figure 2). Such modifications will suppress proteolytic cleavage while maintaining T-cell epitopes. One particularly preferred modification is a substitution of Ser-1165 with Ala. One of skill in the art can readily determine portions of the NS3 protease to delete in order to disrupt activity. The presence or absence of activity can be determined using methods known to those of skill in the art.

For example, protease activity or lack thereof may be determined using the procedure described below in the examples, as well as using assays well known in the art. See, e.g., Takeshita et al., *Anal. Biochem.* (1997) 247:242-246; Kakiuchi et al., *J. Biochem.* (1997) 122:749-755; Sali et al., *Biochemistry* (1998) 37:3392-3401; Cho et al., *J. Virol. Meth.* (1998) 72:109-115; Cerretani et al., *Anal. Biochem.* (1999) 266:192-197; Zhang et al., *Anal. Biochem.* (1999) 270:268-275; Kakiuchi et al., *J. Virol. Meth.* (1999) 80:77-84; Fowler et al., *J. Biomol. Screen.* (2000) 5:153-158; and Kim et al., *Anal. Biochem.* (2000) 284:42-48.

Figure 3A-3J shows a representative HCV fusion polypeptide containing modified NS3 polypeptide, with the NS3 protease domain deleted from the N-terminus and including amino acids 1-121 of Core on the C-terminus.

As explained above, it may be desirable to include polypeptides derived from the core region of the HCV polyprotein in the fusions of the invention. This region occurs at amino acid positions 1-191 of the HCV polyprotein, numbered relative to HCV-1. Either the full-length protein, fragments thereof, such as amino acids 1-160, e.g., amino acids 1-150, 1-140, 1-130, 1-120, for example, amino acids 1-121, 1-122, 1-123...1-151, etc., or smaller fragments containing epitopes of the full-length protein may be used in the subject fusions, such as those epitopes found between amino acids 10-53, amino acids 10-45, amino acids 67-88, amino acids 120-130, or any of the core epitopes identified in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al., *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publication No. WO 93/00365;

Chien, D.Y., International Publication No. WO 94/01778; and U.S. Patent Nos. 6,280,927 and 6,150,087. Moreover, a protein resulting from a frameshift in the core region of the polyprotein, such as described in International Publication No. WO 99/63941, may be used. One particularly desirable core polypeptide for use with the present fusions includes the sequence of amino acids depicted at amino acid positions 1772-1892 of Figure 3A-3J. This core polypeptide includes amino acids 1-121 of the HCV polyprotein, with consensus amino acids Arg-9 and Thr-11 (positions 1780 and 1782, respectively, of Figure 3A-3J). Figures 5A-5E (SEQ ID NOS:7 and 8) show the DNA and corresponding amino acid sequence of a representative fusion protein that includes a C-terminally truncated NS5 polypeptide with the C-terminus of the NS5 polypeptide fused to this core polypeptide. The C-terminally truncated NS5 polypeptide includes amino acids 1973-2990 of the HCV polyprotein, numbered relative to HCV-1 (see, Choo et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455), (amino acids 1-1018 of SEQ ID NO:8), fused to a core polypeptide as described above that includes amino acids 1-121 of the HCV polyprotein (amino acids 1019-1139 of SEQ ID NO:8).

If a core polypeptide is present, it can occur at the N-terminus, the C-terminus and/or internal to the fusion. Particularly preferred is a core polypeptide on the C–terminus as this allows for the formation of complexes with certain adjuvants, such as ISCOMs, described further below.

Other useful polypeptides in the HCV fusion include T-cell epitopes derived from any of the various regions in the polyprotein. In this regard, E1, E2, p7 and NS2 are known to contain human T-cell epitopes (both CD4+ and CD8+) and including one or more of these epitopes serves to increase vaccine efficacy as well as to increase protective levels against multiple HCV genotypes. Moreover, multiple copies of specific, conserved T-cell epitopes can also be used in the fusions, such as a composite of epitopes from different genotypes.

For example, polypeptides from the HCV E1 and/or E2 regions can be used in the fusions of the present invention. E2 exists as multiple species (Spaete et al., *Virol.* (1992) 188:819-830; Selby et al., *J. Virol.* (1996) 70:5177-5182; Grakoui et al., *J. Virol.* (1993) 67:1385-1395; Tomei et al., *J. Virol.* (1993) 67:4017-4026) and clipping and proteolysis may occur at the N– and C-termini of the E2 polypeptide. Thus, an E2 polypeptide for use herein may comprise amino acids 405-661, e.g., 400, 401,

402... to 661, as well as polypeptides such as 383 or 384-661, 383 or 384-715, 383 or 384-746, 383 or 384-749 or 383 or 384-809, or 383 or 384 to any C-terminus between 661-809, of an HCV polyprotein, numbered relative to the full-length HCV-1 polyprotein. Preferably, a portion of the E2 polypeptide that includes amino acids 384-715 is included in the HCV fusions polypeptide. Preferably, the E2 polypeptide sequence occurs at the N-terminal of the HCV fusion polypeptide.

Similarly, E1 polypeptides for use herein can comprise amino acids 192-326, 192-330, 192-333, 192-360, 192-363, 192-383, or 192 to any C-terminus between 326-383, of an HCV polyprotein.

Immunogenic fragments of E1 and/or E2 which comprise epitopes may be used in the subject fusions. For example, fragments of E1 polypeptides can comprise from about 5 to nearly the full-length of the molecule, such as 6, 10, 25, 50, 75, 100, 125, 150, 175, 185 or more amino acids of an E1 polypeptide, or any integer between the stated numbers. Similarly, fragments of E2 polypeptides can comprise 6, 10, 25, 50, 75, 100, 150, 200, 250, 300, or 350 amino acids of an E2 polypeptide, or any integer between the stated numbers.

For example, epitopes derived from, e.g., the hypervariable region of E2, such as a region spanning amino acids 384-410 or 390-410, can be included in the fusions. A particularly effective E2 epitope to incorporate into an E2 polypeptide sequence is one which includes a consensus sequence derived from this region, such as the consensus sequence (SEQ ID NO: 9)
Gly-Ser-Ala-Ala-Arg-Thr-Thr-Ser-Gly-Phe-Val-Ser-Leu-Phe-Ala-Pro-Gly-Ala-Lys-Gln-Asn, which represents a consensus sequence for amino acids 390-410 of the HCV type 1 genome. Additional epitopes of E1 and E2 are known and described in, e.g., Chien et al., International Publication No. WO 93/00365.

Moreover, the E1 and/or E2 polypeptides may lack all or a portion of the membrane spanning domain. With E1, generally polypeptides terminating with about amino acid position 370 and higher (based on the numbering of the HCV-1 polyprotein) will be retained by the ER and hence not secreted into growth media. With E2, polypeptides terminating with about amino acid position 731 and higher (also based on the numbering of the HCV-1 polyprotein) will be retained by the ER and not secreted. (See, e.g., International Publication No. WO 96/04301, published February 15, 1996). It should be noted that these amino acid positions are not

absolute and may vary to some degree. Thus, the present invention contemplates the use of E1 and/or E2 polypeptides which retain the transmembrane binding domain, as well as polypeptides which lack all or a portion of the transmembrane binding domain, including E1 polypeptides terminating at about amino acids 369 and lower, and E2 polypeptides, terminating at about amino acids 730 and lower. Furthermore, the C-terminal truncation can extend beyond the transmembrane spanning domain towards the N-terminus. Thus, for example, E1 truncations occurring at positions lower than, e.g., 360 and E2 truncations occurring at positions lower than, e.g., 715, are also encompassed by the present invention. All that is necessary is that the truncated E1 and E2 polypeptides remain functional for their intended purpose. However, particularly preferred truncated E1 constructs are those that do not extend beyond about amino acid 300. Most preferred are those terminating at position 360. Preferred truncated E2 constructs are those with C-terminal truncations that do not extend beyond about amino acid position 715. Particularly preferred E2 truncations are those molecules truncated after any of amino acids 715-730, such as 725.

In certain preferred embodiments, the fusion protein comprises a modified NS3, an NS4 (NS4a and NS4b), a C-terminally truncated NS5 and, optionally, a core polypeptide of an HCV (NS3*NS4NS5t or NS3*NS4NS5tCore fusion proteins, also termed "NS3*45t" and "NS3*45tCore" herein). These fusion polypeptides may also include a portion of the HCV NS2 polypeptide, preferably, the NS2 portion from amino acids 1018 to 1026 (numbered as in the HCV-1 polyprotein). These regions need not be in the order in which they naturally occur in the native HCV polyprotein. Thus, for example, the core polypeptide may be at the N– and/or C-terminus of the fusion. In a particularly preferred embodiment, the NS5t includes amino acids 1973-2990, numbered relative to the full-length HCV-1 polyprotein and the NS3* molecule includes a substitution of Ala for Ser normally found at position 1165, and the regions occur in the following N-terminus to C-terminus order: NS3*NS4NS5t or NS2NS3*NS4NS5t. These fusions can include a core polypeptide at the C-terminus of the molecule. If present, the core polypeptide preferably includes the sequence of amino acids depicted at amino acid positions 1772-1892 of Figure 3A-3J. This core polypeptide includes amino acids 1-121 of the HCV polyprotein, with consensus amino acids Arg-9 and Thr-11 (positions 1780 and 1782, respectively, of Figure 3A-3J).

In another preferred embodiment, the HCV fusion polypeptides described immediately above include an E2 polypeptide at the N-terminus preceding NS3* or NS2. Preferably, the E2 polypeptide is a C-terminally truncated polypeptide and includes amino acids 384-715, numbered relative to the full-length HCV-1 polyprotein. This fusion can also optionally include a core polypeptide as described above.

If desired, the fusion proteins, or the individual components of these proteins, also can contain other amino acid sequences, such as amino acid linkers or signal sequences, as well as ligands useful in protein purification, such as glutathione-S-transferase and staphylococcal protein A.

Polynucleotides Encoding the HCV Fusion Polypeptides

Polynucleotides contain less than an entire HCV genome, or alternatively can include the sequence of the entire polyprotein with a C-terminally truncated NS5 domain, as described above. The polynucleotides can be RNA or single- or double-stranded DNA. Preferably, the polynucleotides are isolated free of other components, such as proteins and lipids. The polynucleotides encode the fusion proteins described above, and thus comprise coding sequences for NS5t and at least one other HCV polypeptide from a different region of the HCV polyprotein, such as polypeptides derived from NS2, p7, E1, E2, NS3, NS4, core, etc. The polynucleotides preferably encode HCV fusion polypeptides comprising or consisting of E2NS2NS3*NS4NS5core, E2NS2NS3*NS4NS5tcore, NS2NS3*NS4NS5core or NS2NS3*NS4NS5tcore. Polynucleotides of the invention can also comprise other nucleotide sequences, such as sequences coding for linkers, signal sequences, or ligands useful in protein purification such as glutathione-S-transferase and staphylococcal protein A.

To aid expression yields, it may be desirable to split the polyprotein into fragments for expression. These fragments can be used in combination in compositions as described herein. Alternatively, these fragments can be joined subsequent to expression. Thus, for example, NS3*NS4 can be expressed as one construct and NS5tCore can be expressed as a second construct and the two proteins subsequently fused or added separately to compositions. Similarly, E2NS3*NS4 can be expressed as one construct and NS5tCore expressed as a second construct. It is to

be understood that the above combinations are merely representative and any combination of fusions can be expressed separately.

It has been previously shown that inclusion of a truncated NS5 (for example, NS5 truncated at amino acid 2990) in a HCV fusion polypeptide often results in a higher level of expression of the fusion polypeptide compared to one with a full-length NS5 included. It has also been previously suggested that addition of HCV core sequences (for example core amino acids 1-121), at the carboxy terminal of an HCV fusion polypeptide results in a higher level of expression than the expression level of HCV fusion polypeptides without core sequences at the carboxy-terminal. The present inventors have now found that addition of certain HCV E2 sequences (for example, amino acids 384-715) at the N-terminal of an HCV fusion polypeptide enhances the recombinant expression level of the fusion polypeptide compared to those not containing E2 sequences at the N-terminal. The invention thus also provides a method of enhancing the recombinant expression of an HCV fusion polypeptide by positioning HCV E2 sequences, preferably amino acids 384-715 (numbered with respect to the HCV-1 polyprotein), at the N-terminal of the fusion polypeptide. It will be apparent that such positioning of the E2 amino acid sequences at the N-terminal can be accomplished by fusing E2 coding sequences to the 5'end of the fusion polypeptide.

Polynucleotides encoding the various HCV polypeptides can be isolated from a genomic library derived from nucleic acid sequences present in, for example, the plasma, serum, or liver homogenate of an HCV infected individual or can be synthesized in the laboratory, for example, using an automatic synthesizer. An amplification method such as PCR can be used to amplify polynucleotides from either HCV genomic DNA or cDNA encoding therefor.

Polynucleotides can comprise coding sequences for these polypeptides which occur naturally or can be artificial sequences which do not occur in nature. These polynucleotides can be ligated to form a coding sequence for the fusion proteins using standard molecular biology techniques. A polynucleotide encoding these proteins can be introduced into an expression vector which can be expressed in a suitable expression system. A variety of bacterial, yeast, mammalian and insect expression systems are available in the art and any such expression system can be used. Optionally, a polynucleotide encoding these proteins can be translated in a cell-free

translation system. Such methods are well known in the art. The proteins also can be constructed by solid phase protein synthesis.

The expression constructs of the present invention, including the desired fusion, or individual expression constructs comprising the individual components of these fusions, may be used for nucleic acid immunization, to stimulate a cellular immune response, using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346, 5,580,859, 5,589,466. Genes can be delivered either directly to the vertebrate subject or, alternatively, delivered *ex vivo*, to cells derived from the subject and the cells reimplanted in the subject. For example, the constructs can be delivered as plasmid DNA, e.g., contained within a plasmid, such as pBR322, pUC, or ColE1

Additionally, the expression constructs can be packaged in liposomes prior to delivery to the cells. Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger et al., in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

Liposomal preparations for use with the polynucleotides of the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with cationic liposomes particularly preferred. Cationic liposomes are readily available. For example, N[1-2,3-dioleyloxy)propyl] -N,N,N-triethyl-ammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available lipids include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boerhinger). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., in METHODS OF IMMUNOLOGY (1983), Vol.

101, pp. 512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos et al., *Biochim. Biophys. Acta* (1975) 394:483; Wilson et al., *Cell* (1979) 17:77); Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro et al., *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley et al., *Proc. Natl. Acad. Sci. USA* (1979) 76:3348); Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145); Fraley et al., *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder et al., *Science* (1982) 215:166.

The DNA can also be delivered in cochleate lipid compositions similar to those described by Papahadjopoulos et al., *Biochem. Biophys. Acta.* (1975) 394:483-491. See, also, U.S. Patent Nos. 4,663,161 and 4,871,488.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems, such as murine sarcoma virus, mouse mammary tumor virus, Moloney murine leukemia virus, and Rous sarcoma virus. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Patent No. 5,219,740; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) 1:5-14; Scarpa et al., *Virology* (1991) 180:849-852; Burns et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) 3:102-109. Briefly, retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses such as FIV, HIV, HIV-1, HIV-2 and SIV (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; 10801 University Blvd., Manassas, VA 20110-2209), or isolated from known sources using commonly available techniques.

A number of adenovirus vectors have also been described, such as adenovirus Type 2 and Type 5 vectors. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with

insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K.L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy* (1993) 4:461-476).

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery.

Members of the Alphavirus genus, such as but not limited to vectors derived from the Sindbis and Semliki Forest viruses, VEE, will also find use as viral vectors for delivering the gene of interest. For a description of Sindbis-virus derived vectors useful for the practice of the instant methods, see, Dubensky et al., *J. Virol.* (1996) 70:508-519; and International Publication Nos. WO 95/07995 and WO 96/17072.

Other vectors can be used, including but not limited to adeno-associated virus vectors, simian virus 40 and cytomegalovirus. Bacterial vectors, such as Salmonella ssp. *Yersinia enterocolitica*, Shigella spp., *Vibrio cholerae*, Mycobacterium strain BCG, and *Listeria monocytogenes* can be used. Minichromosomes such as MC and MC1, bacteriophages, cosmids (plasmids into which phage lambda *cos* sites have been inserted) and replicons (genetic elements that are capable of replication under their own control in a cell) can also be used.

The expression constructs may also be encapsulated, adsorbed to, or associated with, particulate carriers. Such carriers present multiple copies of a selected molecule to the immune system and promote trapping and retention of molecules in local lymph nodes. The particles can be phagocytosed by macrophages and can enhance antigen presentation through cytokine release. Examples of particulate carriers include those derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; and McGee et al., *J. Microencap.* (1996).

A wide variety of other methods can be used to deliver the expression constructs to cells. Such methods include DEAE dextran-mediated transfection, calcium phosphate precipitation, polylysine- or polyornithine-mediated transfection, or precipitation using other insoluble inorganic salts, such as strontium phosphate,

aluminum silicates including bentonite and kaolin, chromic oxide, magnesium silicate, talc, and the like. Other useful methods of transfection include electroporation, sonoporation, protoplast fusion, liposomes, peptoid delivery, or microinjection. See, e.g., Sambrook et al., *supra*, for a discussion of techniques for transforming cells of interest; and Felgner, P.L., *Advanced Drug Delivery Reviews* (1990) 5:163-187, for a review of delivery systems useful for gene transfer. One particularly effective method of delivering DNA using electroporation is described in International Publication No. WO/0045823.

Additionally, biolistic delivery systems employing particulate carriers such as gold and tungsten, are especially useful for delivering the expression constructs of the present invention. The particles are coated with the construct to be delivered and accelerated to high velocity, generally under a reduced atmosphere, using a gun powder discharge from a "gene gun." For a description of such techniques, and apparatuses useful therefore, see, e.g., U.S. Patent Nos. 4,945,050; 5,036,006; 5,100,792; 5,179,022; 5,371,015; and 5,478,744.

Compositions Comprising Fusion Proteins or Polynucleotides

The invention also provides immunogenic compositions comprising the fusion proteins or polynucleotides. The compositions may be used to stimulate an immunological response, as defined above. The compositions may include one or more fusions, so long as one of the fusions includes a C-terminally truncated NS5 domain as described herein. Preferably, the composition will include a HCV fusion polypeptide comprising or consisting of a portion of NS2, NS3 (particularly a modified NS3), NS4, NS5t and core. More preferably, the composition will include a HCV fusion polypeptide comprising or consisting of a portion of E2, NS2, NS3 (particularly a modified NS3), NS4, NS5t and core. Compositions of the invention may also comprise a pharmaceutically acceptable carrier. The carrier should not itself induce the production of antibodies harmful to the host. Pharmaceutically acceptable carriers are well known to those in the art. Such carriers include, but are not limited to, large, slowly metabolized, macromolecules, such as proteins, polysaccharides such as latex functionalized sepharose, agarose, cellulose, cellulose beads and the like, polylactic acids, polyglycolic acids, polymeric amino acids such as polyglutamic acid, polylysine, and the like, amino acid copolymers, and inactive virus particles.

Pharmaceutically acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides, phosphates, or sulfates, as well as salts of organic acids such as acetates, proprionates, malonates, or benzoates. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, and other proteins well known to those of skill in the art. Compositions of the invention can also contain liquids or excipients, such as water, saline, glycerol, dextrose, ethanol, or the like, singly or in combination, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. The proteins or polynucleotides of the invention can also be adsorbed to, entrapped within or otherwise associated with liposomes and particulate carriers such as PLG. Liposomes and other particulate carriers are described above.

If desired, co-stimulatory molecules which improve immunogen presentation to lymphocytes, such as B7-1 or B7-2, or cytokines, lymphokines, and chemokines, including but not limited to cytokines such as IL-2, modified IL-2 (cys125 to ser125), GM-CSF, IL-12, γ- interferon, IP-10, MIP1β, FLP-3, ribavirin and RANTES, may be included in the composition. Optionally, adjuvants can also be included in a composition. Adjuvants which can be used include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% TWEEN 80, and 0.5% SPAN 85 (optionally containing various amounts of MTP-PE), formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% TWEEN 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi^{TM} adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% TWEEN 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox^{TM}); (3) saponin adjuvants, such as QS21 or

Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes), which ISCOMs may be devoid of additional detergent (see, e.g., International Publication No. WO 00/07621); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins, such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 etc. (see, e.g., International Publication No. WO 99/44636), interferons, such as gamma interferon, macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. W093/13202 and W092/19265); (7) monophosporyl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) (see, e.g., GB 2220221; EPA 0689454), optionally in the substantial absence of alum (see, e.g., International Publication No. WO 00/56358); (8) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulations (see, e.g., EPA 0835318; EPA 0735898; EPA 0761231); (9) a polyoxyethylene ether or a polyoxyethylene ester (see, e.g., International Publication No. WO 99/52549); (10) an immunostimulatory oligonucleotide such as a CpG oligonucleotide, or a saponin and an immunostimulatory oligonucleotide, such as a CpG oligonucleotide (see, e.g., International Publication No. WO 00/62800); (11) an immunostimulant and a particle of a metal salt (see, e.g., International Publication No. WO 00/23105); (12) a saponin and an oil-in-water emulsion (see, e.g., International Publication No. WO 99/11241; (13) a saponin (e.g., QS21) + 3dMPL + IL-12 (optionally + a sterol) (see, e.g., International Publication No. WO 98/57659); (14) the MPL derivative RC529; and (15) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), – acetyl-normuramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to nor-MDP),

N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), *etc.*

Moreover, the fusion protein can be adsorbed to, or entrapped within, an ISCOM. Classic ISCOMs are formed by combination of cholesterol, saponin, phospholipid, and immunogens. Generally, immunogens (usually with a hydrophobic region) are solubilized in detergent and added to the reaction mixture, whereby ISCOMs are formed with the immunogen incorporated therein. ISCOM matrix compositions are formed identically, but without viral proteins. Proteins with high positive charge may be electrostatically bound in the ISCOM particles, rather than through hydrophobic forces. For a more detailed general discussion of saponins and ISCOMs, and methods of formulating ISCOMs, see Barr et al. (1998) *Adv. Drug Delivery Reviews* 32:247-271 (1998).

ISCOMs for use with the present invention are produced using standard techniques, well known in the art, and are described in e.g., U.S. Patent Nos. 4,981,684, 5,178,860, 5,679,354 and 6,027,732; European Publ. Nos. EPA 109,942; 180,564 and 231,039; Coulter et al. (1998) *Vaccine* 16:1243. Typically, the term "ISCOM" refers to immunogenic complexes formed between glycosides, such as triterpenoid saponins (particularly Quil A), and antigens which contain a hydrophobic region. See, e.g., European Publ. Nos. EPA 109,942 and 180,564. In this embodiment, the HCV fusions (usually with a hydrophobic region) are solubilized in detergent and added to the reaction mixture, whereby ISCOMs are formed with the fusions incorporated therein. The HCV polypeptide ISCOMs are readily made with HCV polypeptides which show amphipathic properties. However, proteins and peptides which lack the desirable hydrophobic properties may be incorporated into the immunogenic complexes after coupling with peptides having hydrophobic amino acids, fatty acid radicals, alkyl radicals and the like.

As explained in European Publ. No. EPA 231,039, the presence of antigen is not necessary in order to form the basic ISCOM structure (referred to as a matrix or ISCOMATRIX), which may be formed from a sterol, such as cholesterol, a phospholipid, such as phosphatidylethanolamine, and a glycoside, such as Quil A. Thus, the HCV fusion of interest, rather than being incorporated into the matrix, is present on the outside of the matrix, for example adsorbed to the matrix via electrostatic interactions. For example, HCV fusions with high positive charge may

be electrostatically bound to the ISCOM particles, rather than through hydrophobic forces. For a more detailed general discussion of saponins and ISCOMs, and methods of formulating ISCOMs, see Barr et al. (1998) *Adv. Drug Delivery Reviews* 32:247-271 (1998).

The ISCOM matrix may be prepared, for example, by mixing together solubilized sterol, glycoside and (optionally) phospholipid. If phospholipids are not used, two dimensional structures are formed. See, e.g., European Publ. No. EPA 231,039. The term "ISCOM matrix" is used to refer to both the 3-dimensional and 2-dimensional structures. The glycosides to be used are generally glycosides which display amphipathic properties and comprise hydrophobic and hydrophilic regions in the molecule. Preferably saponins are used, such as the saponin extract from Quillaja saponaria Molina and Quil A. Other preferred saponins are aescine from Aesculus hippocastanum (Patt et al. (1960) *Arzneimittelforschung* 10:273-275 and sapoalbin from Gypsophilla struthium (Vochten et al. (1968) *J. Pharm. Belg.* 42:213-226.

In order to prepare the ISCOMs, glycosides are used in at least a critical micelle-forming concentration. In the case of Quil A, this concentration is about 0.03% by weight. The sterols used to produce ISCOMs may be known sterols of animal or vegetable origin, such as cholesterol, lanosterol, lumisterol, stigmasterol and sitosterol. Suitable phospholipids include phosphatidylcholine and phosphatidylethanolamine. Generally, the molar ratio of glycoside (especially when it is Quil A) to sterol (especially when it is cholesterol) to phospholipid is 1:1:0-1, ± 20% (preferably not more than ±10%) for each figure. This is equivalent to a weight ratio of about 5:1 for the Quil A:cholesterol.

