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(71) Applicant *(for all designated States except US)*: **THE MACFARLANE BURNET INSTITUTE FOR MEDICAL RESEARCH AND PUBLIC HEALTH LIMITED** [AU/AU]; 23-87 Commercial Road, Melbourne, Victoria 3004 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MC CAFFREY, Kathleen** [AU/AU]; 156 Charles Street, Abbotsford, Victoria 3067 (AU). **DRUMMER, Heidi** [AU/AU]; 49 Fran Street, Glenroy, Victoria 3046 (AU). **POUMBOURIOS, Pantelis** [AU/AU]; 112 Bayview Street, Williamstown, Victoria 3016 (AU).

(74) Agents: **SLATTERY, John** et al.; Davies Collison Cave, 1 Nicholson Street, Melbourne, Victoria 3000 (AU).

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(54) Title: RECOMBINANT HCV E2 GLYCOPROTEIN

(57) Abstract: The invention provides modified hepatitis C virus (HCV) E2 glycoproteins comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions wherein in at least one of said variable regions at least a part of the variable region is replaced with a flexible linker sequence. The invention also provides vaccine compositions comprising the modified glycoproteins as well as methods of use thereof.

Recombinant HCV E2 Glycoprotein

Field of Invention

The present invention relates to a new and improved treatment for viral hepatitis C (HCV) infection. The present invention particularly relates to vaccine compositions for prevention and therapeutic treatment of HCV infection based on administering a recombinant HCV polyprotein.

Background of the Invention

According to the World Health Organisation, hepatitis C virus (HCV) infects approximately 170 million to 200 million people worldwide. While governments have increased education about how HCV is transmitted, and despite prevention programs, HCV continues to proliferate. Approximately 80% of those who are infected with HCV remain carriers of the virus. In Australia about 16,000 new cases of HCV infection are reported each year, the new infections being most prevalent amongst injection drug users. HCV is the most common blood-borne viral infection, causing the death of a substantial proportion of the population.

HCV is known to infect the liver and certain immune cells of a sufferer. As a result, HCV leads to serious liver disease such as fibrosis, cirrhosis, steatosis and heptocellular carcinoma (liver cancer) more frequently than other forms of hepatitis. HCV is a leading cause for the requirement of liver transplants. It is generally believed that the acute phase of the infection is often unrecognised due to the sub-clinical nature of the infection, and 80% of individuals progress to a chronic condition. Chronic infection is a result of the immune system's failure to generate an adequate immune response against the virus.

Currently there is no vaccine for HCV and the only available therapy for treatment of HCV has relied on development of antiviral drugs and drug combinations. The general idea behind antiviral drug design is to identify viral proteins, or parts of proteins, that can be disabled or inhibited. A standard treatment of choice for patients suffering moderate or

severe fibrosis includes a combination of alpha- interferon and ribavirin. The antiviral effects of combination alpha-interferon and ribavirin therapy cause a rapid decrease in HCV levels in the blood, even after a single dose. Conventional alpha- interferon treatment for HCV however suffers several drawbacks. For example, (i) when alpha-interferon treatment is stopped after a few weeks or months of treatment, the viral load level is known to re-establish rapidly; (ii) treatment with alpha-interferon/ribavirin is associated with severe side effects, including flu-like symptoms, reduced red or white cell counts, bone marrow suppression, neuropsychiatric effects, particularly depression and anemia; (iii) effective treatment requires patient adherence to a frequent dosing regimen since alpha-interferon is absorbed and eliminated from the body rapidly; and (v) high cost of such treatments.

Some of the above drawbacks, referring particularly to item (iii) above, have been addressed by subjecting alpha-interferon to 'pegylation' in which polyethylene glycol molecules are attached to the interferon. The administration of pegylated interferon in combination with ribavirin increases the half-life of interferon and has the advantage of decreasing the frequency of dosing, hence patient compliance. Such treatment however has proven to be efficacious in less than 50% of treated patients. Given the increasing number of chronic sufferers of HCV, there is a need to develop a vaccine for both prophylactic and therapeutic purposes.

Development of a successful vaccine to protect against HCV infection has been elusive. One proposed reason for this difficulty is that HCV, being an RNA virus, is genetically unstable allowing it to achieve a high rate of viral mutation to evade the body's immune response. It is therefore a challenge for researchers to identify a portion of the virus that is conserved.

HCV has been classified in a separate genus (Hepacivirus) of the Flaviviridae family. HCV is non-cytopathic and rather triggers an immune response that either rapidly clears the infection or initiates an inflammatory response leading to chronic infection and liver injury. Spontaneously resolving infections that permanently clear HCV RNA without treatment occur in ~30% of acute cases suggesting a natural immunity to HCV and is thus

encouraging for the prospect of vaccine development. However, the determinants for this outcome of HCV infection are unknown.