A solubilizing agent may also be present and may be, for example a detergent, urea or guanidine. Generally, a non-ionic, ionic or zwitter-ionic detergent or a cholic acid based detergent, such as sodium desoxycholate, cholate and CTAB (cetyltriammonium bromide), can be used for this purpose. Examples of suitable detergents include, but are not limited to, octylglucoside, nonyl N-methyl glucamide or decanoyl N-methyl glucamide, alkylphenyl polyoxyethylene ethers such as a polyethylene glycol p-isooctyl-phenylether having 9 to 10 oxyethylene groups (commercialized under the trade name TRITON X-100R™), acylpolyoxyethylene esters such as acylpolyoxyethylene sorbitane esters (commercialized under the trade

name TWEEN 20™ , TWEEN 80™, and the like). The solubilizing agent is generally removed for formation of the ISCOMs, such as by ultrafiltration, dialysis, ultracentrifugation or chromatography, however, in certain methods, this step is unnecessary. (See, e.g., U.S. Patent No. 4,981,684).

Generally, the ratio of glycoside, such as QuilA, to HCV fusion by weight is in the range of 5:1 to 0.5:1. Preferably the ratio by weight is approximately 3:1 to 1:1, and more preferably the ratio is 2:1.

Once the ISCOMs are formed, they may be formulated into compositions and administered to animals, as described herein. If desired, the solutions of the immunogenic complexes obtained may be lyophilized and then reconstituted before use.

The HCV fusion polypeptides and compositions including the fusion polypeptides or the polynucleotides encoding the HCV fusion polypeptides, described above, can be used in combination with other HCV immunogenic proteins, and/or compositions comprising the same. For example, the HCV fusion polypeptides can be used in combination with any of the various HCV immunogenic proteins derived from one or more of the regions of the HCV polyprotein described in Table 1. One particular HCV antigen for use with the subject fusion polypeptide and/or composition comprising the fusion polypeptide, is an HCV E1E2 antigen. HCV E1E2 antigens are known, including complexes of HCV E1 with HCV E2, optionally containing part or all of the p7 region, such as HCV E1E2 complexes as described in PCT Publication No. WO 03/002065. The additional HCV immunogenic proteins can be provided in compositions with excipients, adjuvants, immunstimulatory molecules and the like, as described above. For example, the E1E2 complexes can be provided in compositions that include a submicron oil-in-water emulsion such as MF59 and/or oligonucleotides containing immunostimulatory nucleic acid sequences (ISS), such as CpY, CpR and unmethylated CpG motifs (a cytosine followed by guanosine and linked by a phosphate bond). Such compositions are described in detail in PCT Publication No. WO 03/002065.

Thus, it is readily apparent that the compositions of the present invention may be administered in conjunction with a number of immunoregulatory agents and will usually include an adjuvant. Such agents and adjuvants for use with the compositions

include, but are not limited to, any of those substances described above, as well as one or more of the following set forth below.

A. Mineral Containing Compositions

Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminum salts and calcium salts. The invention includes mineral salts such as hydroxides (e.g. oxyhydroxides), phosphates (e.g. hydroxyphosphates, orthophosphates), sulfates, etc. (e.g. see chapters 8 & 9 of *Vaccine Design* (1995) eds. Powell & Newman. ISBN: 030644867X. Plenum), or mixtures of different mineral compounds (e.g. a mixture of a phosphate and a hydroxide adjuvant, optionally with an excess of the phosphate), with the compounds taking any suitable form (e.g. gel, crystalline, amorphous, etc.), and with adsorption to the salt(s) being preferred. The mineral containing compositions may also be formulated as a particle of metal salt (PCT Publication No. WO00/23105).

Aluminum salts may be included in compositions of the invention such that the dose of Al^{3+} is between 0.2 and 1.0 mg per dose. In one embodiment, the aluminum- based adjuvant for use in the present compositions is alum (aluminum potassium sulfate ($AlK(SO_4)_2$)), or an alum derivative, such as that formed *in situ* by mixing an antigen in phosphate buffer with alum, followed by titration and precipitation with a base such as ammonium hydroxide or sodium hydroxide.

Another aluminum-based adjuvant for use in vaccine formulations of the present invention is aluminum hydroxide adjuvant ($Al(OH)_3$) or crystalline aluminum oxyhydroxide (AlOOH), which is an excellent adsorbant, having a surface area of approximately $500m^2/g$. Alternatively, aluminum phosphate adjuvant ($AlPO_4$) or aluminum hydroxyphosphate, which contains phosphate groups in place of some or all of the hydroxyl groups of aluminum hydroxide adjuvant is provided. Preferred aluminum phosphate adjuvants provided herein are amorphous and soluble in acidic, basic and neutral media.

In another embodiment, the adjuvant for use with the present compositions comprises both aluminum phosphate and aluminum hydroxide. In a more particular embodiment thereof, the adjuvant has a greater amount of aluminum phosphate than aluminum hydroxide, such as a ratio of 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 or greater than 9:1, by weight aluminum phosphate to aluminum hydroxide. More particularly,

aluminum salts may be present at 0.4 to 1.0 mg per vaccine dose, or 0.4 to 0.8 mg per vaccine dose, or 0.5 to 0.7 mg per vaccine dose, or about 0.6 mg per vaccine dose.

Generally, the preferred aluminum-based adjuvant(s), or ratio of multiple aluminum-based adjuvants, such as aluminum phosphate to aluminum hydroxide is selected by optimization of electrostatic attraction between molecules such that the antigen carries an opposite charge as the adjuvant at the desired pH. For example, aluminum phosphate adjuvant (iep = 4) adsorbs lysozyme, but not albumin at pH 7.4. Should albumin be the target, aluminum hydroxide adjuvant would be selected (iep 11.4). Alternatively, pretreatment of aluminum hydroxide with phosphate lowers its isoelectric point, making it a preferred adjuvant for more basic antigens.

B. Oil Emulsions

Oil emulsion compositions suitable for use as adjuvants in the compositions include squalene-water emulsions. Particularly preferred adjuvants are submicron oil-in-water emulsions. Preferred submicron oil-in-water emulsions for use herein are squalene/water emulsions optionally containing varying amounts of MTP-PE, such as a submicron oil-in-water emulsion containing 4-5% w/v squalene, 0.25-1.0% w/v Tween 80™ (polyoxyelthylenesorbitan monooleate), and/or 0.25-1.0% Span 85™ (sorbitan trioleate), and, optionally, N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphophoryloxy)-ethylamine (MTP-PE), for example, the submicron oil-in-water emulsion known as "MF59" (International Publication No. WO90/14837; US Patent Nos. 6,299,884 and 6,451,325, and Ott et al., "MF59 -- Design and Evaluation of a Safe and Potent Adjuvant for Human Vaccines" in *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M.F. and Newman, M.J. eds.) Plenum Press, New York, 1995, pp. 277-296). MF59 contains 4-5% w/v Squalene (e.g. 4.3%), 0.25-0.5% w/v Tween 80™, and 0.5% w/v Span 85™ and optionally contains various amounts of MTP-PE, formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA). For example, MTP-PE may be present in an amount of about 0-500 μg/dose, more preferably 0-250 μg/dose and most preferably, 0-100 μg/dose. As used herein, the term "MF59-0" refers to the above submicron oil-in-water emulsion lacking MTP-PE, while the term MF59-MTP

denotes a formulation that contains MTP-PE. For instance, "MF59-100" contains 100 μg MTP-PE per dose, and so on. MF69, another submicron oil-in-water emulsion for use herein, contains 4.3% w/v squalene, 0.25% w/v Tween 80™, and 0.75% w/v Span 85™ and optionally MTP-PE. Yet another submicron oil-in-water emulsion is MF75, also known as SAF, containing 10% squalene, 0.4% Tween 80™, 5% pluronic-blocked polymer L121, and thr-MDP, also microfluidized into a submicron emulsion. MF75-MTP denotes an MF75 formulation that includes MTP, such as from 100-400 μg MTP-PE per dose.

Submicron oil-in-water emulsions, methods of making the same and immunostimulating agents, such as muramyl peptides, for use in the compositions, are described in detail in International Publication No. WO90/14837 and US Patent Nos. 6,299,884 and 6,451,325.

Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) may also be used as adjuvants in the subject compositions.

C. Saponin Formulations

Saponin formulations, may also be used as adjuvants in the compositions. Saponins are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponins isolated from the bark of the *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponins can also be commercially obtained from *Smilax ornata* (sarsaprilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs.

Saponin compositions have been purified using High Performance Thin Layer Chromatography (HP-TLC) and Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in US Patent No. 5,057,540. Saponin formulations may also comprise a sterol, such as cholesterol (see, PCT Publication No. WO96/33739).

Combinations of saponins and cholesterols can be used to form unique particles called Immunostimulating Complexes (ISCOMs). ISCOMs typically also

include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of Quil A, QHA and QHC. ISCOMs are further described in EP0109942, WO96/11711 and WO96/33739. Optionally, the ISCOMS may be devoid of (an) additional detergent(s). See WO00/07621.

A review of the development of saponin-based adjuvants can be found in Barr, et al., "ISCOMs and other saponin based adjuvants", Advanced Drug Delivery Reviews (1998) 32:247-271. See also Sjolander, et al., "Uptake and adjuvant activity of orally delivered saponin and ISCOM vaccines", Advanced Drug Delivery Reviews (1998) 32:321-338.

D. Virosomes and Virus Like Particles (VLPs)

Virosomes and Virus Like Particles (VLPs) can also be used as adjuvants with the present compositions. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins may be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, Qß-phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein p1). VLPs are discussed further in WO03/024480, WO03/024481, and Niikura et al., "Chimeric Recombinant Hepatitis E Virus-Like Particles as an Oral Vaccine Vehicle Presenting Foreign Epitopes", Virology (2002) 293:273-280; Lenz et al., "Papillomarivurs-Like Particles Induce Acute Activation of Dendritic Cells", Journal of Immunology (2001) 5246-5355; Pinto, et al., "Cellular Immune Responses to Human Papillomavirus (HPV)-16 L1 Healthy Volunteers Immunized with Recombinant HPV-16 L1 Virus-Like Particles", Journal of Infectious Diseases (2003) 188:327-338; and Gerber et al., "Human Papillomavrisu Virus-Like Particles Are Efficient Oral Immunogens when Coadministered with Escherichia coli Heat-Labile Entertoxin Mutant R192G or CpG", Journal of Virology (2001) 75(10):4752-4760. Virosomes are discussed

further in, for example, Gluck et al., "New Technology Platforms in the Development of Vaccines for the Future", Vaccine (2002) 20:B10 –B16. Immunopotentiating reconstituted influenza virosomes (IRIV) are used as the subunit antigen delivery system in the intranasal trivalent INFLEXAL™ product {Mischler & Metcalfe (2002) *Vaccine* 20 Suppl 5:B17-23} and the INFLUVAC PLUS™ product.

E. Bacterial or Microbial Derivatives

Adjuvants suitable for use in the present compositions include bacterial or microbial derivatives such as:

(1) *Non-toxic derivatives of enterobacterial lipopolysaccharide (LPS)*

Such derivatives include Monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3dMPL). 3dMPL is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in EP 0 689 454. Such "small particles" of 3dMPL are small enough to be sterile filtered through a 0.22 micron membrane (see EP 0 689 454). Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529. See Johnson *et al.* (1999) *Bioorg Med Chem Lett* 9:2273-2278.

(2) *Lipid A Derivatives*

Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in Meraldi et al., "OM-174, a New Adjuvant with a Potential for Human Use, Induces a Protective Response with Administered with the Synthetic C-Terminal Fragment 242-310 from the circumsporozoite protein of Plasmodium berghei", Vaccine (2003) 21:2485-2491; and Pajak, et al., "The Adjuvant OM-174 induces both the migration and maturation of murine dendritic cells in vivo", Vaccine (2003) 21:836-842.

(3) *Immunostimulatory oligonucleotides*

Immunostimulatory oligonucleotides suitable for use as adjuvants include nucleotide sequences containing a CpG motif (a sequence containing an unmethylated cytosine followed by guanosine and linked by a phosphate bond). Bacterial double stranded RNA or oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. Optionally, the guanosine may be replaced with an analog such as 2'-deoxy-7-deazaguanosine. See, Kandimalla, et al., "Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles", Nucleic Acids Research (2003) 31(9): 2393-2400; WO02/26757 and WO99/62923 for examples of possible analog substitutions. The adjuvant effect of CpG oligonucleotides is further discussed in Krieg, "CpG motifs: the active ingredient in bacterial extracts?", Nature Medicine (2003) 9(7): 831-835; McCluskie, et al., "Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA", FEMS Immunology and Medical Microbiology (2002) 32:179-185; WO98/40100; US Patent No. 6,207,646; US Patent No. 6,239,116 and US Patent No. 6,429,199.

The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT. See, Kandimalla, et al., "Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic CpG DNAs", Biochemical Society Transactions (2003) 31 (part 3): 654-658. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in Blackwell, et al., "CpG-A-Induced Monocyte IFN-gamma-Inducible Protein-10 Production is Regulated by Plasmacytoid Dendritic Cell Derived IFN-alpha", J. Immunol. (2003) 170(8):4061-4068; Krieg, "From A to Z on CpG", TRENDS in Immunology (2002) 23(2): 64-65 and WO01/95935. Preferably, the CpG is a CpG-A ODN.

Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, Kandimalla, et al., "Secondary structures in CpG oligonucleotides affect immunostimulatory activity", BBRC (2003) 306:948-953; Kandimalla, et al., "Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic GpG DNAs", Biochemical Society Transactions (2003) 31(part 3):664-658; Bhagat et

al., "CpG penta- and hexadeoxyribonucleotides as potent immunomodulatory agents" BBRC (2003) 300:853-861 and WO03/035836.

(4) *ADP-ribosylating toxins and detoxified derivatives thereof.*

Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the compositions. Preferably, the protein is derived from *E. coli* (i.e., *E. coli* heat labile enterotoxin "LT), cholera ("CT"), or pertussis ("PT"). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in WO95/17211 and as parenteral adjuvants in WO98/42375. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LTR192G. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in the following references: Beignon, et al., "The LTR72 Mutant of Heat-Labile Enterotoxin of Escherichia coli Enahnces the Ability of Peptide Antigens to Elicit CD4+ T Cells and Secrete Gamma Interferon after Coapplication onto Bare Skin", Infection and Immunity (2002) 70(6):3012-3019; Pizza, et al., "Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants", Vaccine (2001) 19:2534-2541; Pizza, et al., "LTK63 and LTR72, two mucosal adjuvants ready for clinical trials" Int. J. Med. Microbiol (2000) 290(4-5):455-461; Scharton-Kersten et al., "Transcutaneous Immunization with Bacterial ADP-Ribosylating Exotoxins, Subunits and Unrelated Adjuvants", Infection and Immunity (2000) 68(9):5306-5313; Ryan et al., "Mutants of Escherichia coli Heat-Labile Toxin Act as Effective Mucosal Adjuvants for Nasal Delivery of an Acellular Pertussis Vaccine: Differential Effects of the Nontoxic AB Complex and Enzyme Activity on Th1 and Th2 Cells" Infection and Immunity (1999) 67(12):6270-6280; Partidos et al., "Heat-labile enterotoxin of Escherichia coli and its site-directed mutant LTK63 enhance the proliferative and cytotoxic T-cell responses to intranasally co-immunized synthetic peptides", Immunol. Lett. (1999) 67(3):209-216; Peppoloni et al., "Mutants of the Escherichia coli heat-labile enterotoxin as safe and strong adjuvants for intranasal delivery of vaccines", Vaccines (2003) 2(2):285-293; and Pine et al., (2002) "Intranasal immunization with influenza vaccine and a detoxified mutant of heat labile enterotoxin from Escherichia coli (LTK63)" J. Control Release (2002) 85(1-3):263-270. Numerical reference for amino acid substitutions is

preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in Domenighini et al., Mol. Microbiol (1995) 15(6):1165-1167.

F. Bioadhesives and Mucoadhesives

Bioadhesives and mucoadhesives may also be used as adjuvants in the subject compositions. Suitable bioadhesives include esterified hyaluronic acid microspheres (Singh *et al.* (2001) *J. Cont. Rele.* 70:267-276) or mucoadhesives such as cross-linked derivatives of polyacrylic acid, polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the compositions. See, e.g., WO99/27960.

G. Microparticles

Microparticles may also be used as adjuvants in the compositions. Microparticles (i.e. a particle of ~100 nm to ~150 μm in diameter, more preferably ~200 nm to ~30 μm in diameter, and most preferably ~500 nm to ~10 μm in diameter) formed from materials that are biodegradable and non-toxic (e.g. a poly(α-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, etc.), with poly(lactide-co-glycolide) are preferred, optionally treated to have a negatively-charged surface (e.g. with SDS) or a positively-charged surface (e.g. with a cationic detergent, such as CTAB).

H. Liposomes

Examples of liposome formulations suitable for use as adjuvants are described in US Patent No. 6,090,406, US Patent No. 5,916,588, and EP 0 626 169.

I. Polyoxyethylene ether and Polyoxyethylene Ester Formulations

Adjuvants suitable for use in the compositions include polyoxyethylene ethers and polyoxyethylene esters. See, e.g., WO99/52549. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol (WO01/21657) as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol (WO01/21152). Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-steoryl ether,

polyoxytheylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

J. Polyphosphazene (PCPP)

PCPP formulations are described, for example, in Andrianov et al., "Preparation of hydrogel microspheres by coacervation of aqueous polyphophazene solutions", Biomaterials (1998) 19(1-3):109-115 and Payne et al., "Protein Release from Polyphosphazene Matrices", Adv. Drug. Delivery Review (1998) 31(3):185-196.

K. Muramyl peptides

Examples of muramyl peptides suitable for use as adjuvants include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-l-alanyl-d-isoglutamine (nor-MDP), and N-acetylmuramyl-l-alanyl-d-isoglutaminyl-l-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

L. Imidazoquinoline Compounds

Examples of imidazoquinoline compounds suitable for use as adjuvants in the compositions include Imiquimod and its analogues, described further in Stanley, "Imiquimod and the imidazoquinolines: mechanism of action and therapeutic potential" Clin Exp Dermatol (2002) 27(7):571-577; Jones, "Resiquimod 3M", Curr Opin Investig Drugs (2003) 4(2):214-218; and U.S. Patent Nos. 4,689,338, 5,389,640, 5,268,376, 4,929,624, 5,266,575, 5,352,784, 5,494,916, 5,482,936, 5,346,905, 5,395,937, 5,238,944, and 5,525,612.

M. Thiosemicarbazone Compounds

Examples of thiosemicarbazone compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the compositions include those described in WO04/60308. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

N. Tryptanthrin Compounds

Examples of tryptanthrin compounds, as well as methods of formulating, manufacturing, and screening for compounds all suitable for use as adjuvants in the

compositions include those described in WO04/64759. The tryptanthrin compounds are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF-α.

O. Human Immunomodulators

Human immunomodulators suitable for use as adjuvants in the compositions include cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*e.g.* interferon-γ), macrophage colony stimulating factor, and tumor necrosis factor.

The compositions may also comprise combinations of aspects of one or more of the adjuvants identified above. For example, the following adjuvant compositions may be used in the invention:

(1) a saponin and an oil-in-water emulsion (WO99/11241);

(2) a saponin (e.g.., QS21) + a non-toxic LPS derivative (e.g. 3dMPL) (see WO94/00153);

(3) a saponin (e.g.., QS21) + a non-toxic LPS derivative (e.g. 3dMPL) + a cholesterol;

(4) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) (WO98/57659);

(5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions (See European patent applications 0835318, 0735898 and 0761231);

(6) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion.

(7) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); and

(8) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dPML).

(9) one or more mineral salts (such as an aluminum salt) + an immunostimulatory oligonucleotide (such as a nucleotide sequence including a CpG motif).

Aluminum salts and MF59 are preferred adjuvants for use with injectable vaccines. Bacterial toxins and bioadhesives are preferred adjuvants for use with mucosally-delivered vaccines, such as nasal vaccines.

Methods of Producing HCV-Specific Antibodies

The HCV fusion polypeptides can be used to produce HCV-specific polyclonal and monoclonal antibodies. HCV-specific polyclonal and monoclonal antibodies specifically bind to HCV antigens. Polyclonal antibodies can be produced by administering the fusion protein to a mammal, such as a mouse, a rabbit, a goat, or a horse. Serum from the immunized animal is collected and the antibodies are purified from the plasma by, for example, precipitation with ammonium sulfate, followed by chromatography, preferably affinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art.

Monoclonal antibodies directed against HCV-specific epitopes present in the fusion polypeptides can also be readily produced. Normal B cells from a mammal, such as a mouse, immunized with an HCV fusion polypeptide, can be fused with, for example, HAT-sensitive mouse myeloma cells to produce hybridomas. Hybridomas producing HCV-specific antibodies can be identified using RIA or ELISA and isolated by cloning in semi-solid agar or by limiting dilution. Clones producing HCV-specific antibodies are isolated by another round of screening.

Antibodies, either monoclonal and polyclonal, which are directed against HCV epitopes, are particularly useful for detecting the presence of HCV or HCV antigens in a sample, such as a serum sample from an HCV-infected human. An immunoassay for an HCV antigen may utilize one antibody or several antibodies. An immunoassay for an HCV antigen may use, for example, a monoclonal antibody directed towards an HCV epitope, a combination of monoclonal antibodies directed towards epitopes of one HCV polypeptide, monoclonal antibodies directed towards epitopes of different HCV polypeptides, polyclonal antibodies directed towards the same HCV antigen, polyclonal antibodies directed towards different HCV antigens, or a combination of monoclonal and polyclonal antibodies. Immunoassay protocols may

be based, for example, upon competition, direct reaction, or sandwich type assays using, for example, labeled antibody. The labels may be, for example, fluorescent, chemiluminescent, or radioactive.

The polyclonal or monoclonal antibodies may further be used to isolate HCV particles or antigens by immunoaffinity columns. The antibodies can be affixed to a solid support by, for example, adsorption or by covalent linkage so that the antibodies retain their immunoselective activity. Optionally, spacer groups may be included so that the antigen binding site of the antibody remains accessible. The immobilized antibodies can then be used to bind HCV particles or antigens from a biological sample, such as blood or plasma. The bound HCV particles or antigens are recovered from the column matrix by, for example, a change in pH.

HCV-Specific T cells

HCV-specific T cells that are activated by the above-described fusions, including the NS2NS3*NS4NS5t fusion protein or E2NS2NS3*NS4NS5t fusion protein, with or without a core polypeptide, as well as any of the other various fusions described herein, expressed *in vivo* or *in vitro*, preferably recognize an epitope of an HCV polypeptide such as an NS2, p7, E1, E2, NS3, NS4, NS5a or NS5b polypeptide, including an epitope of a fusion of one or more of these peptides with an NS5t, with or without a core polypeptide. HCV-specific T cells can be $CD8^+$ or $CD4^+$. In other embodiments, the NS5 portion may not be truncated.

The invention provides novel HCV fusion polypeptides that include the naturally occurring methionine of the NS2 protein found at amino acid position 1018 fused to at least the NS345 protein sequences, wherein the NS3 sequence is mutated to remove the inherent protease function of NS3. These compositions can further comprise E2 and core.

Adding E2 and/or core to the fusion proteins provides additional T-cell epitopes for the immune system to recognize. By not including E1, P7 and the major portion of NS2, expression is optimized due to loss of hydrophobic regions.

Expressing HCV proteins without the terminus of NS5b also enhances protein expression. (See, US20060088819-A1)

HCV-specific $CD8^+$ T cells can be cytotoxic T lymphocytes (CTL) which can kill HCV-infected cells that display any of these epitopes complexed with an MHC class I molecule. HCV-specific $CD8^+$ T cells can be detected by, for example, ^{51}Cr release assays (see the examples). ^{51}Cr release assays measure the ability of HCV-specific $CD8^+$ T cells to lyse target cells displaying one or more of these epitopes. HCV-specific $CD8^+$ T cells which express antiviral agents, such as IFN-γ, are also contemplated herein and can also be detected by immunological methods, preferably by intracellular staining for IFN-γ or like cytokine after *in vitro* stimulation with one or more of the HCV polypeptides, such as but not limited to an E2, NS3, NS4, NS5a, or NS5b polypeptide or core (see the examples).

HCV-specific $CD4^+$ cells activated by the above-described fusions, such as but not limited to an NS2NS3*NS4NS5t fusion polypeptide or an E2NS2NS3*NS4NS5t fusion polypeptide, with or without a core polypeptide, expressed *in vivo* or *in vitro*, preferably recognize an epitope of an HCV polypeptide, such as but not limited to an NS2, E2, NS3, NS4, NS5a, or NS5b or core polypeptide, including an epitope of fusions thereof, bound to an MHC class II molecule on an HCV-infected cell and proliferate in response to stimulating, e.g., NS2NS3*NS4NS5t or E2NS2NS3*NS4NS5t fusion polypeptide, with or without a core polypeptide.

HCV-specific $CD4^+$ T cells can be detected by a lymphoproliferation assay (see the examples). Lymphoproliferation assays measure the ability of HCV-specific $CD4^+$ T cells to proliferate in response to, e.g., an NS2, E2, NS3, an NS4, an NS5a, and/or an NS5b or core epitope.

Methods of Activating HCV-Specific T Cells.

The HCV fusion polypeptides or polynucleotides can be used to activate HCV-specific T cells either *in vitro* or *in vivo*. Activation of HCV-specific T cells can be used, *inter alia*, to provide model systems to optimize CTL responses to HCV and to provide prophylactic or therapeutic treatment against HCV infection. For *in vitro* activation, proteins are preferably supplied to T cells via a plasmid or a viral vector, such as an adenovirus vector, as described above.

Polyclonal populations of T cells can be derived from the blood, and preferably from peripheral lymphoid organs, such as lymph nodes, spleen, or thymus, of mammals that have been infected with an HCV. Preferred mammals include mice, chimpanzees, baboons, and humans. The HCV serves to expand the number of activated HCV-specific T cells in the mammal. The HCV-specific T cells derived from the mammal can then be restimulated *in vitro* by adding an HCV fusion polypeptide as described herein, such as but not limited to an HCV NS2NS3*NS4NS5t fusion protein or an E2NS2NS3*NS4NS5t fusion protein, with or without a core polypeptide, to the T cells. The HCV-specific T cells can then be tested for, *inter alia*, proliferation, the production of IFN-γ, and the ability to lyse target cells displaying HCV epitopes *in vitro*.

In a lymphoproliferation assay, HCV-activated $CD4^+$ T cells proliferate when cultured with an HCV polypeptide, such as but not limited to an NS3, NS4, NS5a, NS5b, NS3NS4NS5, E2NS3NS4NS5, or E2NS2NS3NS4NS5, or E2NS3NS4NS5t, or E2NS2NS3NS4NS5t, or E2NS3NS4NS5core, or E2NS2NS3NS4NS5core, or E2NS3NS4NS5tcore, or E2NS2NS3NS4NS5tcore epitopic peptide, but not in the absence of an epitopic peptide. Thus, particular HCV epitopes, such as NS2, E2, NS3, NS4, NS5a, NS5b, or core and fusions of these epitopes, such as but not limited to NS3NS4NS5 and E2NS3NS4NS5 epitopes that are recognized by HCV-specific $CD4^+$ T cells can be identified using a lymphoproliferation assay.

Similarly, detection of IFN-γ in HCV-specific CD4+ and/or $CD8^+$ T cells after *in vitro* stimulation with the above-described fusion proteins, can be used to identify, for example, fusion protein epitopes, such as but not limited to epitopes of NS2, p7, E1, E2, NS3, NS4, NS5a, NS5b, and fusions of these epitopes, such as but not limited to NS3NS4NS5, and E2NS3NS4NS5 epitopes that are particularly effective at stimulating CD4+ and/or $CD8^+$ T cells to produce IFN-γ (see Example 2).

Further, ^{51}Cr release assays are useful for determining the level of CTL response to HCV. *See* Cooper *et al.* Immunity 10:439-449. For example, HCV-specific $CD8^+$ T cells can be derived from the liver of an HCV infected mammal. These T cells can be tested in ^{51}Cr release assays against target cells displaying, e.g., E2NS2NS3NS4NS5 or NS2NS3NS4NS5 epitopes. Several target cell populations expressing different NS2NS3NS4NS5 or E2NS2NS3NS4NS5

epitopes can be constructed so that each target cell population displays different epitopes of NS2NS3NS4NS5 or E2NS2NS3NS4NS5. The HCV-specific $CD8^+$ cells can be assayed against each of these target cell populations. The results of the ^{51}Cr release assays can be used to determine which epitopes of NS2NS3NS4NS5 or E2NS2NS3NS4NS5 are responsible for the strongest CTL response to HCV. NS2NS3*NS4NS5t fusion proteins or E2NS2NS3*NS4NS5t fusion proteins, with or without core polypeptides, which contain the epitopes responsible for the strongest CTL response can then be constructed using the information derived from the ^{51}Cr release assays.

An HCV fusion polypeptide as described above, or polynucleotide encoding such a fusion polypeptide, can be administered to a mammal, such as a mouse, baboon, chimpanzee, or human, to stimulate a humoral and/or cellular immune response, such as to activate HCV-specific T cells *in vivo*. Administration can be by any means known in the art, including parenteral, intranasal, intramuscular or subcutaneous injection, including injection using a biological ballistic gun ("gene gun"), as discussed above.

Preferably, injection of a polynucleotide encoding an HCV fusion polypeptide is used to activate T cells. In addition to the practical advantages of simplicity of construction and modification, injection of the polynucleotides results in the synthesis of a fusion protein in the host. Thus, these immunogens are presented to the host immune system with native post-translational modifications, structure, and conformation. The polynucleotides are preferably injected intramuscularly to a large mammal, such as a human, at a dose of 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5 or 10 mg/kg.

A composition of the invention comprising an HCV fusion polypeptide or polynucleotide encoding same is administered in a manner compatible with the particular composition used and in an amount which is effective to activate HCV-specific T cells as measured by, *inter alia*, a ^{51}Cr release assay, a lymphoproliferation assay, or by intracellular staining for IFN-γ. The proteins and/or polynucleotides can be administered either to a mammal which is not infected with an HCV or can be administered to an HCV-infected mammal. The particular dosages of the polynucleotides or fusion proteins in a composition will depend on many factors including, but not limited to the species, age, and general condition of the mammal to

which the composition is administered, and the mode of administration of the composition. An effective amount of the composition of the invention can be readily determined using only routine experimentation. *In vitro* and *in vivo* models described above can be employed to identify appropriate doses. The amount of polynucleotide used in the example described below provides general guidance which can be used to optimize the activation of HCV-specific T cells either *in vivo* or *in vitro*. Generally, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5 or 10 mg of an HCV fusion polypeptide or polynucleotide will be administered to a large mammal, such as a baboon, chimpanzee, or human. If desired, co-stimulatory molecules or adjuvants can also be provided before, after, or together with the compositions.

Immune responses of the mammal generated by the delivery of a composition of the invention, including activation of HCV-specific T cells, can be enhanced by varying the dosage, route of administration, or boosting regimens. Compositions of the invention may be given in a single dose schedule, or preferably in a multiple dose schedule in which a primary course of vaccination includes 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and/or reinforce an immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose or doses after several months.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Those of skill in the art will readily appreciate that the invention may be practiced in a variety of ways given the teaching of this disclosure.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

Cloning of Polynucleotides Encoding HCV Fusion Polypeptides

Synthetic nucleic acid sequences encoding, in an amino-terminal to carboxy-terminal direction, the carboxy terminus of NS2 from amino acid 1018 to 1026, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 or 3011 of NS5 and optionally amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence if present is arginine and amino acid 11 of the core sequence is threonine, and optionally containing an E2 sequence from amino acid 384 to amino acid 715, were constructed following general methods and those outlined below. The various constructs are represented schematically in Figure 11 with amino acid numberings relative to the HCV-1 sequence. The nucleic acid sequences were cloned into plasmid vectors, and fusion polypeptides were expressed from host cells transformed with the plasmid vectors containing the fusion-polypeptide-encoding DNA insert, by utilizing standard recombinant cloning techniques and in particular those methods described previously in US2006-0088819A1, WO01/38360 and WO2004/005473.

A detailed example is given below for construction of one embodiment. Similar approaches were used to construct plasmid vectors containing the other nucleic acid sequences described above.

Cloning of Polynucleotides Encoding e2ns3$_m$ns5tr.c121

A synthetic HCV1a nucleic acid was constructed to encode an HCV fusion polypeptide consisting of, in an amino-terminal to carboxy-terminal direction, a methionine, amino acids 384-715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is arginine and amino acid 11 of the core sequence is threonine,. The fusion protein encoded by this nucleic acid sequence is represented schematically in Figure 7 with amino acid numberings relative to the HCV-1 sequence, and it is designated herein as "e2ns3$_m$ns5tr.c121", "e2.ns3m-ns5tr.core121", or "E2NS3*NS4NS5tr.core121" or "E2NS2NS3*NS4NS5tr.core121".

The e2ns3$_m$ns5tr.c121 fusion polypeptide was genetically engineered for expression in *Saccharomyces cerevisiae* using the yeast expression vector pBS24.1

(United States Patent 6,458,527 section 4.2.4.2 and United States Patent 5,635,374, illustrated in Figure 6a). This vector contains the 2μ sequence for autonomous replication in yeast and the yeast genes *leu2d* and URA3 as selectable markers. The α-factor terminator, β-lactamase gene and the *Col*E1 origin of replication, required for plasmid replication in bacteria, are also present in this expression vector.

The following steps were taken to construct the expression cassette for the e2ns3$_m$ns5tr.c121 polyprotein (illustrated in Figures 6a-6f):

First, to assemble the N-terminus region, a HindIII/AclI fragment of 819bp was gel isolated from pGEM7.d.E2 (HindIII/XhoI) subclone #3. The 5' HindIII cloning site is followed by the sequence ACAAAACAAA, the inititator ATG, and codons for the HCV-1 E2 ectodomain, beginning at aa384 and continuing to an AclI restriction site at aa650. The HindIII/AclI fragment and a 34bp AclI/CelII kinased synthetic fragment, corresponding to aa651- aa662 of the E2 ectodomain, were ligated into a pT7Blue2 HindIII/CelII vector containing a 228bp CelII/BlnI fragment which encodes aa662 to aa715 of the HCV-1 E2 ectodomain sequence, followed by codons for aa1018 – aa1039 of HCV-1 NS2 and NS3. The ligation mixture was transformed into HB101 competent cells and plated onto Luria-ampicillin agar plates (100μg/ml). After miniprep DNA analysis, identification of the desired clones and sequence confirmation, pT7Blue2.E2/ns2.3 #23 was digested with HindIII and BlnI to isolate a 1081bp fragment which encodes E2/NS2/NS3.

Secondly, to introduce the Ser$_{1165}$-Ala mutation in the NS3 domain, a BlnI/ClaI fragment of 703bp was gel isolated from pSP72 HindIII/ClaI.ns3mut 1165 #15. This 703bp fragment encodes aa1040-aa1274 of the HCV-1 genome in which Ser$_{1165}$ was mutated to Ala by site-directed mutagenesis.

Third, to facilitate the cloning of the e2.ns3$_m$-ns5core121 expression cassette, the 1081bp HindIII/BlnI fragment (encoding E2/NS2/NS3) and the 703bp Bln/ClaI fragment (encoding NS3m Ser1165-Ala) were ligated into the pSP72 HindIII/ClaI vector. The ligation mixture was transformed as above, and after DNA analysis the resultant clone was named pSP72.HindIII/Cla e2.ns3$_m$ #1

Fourth, a 1784bp HindIII/ClaI fragment, encoding E2/NS2/NS3m, was gel purified from pSP72.HindIII/Cla e2.ns3$_m$#1 described above. A ClaI/NheI 2787bp fragment encoding aa1274-aa2202 from NS3-NS5a of HCV-1 was isolated from a full-length HCV-1 clone, pUC.HCV3. A 2732bp Nhe/SalI fragment was gel isolated

from a pSP72.HindIII/SalI.ns5ab.2990.core121 #27 subclone. The Nhe/Sal fragment corresponds to aa2203-2990 of NS5a and NS5b, followed by aa1-121 of the core domain. Within the HCV-1 core sequence, consensus aa were incorporated at position 9 (Arg instead of the Lys of HCV-1 core sequence) and position 11 (Thr instead of the Asn of HCV-1).

Lastly, a 1366bp BamHI/HindIII ADH2/GAPDH promoter fragment, described in the United States Patent 6,183,985, was ligated with the 1784bp HindIII/ClaI fragment, the 2787bp Cla/NheI fragment, and the 2732bp NheI/SalI fragment into the pBS24.1 BamHI/SalI yeast expression vector, thereby creating plasmid pd.e2ns3$_m$ns5tr.c121 (see Figure 6f).

Using similar approaches, other polynucleotides were constructed to encode other fusion polypeptides as shown in Figure 11 and inserted into the expression plasmid vector pBS24.1. These other fusion proteins are:

Δns3ns5core121 or ΔNS3-NS5.core121 (aa1242-aa1657 of NS3, aa1658-aa1972 of NS4, aa1973-aa2990 of NS5 and aa1-aa121 of core);

ns3m.ns5-core or NS3m-NS5 (aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4 and aa1973-aa3011 of NS5);

ns3m.ns5tr-core or NS3m-NS5tr (aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4 and aa1973-aa2990 of NS5);

ns3m.ns5+core121 or NS3m-NS5.core121 (aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4, aa1973-aa3011 of NS5 and aa1-aa121 of core);

ns3m.ns5tr+core or NS3m-NS5tr.core121 (aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4, aa1973-aa2990 of NS5 and aa1-aa121 of core);

e2.ns3m.ns5-core or E2.NS3m-NS5 (methionine, aa384-aa715 of E2, aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4 and aa1973-aa3011 of NS5);

e2.ns3m.ns5tr-core or E2.NS3m-NS5tr (methionine, aa384-aa715 of E2, aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4 and aa1973-aa2990 of NS5); and

e2.ns3m.ns5+core or E2.NS3m-NS5.core121 (methionine, aa384-aa715 of E2, aa1018-aa1026 of NS2, aa1027-aa1657 of NS3, aa1658-aa1972 of NS4, aa1973-aa3011 of NS5 and aa1-aa121 of core).

All of these fusion proteins had Arg as amino acid 9 of the core sequence and Thr as amino acid 11 of the core sequence. All except Δns3ns5core121 had the Ser1165 – Ala mutation in NS3.

Expression and Detection of HCV Fusion Proteins

The expression plasmids with the various constructs described above were transformed into yeast and expressed as demonstrated in Figures 10a to 10d.

The fusion proteins were expressed from the yeast expression plasmids using the ADH2/GAPDH promoter. For example, the E2NS2NS3*NS4NS5tr.core121 fusion protein was expressed from plasmid pd.e2ns3$_m$ns5tr.c121.

S. cerevisiae strain AD3* (genotype matα,leu2,trp1,ura3-52,prb-1122,pep4-3,prc1-407, cir°,trp+, :DM15[GAP/ADR], originally derived from strain BJ2168 as described in United States Patent 6,458,527 section 4.2.4.4), was transformed with the pd.e2ns3$_m$ns5tr.c121 yeast expression plasmid or other plasmids as described above. Yeast cells were transformed with the expression plasmids using a lithium acetate protocol. Ura- transformants were streaked for single colonies and patched onto leu$^-$ /8% glucose plates to increase plasmid copy number. Leu$^-$starter cultures were grown for 24-48 hours at 30° C and then diluted 1:20 in YEPD (yeast extract bactopeptone 2% glucose) media. Cells were grown at either 25°C or 30°C for 48 hours and harvested after depletion of glucose in the medium.

For experiments whose results are shown in Figures 10a-10d, to test for expression of the HCV fusion polypeptide encoded by plasmid e2ns3$_m$ns5tr.c121 as well as the other fusion polypeptides as described above, yeast transformants were inoculated into 3ml of leu$^-$/8% glucose media from either freshly grown single colonies, frozen glycerol stocks or days-old liquid cultures. These cultures, referred to as "starter cultures", were grown at 30°C for 36-48 hrs. Then 1.5ml of each starter culture was inoculated into 28.5ml YEPD and grown at 25°C for 48-50 hours. Equal aliquots of cells (same volumes of packed cells) were lysed with glass beads in lysis buffer (10mM Tris-Cl pH 7.5, 1mM EDTA, 10 mM DTT, 1 mM PMSF). Lysed yeast cell samples were centrifuged for 30min. at 14K rpm, supernatant was discarded and

insoluble yeast pellet (IP) was resuspended in 500µl pH 12 SDS sample buffer + 50mM DTT, placed on a tilt shaker for 1hr. The resuspended IP samples were sonicated for 8-10 seconds (Virsonic 60, set at 17), and additional 500µl pH12 SDS sample buffer + 50mM DTT were added to the sonicated samples. The samples were centrifuged for 1 min. in a microfuge to pellet debris. Five microliters of each sample were loaded on SDS Tris-Glycine gels (4-20%) without boiling.

After electrophoresis, gels were either stained with Coomassie blue (shown on the left in each of Figures 10a-10d) or blotted to nitrocellulose filter paper and incubated with a rabbit anti-HCV Helicase antibody using the Western Blotting technique (shown on the right in each of Figures 10a-10d). The primary antibody, rabbit anti-Helicase #1 antibody (BAbCO, Berkeley Antibody Company, Richmond, California) was used at a dilution of 1:10,000. The blots were then detected with a goat anti-rabbit IgG (H+L) HRP-conjugate at a 1:1000 dilution and developed with HRP color development reagent.

Figure 10A is a comparison of the expression levels of the HCV fusion polypeptides that do not have the E2 ectodomain of aa384-715 at the amino terminus. The starter cultures for all the samples shown in this figure were inoculated from the freshly grown single colonies. All samples represent the insoluble pellet (IP), resuspended in 1ml pH12 SB+DTT for 1 hr and sonicated for ~8 seconds. The samples shown in Figure 10A are:

Lane ST, molecular weight standard;
Lane C, pAB24 plasmid vector control;
Lane 1, ns3m-ns5 (219.3 kD), colony A;
Lane 2, ns3m-ns5 (219.3 kD), colony B;
Lane 3, ns3m-ns5tr (217.0 kD), colony A;
Lane 4, ns3m-ns5tr (217.0 kD), colony B;
Lane 5, ns3m-ns5.core121 (233.6 kD), colony A;
Lane 6, ns3m-ns5.core121 (233.6 kD), colony B;
Lane 7, ns3m-ns5tr.core121 (230.3 kD), colony A;
Lane 8, ns3m-ns5tr.core121 (230.3 kD), colony B;
Lane 9, Δns3-ns5.core121 (208.0 kD), colony A;
Lane 10, Δns3-ns5.core121 (208.0 kD), colony B.

Figure 10B is a comparison of the expression levels of the fusion polypeptides that have the E2 ectodomain of aa384-715 at the amino terminus with each other and with the Δns3-ns5.core121 polypeptide which serves as a control. The starter cultures for all the samples shown in this figure were inoculated from the freshly grown single colonies. All samples represent the insoluble pellet (IP), resuspended in 1ml pH12 SB+DTT for 1 hr and sonicated for ~8 seconds. The samples shown in Figure 10B are:

Lane C, pAB24 plasmid vector control;
Lane ST, molecular weight standard;
Lane 1, e2.ns3m-ns5 (256.0 kD), colony A;
Lane 2, e2.ns3m-ns5 (256.0 kD), colony B;
Lane 3, e2.ns3m-ns5tr (253.6 kD), colony A;
Lane 4, e2.ns3m-ns5tr (253.6 kD), colony B;
Lane 5, e2.ns3m-ns5.core121 (269.2 kD), colony A;
Lane 6, e2.ns3m-ns5.core121 (269.2 kD), colony B;
Lane 7, e2.ns3m-ns5tr.core121 (266.3 kD), colony A;
Lane 8, e2.ns3m-ns5tr.core121 (266.3 kD), colony B;
Lane 9, Δns3-ns5.core121 (208.0 kD), colony A;
Lane 10, Δns3-ns5.core121 (208.0 kD), colony B.

Figure 10C is a comparison of expression and detection of various fusion polypeptides with inocula from frozen glycerol stocks except pAB24 which was inoculated from a freshly grown colony. Figure 10D is a comparison of the same fusion proteins with inocula from days-old liquid cultures instead of frozen stocks or single colonies. These were performed to confirm that future expression and detection levels of the desired fusion polypeptides can be maintained through multiple generational growths from both frozen stocks, previously prepared liquid cultures and fresh single colonies.

The samples shown in Figure 10C are:
Lane ST, molecular weight standard;
Lane C, pAB24 plasmid vector control;
Lane 1, ns3m-ns5 (219.3 kD), frozen stock A;

Lane 2, ns3m-ns5 (219.3 kD), frozen stock B;
Lane 3, ns3m-ns5tr.core121 (230.3 kD), frozen stock A;
Lane 4, ns3m-ns5tr.core121 (230.3 kD), frozen stock B;
Lane 5, e2.ns3m-ns5 (256.0 kD), frozen stock A;
Lane 6, e2.ns3m-ns5 (256.0 kD), frozen stock B;
Lane 7, e2.ns3m-ns5tr.core121 (266.3 kD), frozen stock A;
Lane 8, e2.ns3m-ns5tr.core121 (266.3 kD), frozen stock B;
Lane 9, e2.ns3m-ns5tr.core121 (266.3 kD), frozen stock C;
Lane 10, Δns3-ns5.core121 (208.0 kD), colony.

The samples shown in Figure 10D are:
Lane ST, molecular weight standard;
Lane C, pAB24 plasmid vector control;
Lane 1, ns3m-ns5 (219.3 kD), liquid culture A;
Lane 2, ns3m-ns5 (219.3 kD), liquid culture B;
Lane 3, ns3m-ns5tr.core121 (230.3 kD), liquid culture A;
Lane 4, ns3m-ns5tr.core121 (230.3 kD), liquid culture B;
Lane 5, e2.ns3m-ns5 (256.0 kD), liquid culture A;
Lane 6, e2.ns3m-ns5 (256.0 kD), liquid culture B;
Lane 7, e2.ns3m-ns5tr.core121 (266.3 kD), liquid culture A;
Lane 8, e2.ns3m-ns5tr.core121 (266.3 kD), liquid culture B;

Using Δns3-ns5.core121 as the control for the amount of expression level detected by Western Analysis, the amounts of fusion polypeptides detected in Figure 10a and 10b were estimated by visual inspection, and the relative amounts are summarized in Table 2 below.

Table 2. The summary of HCV fusion polypeptide expression levels detected by Western Analysis, compared to the Δns3-ns5.core121 fusion polyprotein control.

Fusion Protein	Expression Levels vs. Control (Western Analysis)
Δns3-ns5.core121 control	1X
ns3m-ns5	1/3X*

ns3m-ns5tr	1/4X*
ns3m-ns5.core121	1/5X*
ns3m-ns5tr.core121	1/3X*
e2.ns3m-ns5	2X
e2.ns3m-ns5tr	1/4X
e2.ns3m-ns5.core121	1/4X
e2.ns3m-ns5tr.core121	2X

*Degradation amounts and patterns consistent throughout all samples in Western Analysis

The results shown in Figures 10A, 10B and Table 2 indicate that e2.ns3m-ns5 and e2.ns3m-ns5tr core121 were expressed at particularly high levels, especially when compared to ns3m-ns5 and ns3m-ns5tr core121 which do not have the E2 ectodomain of aa384-715 at the amino terminus.

Production of the HCV Fusion Polypeptide-ISCOM Formulations

The E2NS2NS3*NS4NS5tcore121 fusion protein, or e2.ns3m-ns5tr.core121, produced as described above was used to produce HCV fusion-ISCOMs as follows. The fusion-ISCOM formulations were prepared by mixing the fusion protein with a preformed ISCOMATRIX (empty ISCOMs) utilizing ionic interactions to maximize association between the fusion protein and the adjuvant. ISCOMATRIX is prepared essentially as described in Coulter et al. (1998) *Vaccine* 16:1243. Further methods for production of HCV fusion polypeptides plus ISCOMs are described herein. The fusion-ISCOM formulations are also referred to herein as "IMX/poly" or "IMX-poly". In one embodiment, CpG was added to the formulation, and the complete formulation was named "IMX/poly/CpG".

EXAMPLE 2

Ability of Fusion Polypeptide Vaccine Formulations to Prime T-cell Responses

Immunization

The following studies were conducted to determine the ability of E2NS3*NS4NS5tcore121/ISCOMS, or IMX/poly, with or without CpG, to prime HCV-specific immune responses, especially T cell responses. In addition, primary

immunizations with IMX/poly followed by boosts with alphavirus replicons encoding wildtype NS345 ("α-NS345"), or vice versa (priming with α-NS345 and boosting with fusion-ISCOM formulations), were tested for their effects on HCV-specific T cell responses. The results are shown in Figure 8 ($CD4^+$ T cell responses) and Figure 9 ($CD8^+$ T cell responses).

10 female BALB/c mice per group were injected intra-muscularly (IM) in the tibialis anterior muscle in a total volume of 100 μl (i.e. 50 μl per thigh) with the indicated vaccine formulations (Figures 8 and 9) at weeks 0, 3, and 6, and the sera were collected at weeks 2, 5, and 8. For the prime-boost studies, the mice were primed at week 0 and 3, and boosted at week 6. For non-structural protein (NS345), 5E6 replication particles of VEE/SIN-NS345 and 50 μg of polyprotein were mixed with 5 μg of IMX (Pearse, M. J., and D. Drane. 2005. Adv Drug Deliv Rev 57:465-74; Pearse, M. J., and D. Drane. 2004.Vaccine 22:2391-5; Polakos, et al. 2001.J Immunol 166:3589-98) with or without 10 μg of CpG for injection. The mice were sacrificed at week 8 for detecting T cell responses in spleen and antibody responses in serum.