The HCV virion contains a positive-sense single stranded RNA genome of about 9.5 kb. The genome encodes a single polyprotein of 3,010 to 3,030 amino acids. The structural proteins comprise a core protein forming the viral nucleocapsid and two envelope glycoproteins, E1 and E2. Some recent efforts towards the development of a HCV vaccine have focused on HCV envelope glycoproteins E1 and E2. It has been found that E1 and E2 form non-covalently associated heterodimers on the surface of the virion that mediate both viral attachment and entry and thus present targets for the host immune response.

Recent studies have suggested that envelope glycoprotein E2 binds to CD81 on the surface of CD4+ T cells. During the binding process, E2 undergoes rapid conformational change. To date no research has been able to provide a suitable modified envelope glycoprotein, which can exhibit "wild-type" levels of CD81 binding.

Throughout this specification, including the claims, all numbering of polypeptide residues of the HCV envelope glycoproteins E1 and E2 is based on the prototype HCV-H77 polyprotein sequence, Genbank Accession No. AF 009606. The mature form of glycoprotein E1 is encompassed by polyprotein residues 191 and 383, and the mature form of glycoprotein E2 is encompassed by polyprotein residues 384 and 746.

The receptor-binding domain (RBD) of E2 is encompassed by polyprotein residues 384-661 ($E2_{661}$). Recombinant forms of $E2_{661}$ RBD are efficiently secreted from transfected cells and are able to interact with CD81 and other cell surface molecules. The E2 RBD contains two variable regions, HVR1 (384-410) and HVR2 (474-482).

Variable region 1, located at the N-terminus of E2, is the most variable region in the HCV genome, is highly immunogenic and rapidly accumulates neutralization escape mutations. Despite the high level of amino acid variability in HVR1, there is an overall conservation of basic residues that are important for viral entry.

Variable region 2 is located within the region flanked by Cys-459 to Cys-486. Although originally described as a 7-residue sequence, comparison of E2 sequences from different HCV genotypes suggests it may extend from residues 461-481. In comparison to HVR1, the sequence of HVR2 is relatively stable within HCV infected people, although an accumulation of mutations at this location has been shown to correlate with responsiveness to interferon-α treatment.

In work leading to the present invention, the inventors have observed that alignment of E2 sequences representing the six major genotypes of HCV reveals a previously undescribed variable region between polyprotein residues 570-580 that is relatively conserved within a genotype but varies across genotypes due to amino acid insertions and deletions. Accordingly, amino acids 570-580 have been denoted the intergenotypic variable region (igVR). Examination of the corresponding region from all 6 genotypes of HCV, and divergent isolates therein, show that igVR is also flanked by conserved cysteine residues (Cys-569 and Cys-581), suggesting that these sequences form disulfide-constrained loops.

To date no vaccine treatment for HCV using the adaptive immune response route has been successful. Given the drawbacks of current and experimental therapies for treatment of HCV, there is an unmet need for providing a cell-mediated immune response to treat HCV infection.

It is one object of the present invention to provide an immunotherapeutic approach to prevent or treat HCV infection. A further object of the present invention is to provide an immunotherapeutic approach to prevent or treat HCV infectiona . A further object of the present invention is to provide a modified E2 glycoprotein, which approaches 'wild-type' binding levels to natural cellular receptors of HCV infection.

International Patent Publication No. WO 02/22155 (Hawaii Biotechnology Group, Inc.) discloses a truncated HCV E2 polypeptide which lacks the HVR1 region and is capable of secretion into growth medium when expressed in recombinant form in a host cell. The polypeptide may also lack its C-terminus after residue 662. International Patent

Publication No. WO 03/022880 (XTL Biopharmaceuticals Ltd.) also discloses a truncated version of the E2 protein lacking HVR1 region.

The foregoing discussion is intended to introduce the field of the present invention and should not be construed in any way an admission of the state of common general knowledge in this art. Bibliographic details of publications referred to in this specification are set out at the end of the description. The reference to any prior art document in the specification is not, and should not be taken as, an acknowledgement or any form of suggestion that the document forms part of the common general knowledge.

Summary of the Invention

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

In one aspect, the present invention provides a modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein in at least one of said variable regions at least a part of the variable region is replaced with a flexible linker sequence.

In another aspect, the present invention provides a modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein at least a part of the HVR2 variable region is removed or is replaced with a flexible linker sequence.

In yet another aspect, the present invention provides a modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein at least a part of the igVR variable region is removed or is replaced with a flexible linker sequence.

The modified HCV E2 glycoproteins as broadly described above are glycoproteins which substantially approach HCV virion wild-type conformation, and retain the ability to bind to the HCV receptor CD81 and conformation-dependent antibodies.

The present invention also provides a composition comprising a modified HCV E2 glycoprotein as broadly described above, together with a pharmaceutically acceptable carrier or diluent.

Such a composition may be formulated as a vaccine composition, preferably including an adjuvant.