Intracellular staining (ICS)

Spleen cells (1E6) were stimulated with 10 μg/ml of the peptides or proteins indicated in Table 3 for 6 hours at 37°C in the presence of anti-CD28 antibody (1μg/ml) (BD Biosciences, San Jose, CA) and Brefeldin A (BD Biosciences, San Jose, CA), and then stained with antibodies against CD4 (anti-CD4 allophycocyanin conjugate, clone SK3, Becton Dickinson, San Jose, CA) and CD8 (anti-CD8α PerCP conjugate, clone SK1, Becton Dickinson), permeabilized with Cytofix/Cytoperm (Pharmingen), and IFN-γ (clone 4S.B3, phycoerythrin conjugate, Pharmingen). Stained cells were analyzed with a FACSCalibur™ flow cytometer (Becton Dickinson). The mean frequencies of cytokine-positive cells were calculated for each pair of duplicates. The antigen-specific frequency was determined by comparing unstimulated mean frequency (no peptide) with the stimulated mean frequency (with HCV peptides, Table 3), and $p<0.05$ is considered statistically significant by t-test.

Table 3: Peptides or proteins used to stimulate T cells.

Name	Peptide or protein information	Reference
NS3 pool	20 mer overlapping peptides covering NS3	
NS4 pool	20 mer overlapping peptides covering NS4	
NS5A pool	20 mer overlapping peptides covering NS5A	
NS5B pool	20 mer overlapping peptides covering NS5B	
NS3-1 pep	LVALGINAVAYYRGL	(6)
NS3-2 pep	TTVRLRAYMNTPGLP	(6)
NS3-3 pep	SSPPVVPQSF	(1, 2)
NS5B pep	MSYSWTGALVTPCAAE	(7)
SOD-C100 (NS4)	Recombinant NS4 protein purified from yeast (HCV 1a a.a. 1569-1931)	(3, 4)
SOD-NS5	Recombinant NS5A/B protein purified from yeast (HCV 1a a.a. 2054-2995)	(5)

References for Example 2:

1. Arribillaga, L., et al 2002. Vaccine 21:202-10.
2. Arribillaga, L., et al 2005. Vaccine 23:3493-9.
3. Kuo, G., et al. 1989. Science 244:362-4.
4. Minutello, M. A.,et al. 1993.. J Exp Med 178:17-25.
5. Saracco, G., et al. 1994. Liver 14:65-70.
6. Simon, B. E., et al. 2003. Infect Immun 71:6372-80.
7. Uno-Furuta, S., et al.. 2003. Vaccine 21:3149-56.

The results shown in Figure 8 indicate that IMX-poly without CpG was able to induce HCV-specific $CD4^+$ T cell responses as compared to the negative control. Addition of CpG to IMX-poly increased $CD4^+$ T cell responses further. Priming and boosting with IMX-poly and α-NS345 in various orders with or without CpG also induced significant $CD4^+$ T cell responses.

EXAMPLE 3

Alphaviruses Expressing Corresponding NS epitopes

Alphavirus replicon particles, for example, SINCR (DC+) and SINCR (LP) are prepared as described in Polo et al., *Proc. Natl. Acad. Sci. USA* (1999) 96:4598-4603. The alphavirus replicons can contain all or part or include additional HCV epitopes when compared to the amino acids of the immunogenic HCV fusion polypeptide compositions described herein. The α-NS345 replicons used in Example

2 contained complete sequences of NS3, 4 and 5 (amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5), and there was no mutation in NS3. Such alphavirus particles can be used in combination with the HCV fusion polypeptides of the invention as part of a prime boost immunization strategy, as shown in Example 2.

EXAMPLE 4

Production of modified E2NS2NS3*NS4NS5t and E2NS2NS3*NS4NS5tCore Polynucleotides and Polypeptides

E2 in the following examples represents a C-terminally truncated E2 molecule that includes amino acids 384-715, numbered relative to the full-length HCV-1 polyprotein. The E2 nucleic acid in one embodiment was fused to a nucleic acid encoding amino acids 1018 to 1026 of the NS2 protein, which is fused to a nucleic acid encoding amino acids 1027 to 3011 or 2990 of the polyprotein, fused to a nucleic acid encoding the core 121 amino acids of the polyprotein.

These two versions of HCV fusion polypeptides are found in Figure 11 as "e2.ns3m.ns5-/-core 121" (the full length version of NS5b) and "e2.ns3m.ns5tr-/-core 121" (truncated NS5b) modified NS3 polypeptide. The constructs comprising E2 amino acids sequences shown include core 1 to 121 as described herein.

The portion of the nucleic acid encoding amino acids 1027 to 2990 of the polyprotein encodes a modified NS3 protein (1027-1657), NS4aNS4b (1658-1972) and NS5aNS5b(1973-2090), wherein the NS5b protein is truncated.

The nucleic acid encoding amino acids 1027 to 3011 of the polyprotein encodes a modified NS3 protein (1027-1657), NS4aNS4b (1658-1972) and NS5aNS5b(1973-3011)" wherein the NS5b is full length.

The modified NS3 portions of the HCV fusion polypeptides comprise a Ser1165 to Alanine mutation that results in loss of protease activity.

Additional fusion proteins shown in Figure 11 contain the last nine amino acids of NS2 fused to (1) the nucleic acid encoding amino acids 1027 to 3011 of the polyprotein encodes a modified NS3 protein (1027-1657), NS4aNS4b (1658-1972) and NS5aNS5b(1973-3011), wherein the NS5b is full length ("ns3m.ns5+/-core 121"), or (2) the nucleic acid encoding amino acids 1027 to 2990 of the polyprotein

encodes a modified NS3 protein (1027-1657), NS4aNS4b (1658-1972) and NS5aNS5b(1973-2090), wherein the NS5b protein is truncated ("ns3m.ns5tr+/-core 121").

Addition of the last nine amino acids of NS2 to the fusion proteins described herein provides a naturally occurring methionine upstream of the NS3 gene portion and downstream of the E2 portion of the polyprotein, thus minimizing changes in epitopes from the naturally occurring HCV polyprotein.

In one embodiment, the invention provides modified HCV fusion polypeptides that allow for improved protein expression in yeast.

Since the N-terminus of the HCV-1 NS3 domain (aa1027-aa1657) encodes a trypsin-like serine protease, the natural Ser_{1165} of the protease catalytic triad is mutated to Ala to prevent autoproteolysis of the HCV e2ns3$_m$ns5tr.c121 fusion polypeptide.

Thus, nucleic acids encoding and HCV fusion polypeptides and polynucleotides encoding the polypeptides are disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the claims.

What is claimed is :

1. An immunogenic composition comprising an isolated HCV fusion polypeptide consisting of a polypeptide selected from the group of polypeptides consisting of:

(1) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine;

(2) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine;

(3) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine; and

(4) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine.

2. An immunogenic composition comprising an isolated HCV fusion polypeptide consisting of a polypeptide selected from the group of polypeptides consisting of:

(1) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence

is replaced with an alanine, amino acid 9 of the core sequence is arginine and amino acid 11 of the core sequence is threonine;

(2) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine;

(3) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine; and

(4) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine.

3. The composition of claim 1 or claim 2, further comprising an additional HCV immunogenic polypeptide.

4. The composition of claim 3, wherein the additional HCV immunogenic polypeptide comprises an E1E2 complex.

5. The composition of any of claims 1 to 4 further comprising an adjuvant.

6. The composition of any of claims 1 to 5 further comprising an immunostimulatory molecule.

7. The composition of claim 6 wherein the immunostimulatory molecule is a CpG.

8. A method of stimulating a cellular immune response in a vertebrate subject comprising administering to the subject a therapeutically effective amount of the composition of any of claims 1 to 7.

9. Use of a composition according to any of claims 1 to 7 in a method of stimulating a cellular immune response in a vertebrate subject.

10. Use of a composition according to any of claims 1 to 7, in the manufacture of a medicament for stimulating a cellular immune response in a subject.

11. A method for producing a composition comprising combining a composition according to any of claims 1 to 7, with a pharmaceutically acceptable excipient.

12. A polynucleotide comprising a coding sequence encoding an HCV fusion polypeptide consisting of polypeptide selected from the group of polypeptides consisting of:

(1) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine;

(2) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine;

(3) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine

at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine; and

(4) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a lysine and amino acid 11 of the core sequence is asparagine.

13. A polynucleotide comprising a coding sequence encoding an HCV fusion polypeptide consisting of a polypeptide selected from the group of polypeptides consisting of:

(1) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is arginine and amino acid 11 of the core sequence is threonine;

(2) in an amino terminal to carboxy terminal direction, a methionine, amino acids 384 to 715 of E2, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine;

(3) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 2990 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine; and

(4) in an amino terminal to carboxy terminal direction, amino acids 1018 to 1026 of NS2, amino acids 1027 to 1657 of NS3, amino acids 1658 to 1972 of NS4, amino acids 1973 to 3011 of NS5 and amino acids 1 to 121 of core, wherein the serine at position 1165 of the NS3 sequence is replaced with an alanine, amino acid 9 of the core sequence is a arginine and amino acid 11 of the core sequence is threonine.

14. An isolated nucleic acid encoding

(a) the polynucleotide according to claim 12 or claim 13; and

(b) at least one control element operably linked to the polynucleotide, whereby the coding sequence can be transcribed and translated in a host cell.

15. A host cell transformed with the nucleic acid of claim 14.

16. A method for producing an HCV fusion polypeptide, said method comprising culturing a population of host cells according to claim 15 under conditions for producing said polypeptide.

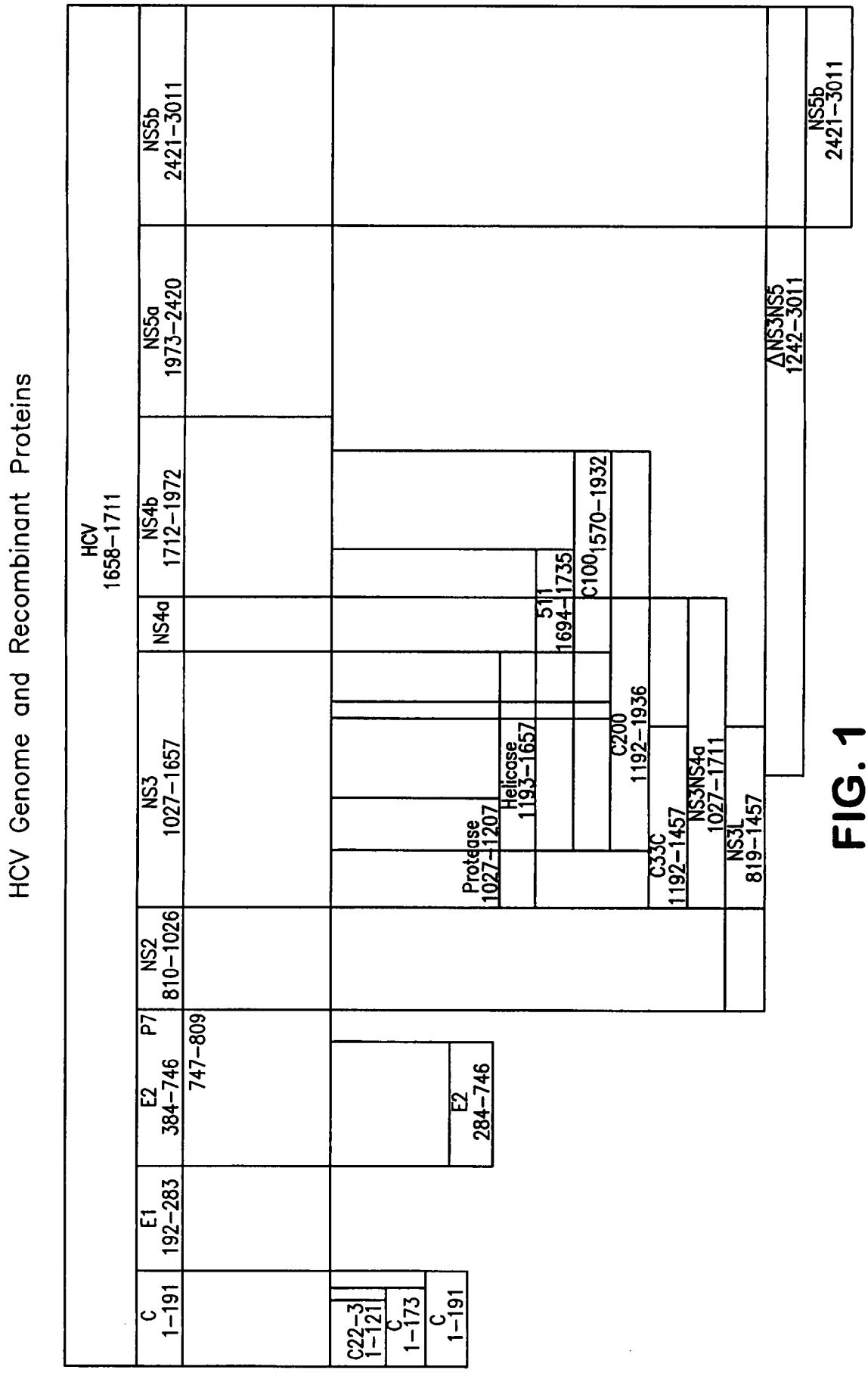
HCV Genome and Recombinant Proteins
HCV
1658–1711
C
1–191
E1
192–283
E2
384–746
P7
747–809
NS2
810–1026
NS3
1027–1657
NS4a
NS4b
1712–1972
NS5a
1973–2420
NS5b
2421–3011
C22–3
1–121
C
1–173
C
1–191
E2
284–746
Protease
1027–1207
Helicase
1193–1657
5'1
1694–1735
C100
1570–1932
C200
1192–1936
C33C
1192–1457
NS3NS4a
1027–1711
NS3L
819–1457
ΔNS3NS5
1242–3011
NS5b
2421–3011

FIG. 1

```
                                    M   A   P   I   T   A   Y   A   Q   Q
                                    1                                   10
                                   ATG GCG CCC ATC ACG GCG TAC GCC CAG CAG

 T   R   G   L   L   G   C   I   I   T   S   L   T   G   R
ACA AGG GGC CTC CTA GGG TGC ATA ATC ACC AGC CTA ACT GGC CGG
                                    20

 D   K   N   Q   V   E   G   E   V   Q   I   V   S   T   A
GAC AAA AAC CAA GTG GAG GGT GAG GTC CAG ATT GTG TCA ACT GCT
            30                                              40

 A   Q   T   F   L   A   T   C   I   N   G   V   C   W   T
GCC CAA ACC TTC CTG GCA ACG TGC ATC AAT GGG GTG TGC TGG ACT
                                    50

 V   Y   H   G   A   G   T   R   T   I   A   S   P   K   G
GTC TAC CAC GGG GCC GGA ACG AGG ACC ATC GCG TCA CCC AAG GGT
            60                                              70

 P   V   I   Q   M   Y   T   N   V   D   Q   D   L   V   G
CCT GTC ATC CAG ATG TAT ACC AAT GTA GAC CAA GAC CTT GTG GGC
                                    80

 W   P   A   P   Q   G   S   R   S   L   T   P   C   T   C
TGG CCC GCT CCG CAA GGT AGC CGA TCA TTG ACA CCC TGC ACT TGC
            90                                             100

 G   S   S   D   L   Y   L   V   T   R   H   A   D   V   I
GGC TCC TCG GAC CTT TAC CTG GTC ACG AGG CAC GCC GAT GTC ATT
                                   110

 P   V   R   R   R   G   D   S   R   G   S   L   L   S   P
CCC GTG CGC CGG CGG GGT GAT AGC AGG GGC AGC CTG CTG TCG CCC
            120                                            130

 R   P   I   S   Y   L   K   G   S   S   G   G   P   L   L
CGG CCC ATT TCC TAC TTG AAA GGC TCC TCG GGG GGT CCG CTG TTG
                                   140

 C   P   A   G   H   A   V   G   I   F   R   A   A   V   C
TGC CCC GCG GGG CAC GCC GTG GGC ATA TTT AGG GCC GCG GTG TGC
            150                                            160

 T   R   G   V   A   K   A   V   D   F   I   P   V   E   N
ACC CGT GGA GTG GCT AAG GCG GTG GAC TTT ATC CCT GTG GAG AAC
                                   170

 L   E   T   T   M   R   S
CTA GAG ACA ACC ATG AGG TCC
            180
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FIG. 2

```
                                    1                                  10
                                    M   A   A   Y   A   A   Q   G   Y   K
                                   ATG GCT GCA TAT GCA GCT CAG GGC TAT AAG

                                                       20
 V   L   V   L   N   P   S   V   A   A   T   L   G   F   G
GTG CTA GTA CTC AAC CCC TCT GTT GCT GCA ACA CTG GGC TTT GGT

                        30                                 40
 A   Y   M   S   K   A   H   G   I   D   P   N   I   R   T
GCT TAC ATG TCC AAG GCT CAT GGG ATC GAT CCT AAC ATC AGG ACC

                                    50
 G   V   R   T   I   T   T   G   S   P   I   T   Y   S   T
GGG GTG AGA ACA ATT ACC ACT GGC AGC CCC ATC ACG TAC TCC ACC

                    60                                     70
 Y   G   K   F   L   A   D   G   G   C   S   G   G   A   Y
TAC GGC AAG TTC CTT GCC GAC GGC GGG TGC TCG GGG GGC GCT TAT

                                    80
 D   I   I   I   C   D   E   C   H   S   T   D   A   T   S
GAC ATA ATA ATT TGT GAC GAG TGC CAC TCC ACG GAT GCC ACA TCC

                        90                                100
 I   L   G   I   G   T   V   L   D   Q   A   E   T   A   G
ATC TTG GGC ATT GGC ACT GTC CTT GAC CAA GCA GAG ACT GCG GGG

                                   110
 A   R   L   V   V   L   A   T   A   T   P   P   G   S   V
GCG AGA CTG GTT GTG CTC GCC ACC GCC ACC CCT CCG GGC TCC GTC

                    120                                    130
 T   V   P   H   P   N   I   E   E   V   A   L   S   T   T
ACT GTG CCC CAT CCC AAC ATC GAG GAG GTT GCT CTG TCC ACC ACC

                                   140
 G   E   I   P   F   Y   G   K   A   I   P   L   E   V   I
GGA GAG ATC CCT TTT TAC GGC AAG GCT ATC CCC CTC GAA GTA ATC

                        150                                160
 K   G   G   R   H   L   I   F   C   H   S   K   K   K   C
AAG GGG GGG AGA CAT CTC ATC TTC TGT CAT TCA AAG AAG AAG TGC

                                   170
 D   E   L   A   A   K   L   V   A   L   G   I   N   A   V
GAC GAA CTC GCC GCA AAG CTG GTC GCA TTG GGC ATC AAT GCC GTG

                    180                                    190
 A   Y   Y   R   G   L   D   V   S   V   I   P   T   S   G
GCC TAC TAC CGC GGT CTT GAC GTG TCC GTC ATC CCG ACC AGC GGC

                                   200                      .
 D   V   V   V   V   A   T   D   A   L   M   T   G   Y   T
GAT GTT GTC GTC GTG GCA ACC GAT GCC CTC ATG ACC GGC TAT ACC
```

FIG. 3A

```
                                  210                                     220
 G   D   F   D   S   V   I   D   C   N   T   C   V   T   Q
GGC GAC TTC GAC TCG GTG ATA GAC TGC AAT ACG TGT GTC ACC CAG

                                  230
 T   V   D   F   S   L   D   P   T   F   T   I   E   T   I
ACA GTC GAT TTC AGC CTT GAC CCT ACC TTC ACC ATT GAG ACA ATC

                  240                                     250
 T   L   P   Q   D   A   V   S   R   T   Q   R   R   G   R
ACG CTC CCC CAA GAT GCT GTC TCC CGC ACT CAA CGT CGG GGC AGG

                              260
 T   G   R   G   K   P   G   I   Y   R   F   V   A   P   G
ACT GGC AGG GGG AAG CCA GGC ATC TAC AGA TTT GTG GCA CCG GGG

                  270                                     280
 E   R   P   S   G   M   F   D   S   S   V   L   C   E   C
GAG CGC CCC TCC GGC ATG TTC GAC TCG TCC GTC CTC TGT GAG TGC

                              290
 Y   D   A   G   C   A   W   Y   E   L   T   P   A   E   T
TAT GAC GCA GGC TGT GCT TGG TAT GAG CTC ACG CCC GCC GAG ACT

                  300                                     310
 T   V   R   L   R   A   Y   M   N   T   P   G   L   P   V
ACA GTT AGG CTA CGA GCG TAC ATG AAC ACC CCG GGG CTT CCC GTG

                              320
 C   Q   D   H   L   E   F   W   E   G   V   F   T   G   L
TGC CAG GAC CAT CTT GAA TTT TGG GAG GGC GTC TTT ACA GGC CTC

                  330                                     340
 T   H   I   D   A   H   F   L   S   Q   T   K   Q   S   G
ACT CAT ATA GAT GCC CAC TTT CTA TCC CAG ACA AAG CAG AGT GGG

                              350
 E   N   L   P   Y   L   V   A   Y   Q   A   T   V   C   A
GAG AAC CTT CCT TAC CTG GTA GCG TAC CAA GCC ACC GTG TGC GCT

                  360                                     370
 R   A   Q   A   P   P   P   S   W   D   Q   M   W   K   C
AGG GCT CAA GCC CCT CCC CCA TCG TGG GAC CAG ATG TGG AAG TGT

                              380
 L   I   R   L   K   P   T   L   H   G   P   T   P   L   L
TTG ATT CGC CTC AAG CCC ACC CTC CAT GGG CCA ACA CCC CTG CTA

                  390                                     400
 Y   R   L   G   A   V   Q   N   E   I   T   L   T   H   P
TAC AGA CTG GGC GCT GTT CAG AAT GAA ATC ACC CTG ACG CAC CCA

                              410
 V   T   K   Y   I   M   T   C   M   S   A   D   L   E   V
GTC ACC AAA TAC ATC ATG ACA TGC ATG TCG GCC GAC CTG GAG GTC
```

FIG. 3B

```
                                420                                 430
 V   T   S   T   W   V   L   V   G   G   V   L   A   A   L
GTC ACG AGC ACC TGG GTG CTC GTT GGC GGC GTC CTG GCT GCT TTG

                                                440
 A   A   Y   C   L   S   T   G   C   V   V   I   V   G   R
GCC GCG TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG

                            450                             460
 V   V   L   S   G   K   P   A   I   I   P   D   R   E   V
GTC GTC TTG TCC GGG AAG CCG GCA ATC ATA CCT GAC AGG GAA GTC

                                            470
 L   Y   R   E   F   D   E   M   E   E   C   S   Q   H   L
CTC TAC CGA GAG TTC GAT GAG ATG GAA GAG TGC TCT CAG CAC TTA

                        480                                 490
 P   Y   I   E   Q   G   M   M   L   A   E   Q   F   K   Q
CCG TAC ATC GAG CAA GGG ATG ATG CTC GCC GAG CAG TTC AAG CAG

                                        500
 K   A   L   G   L   L   Q   T   A   S   R   Q   A   E   V
AAG GCC CTC GGC CTC CTG CAG ACC GCG TCC CGT CAG GCA GAG GTT

                        510                             520
 I   A   P   A   V   Q   T   N   W   Q   K   L   E   T   F
ATC GCC CCT GCT GTC CAG ACC AAC TGG CAA AAA CTC GAG ACC TTC

                                    530
 W   A   K   H   M   W   N   F   I   S   G   I   Q   Y   L
TGG GCG AAG CAT ATG TGG AAC TTC ATC AGT GGG ATA CAA TAC TTG

                            540                             550
 A   G   L   S   T   L   P   G   N   P   A   I   A   S   L
GCG GGC TTG TCA ACG CTG CCT GGT AAC CCC GCC ATT GCT TCA TTG

                                    560
 M   A   F   T   A   A   V   T   S   P   L   T   T   S   Q
ATG GCT TTT ACA GCT GCT GTC ACC AGC CCA CTA ACC ACT AGC CAA

                        570                             580
 T   L   L   F   N   I   L   G   G   W   V   A   A   Q   L
ACC CTC CTC TTC AAC ATA TTG GGG GGG TGG GTG GCT GCC CAG CTC

                                    590
 A   A   P   G   A   A   T   A   F   V   G   A   G   L   A
GCC GCC CCC GGT GCC GCT ACT GCC TTT GTG GGC GCT GGC TTA GCT

                            600                             610
 G   A   A   I   G   S   V   G   L   G   K   V   L   I   D
GGC GCC GCC ATC GGC AGT GTT GGA CTG GGG AAG GTC CTC ATA GAC

                                    620
 I   L   A   G   Y   G   A   G   V   A   G   A   L   V   A
ATC CTT GCA GGG TAT GGC GCG GGC GTG GCG GGA GCT CTT GTG GCA
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FIG. 3C

```
                        630                                         640
 F   K   I   M   S   G   E   V   P   S   T   E   D   L   V
TTC AAG ATC ATG AGC GGT GAG GTC CCC TCC ACG GAG GAC CTG GTC

                                    650
 N   L   L   P   A   I   L   S   P   G   A   L   V   V   G
AAT CTA CTG CCC GCC ATC CTC TCG CCC GGA GCC CTC GTA GTC GGC

                    660                                     670
 V   V   C   A   A   I   L   R   R   H   V   G   P   G   E
GTG GTC TGT GCA GCA ATA CTG CGC CGG CAC GTT GGC CCG GGC GAG

                                680
 G   A   V   Q   W   M   N   R   L   I   A   F   A   S   R
GGG GCA GTG CAG TGG ATG AAC CGG CTG ATA GCC TTC GCC TTC CGG

                    690                                         700
 G   N   H   V   S   P   T   H   Y   V   P   E   S   D   A
GGG AAC CAT GTT TCC CCC ACG CAC TAC GTG CCG GAG AGC GAT GCA

                                710
 A   A   R   V   T   A   I   L   S   S   L   T   V   T   Q
GCT GCC CGC GTC ACT GCC ATA CTC AGC AGC CTC ACT GTA ACC CAG

                    720                                     730
 L   L   R   R   L   H   Q   W   I   S   S   E   C   T   T
CTC CTG AGG CGA CTG CAC CAG TGG ATA AGC TCG GAG TGT ACC ACT

                                740
 P   C   S   G   S   W   L   R   D   I   W   D   W   I   C
CCA TGC TCC GGT TCC TGG CTA AGG GAC ATC TGG GAC TGG ATA TGC

                    750                                     760
 E   V   L   S   D   F   K   T   W   L   K   A   K   L   M
GAG GTG TTG AGC GAC TTT AAG ACC TGG CTA AAA GCT AAG CTC ATG

                                770
 P   Q   L   P   G   I   P   F   V   S   C   Q   R   G   Y
CCA CAG CTG CCT GGG ATC CCC TTT GTG TCC TGC CAG CGC GGG TAT

                    780                                     790
 K   G   V   W   R   G   D   G   I   M   H   T   R   C   H
AAG GGG GTC TGG CGA GGG GAC GGC ATC ATG CAC ACT CGC TGC CAC

                                800
 C   G   A   E   I   T   G   H   V   K   N   G   T   M   R
TGT GGA GCT GAG ATC ACT GGA CAT GTC AAA AAC GGG ACG ATG AGG

                    810                                     820
 I   V   G   P   R   T   C   R   N   M   W   S   G   T   F
ATC GTC GGT CCT AGG ACC TGC AGG AAC ATG TGG AGT GGG ACC TTC

                                830
 P   I   N   A   Y   T   T   G   P   C   T   P   L   P   A
CCC ATT AAT GCC TAC ACC ACG GGC CCC TGT ACC CCC CTT CCT GCG
```

FIG. 3D

```
                                840                                 850
 P   N   Y   T   F   A   L   W   R   V   S   A   E   E   Y
CCG AAC TAC ACG TTC GCG CTA TGG AGG GTG TCT GCA GAG GAA TAC

                                            860
 V   E   I   R   Q   V   G   D   F   H   Y   V   T   G   M
GTG GAG ATA AGG CAG GTG GGG GAC TTC CAC TAC GTG ACG GGT ATG

                            870                                 880
 T   T   D   N   L   K   C   P   C   Q   V   P   S   P   E
ACT ACT GAC AAT CTT AAA TGC CCG TGC CAG GTC CCA TCG CCC GAA

                                        890
 F   F   T   E   L   D   G   V   R   L   H   R   F   A   P
TTT TTC ACA GAA TTG GAC GGG GTG CGC CTA CAT AGG TTT GCG CCC

                            900                                 910
 P   C   K   P   L   L   R   E   E   V   S   F   R   V   G
CCC TGC AAG CCC TTG CTG CGG GAG GAG GTA TCA TTC AGA GTA GGA

                                        920
 L   H   E   Y   P   V   G   S   Q   L   P   C   E   P   E
CTC CAC GAA TAC CCG GTA GGG TCG CAA TTA CCT TGC GAG CCC GAA

                            930                                 940
 P   D   V   A   V   L   T   S   M   L   T   D   P   S   H
CCG GAC GTG GCC GTG TTG ACG TCC ATG CTC ACT GAT CCC TCC CAT

                                        950
 I   T   A   E   A   A   G   R   R   L   A   R   G   S   P
ATA ACA GCA GAG GCG GCC GGG CGA AGG TTG GCG AGG GGA TCA CCC

                            960                                 970
 P   S   V   A   S   S   S   A   S   Q   L   S   A   P   S
CCC TCT GTG GCC AGC TCC TCG GCT AGC CAG CTA TCC GCT CCA TCT

                                    980
 L   K   A   T   C   T   A   N   H   D   S   P   D   A   E
CTC AAG GCA ACT TGC ACC GCT AAC CAT GAC TCC CCT GAT GCT GAG

                            990                                1000
 L   I   E   A   N   L   L   W   R   Q   E   M   G   G   N
CTC ATA GAG GCC AAC CTC CTA TGG AGG CAG GAG ATG GGC GGC AAC

                                        1010
 I   T   R   V   E   S   E   N   K   V   V   I   L   D   S
ATC ACC AGG GTT GAG TCA GAA AAC AAA GTG GTG ATT CTG GAC TCC

                            1020                                1030
 F   D   P   L   V   A   E   E   D   E   R   E   I   S   V
TTC GAT CCG CTT GTG GCG GAG GAG GAC GAG CGG GAG ATC TCC GTA

                                    1040
 P   A   E   I   L   R   K   S   R   R   F   A   Q   A   L
CCC GCA GAA ATC CTG CGG AAG TCT CGG AGA TTC GCC CAG GCC CTG
```

FIG. 3E

```
                                1050                                1060
 P   V   W   A   R   P   D   Y   N   P   P   L   V   E   T
CCC GTT TGG GCG CGG CCG GAC TAT AAC CCC CCG CTA GTG GAG ACG

                                1070
 W   K   K   P   D   Y   E   P   P   V   V   H   G   C   P
TGG AAA AAG CCC GAC TAC GAA CCA CCT GTG GTC CAT GGC TGC CCG

                1080                                        1090
 L   P   P   P   K   S   P   P   V   P   P   P   R   K   K
CTT CCA CCT CCA AAG TCC CCT CCT GTG CCT CCG CCT CGG AAG AAG

                                1100
 R   T   V   V   L   T   E   S   T   L   S   T   A   L   A
CGG ACG GTG GTC CTC ACT GAA TCA ACC CTA TCT ACT GCC TTG GCC

                1110                                        1120
 E   L   A   T   R   S   F   G   S   S   S   T   S   G   I
GAG CTC GCC ACC AGA AGC TTT GGC AGC TCC TCA ACT TCC GGC ATT

                                1130
 T   G   D   N   T   T   T   S   S   E   P   A   P   S   G
ACG GGC GAC AAT ACG ACA ACA TCC TCT GAG CCC GCC CCT TCT GGC

                1140                                        1150
 C   P   P   D   S   D   A   E   S   Y   S   S   M   P   P
TGC CCC CCC GAC TCC GAC GCT GAG TCC TAT TCC TCC ATG CCC CCC

                                1160
 L   E   G   E   P   G   D   P   D   L   S   D   G   S   W
CTG GAG GGG GAG CCT GGG GAT CCG GAT CTT AGC GAC GGG TCA TGG

                1170                                        1180
 S   T   V   S   S   E   A   N   A   E   D   V   V   C   C
TCA ACG GTC AGT AGT GAG GCC AAC GCG GAG GAT GTC GTG TGC TGC

                                1190
 S   M   S   Y   S   W   T   G   A   L   V   T   P   C   A
TCA ATG TCT TAC TCT TGG ACA GGC GCA CTC GTC ACC CCG TGC GCC

                1200                                        1210
 A   E   E   Q   K   L   P   I   N   A   L   S   N   S   L
GCG GAA GAA CAG AAA CTG CCC ATC AAT GCA CTA AGC AAC TCG TTG

                                1220
 L   R   H   H   N   L   V   Y   S   T   T   S   R   S   A
CTA CGT CAC CAC AAT TTG GTG TAT TCC ACC ACC TCA CGC AGT GCT

                1230                                        1240
 C   Q   R   Q   K   K   V   T   F   D   R   L   Q   V   L
TGC CAA AGG CAG AAG AAA GTC ACA TTT GAC AGA CTG CAA GTT CTG

                                1250
 D   S   H   Y   Q   D   V   L   K   E   V   K   A   A   A
GAC AGC CAT TAC CAG GAC GTA CTC AAG GAG GTT AAA GCA GCG GCG
```

FIG. 3F

```
                                1260                                1270
 S   K   V   K   A   N   L   L   S   V   E   E   A   C   S
TCA AAA GTG AAG GCT AAC TTG CTA TCC GTA GAG GAA GCT TGC AGC

                                1280
 L   T   P   P   H   S   A   K   S   K   F   G   Y   G   A
CTG ACG CCC CCA CAC TCA GCC AAA TCC AAG TTT GGT TAT GGG GCA

                1290                                        1300
 K   D   V   R   C   H   A   R   K   A   V   T   H   I   N
AAA GAC GTC CGT TGC CAT GCC AGA AAG GCC GTA ACC CAC ATC AAC

                                1310
 S   V   W   K   D   L   L   E   D   N   V   T   P   I   D
TCC GTG TGG AAA GAC CTT CTG GAA GAC AAT GTA ACA CCA ATA GAC

                1320                                        1330
 T   T   I   M   A   K   N   E   V   F   C   V   Q   P   E
ACT ACC ATC ATG GCT AAG AAC GAG GTT TTC TGC GTT CAG CCT GAG

                                1340
 K   G   G   R   K   P   A   R   L   I   V   F   P   D   L
AAG GGG GGT CGT AAG CCA GCT CGT CTC ATC GTG TTC CCC GAT CTG

                1350                                        1360
 G   V   R   V   C   E   K   M   A   L   Y   D   V   V   T
GGC GTG CGC GTG TGC GAA AAG ATG GCT TTG TAC GAC GTG GTT ACA

                                1370
 K   L   P   L   A   V   M   G   S   S   Y   G   F   Q   Y
AAG CTC CCC TTG GCC GTG ATG GGA AGC TCC TAC GGA TTC CAA TAC

                1380                                        1390
 S   P   G   Q   R   V   E   F   L   V   Q   A   W   K   S
TCA CCA GGA CAG CGG GTT GAA TTC CTC GTG CAA GCG TGG AAG TCC

                                1400
 K   K   T   P   M   G   F   S   Y   D   T   R   C   F   D
AAG AAA ACC CCA ATG GGG TTC TCG TAT GAT ACC CGC TGC TTT GAC

                1410                                        1420
 S   T   V   T   E   S   D   I   R   T   E   E   A   I   Y
TCC ACA GTC ACT GAG AGC GAC ATC CGT ACG GAG GAG GCA ATC TAC

                                1430
 Q   C   C   D   L   D   P   Q   A   R   V   A   I   K   S
CAA TGT TGT GAC CTC GAC CCC CAA GCC CGC GTG GCC ATC AAG TCC

                1440                                        1450
 L   T   E   R   L   Y   V   G   G   P   L   T   N   S   R
CTC ACC GAG AGG CTT TAT GTT GGG GGC CCT CTT ACC AAT TCA AGG

                                1460
 G   E   N   C   G   Y   R   R   C   R   A   S   G   V   L
GGG GAG AAC TGC GGC TAT CGC AGG TGC CGC GCG AGC GGC GTA CTG
```

FIG. 3G

```
                    1470                                            1480
 T   T   S   C   G   N   T   L   T   C   Y   I   K   A   R
ACA ACT AGC TGT GGT AAC ACC CTC ACT TGC TAC ATC AAG GCC CGG

                                1490
 A   A   C   R   A   A   G   L   Q   D   C   T   M   L   V
GCA GCC TGT CGA GCC GCA GGG CTC CAG GAC TGC ACC ATG CTC GTG

                    1500                                            1510
 C   G   D   D   L   V   V   I   C   E   S   A   G   V   Q
TGT GGC GAC GAC TTA GTC GTT ATC TGT GAA AGC GCG GGG GTC CAG

                                1520
 E   D   A   A   S   L   R   A   F   T   E   A   M   T   R
GAG GAC GCG GCG AGC CTG AGA GCC TTC ACG GAG GCT ATG ACC AGG

                    1530                                            1540
 Y   S   A   P   P   G   D   P   P   Q   P   E   Y   D   L
TAC TCC GCC CCC CCT GGG GAC CCC CCA CAA CCA GAA TAC GAC TTG

                                1550
 E   L   I   T   S   C   S   S   N   V   S   V   A   H   D
GAG CTC ATA ACA TCA TGC TCC TCC AAC GTG TCA GTC GCC CAC GAC

                    1560                                            1570
 G   A   G   K   R   V   Y   Y   L   T   R   D   P   T   T
GGC GCT GGA AAG AGG GTC TAC TAC CTC ACC CGT GAC CCT ACA ACC

                                1580
 P   L   A   R   A   A   W   E   T   A   R   H   T   P   V
CCC CTC GCG AGA GCT GCG TGG GAG ACA GCA AGA CAC ACT CCA GTC

                    1590                                            1600
 N   S   W   L   G   N   I   I   M   F   A   P   T   L   W
AAT TCC TGG CTA GGC AAC ATA ATC ATG TTT GCC CCC ACA CTG TGG

                                1610
 A   R   M   I   L   M   T   H   F   F   S   V   L   I   A
GCG AGG ATG ATA CTG ATG ACC CAT TTC TTT AGC GTC CTT ATA GCC

                    1620                                            1630
 R   D   Q   L   E   Q   A   L   D   C   E   I   Y   G   A
AGG GAC CAG CTT GAA CAG GCC CTC GAT TGC GAG ATC TAC GGG GCC

                                1640
 C   Y   S   I   E   P   L   D   L   P   P   I   I   Q   R
TGC TAC TCC ATA GAA CCA CTG GAT CTA CCT CCA ATC ATT CAA AGA

                    1650                                            1660
 L   H   G   L   S   A   F   S   L   H   S   Y   S   P   G
CTC CAT GGC CTC AGC GCA TTT TCA CTC CAC AGT TAC TCT CCA GGT

                                1670
 E   I   N   R   V   A   A   C   L   R   K   L   G   V   P
GAA ATC AAT AGG GTG GCC GCA TGC CTC AGA AAA CTT GGG GTA CCG
```

FIG. 3H

```
                                1680                                1690
 P   L   R   A   W   R   H   R   A   R   S   V   R   A   R
CCC TTG CGA GCT TGG AGA CAC CGG GCC CGG AGC GTC CGC GCT AGG

 L   L   A   R   G   G   R   A   A   I   C   G   K   Y   L
CTT CTG GCC AGA GGA GGC AGG GCT GCC ATA TGT GGC AAG TAC CTC

 F   N   W   A   V   R   T   K   L   K   L   T   P   I   A
TTC AAC TGG GCA GTA AGA ACA AAG CTC AAA CTC ACT CCA ATA GCG

 A   A   G   Q   L   D   L   S   G   W   F   T   A   G   Y
GCC GCT GGC CAG CTG GAC TTG TTC GGC TGG TTC ACG GCT GGC TAC

 S   G   G   D   I   Y   H   S   V   S   H   A   R   P   R
AGC GGG GGA GAC ATT TAT CAC AGC GTG TCT CAT GCC CGG CCC CGC

 W   I   W   F   C   L   L   L   L   A   A   G   V   G   I
TGG ATC TGG TTT TGC CTA CTC CTG CTT GCT GCA GGG GTA GGC ATC

 Y   L   L   P   N   R   M   S   T   N   P   K   P   Q   R
TAC CTC CTC CCC AAC CGA ATG AGC ACG AAT CCT AAA CCT CAA AGA

 K   T   K   R   N   T   N   R   R   P   Q   D   V   K   F
AAG ACC AAA CGT AAC ACC AAC CGG CGG CCG CAG GAC GTC AAG TTC

 P   G   G   G   Q   I   V   G   G   V   Y   L   L   P   R
CCG GGT GGC GGT CAG ATC GTT GGT GGA GTT TAC TTG TTG CCG CGC

 R   G   P   R   L   G   V   R   A   T   R   K   T   S   E
AGG GGC CCT AGA TTG GGT GTG CGC GCG ACG AGA AAG ACT TCC GAG

 R   S   Q   P   R   G   R   R   Q   P   I   P   K   A   R
CGG TCG CAA CCT CGA GGT AGA CGT CAG CCT ATC CCC AAG GCT CGT

 R   P   E   G   R   T   W   A   Q   P   G   Y   P   W   P
CGG CCC GAG GGC AGG ACC TGG GCT CAG CCC GGG TAC CCT TGG CCC

 L   Y   G   N   E   G   C   G   W   A   G   W   L   L   S
CTC TAT GGC AAT GAG GGC TGC GGG TGG GCG GGA TGG CTC CTG TCT

 P   R   G   S   R   P   S   W   G   P   T   D   P   R   R
CCC CGT GGC TCT CGG CCT AGC TGG GGC CCC ACA GAC CCC CGG CGT
```

FIG. 31

```
                          1890          1892
 R   S   R   N    L   G    K    OC
AGG TCG CGC AAT TTG GGT AAG TAA
```

FIG. 3J

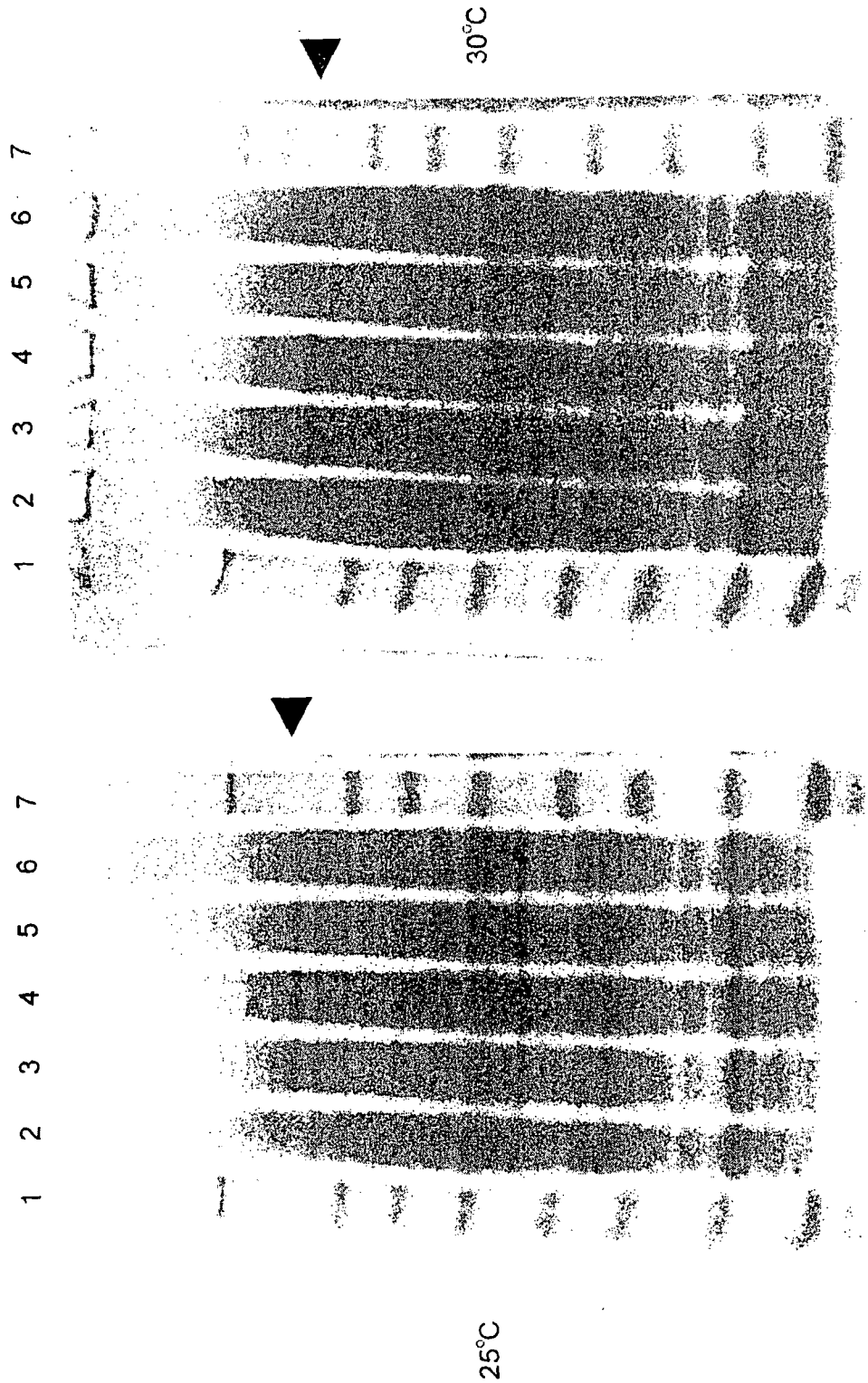


FIG. 4

NS5a
1973
SerGlySerTrpLeuArgAspIleTrpAspTrpIleCysGlu
TCCGGTTCCTGGCTAAGGGACATCTGGGACTGGATATGCGAG
AGGCCAAGGACCGATTCCCTGTAGACCCTGACCTATACGCTC

ValLeuSerAspPheLysThrTrpLeuLysAlaLysLeuMetProGlnLeuProGlyIle
GTGTTGAGCGACTTTAAGACCTGGCTAAAAGCTAAGCTCATGCCACAGCTGCCTGGGATC
CACAACTCGCTGAAATTCTGGACCGATTTTCGATTCGAGTACGGTGTCGACGGACCCTAG

ProPheValSerCysGlnArgGlyTyrLysGlyValTrpArgGlyAspGlyIleMetHis
CCCTTTGTGTCCTGCCAGCGCGGGTATAAGGGGGTCTGGCGAGGGGACGGCATCATGCAC
GGGAAACACAGGACGGTCGCGCCCATATTCCCCCAGACCGCTCCCCTGCCGTAGTACGTG

ThrArgCysHisCysGlyAlaGluIleThrGlyHisValLysAsnGlyThrMetArgIle
ACTCGCTGCCACTGTGGAGCTGAGATCACTGGACATGTCAAAAACGGGACGATGAGGATC
TGAGCGACGGTGACACCTCGACTCTAGTGACCTGTACAGTTTTTGCCCTGCTACTCCTAG

ValGlyProArgThrCysArgAsnMetTrpSerGlyThrProProIleAsnAlaTyrThr
GTCGGTCCTAGGACCTGCAGGAACATGTGGAGTGGGACCTTCCCCATTAATGCCTACACC
CAGCCAGGATCCTGGACGTCCTTGTACACCTCACCCTGGAAGGGGTAATTACGGATGTGG

ThrGlyProCysThrProLeuProAlaProAsnTyrThrPheAlaLeuTrpArgValSer
ACGGGCCCCTGTACCCCCCTTCCTGCGCCGAACTACACGTTCGCGCTATGGAGGGTGTCT
TGCCCGGGGACATGGGGGGAAGGACGCGGCTTGATGTGCAAGCGCGATACCTCCCACAGA

AlaGluGluTyrValGluIleArgGlnValGlyAspPheHisTyrValThrGlyMetThr
GCAGAGGAATACGTGGAGATAAGGCAGGTGGGGGACTTCCACTACGTGACGGGTATGACT
CGTCTCCTTATGCACCTCTATTCCGTCCACCCCCTGAAGGTGATGCACTGCCCATACTGA

ThrAspAsnLeuLysCysProCysGlnValProSerProGluPhePheThrGluLeuAsp
ACTGACAATCTTAAATGCCCGTGCCAGGTCCCATCGCCCGAATTTTTCACAGAATTGGAC
TGACTGTTAGAATTTACGGGCACGGTCCAGGGTAGCGGGCTTAAAAAGTGTCTTAACCTG

GlyValArgLeuHisArgPheAlaProProCysLysProLeuLeuArgGluGluValSer
GGGGTGCGCCTACATAGGTTTGCGCCCCCCTGCAAGCCCTTGCTGCGGGAGGAGGTATCA
CCCCACGCGGATGTATCCAAACGCGGGGGGACGTTCGGGAACGACGCCCTCCTCCATAGT

PheArgValGlyLeuHisGluTyrProValGlySerGlnLeuProCysGluProGluPro
TTCAGAGTAGGACTCCACGAATACCCGGTAGGGTCGCAATTACCTTGCGAGCCCGAACCG
AAGTCTCATCCTGAGGTGCTTATGGGCCATCCCAGCGTTAATGGAACGCTCGGGCTTGGC