In yet another aspect, the present invention also provides a method of eliciting an immune response in a patient, which comprises administration to the patient of an effective amount of a modified HCV E2 glycoprotein as broadly described above.

In this aspect, the invention includes a method for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises administration to the patient of an effective amount of a modified HCV E2 glycoprotein as broadly described above.

In a further aspect, this invention provides the use of a modified HCV E2 glycoprotein as broadly described above in, or in the manufacture of a medicament for, eliciting an immune response in a patient.

In this further aspect, this invention includes the use of a modified HCV E2 glycoprotein as broadly described above in, or in the manufacture of a medicament for, prophylactic or therapeutic treatment of HCV infection in a patient.

In yet a further aspect, this invention provides an agent for eliciting an immune response in a patient, which comprises a modified HCV E2 glycoprotein as broadly described above.

In this further aspect, the invention includes an agent for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises a modified HCV E2 glycoprotein as broadly described above.

The present invention also provides an isolated antibody raised against a modified HCV E2 glycoprotein as broadly described above. The antibody may be monoclonal or polyclonal.

In this aspect, the invention also provides a method for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises administration to the patient of an effective amount of an antibody as described above.

The invention also provides the use of an antibody as described above in, or in the manufacture of a medicament for, prophylactic or therapeutic treatment of HCV infection in a patient.

In this aspect also, the invention provides an agent for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises an antibody as described above.

Further, the invention provides a method of detecting HCV infection in a patient, comprising contacting a biological sample from the patient with an antibody as described above under conditions which allow formation of an antibody-antigen complex, and detecting said complex, wherein formation of said complex is indicative of the presence of HCV in the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the expression and heterodimerization of E1E2 containing single variable region deletions in cell lysates. Metabolically-labelled cell lysates of 293T cells transfected with wild-type (pE1E2), pE1E2 containing one variable region deletion, or empty vector were immunoprecipitated with A. anti-E1E2 polyclonal antibody (779), B. anti-E1 monoclonal antibody (A4), C. anti-E2 monoclonal antibody (A11) and D. anti-E2 conformation-dependent monoclonal antibody (H53) under both **i)** non-reducing and **ii)**

reducing conditions (+ β-mercaptopethanol). All samples were run on 10-15% SDS-PAGE gradient gels visualized using a phosphoimager. This data is representative of two independent experiments.

Figure 2 shows a ClustalX alignment of diverse HCV E2 glycoprotein sequences from genotypes 1-6. The two recognized hypervariable regions, HVR1 and HVR2, and the novel variable region igVR are highlighted (grey). The conserved cysteine residues that flank the HVR2 and igVR regions, as well as the first conserved cysteine residue proposed to anchor the N-terminal region to the rest of the E2 glycoprotein, are also indicated (bold type). The positions of the CD81-binding determinants (boxed) and the epitope for the broadly neutralizing antibody AP33 are indicated. N-linked glycosylation sites associated with variable regions are also shown (tree). Predicted transmembrane domain is underlined.

Figure 3 shows the incorporation of E1E2 glycoproteins containing single variable region deletions into retroviral pseudotyped HIV-1 particles. A. Metabolically labelled HCV glycoprotein pseudotyped HIV-1 particles were pelleted from the tissue culture fluid of 293T cells transfected with wild-type (pE1E2), pE1E2 containing a variable region deletion or empty vector prior to lysis. E1E2 heterodimers were immunoprecipitated with the conformation-dependent anti-E2 monoclonal antibody H53. All samples were separated on a 10-15% SDS-PAGE gradient gel under non-reducing conditions and visualized using a phosphoimager. B. Processing and incorporation of HIV-1 structural proteins into retroviral pseudotyped HIV-1 particles. HIV-1 structural proteins $Pr55^{Gag}$, $p24^{CA}$ $p66^{RT}$ and $p17^{MA}$ from lysed metabolically-labelled HCV glycoprotein pseudotyped HIV-1 particles were immunoprecipitated using IgG from an HIV-1 infected individual (IgG14). All samples were separated on a 7.5-15% SDS-PAGE gradient gel and visualized using a phosphoimager. This data is representative of two independent experiments.

Figure 4 shows the ability of HCV glycoprotein pseudotyped HIV-1 particles containing single variable region deletions to enter Huh7 cells. The tissue culture fluid from 293T cells co-transfected with the vector NL4-3.LUC.R-E- and either wild-type (pE1E2), pE1E2 containing a deletion in a variable region, or empty vector was used to

infect Huh7 cells in triplicate. The Huh7 cells were lysed and the luciferase activity (relative light units) measured using a Fluostar fitted with luminescence optics. Mean and standard deviation was calculated from triplicate infections. Data is representative of three independent experiments.

Figure 5 shows the ability of HCV E1E2 containing single variable region deletions to bind CD81-LEL. A. Ability of intracellular forms of E2 to bind to CD81-LEL. Metabolically-labelled cell lysates of 293T cells transfected with wild-type (pE1E2), pE1E2 with a deletion of a variable region, or empty vector were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected using anti-E2 conformation-dependent antibody H53 and rabbit anti-mouse horseradish peroxidase (HRP) conjugate. Absorbance values (optical density) were read at 450 nm and the 620 nm background subtracted. B. Virion-incorporated E2 glycoprotein binding to CD81-LEL. Lysates of metabolically-labelled HCV glycoprotein-pseudotyped HIV-1 particles were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected as described for A. Data is representative of two independent experiments.

Figure 6 shows the expression and heterodimerization of E1E2 containing modified single variable region deletions in cell lysates. Metabolically-labelled cell lysates of 293T cells transfected with wild-type (pE1E2), or pE1E2 containing single variable region deletions with an extended linker, or empty vector were immunoprecipitated with A. anti-E1E2 polyclonal antibody (779), B. anti-E1 monoclonal antibody (A4), C. anti-E2 monoclonal antibody (A11) and D. anti-E2 conformation-dependent monoclonal antibody (H53) under both i) non-reducing and ii) reducing conditions (+ β-mercaptopethanol). All samples were run on 10-15% SDS-PAGE gradient gels and visualized using a phosphorimager. Data is representative of two independent experiments.

Figure 7 shows the incorporation of E1E2 glycoproteins containing modified single variable region deletions into HCV glycoprotein pseudotyped HIV-1 particles. A. Metabolically labelled E1E2-pseudotyped HIV-1 particles were pelleted from the tissue

culture fluid of 293T cells transfected with NL4-3.LUC.R-E- and wild-type (pE1E2), containing single variable region deletions with an extended linker, or empty vector prior to lysis. E1E2 heterodimers were immunoprecipitated with the conformation-dependent anti-E2 monoclonal antibody H53. All samples were separated under non-reducing conditions on a 10-15% SDS-PAGE gradient gel and visualized using a phosphoimager. B. Processing and incorporation of HIV-1 structural proteins into HCV glycoprotein-pseudotyped HIV-1 particles. HIV-1 structural proteins $Pr55^{Gag}$, $p17^{MA}$, $p24^{CA}$ and $p66^{RT}$ from lysed metabolically-labelled E1E2-pseudotyped HIV-1 particles were immunoprecipitated using IgG from an HIV-1 infected individual (IgG14) . All samples were separated on a 7.5-15% SDS-PAGE gradient gel and visualized using a phosphorimager. Data is representative of two independent experiments.

Figure 8 shows the ability of HCV glycoprotein-pseudotyped HIV-1 particles containing single modified variable region deletions to enter Huh7 cells. The tissue culture fluid from 293T cells co-transfected with NL4-3.LUC.R-E- and either wild-type (pE1E2), containing single variable region deletions with an extended linker, or empty (pCDNA4) vector was used to infect Huh7 cells in triplicate. The Huh7 cells were lysed and the luciferase activity (relative light units) measured using a Fluostar fitted with luminescence optics. Mean and standard deviation was calculated from triplicate infections. *p*-value was calculated using the student's *t*-test. Data is representative of three independent experiments.

Figure 9 shows the ability of E2 containing single modified deletions of one variable region to bind CD81-LEL. A. Metabolically-labelled cell lysates of 293T cells transfected with wild-type (pE1E2), pE1E2 containing a modified deletion of one variable region, or empty (pCDNA4) were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected using the anti-E2 conformation-dependent monoclonal antibody H53 and rabbit anti-mouse horseradish peroxidase (HRP) conjugate. Absorbance values were read at 450nm-620nm (background). Data is representative of two independent experiments. B. Virion-incorporated E2 glycoprotein binding to CD81-LEL in viral lysates. Lysates of metabolically-labelled E1E2-pseudotyped HIV-1 particles were applied to CD81 MBP-

LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions and bound E2 was detected as described above. Absorbance values were read at 450nm-620nm (background) C. Independent repeat of B.

Figure 10 shows the expression and heterodimerization of immature forms of E1E2 containing multiple variable region deletions. Metabolically-labelled cell lysates of 293T cells transfected with wild-type (pE1E2), pE1E2 containing multiple variable region deletions, or empty vector were immunoprecipitated with A. anti-E1E2 polyclonal antibody (779), B. anti-E1 monoclonal antibody (A4), C. anti-E2 monoclonal antibody (A11) and D. anti-E2 conformation-dependent monoclonal antibody (H53) under both i) non-reducing and ii) reducing conditions (+ β-mercaptopethanol). All samples were run on 10-15% SDS-PAGE gradient gels and visualized using a phosphorimager. Data is representative of two independent experiments.