AspValAlaValLeuThrSerMetLeuThrAspProSerHisIleThrAlaGluAlaAla
GACGTGGCCGTGTTGACGTCCATGCTCACTGATCCCTCCCATATAACAGCAGAGGCGGCC
CTGCACCGGCACAACTGCAGGTACGAGTGACTAGGGAGGGTATATTGTCGTCTCCGCCGG

GlyArgArgLeuAlaArgGlySerProProSerValAlaSerSerSerAlaSerGlnLeu
GGGCGAAGGTTGGCGAGGGGATCACCCCCCTCTGTGGCCAGCTCCTCGGCTAGCCAGCTA
CCCGCTTCCAACCGCTCCCCTAGTGGGGGGAGACACCGGTCGAGGAGCCGATCGGTCGAT

FIGURE 5A

```
SerAlaProSerLeuLysAlaThrCysThrAlaAsnHisAspSerProAspAlaGluLeu
TCCGCTCCATCTCTCAAGGCAACTTGCACCGCTAACCATGACTCCCCTGATGCTGAGCTC
AGGCGAGGTAGAGAGTTCCGTTGAACGTGGCGATTGGTACTGAGGGGACTACGACTCGAG

IleGluAlaAsnLeuLeuTrpArgGlnGluMetGlyGlyAsnIleThrArgValGluSer
ATAGAGGCCAACCTCCTATGGAGGCAGGAGATGGGCGGCAACATCACCAGGGTTGAGTCA
TATCTCCGGTTGGAGGATACCTCCGTCCTCTACCCGCCGTTGTAGTGGTCCCAACTCAGT

GluAsnLysValValIleLeuAspSerPheAspProLeuValAlaGluGluAspGluArg
GAAAACAAAGTGGTGATTCTGGACTCCTTCGATCCGCTTGTGGCGGAGGAGGACGAGCGG
CTTTTGTTTCACCACTAAGACCTGAGGAAGCTAGGCGAACACCGCCTCCTCCTGCTCGCC

GluIleSerValProAlaGluIleLeuArgLysSerArgArgPheAlaGlnAlaLeuPro
GAGATCTCCGTACCCGCAGAAATCCTGCGGAAGTCTCGGAGATTCGCCCAGGCCCTGCCC
CTCTAGAGGCATGGGCGTCTTTAGGACGCCTTCAGAGCCTCTAAGCGGGTCCGGGACGGG

ValTrpAlaArgProAspTyrAsnProProLeuValGluThrTrpLysLysProAspTyr
GTTTGGGCGCGGCCGGACTATAACCCCCCGCTAGTGGAGACGTGGAAAAAGCCCGACTAC
CAAACCCGCGCCGGCCTGATATTGGGGGGCGATCACCTCTGCACCTTTTTCGGGCTGATG

GluProProValValHisGlyCysProLeuProProProLysSerProProValProPro
GAACCACCTGTGGTCCATGGCTGCCCGCTTCCACCTCCAAAGTCCCCTCCTGTGCCTCCG
CTTGGTGGACACCAGGTACCGACGGGCGAAGGTGGAGGTTTCAGGGGAGGACACGGAGGC

ProArgLysLysArgThrValValLeuThrGluSerThrLeuSerThrAlaLeuAlaGlu
CCTCGGAAGAAGCGGACGGTGGTCCTCACTGAATCAACCCTATCTACTGCCTTGGCCGAG
GGAGCCTTCTTCGCCTGCCACCAGGAGTGACTTAGTTGGGATAGATGACGGAACCGGCTC

LeuAlaThrArgSerPheGlySerSerSerThrSerGlyIleThrGlyAspAsnThrThr
CTCGCCACCAGAAGCTTTGGCAGCTCCTCAACTTCCGGCATTACGGGCGACAATACGACA
GAGCGGTGGTCTTCGAAACCGTCGAGGAGTTGAAGGCCGTAATGCCCGCTGTTATGCTGT

ThrSerSerGluProAlaProSerGlyCysProProAspSerAspAlaGluSerTyrSer
ACATCCTCTGAGCCCGCCCCTTCTGGCTGCCCCCCCGACTCCGACGCTGAGTCCTATTCC
TGTAGGAGACTCGGGCGGGGAAGACCGACGGGGGGGCTGAGGCTGCGACTCAGGATAAGG

SerMetProProLeuGluGlyGluProGlyAspProAspLeuSerAspGlySerTrpSer
TCCATGCCCCCCCTGGAGGGGGAGCCTGGGGATCCGGATCTTAGCGACGGGTCATGGTCA
AGGTACGGGGGGGACCTCCCCCTCGGACCCCTAGGCCTAGAATCGCTGCCCAGTACCAGT

ThrValSerSerGluAlaAsnAlaGluAspValValCysCysSerMetSerTyrSerTrp
ACGGTCAGTAGTGAGGCCAACGCGGAGGATGTCGTGTGCTGCTCAATGTCTTACTCTTGG
TGCCAGTCATCACTCCGGTTGCGCCTCCTACAGCACACGACGAGTTACAGAATGAGAACC

ThrGlyAlaLeuValThrProCysAlaAlaGluGluGlnLysLeuProIleAsnAlaLeu
ACAGGCGCACTCGTCACCCCGTGCGCCGCGGAAGAACAGAAACTGCCCATCAATGCACTA
TGTCCGCGTGAGCAGTGGGGCACGCGGCGCCTTCTTGTCTTTGACGGGTAGTTACGTGAT

SerAsnSerLeuLeuArgHisHisAsnLeuValTyrSerThrThrSerArgSerAlaCys
AGCAACTCGTTGCTACGTCACCACAATTTGGTGTATTCCACCACCTCACGCAGTGCTTGC
TCGTTGAGCAACGATGCAGTGGTGTTAAACCACATAAGGTGGTGGAGTGCGTCACGAACG
```

FIGURE 5B

```
GlnArgGlnLysLysValThrPheAspArgLeuGlnValLeuAspSerHisTyrGlnAsp
CAAAGGCAGAAGAAAGTCACATTTGACAGACTGCAAGTTCTGGACAGCCATTACCAGGAC
GTTTCCGTCTTCTTTCAGTGTAAACTGTCTGACGTTCAAGACCTGTCGGTAATGGTCCTG

ValLeuLysGluValLysAlaAlaAlaSerLysValLysAlaAsnLeuLeuSerValGlu
GTACTCAAGGAGGTTAAAGCAGCGGCGTCAAAAGTGAAGGCTAACTTGCTATCCGTAGAG
CATGAGTTCCTCCAATTTCGTCGCCGCAGTTTTCACTTCCGATTGAACGATAGGCATCTC

GluAlaCysSerLeuThrProProHisSerAlaLysSerLysPheGlyTyrGlyAlaLys
GAAGCTTGCAGCCTGACGCCCCCACACTCAGCCAAATCCAAGTTTGGTTATGGGGCAAAA
CTTCGAACGTCGGACTGCGGGGGTGTGAGTCGGTTTAGGTTCAAACCAATACCCCGTTTT

AspValArgCysHisAlaArgLysAlaValThrHisIleAsnSerValTrpLysAspLeu
GACGTCCGTTGCCATGCCAGAAAGGCCGTAACCCACATCAACTCCGTGTGGAAAGACCTT
CTGCAGGCAACGGTACGGTCTTTCCGGCATTGGGTGTAGTTGAGGCACACCTTTCTGGAA

LeuGluAspAsnValThrProIleAspThrThrIleMetAlaLysAsnGluValPheCys
CTGGAAGACAATGTAACACCAATAGACACTACCATCATGGCTAAGAACGAGGTTTTCTGC
GACCTTCTGTTACATTGTGGTTATCTGTGATGGTAGTACCGATTCTTGCTCCAAAAGACG

ValGlnProGluLysGlyGlyArgLysProAlaArgLeuIleValPheProAspLeuGly
GTTCAGCCTGAGAAGGGGGGTCGTAAGCCAGCTCGTCTCATCGTGTTCCCCGATCTGGGC
CAAGTCGGACTCTTCCCCCCAGCATTCGGTCGAGCAGAGTAGCACAAGGGGCTAGACCCG

ValArgValCysGluLysMetAlaLeuTyrAspValValThrLysLeuProLeuAlaVal
GTGCGCGTGTGCGAAAAGATGGCTTTGTACGACGTGGTTACAAAGCTCCCCTTGGCCGTG
CACGCGCACACGCTTTTCTACCGAAACATGCTGCACCAATGTTTCGAGGGGAACCGGCAC

MetGlySerSerTyrGlyPheGlnTyrSerProGlyGlnArgValGluPheLeuValGln
ATGGGAAGCTCCTACGGATTCCAATACTCACCAGGACAGCGGGTTGAATTCCTCGTGCAA
TACCCTTCGAGGATGCCTAAGGTTATGAGTGGTCCTGTCGCCCAACTTAAGGAGCACGTT

AlaTrpLysSerLysLysThrProMetGlyPheSerTyrAspThrArgCysPheAspSer
GCGTGGAAGTCCAAGAAAACCCCAATGGGGTTCTCGTATGATACCCGCTGCTTTGACTCC
CGCACCTTCAGGTTCTTTTGGGGTTACCCCAAGAGCATACTATGGGCGACGAAACTGAGG

ThrValThrGluSerAspIleArgThrGluGluAlaIleTyrGlnCysCysAspLeuAsp
ACAGTCACTGAGAGCGACATCCGTACGGAGGAGGCAATCTACCAATGTTGTGACCTCGAC
TGTCAGTGACTCTCGCTGTAGGCATGCCTCCTCCGTTAGATGGTTACAACACTGGAGCTG

ProGlnAlaArgValAlaIleLysSerLeuThrGluArgLeuTyrValGlyGlyProLeu
CCCCAAGCCCGCGTGGCCATCAAGTCCCTCACCGAGAGGCTTTATGTTGGGGGCCCTCTT
GGGGTTCGGGCGCACCGGTAGTTCAGGGAGTGGCTCTCCGAAATACAACCCCCGGGAGAA

ThrAsnSerArgGlyGluAsnCysGlyTyrArgArgCysArgAlaSerGlyValLeuThr
ACCAATTCAAGGGGGGAGAACTGCGGCTATCGCAGGTGCCGCGCGAGCGGCGTACTGACA
TGGTTAAGTTCCCCCCTCTTGACGCCGATAGCGTCCACGGCGCGCTCGCCGCATGACTGT

ThrSerCysGlyAsnThrLeuThrCysTyrIleLysAlaArgAlaAlaCysArgAlaAla
ACTAGCTGTGGTAACACCCTCACTTGCTACATCAAGGCCCGGGCAGCCTGTCGAGCCGCA
TGATCGACACCATTGTGGGAGTGAACGATGTAGTTCCGGGCCCGTCGGACAGCTCGGCGT
```

FIGURE 5C

```
GlyLeuGlnAspCysThrMetLeuValCysGlyAspAspLeuValValIleCysGluSer
GGGCTCCAGGACTGCACCATGCTCGTGTGTGGCGACGACTTAGTCGTTATCTGTGAAAGC
CCCGAGGTCCTGACGTGGTACGAGCACACACCGCTGCTGAATCAGCAATAGACACTTTCG

AlaGlyValGlnGluAspAlaAlaSerLeuArgAlaPheThrGluAlaMetThrArgTyr
GCGGGGGTCCAGGAGGACGCGGCGAGCCTGAGAGCCTTCACGGAGGCTATGACCAGGTAC
CGCCCCCAGGTCCTCCTGCGCCGCTCGGACTCTCGGAAGTGCCTCCGATACTGGTCCATG

SerAlaProProGlyAspProProGlnProGluTyrAspLeuGluLeuIleThrSerCys
TCCGCCCCCCCTGGGGACCCCCCACAACCAGAATACGACTTGGAGCTCATAACATCATGC
AGGCGGGGGGGACCCCTGGGGGGTGTTGGTCTTATGCTGAACCTCGAGTATTGTAGTACG

SerSerAsnValSerValAlaHisAspGlyAlaGlyLysArgValTyrTyrLeuThrArg
TCCTCCAACGTGTCAGTCGCCCACGACGGCGCTGGAAAGAGGGTCTACTACCTCACCCGT
AGGAGGTTGCACAGTCAGCGGGTGCTGCCGCGACCTTTCTCCCAGATGATGGAGTGGGCA

AspProThrThrProLeuAlaArgAlaAlaTrpGluThrAlaArgHisThrProValAsn
GACCCTACAACCCCCCTCGCGAGAGCTGCGTGGGAGACAGCAAGACACACTCCAGTCAAT
CTGGGATGTTGGGGGGAGCGCTCTCGACGCACCCTCTGTCGTTCTGTGTGAGGTCAGTTA

SerTrpLeuGlyAsnIleIleMetPheAlaProThrLeuTrpAlaArgMetIleLeuMet
TCCTGGCTAGGCAACATAATCATGTTTGCCCCCACACTGTGGGCGAGGATGATACTGATG
AGGACCGATCCGTTGTATTAGTACAAACGGGGGTGTGACACCCGCTCCTACTATGACTAC

ThrHisPhePheSerValLeuIleAlaArgAspGlnLeuGluGlnAlaLeuAspCysGlu
ACCCATTTCTTTAGCGTCCTTATAGCCAGGGACCAGCTTGAACAGGCCCTCGATTGCGAG
TGGGTAAAGAAATCGCAGGAATATCGGTCCCTGGTCGAACTTGTCCGGGAGCTAACGCTC

IleTyrGlyAlaCysTyrSerIleGluProLeuAspLeuProProIleIleGlnArgLeu
ATCTACGGGGCCTGCTACTCCATAGAACCACTGGATCTACCTCCAATCATTCAAAGACTC
TAGATGCCCCGGACGATGAGGTATCTTGGTGACCTAGATGGAGGTTAGTAAGTTTCTGAG

HisGlyLeuSerAlaPheSerLeuHisSerTyrSerProGlyGluIleAsnArgValAla
CATGGCCTCAGCGCATTTTCACTCCACAGTTACTCTCCAGGTGAAATCAATAGGGTGGCC
GTACCGGAGTCGCGTAAAAGTGAGGTGTCAATGAGAGGTCCACTTTAGTTATCCCACCGG

AlaCysLeuArgLysLeuGlyValProProLeuArgAlaTrpArgHisArgAlaArgSer
GCATGCCTCAGAAAACTTGGGGTACCGCCCTTGCGAGCTTGGAGACACCGGGCCCGGAGC
CGTACGGAGTCTTTTGAACCCCATGGCGGGAACGCTCGAACCTCTGTGGCCCGGGCCTCG

ValArgAlaArgLeuLeuAlaArgGlyGlyArgAlaAlaIleCysGlyLysTyrLeuPhe
GTCCGCGCTAGGCTTCTGGCCAGAGGAGGCAGGGCTGCCATATGTGGCAAGTACCTCTTC
CAGGCGCGATCCGAAGACCGGTCTCCTCCGTCCCGACGGTATACACCGTTCATGGAGAAG

AsnTrpAlaValArgThrLysLeuLysLeuThrProIleAlaAlaAlaGlyGlnLeuAsp
AACTGGGCAGTAAGAACAAAGCTCAAACTCACTCCAATAGCGGCCGCTGGCCAGCTGGAC
TTGACCCGTCATTCTTGTTTCGAGTTTGAGTGAGGTTATCGCCGGCGACCGGTCGACCTG

LeuSerGlyTrpPheThrAlaGlyTyrSerGlyGlyAspIleTyrHisSerValSerHis
TTGTCCGGCTGGTTCACGGCTGGCTACAGCGGGGGAGACATTTATCACAGCGTGTCTCAT
AACAGGCCGACCAAGTGCCGACCGATGTCGCCCCCTCTGTAAATAGTGTCGCACAGAGTA
```

FIGURE 5D

```
        2990core---------->
AlaArgProArgMetSerThrAsnProLysProGlnArgLysThrLysArgAsnThrAsn
GCCCGGCCCCGCATGAGCACGAATCCTAAACCTCAAAGAAAGACCAAACGTAACACCAAC
CGGGCCGGGGCGTACTCGTGCTTAGGATTTGGAGTTTCTTTCTGGTTTGCATTGTGGTTG

ArgArgProGlnAspValLysPheProGlyGlyGlyGlnIleValGlyGlyValTyrLeu
CGGCGGCCGCAGGACGTCAAGTTCCCGGGTGGCGGTCAGATCGTTGGTGGAGTTTACTTG
GCCGCCGGCGTCCTGCAGTTCAAGGGCCCACCGCCAGTCTAGCAACCACCTCAAATGAAC

LeuProArgArgGlyProArgLeuGlyValArgAlaThrArgLysThrSerGluArgSer
TTGCCGCGCAGGGGCCCTAGATTGGGTGTGCGCGCGACGAGAAAGACTTCCGAGCGGTCG
AACGGCGCGTCCCCGGGATCTAACCCACACGCGCGCTGCTCTTTCTGAAGGCTCGCCAGC

GlnProArgGlyArgArgGlnProIleProLysAlaArgArgProGluGlyArgThrTrp
CAACCTCGAGGTAGACGTCAGCCTATCCCCAAGGCTCGTCGGCCCGAGGGCAGGACCTGG
GTTGGAGCTCCATCTGCAGTCGGATAGGGGTTCCGAGCAGCCGGGCTCCCGTCCTGGACC

AlaGlnProGlyTyrProTrpProLeuTyrGlyAsnGluGlyCysGlyTrpAlaGlyTrp
GCTCAGCCCGGGTACCCTTGGCCCCTCTATGGCAATGAGGGCTGCGGGTGGGCGGGATGG
CGAGTCGGGCCCATGGGAACCGGGGAGATACCGTTACTCCCGACGCCCACCCGCCCTACC

LeuLeuSerProArgGlySerArgProSerTrpGlyProThrAspProArgArgArgSer
CTCCTGTCTCCCCGTGGCTCTCGGCCTAGCTGGGGCCCCACAGACCCCCGGCGTAGGTCG
GAGGACAGAGGGGCACCGAGAGCCGGATCGACCCCGGGGTGTCTGGGGGCCGCATCCAGC

           121
ArgAsnLeuGlyLys
CGCAATTTGGGTAAG
GCGTTAAACCCATTC
```

FIGURE 5E

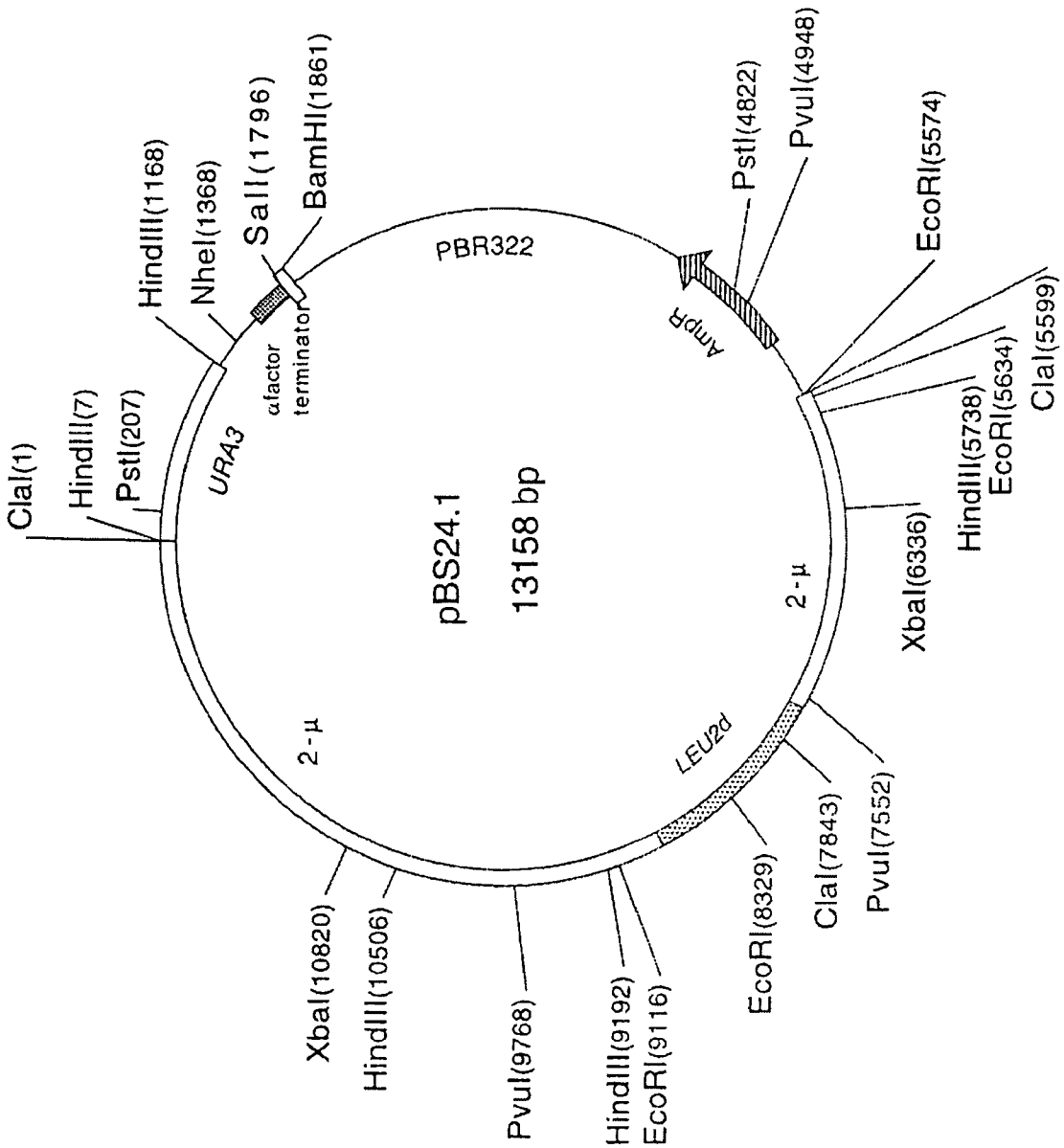
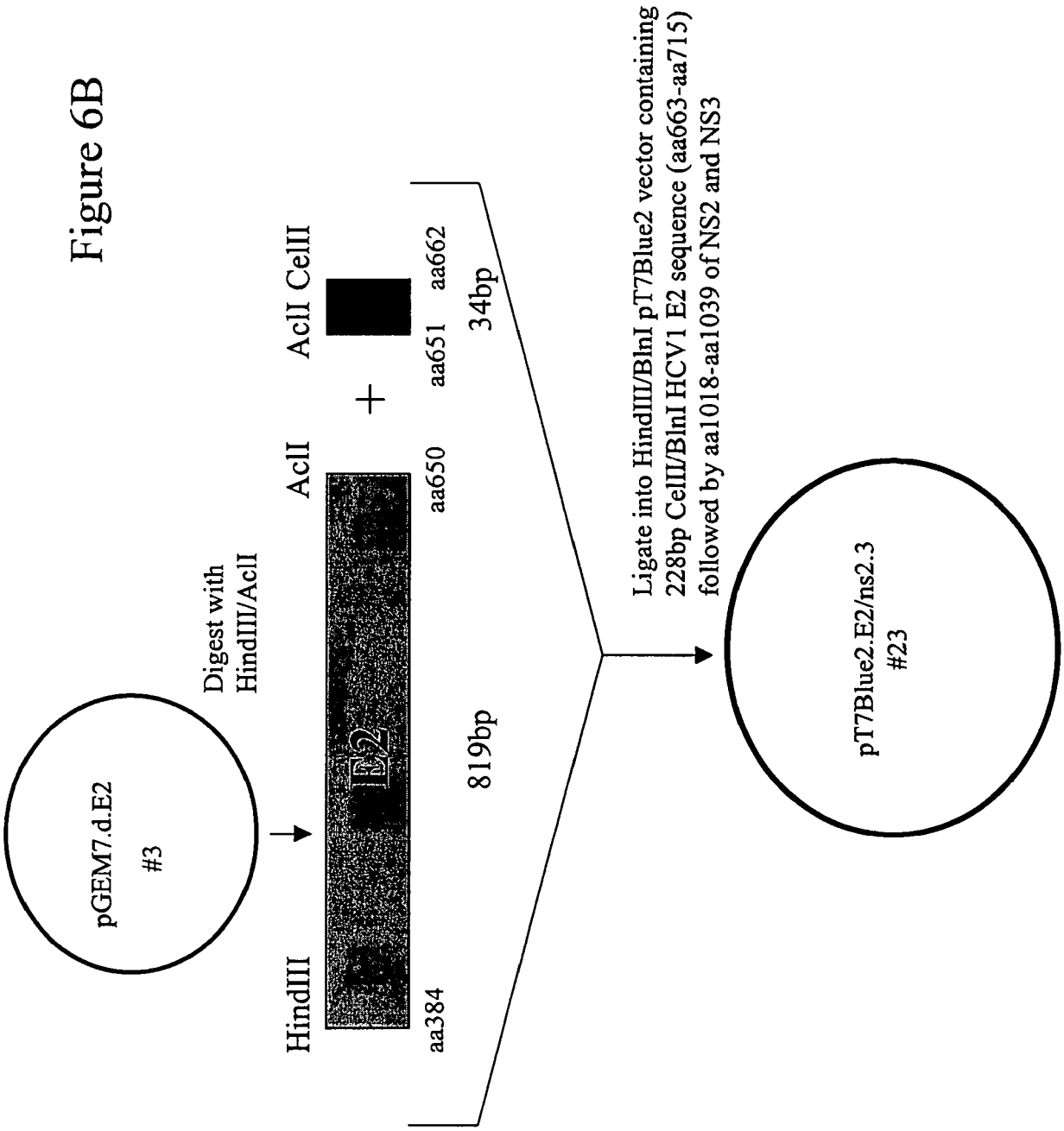

pBS24.1
13158 bp
PBR322
URA3
αfactor terminator
AmpR
LEU2d
2-μ
2-μ
ClaI(1)
HindIII(7)
PstI(207)
HindIII(1168)
NheI(1368)
SalI(1796)
BamHI(1861)
PstI(4822)
PvuI(4948)
EcoRI(5574)
ClaI(5599)
HindIII(5738)
EcoRI(5634)
XbaI(6336)
ClaI(7843)
PvuI(7552)
EcoRI(8329)
HindIII(9192)
EcoRI(9116)
PvuI(9768)
HindIII(10506)
XbaI(10820)

Figure 6A

Figure 6B


pGEM7.d.E2
#3
Digest with
HindIII/AclI
HindIII
AclI
AclI CelII
E2
aa384
aa650
aa651
aa662
819bp
34bp
Ligate into HindIII/BlnI pT7Blue2 vector containing
228bp CelII/BlnI HCV1 E2 sequence (aa663-aa715)
followed by aa1018-aa1039 of NS2 and NS3
pT7Blue2.E2/ns2.3
#23

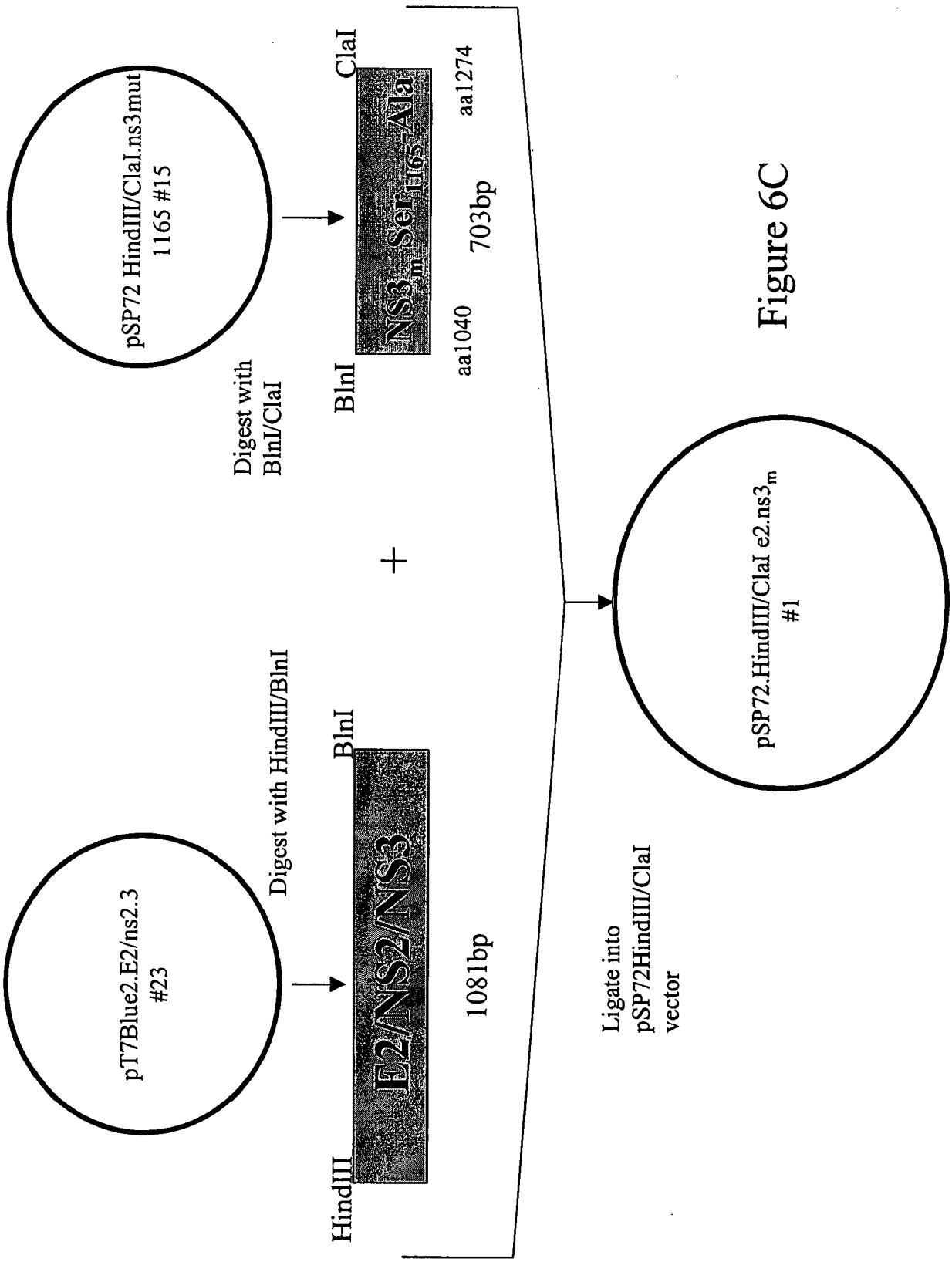

pT7Blue2.E2/ns2.3
#23
Digest with HindIII/BlnI
HindIII
BlnI
E2/NS2/NS3
1081bp
+
pSP72 HindIII/ClaI.ns3mut
1165 #15
Digest with
BlnI/ClaI
BlnI
ClaI
NS3m Ser1165-Ala
aa1040
703bp
aa1274
Ligate into
pSP72HindIII/ClaI
vector
pSP72.HindIII/ClaI e2.ns3m
#1

Figure 6C

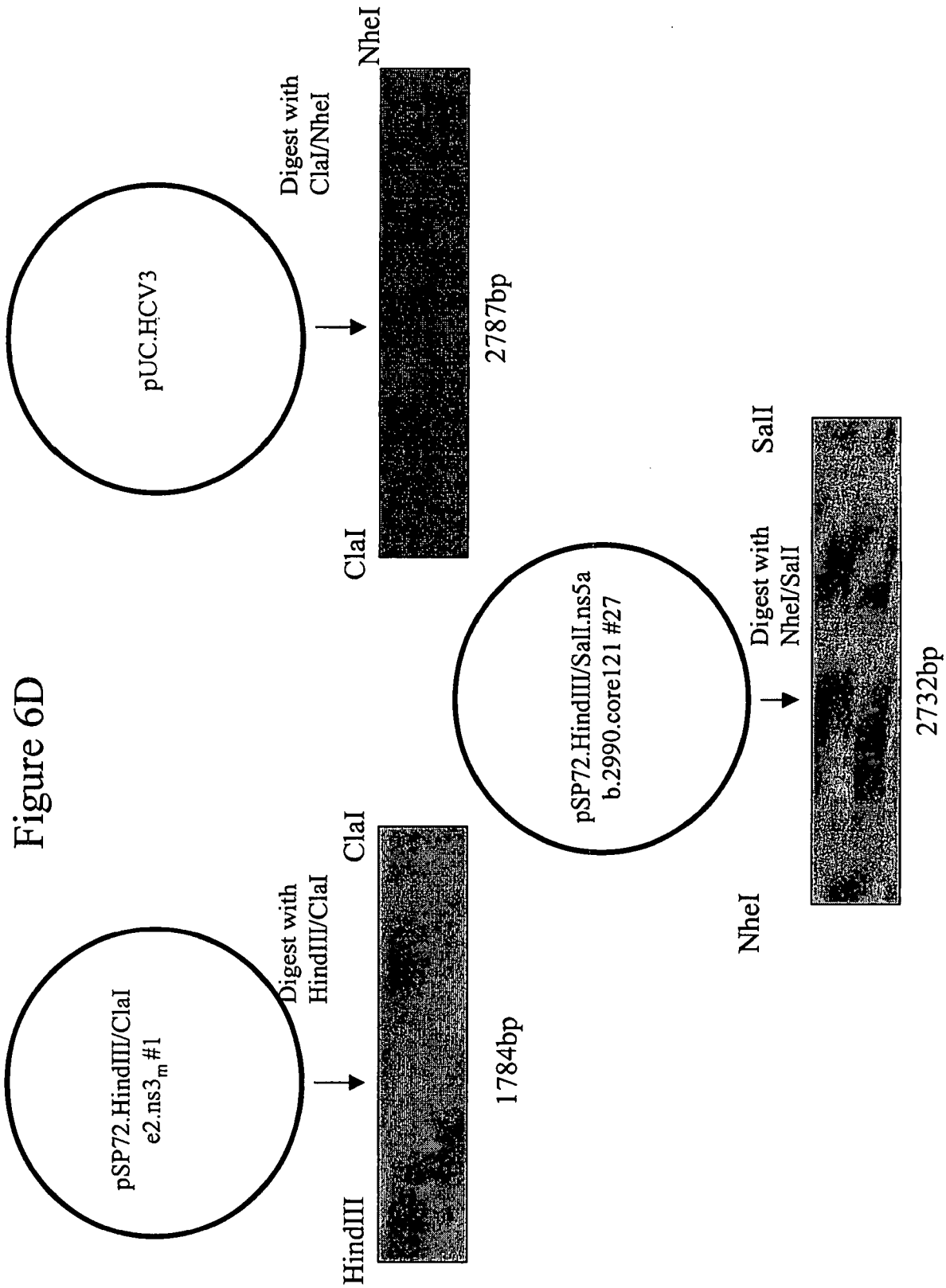
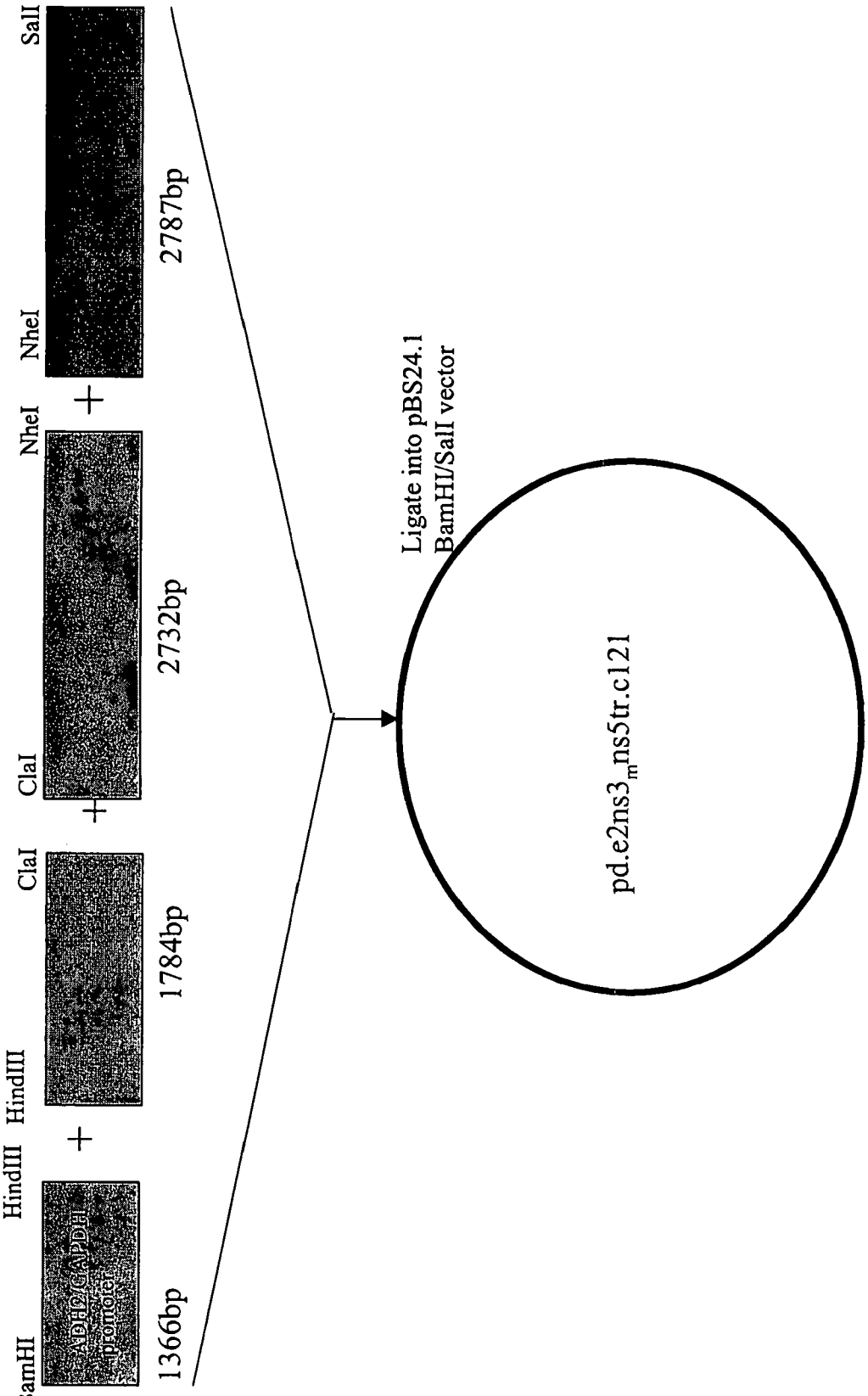

pUC.HCV3
Digest with ClaI/NheI
NheI
ClaI
2787bp
pSP72.HindIII/ClaI e2.ns3m#1
Digest with HindIII/ClaI
ClaI
HindIII
1784bp
pSP72.HindIII/SalI.ns5a b.2990.core121 #27
Digest with NheI/SalI
SalI
NheI
2732bp

Figure 6D

Figure 6E


BamHI
HindIII
ADH2/GAPDH promoter
HindIII
HindIII
ClaI
1366bp
1784bp
ClaI
NheI
2732bp
NheI
NheI
SalI
2787bp
Ligate into pBS24.1
BamHI/SalI vector
pd.e2ns3_m ns5tr.c121

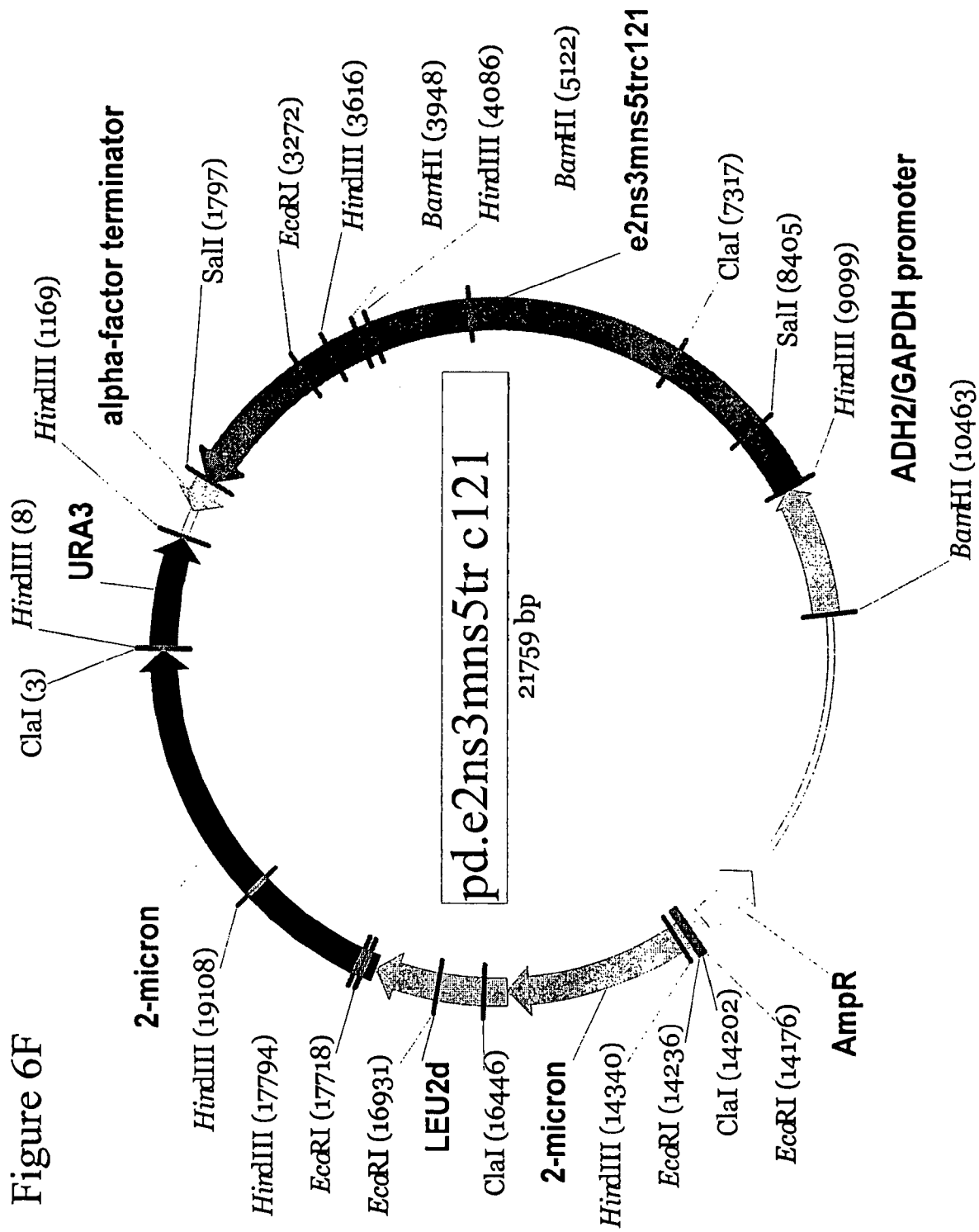

pd.e2ns3mns5tr c121
21759 bp
ClaI (3)
HindIII (8)
URA3
HindIII (1169)
alpha-factor terminator
SalI (1797)
EcoRI (3272)
HindIII (3616)
BamHI (3948)
HindIII (4086)
BamHI (5122)
e2ns3mns5trc121
ClaI (7317)
SalI (8405)
HindIII (9099)
ADH2/GAPDH promoter
BamHI (10463)
AmpR
EcoRI (14176)
ClaI (14202)
EcoRI (14236)
HindIII (14340)
2-micron
ClaI (16446)
LEU2d
EcoRI (16931)
EcoRI (17718)
HindIII (17794)
HindIII (19108)
2-micron

Figure 6F

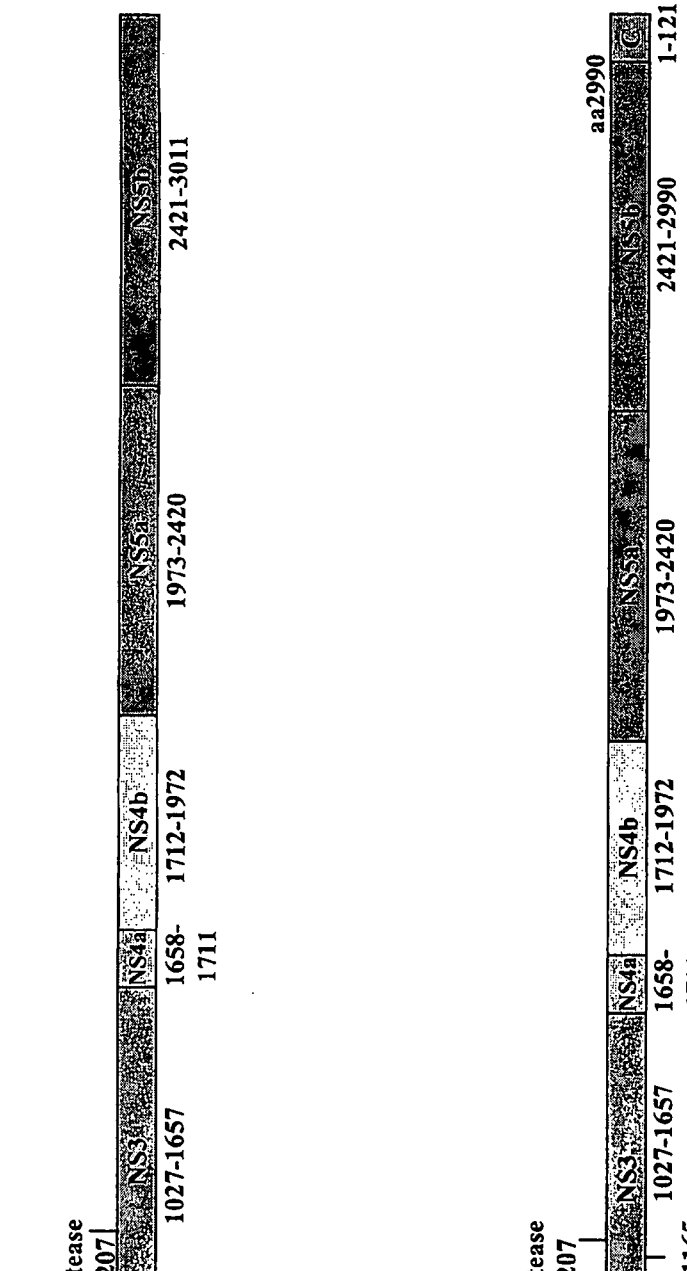

HCV
NS3 Protease
1027-1207
C
E1
E2
p7
NS2
NS3
NS4a
NS4b
NS5a
NS5b
1-191
192-383
384-746
747-809
810-1026
1027-1657
1658-1711
1712-1972
1973-2420
2421-3011
e2.ns3m.ns5tr +/- core 121
NS3 Protease
1027-1207
aa1018
aa2990
E2
NS2
NS3
NS4a
NS4b
NS5b
C
384-715
1027-1657
1658-1711
1712-1972
1973-2420
2421-2990
1-121
S1165 → A

Figure 7

FIGURE 8

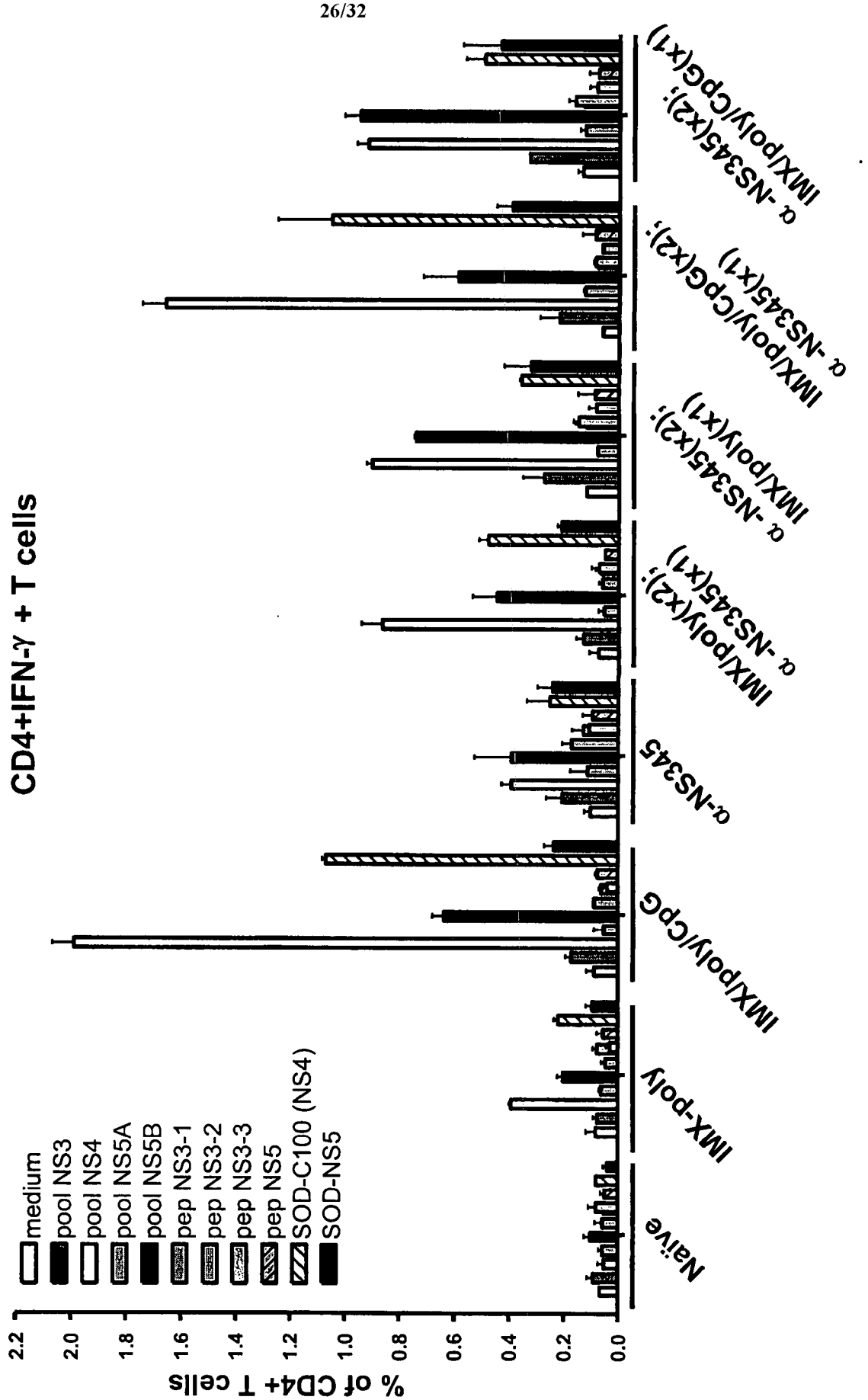

CD4+IFN-γ + T cells
% of CD4+ T cells
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
medium
pool NS3
pool NS4
pool NS5A
pool NS5B
pep NS3-1
pep NS3-2
pep NS3-3
pep NS5
SOD-C100 (NS4)
SOD-NS5
Naïve
IMX-poly
IMX/poly/CPG
α-NS345
IMX/poly(x2); α-NS345(x1)
IMX/poly(x1); α-NS345(x2)
IMX/poly/CPG(x2); α-NS345(x1)
IMX/poly/CPG(x1); α-NS345(x2)