Figure 11 shows the incorporation of E1E2 glycoproteins containing multiple variable region deletions into HCV glycoprotein-pseudotyped HIV-1 particles. Metabolically labelled E1E2-pseudotyped HIV-1 particles were pelleted from the tissue culture fluid of 293T cells co-transfected with NL4-3.LUC.R-E- and either wild-type (pE1E2), pE1E2 containing multiple variable region deletions, or empty vector prior to lysis. E1E2 heterodimers were immunoprecipitated with conformation-dependent anti-E2 monoclonal antibody H53. All samples were separated under non-reducing conditions on a 10-15% SDS-PAGE gradient gel and visualized using a phosphorimager. B. Processing and incorporation of HIV-1 structural proteins into HCV glycoprotein-pseudotyped HIV-1 particles. HIV-1 structural proteins $Pr55^{Gag}$, $p17^{MA}$, $p24^{CA}$ and $p66^{RT}$ from lysed metabolically-labelled E1E2-pseudotyped HIV-1 particles were immunoprecipitated using IgG from an HIV-1 infected individual (IgG14). Samples were separated under reducing conditions on a 7.5-15% SDS-PAGE gradient gel and visualized using a phosphorimager. Data is representative of two independent experiments.

Figure 12 shows the ability of E1E2-pseudotyped HIV-1 particles containing multiple variable region deletions to enter Huh7 cells. The tissue culture fluid from 293T cells co-transfected with the HIV-1 firefly luciferase vector (NL4-3.LUC.R-E-) and either wild-

type (pE1E2), pE1E2 containing multiple variable region deletions, or empty (pCDNA4) vector was used to infect Huh7 cells in triplicate. The Huh7 cells were lysed and the luciferase activity measured using a Fluostar fitted with luminescence optics. The mean and standard deviation was calculated from triplicate infections. Data is representative of three independent experiments.

Figure 13 shows the ability of E2 containing multiple variable region deletions to bind to CD81-LEL. A. Metabolically-labelled cell lysates of 293T cells transfected with wild-type (pE1E2), pE1E2 containing multiple variable region deletions, or empty (pCDNA4) vector were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected using anti-E2 conformation-dependent monoclonal antibody H53 and rabbit anti-mouse horseradish peroxidase (HRP) conjugate. Absorbance values were read at 450nm-620nm(background). B. Ability of virion-incorporated forms of E2 containing multiple variable region deletions to bind CD81-LEL. Lysates of metabolically-labelled E1E2-pseudotyped HIV-1 particles were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected as described above. Absorbance values were read at 450nm-620nm (background). Data is representative of two independent experiments.

Figure 14 shows the expression of E2 RBD (residues 384-661) containing single and multiple variable region deletions in cell lysates. A. Metabolically labelled cell lysates of 293T cells transfected with either wild-type (E2-myc), E2-myc containing single or multiple variable region deletions, or empty vector were immunoprecipitated with anti-E2 conformation-dependent antibody H53. B. Secretion of E2 RBD (residues 384-661) containing single and multiple variable region deletions in supernatant fluid. The supernatant fluid from metabolically-labelled 293T cells transfected with either wild-type (E2-myc), E2-myc containing single or multiple variable region deletions, or empty vector were immunoprecipitated with the anti-E2 conformation dependent antibody, H53. All samples were separated on 10-15% SDS-PAGE gradient gels and visualized using a phosphoimager. Data for the single variable region deletion is from one experiment.

Figure 15 shows the ability of E2 RBD (residues 384-661) containing single variable region deletions to bind CD81-LEL. A. Cell lysates from metabolically-labelled 293T cells transfected with wild-type (E2-myc), E2-myc containing single variable region deletions, or empty vector were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected using anti-E2 conformation-dependent antibody H53 and rabbit anti-mouse horseradish peroxidase (HRP) conjugate. Absorbance values were read at 450nm-620nm (background). B. Ability of secreted E2 RBD (residues 384-661) containing single variable region deletions to bind CD81-LEL. Tissue-culture fluid from metabolically-labelled 293T cells transfected with wild-type (E2-myc), E2-myc containing single variable region deletions were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Bound E2 was detected using anti-E2 conformation-dependent antibody H53 and rabbit anti-mouse horseradish peroxidase (HRP) conjugate. Absorbance values were read at 450nm-620nm (background). Data is from a single experiment.

Figure 16 shows the ability of E2 RBD (residues 384-661) containing multiple variable region deletions to bind CD81-LEL. A. Cell lysates from metabolically-labelled 293T cells transfected with wild-type (E2-myc), E2-myc containing multiple variable region deletions, or empty vector were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Absorbance values were read at 450nm-620nm (background). B. Ability of secreted E2 RBD (residues 384-661) containing multiple variable region deletions to bind CD81-LEL. Supernatant fluid from metabolically-labelled 293T cells transfected with wild-type (E2-myc), E2-myc containing multiple variable region deletions or empty vector were applied to CD81 MBP-LEL (residues 113-201) coated enzyme immunoassay plates at progressive two-fold dilutions. Absorbance values were read at 450nm-620nm (background). Data is from a single experiment.

Figure 17 shows the binding of E2-myc proteins to full length surface expressed CD81. Equivalent amounts of monomeric secreted E2-myc proteins produced from 293T cells were applied to CHO-K1 cells transfected with vector encoding full length CD81. Bound E2-myc was detected with iodinated 9E10 prior to measurement in a gamma

counter. Mean of three independent assays ± standard error. P values derived by comparison with the E2 Δ23-myc E2 construct using the students *t* test assuming unequal variances.

Figure 18 shows the ability of E2-myc and E2 Δ123-myc to be detected with human conformation sensitive monoclonal antibodies. Wild-type (E2-myc) and E2 Δ123-myc were metabolically labelled and immunoprecipitated with the "CBH" panel of conformation dependent human monoclonal antibodies specific to three immunogenic domains (A, B and C) of E2 . Immunoprecipitated proteins were analysed by SDS-PAGE under non-reducing conditions in 10-15% polyacrylamide gradient gels followed by scanning in a phosphorimager.

Figure 19 shows a schematic representation of the E1E2 polyprotein containing single and multiple variable region deletions. The pE1E2 vector encodes the full-length H77c sequence of E1 (dark grey) and E2 (light gray) including their transmembrane domains (horizontal stripes) at the C-terminus of E1 and E2 and the signal peptides with the signal peptidase cleavage sites indicated (arrows). The E2 variable regions (dotted) and the conserved region adjacent to HVR1 (diagonal stripes) are replaced – individually and in combination - with flexible Gly-Ser-Ser-Gly (GSSG) linker motifs as indicated.

Figure 20 shows a schematic representation of overlap extension PCR strategy. The variable regions were deleted from the HCV glycoprotein template sequence (diagonal stripes) using two oligonucleotide pairs: each contains one external and one internal primer that amplifies either the 5' or 3' fragment adjacent to HVR1 as shown. The internal primer sequence introduces the Gly-Ser-Ser-Gly linker motif (bold) and a short 'overlap' sequence complementary to the respective 5' or 3' fragment. These sequences anneal to form the template for the second round of PCR amplification that uses the external primers to amplify the HVR1 deleted glycoprotein sequence.

Figure 21 shows a schematic representation of HCV E1E2 polyprotein containing modified single variable region deletions with extended linkers. The pE1E2 vector encodes the full-length H77c sequence of E1 (dark gray) and E2 (light gray) including their

transmembrane domains (vertical stripes) at the C-terminus of E1 and E2. The signal peptidase cleavage sites are also indicated (arrows). The E2 variable regions and the conserved region adjacent to HVR1 (con) are *partially* deleted and replaced with flexible Gly-Ser-Ser-Gly linker motifs.

Figure 22 shows a schematic representation of the E2 receptor-binding domain (E2 RBD_{661}) containing single and multiple variable region deletions. The pE2661 vector encodes the E2 RBD encoding residues 384-661 (E2-myc). The variable regions (dotted) and a conserved sequence adjacent to HVR1 (diagonal stripes) were replaced individually and in combination with short flexible Gly-Ser-Ser-Gly (GSSG) linker motifs as indicated. A myc epitope tag (vertical stripes) was also introduced at the C-terminus of these constructs.

Figure 23 shows a schematic representation of the strategy used to generate HCV glycoprotein-pseudotyped HIV-1 particles. The HCV glycoprotein expression vector encoding E1E2 (pE1E2) or pE1E2 containing variable region deletions and the retroviral vector (HIV-1 NL4-3.LUC.R^-E^-) lacking its native envelope gene and containing a luciferase reporter gene are co-transfected into 293T cells. As the retroviral core proteins assemble within the cell they acquire an envelope by budding from the plasma membrane, incorporating the HCV E1E2 glycoproteins. These virions are then collected and used to undergo one round of infection and replication in Huh7 cells. The level of E1E2-mediated entry is then quantified by the resulting luciferase activity within these infected Huh7 cells.

Figure 24 shows the ability of E2-myc and E2 Δ123-myc proteins to be immunoprecipitated by a panel of human sera obtained from HCV infected individuals. Immunoprecipitated proteins were analysed by SDS-PAGE under non-reducing conditions in 10-15% polyacrylamide gradient gels followed by scanning in a phosphorimager. Below each lane is the HCV RNA status, presence of HCV specific antibody detected using one of BioRad Monolisa, Abbot Murex or Chiron RIBA assays and the 50% neutralizing antibody titre for that serum sample. Molecular weight markers are indicated to the left.