FIGURE 9

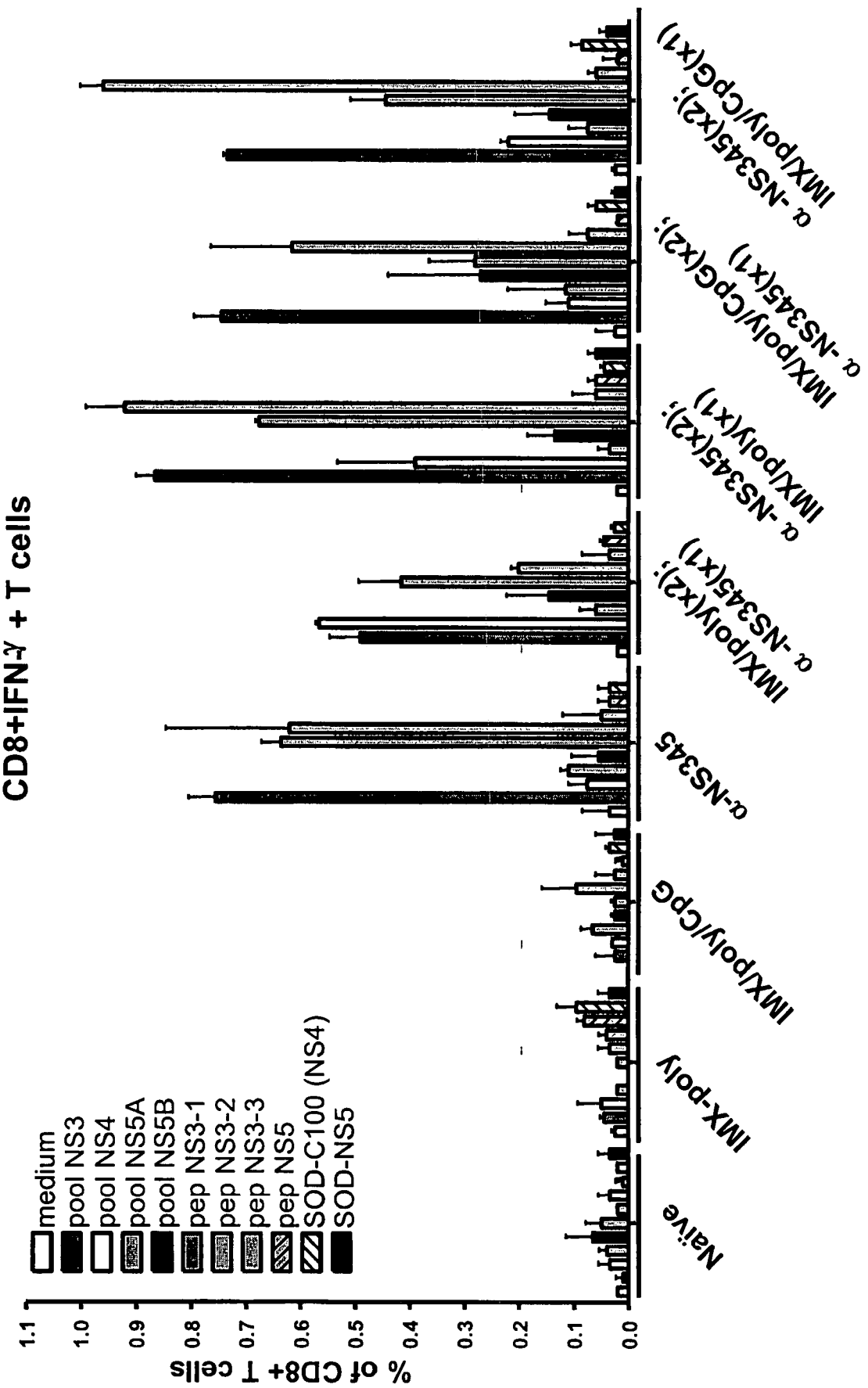
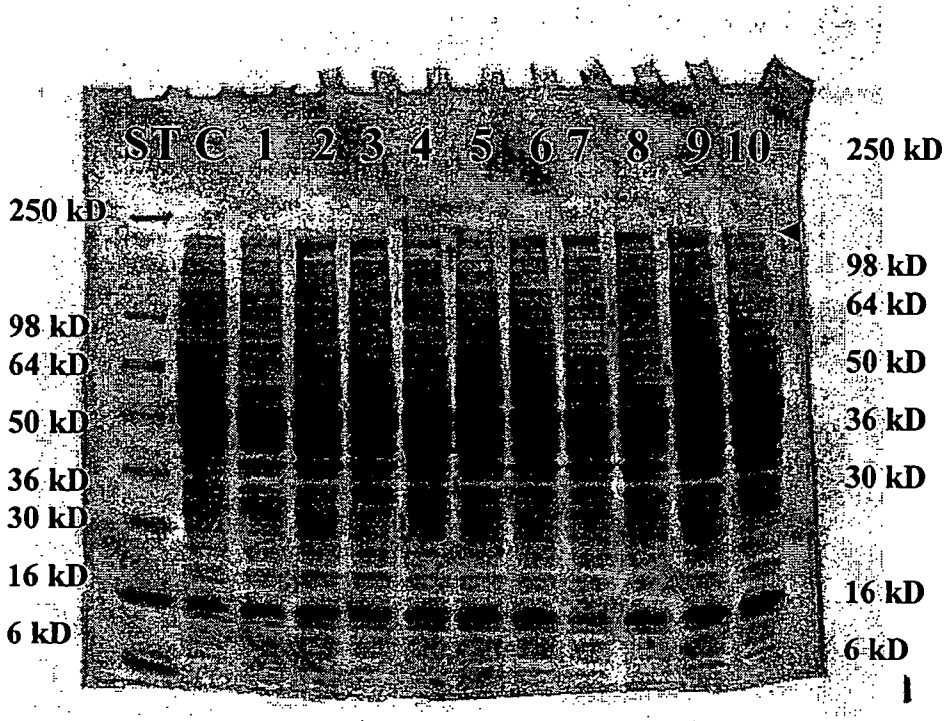
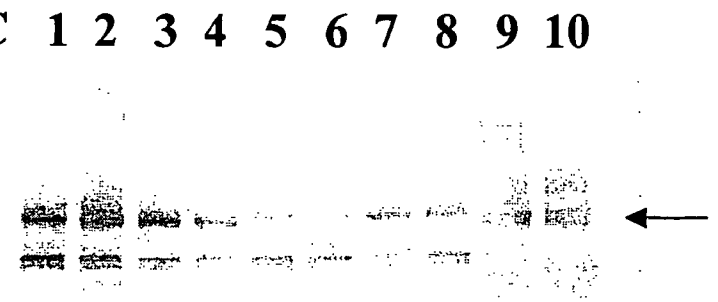

CD8+IFN-γ + T cells
% of CD8+ T cells
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
medium
pool NS3
pool NS4
pool NS5A
pool NS5B
pep NS3-1
pep NS3-2
pep NS3-3
pep NS5
SOD-C100 (NS4)
SOD-NS5
Naïve
IMX-poly
IMX/poly/CpG
α-NS345
IMX/poly(x2);
α-NS345(x1)
IMX/poly(x1);
α-NS345(x2)
IMX/poly/CpG(x2);
α-NS345(x1)
IMX/poly/CpG(x1);
α-NS345(x2)

Figure 10A





ST-See Blue Standard

C-pAB24

1-ns3m-ns5 (219.3 kD)

2-ns3m-ns5 "

3-ns3m-ns5tr (217.0 kD)

4-ns3m-ns5tr "

5-ns3m-ns5core121 (233.6 kD)

6-ns3m-ns5core121 "

7-ns3m-ns5tr.core121 (230.3 kD)

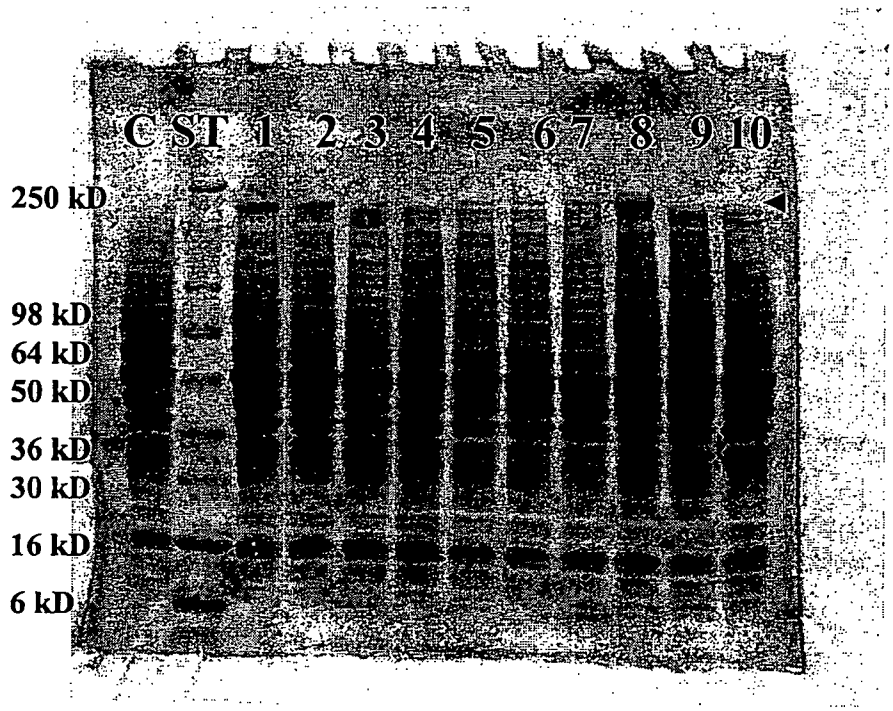
8-ns3m-ns5tr.core121 "

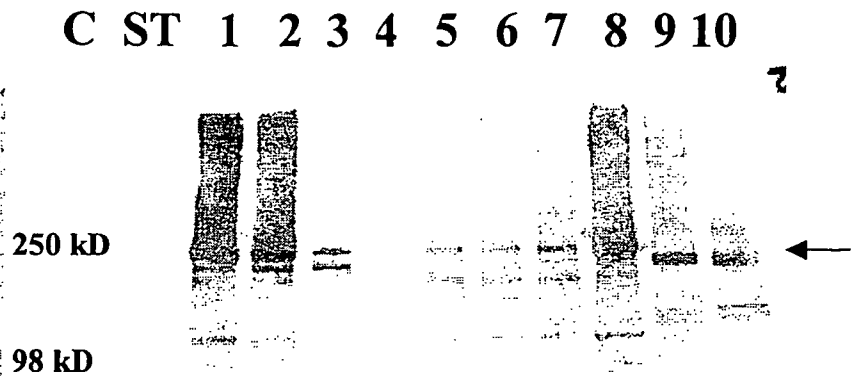
9-Δns3-ns5.core121 (208.0 kD)

10-Δns3-ns5.core121 "

All samples represent the insoluble pellet (IP), resuspended in 1ml pH12 SB+DTT for 1hr and sonicated for ~8 seconds.

Figure 10B





C-pAB24 3-e2.ns3m-ns5tr (253.6 kD) 7-e2.ns3m-ns5tr.core121 (266.3 kD

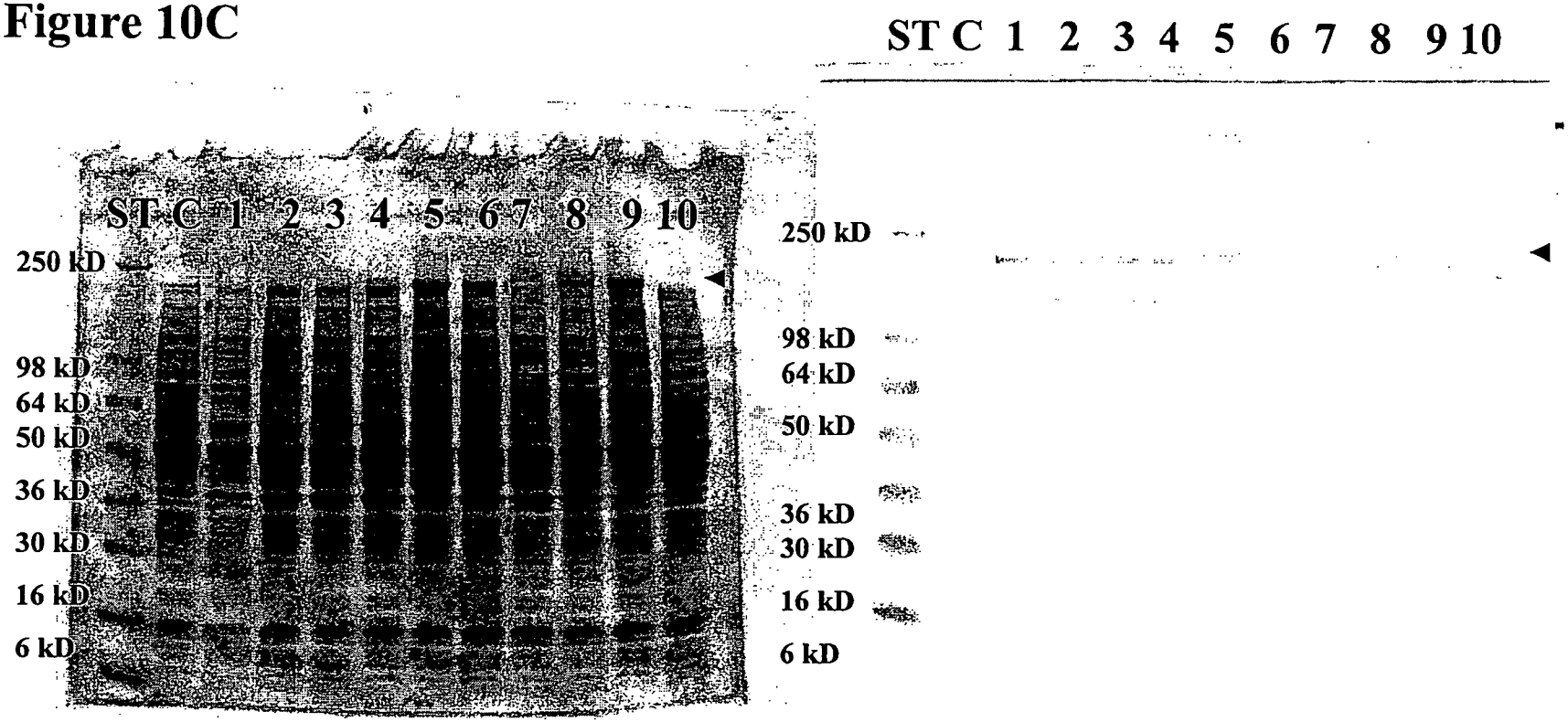
ST-See Blue Standard 4-e2.ns3m-ns5tr " 8-e2.ns3m-ns5tr.core121 "

1-e2.ns3m-ns5 (256.0 kD) 5-e2.ns3m-ns5core121 (269.2 kD) 9-Δns3-ns5.core121 (208.0 kD

2-e2.ns3m-ns5 " 6-e2.ns3m-ns5core121 " 10-Δns3-ns5.core121 "

All samples represent the insoluble pellet IP, resuspended in 1ml pH12 SB+DTT for 1hr and sonicated for ~8 seconds.

Figure 10C



ST-See Blue Standard **3-ns3m-ns5tr.core121(230.3 kD)** **7-e2.ns3m-ns5tr.core121(266.3kD**

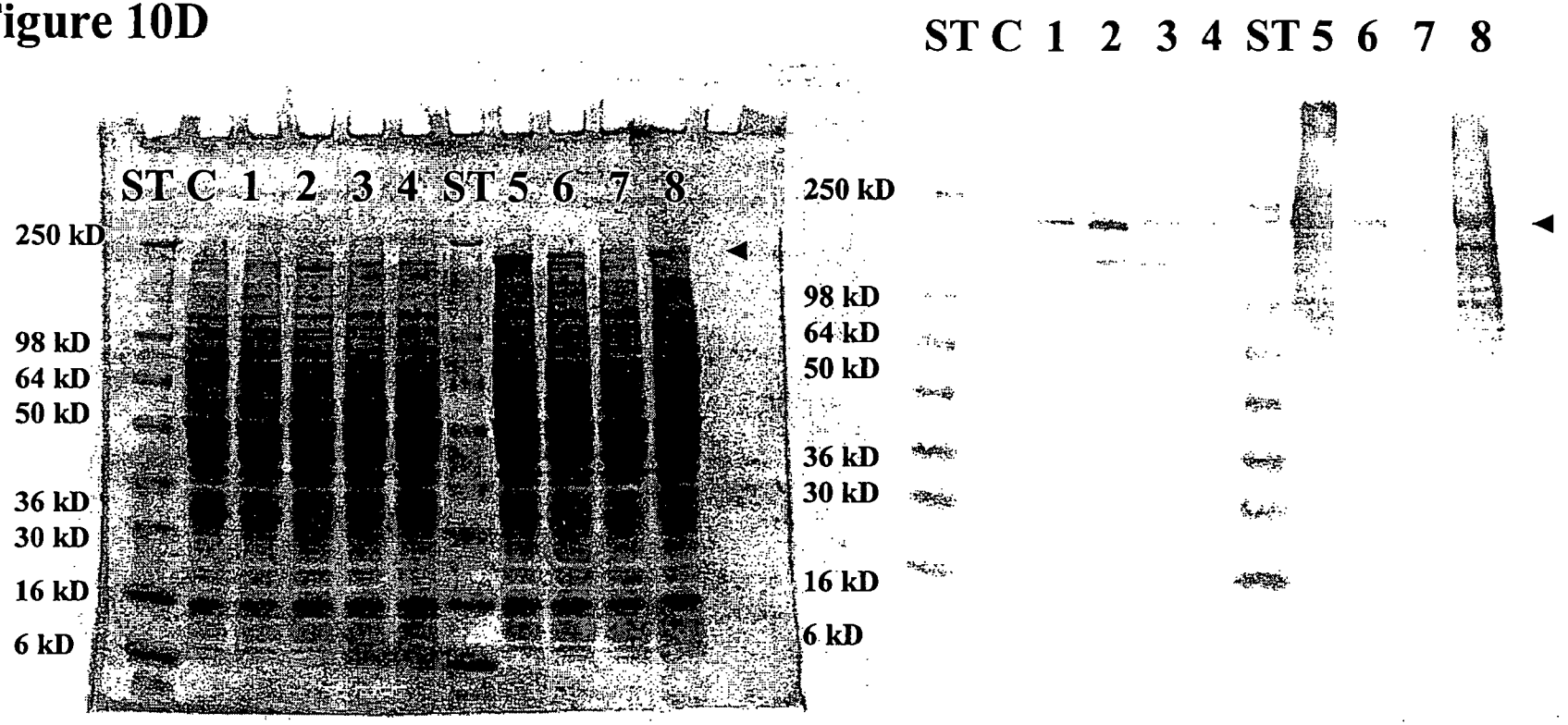
C-pAB24 **4-ns3m-ns5tr.core121 "** **8-e2.ns3m-ns5tr.core121 "**

1-ns3m-ns5 (219.3 kD) **5-e2.ns3m-ns5 (256.0 kD)** **9-e2.ns3m-ns5tr.core121 "**

2-ns3m-ns5 " **6-e2.ns3m-ns5 "** **10-Δns3-ns5.core121 (208.0 kD)**

All samples represent the IP, resuspended in 1ml pH12 SB+DTT for 1hr and sonicated for ~8 seconds. Inocula from *frozen glycerol stocks* (except pAB24).

Figure 10D



ST-See Blue Standard 3-ns3m-ns5tr.core121 (230.3 kD) 6-e2.ns3m-ns5 (256.0 kD)

C-pAB24 4-ns3m-ns5tr.core121 " 7-e2.ns3m-ns5tr.core121 (266.3 kD)

1-ns3m-ns5 (219.3 kD) ST-See Blue Standard 8-e2.ns3m-ns5tr.core121 "

2-ns3m-ns5 " 5-e2ns3m-ns5 (256.0 kD)

All samples represent the IP, resuspended in 1ml pH12 SB+DTT for 1hr and sonicated for ~8 seconds. Inocula from *liquid culture*.

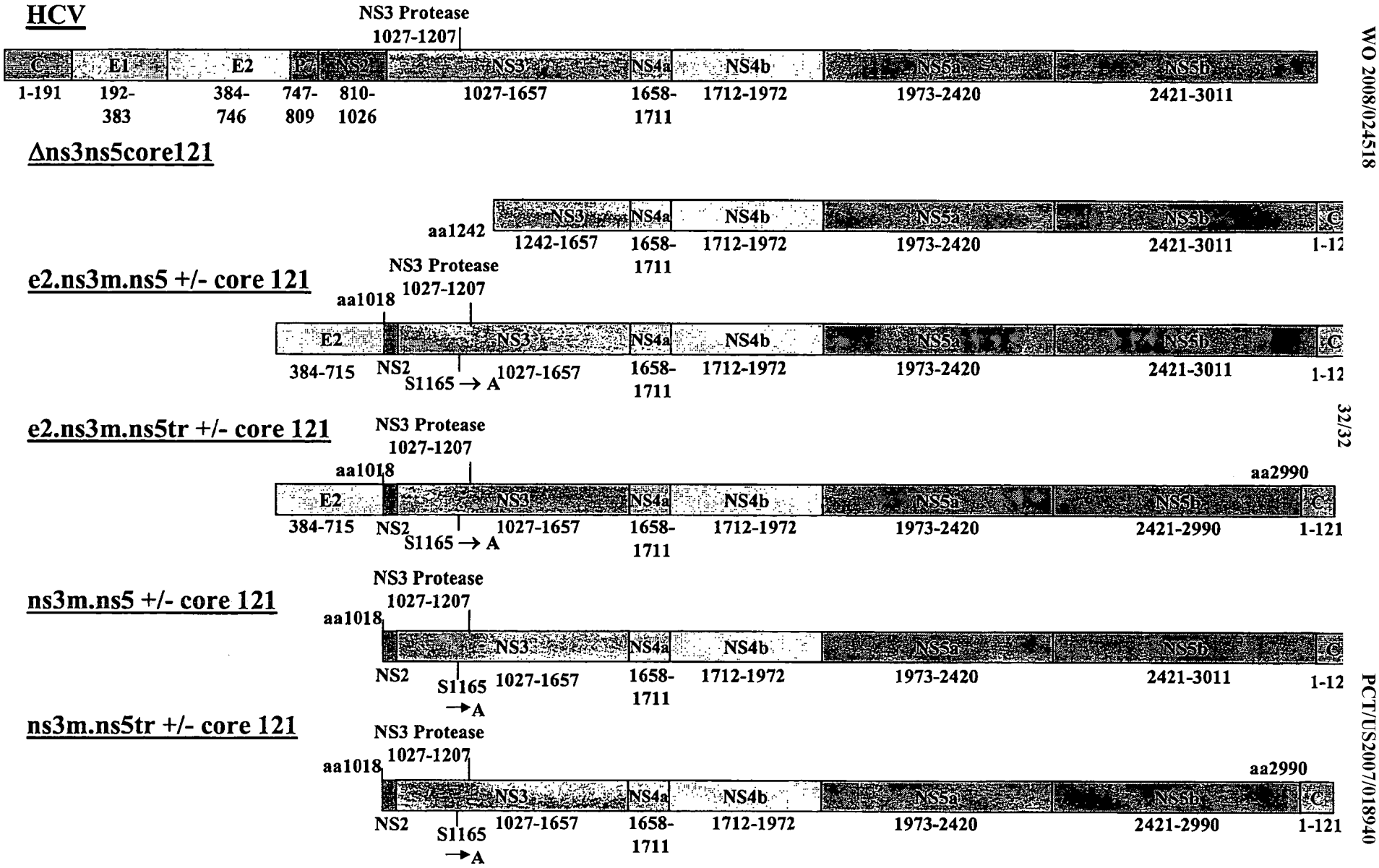


Figure 11