Figure 25 shows the ability of E2-myc proteins containing variable region deletions to interact with recombinant large extracellular loop of CD81. A. Ability of wild-type E2-myc, E2 Δ12-myc, E2 Δ23-myc and E2-myc protein containing the mutation L441M, that disrupts interaction with CD81, to interact with wild-type recombinant MBP-LEL. E2-myc proteins were serially diluted in MBP-LEL coated enzyme immunoassay plates. Data is the average of two to seven independent experiments and is shown as the mean percentage binding relative to wild-type E2-myc ± standard deviation. B. Ability of WT E2-myc, E2 Δ13-myc and E2 Δ123-myc to interact with wild-type recombinant MBP-LEL (solid lines) or recombinant MBP-LEL containing the F186S mutation that disrupts E2 binding (dashed line). Data is the average of two to seven independent experiments and is shown as the mean percentage binding relative to wild-type E2-myc ± standard deviation.

Figure 26 shows the ability of E2-myc proteins containing variable region deletions to interact with full length surface expressed CD81. A. Ability of WT E2-myc, E2 Δ12-myc,E2 Δ23-myc and E2-myc protein containing the mutation L441M to interact with full length CD81 transfected CHO-K1 cells. Dilutions of wild-type or variable region deleted E2-myc proteins were applied to ice cold, human CD81 transfected CHO-K1 cells and incubated on ice for 4 hrs. After washing, 1 x 10^6 CPM ^{125}I-9E10 was added and plates incubated for 1 h at room temperature, washed and counted in a Packard Auto GammaCounter. Data shown is the mean percentage binding relative to wild-type ± standard error of two to five independent experiments. B. Ability of WT E2-myc, E2 Δ123-myc, and E2 Δ13-myc to interact with full length human CD81 (solid line) or F186S-CD81 (dashed line) transfected CHO-K1 cells. Data shown is the mean percentage binding relative to wild-type ± standard error of two to five independent experiments.

Figure 27 shows an SDS-PAGE of purified HCV E2 protein variants in 4-20 % polyacrylamide gradient gel of the E2-his proteins containing none (wild-type) one or more variable region deletions. The proteins were visualized by staining with Coomassie brilliant blue.

Figure 28 shows a blue-native PAGE analysis of E2-his proteins. The purified proteins (10 μg) were electrophoresed in 5-15 % polyacrylamide gradient gels under native

conditions. After electrophoresis, the gels were destained overnight and scanned in a Licor Odyssey scanner at 680 nm. The migration position of protein standards is shown on the right.

Figure 29 shows a Western blot analysis of purified E2-his proteins. Samples of the purified proteins were subjected to reducing SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. E2 proteins were detected with a non-conformation dependent E2 specific monoclonal antibody followed by goat anti-mouse immunoglobulin coupled to Alexafluor 680 nm (Invitrogen). Immunoblots were scanned in an Licor Odyssey scanner. Molecular weight markers are shown on the left (kDa).

Figure 30 shows the ability of HCV E2-his proteins to bind to a recombinant form of the CD81 large extracellular loop (CD81-LEL). Enzyme immunoassay plates were coated with maltose binding protein fused to (A) wild-type large extracellular loop of CD81 (residues 113-201) (CD81-LEL) or (B) CD81-LEL containing an F186S mutation in the E2 binding site of CD81-LEL. Plates were blocked with bovine serum albumin and then incubated with serial dilutions of E2-his proteins (in 50 µl PBS containing 5 mg/ml bovine serum albumin and 0.05 % Tween20) for 2 h. The bound E2-his proteins were detected using an E2 specific monoclonal antibody and rabbit anti-mouse immunoglobulins coupled to horseradish peroxidase (Dako). Plates were developed using tetramethylbenzidine hydrochloride substrate and stopped by the addition of 1M HCl. Absorbance was measured at 450 nm and the background at 620 nm subtracted. Percentage binding was calculated by dividing the absorbance value for each protein by the maximal absorbance obtained for wild-type E2-his and multiplied by 100.

Figure 31 shows the immunoreactivity towards homologous E2-his antigen of mouse sera obtained after 2 immunizations with E2-his proteins. E2-his proteins were captured on 96 well Maxisorb microtitre plates (Nunc) precoated with galanthis nivalis (GNA) lectin. Serial dilutions of immune mouse sera were incubated with the captured corresponding E2 protein variant used for the immunization and bound immunoglobulins detected with rabbit anti-mouse immunoglobulin coupled to horseradish peroxidase. The assay was developed using tetramethylbenzidine hydrochloride substrate and stopped by the addition of 1M

HCl. Absorbance values were measured at 450 nm and the background at 620 nm subtracted in a Fluostar plate reader (BMG technologies). The antibody titres of individual sera were determined as the serum dilution giving 5-times the background absorbance. The maximum (upper error bar), 75th and 25th percentile (upper and lower edges of box, respectively), median (horizontal line within box) and minimum (lower error bar) titres for each immunogen group are shown.

Figure 32 shows the immunoreactivity towards E2-his (A) and E2 Δ123-his (B) antigens of mouse sera obtained after 2 immunizations with E2 protein variants. E2-his and E2 Δ123-his proteins were captured on 96 well Maxisorb microtitre plates and antibody titres of individual sera determined as described in Figure 31. The maximum (upper error bar), 75th and 25th percentile (upper and lower edges of box, respectively), median (horizontal line within box) and minimum (lower error bar) titres for each immunogen group are shown.

Figure 33 shows the immunoreactivity towards homologous E2-his antigen of mouse sera obtained after 3 immunizations with E2-his proteins. E2-his proteins were captured on 96 well Maxisorb microtitre plates and antibody titres of individual sera determined as described in Figure 31. The maximum (upper error bar), 75th and 25th percentile (upper and lower edges of box, respectively), median (horizontal line within box) and minimum (lower error bar) titres for each immunogen group are shown.

Figure 34 shows the immunoreactivity towards E2-his (A) and E2 Δ123-his (B) antigens of mouse sera obtained after 3 immunizations with E2 protein variants. E2-his and E2 Δ123-his proteins were captured on 96 well Maxisorb microtitre plates and antibody titres of individual sera determined as described in Figure 31. The maximum (upper error bar), 75th and 25th percentile (upper and lower edges of box, respectively), median (horizontal line within box) and minimum (lower error bar) titres for each immunogen group are shown.

Figure 35 shows the immunoreactivity towards Con1 E2$_{RBD}$-his (A) and JFH1 E2$_{RBD}$-myc (B) antigens of mouse sera obtained after 3 immunizations with E2 protein variants.

Con1 and JFH1 RBD proteins were captured on 96 well Maxisorb microtitre plates and antibody titres determined for individual sera as described in Figure 31. The maximum (upper error bar), 75th and 25th percentile (upper and lower edges of box, respectively), median (horizontal line within box) and minimum (lower error bar) titres for each immunogen group are shown.

Figure 36 shows the eighty-percent neutralization titres (log_{10}) of mouse sera obtained after 3 immunizations with E2-his proteins. Serial 5-fold dilutions of heat-inactivated immune mouse sera preincubated for 1 h at 37°C with H77c HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses (1 h) and then added to quadruplicate Huh7 cell monolayers in 48-well tissue culture plates. Following a 4 h incubation (37 °C, 5 % CO_2) the cells were washed with PBS and the medium replaced. After an additional 3-day incubation (37 °C in 5 % CO_2), the cells were lysed and luciferase activity assayed in a fluostar fitted with luminescence optics. The neutralization titres of individual sera were determined as the serum dilution giving 80 % neutralization compared to HCV glycoprotein-pseudotyped HIV-1 luciferase reporter virus preincubated with medium alone.

DETAILED DESCRIPTION OF THE INVENTION

The modified E2 core structure of the HCV virion in which at least one of the variable regions, and in particular the variable region igVR identified by the inventors, has been modified by removal or deletion of at least a part of the variable region, and optional insertion of a linker sequence, has practical use as a vaccine that can elicit broadly neutralizing antibodies to diverse strains of HCV. The binding efficiency of the modified E2 glycoprotein exhibits wild-type binding to the HCV receptor CD81, and the modified E2 glycoprotein provides a means of treating HCV infection by mimicking complex conformational changes of the E2 ectodomain required for effective CD81 binding, and thereafter initiating an immune response without cell invasion.

In one aspect, the present invention provides a modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein in at least one of said variable regions at least a part of the variable region is replaced with a flexible linker sequence.

In another aspect, the present invention provides a modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein at least a part of the HVR2 variable region is removed or is replaced with a flexible linker sequence.

In yet another aspect, the present invention provides a modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein at least a part of the igVR variable region is removed or is replaced with a flexible linker sequence.

References herein to the "HVR1" and "HVR2" variable regions are to be understood as references to the two variable regions HVR1 (384-410) and HVR2 (461-481), while references herein to the "igVR" variable region are to be understood as references to the intergenotypic variable region igVR (570-580) identified by the inventors.

The term "flexible linker sequence" is used herein to refer to a short, flexible, polypeptide sequence which permits disulfide bond linkages between cysteine residues in the modified glycoprotein leading to retention of the native or "wild-type" disulfide linkages, and in particular retention of the ability to bind to the HCV CD81 receptor and conformation-dependent antibodies. Suitable linker sequences are discussed in review articles by George and Heringa, 2002, and Argos, 1990, and may consist of up to 20 amino acid residues such as Gly and Ser, and include, and comprise amino acids selected from the sequence group consisting of Gly, Ser, Ala, Thr and Arg, more particularly Gly- and Ser-Ser-Gly (GSSG). Suitable linker sequences include, by way of example, the sequences

$(Gly)_2$-Ala-$(Gly)_2$, $(Gly)_5$ or $(Gly)_8$ (see Sabourin *et al.*, 2007),

$(Gly)_6$, $(Gly)_7$ or $(Gly)_{10}$ (see Yang and Gruebele, 2006),

Gly-Ser-Gly-Ser-Gly (see Dipti *et al.*, 2006),

$(Gly)_4$ (see Anandarao *et al.*, 2006),
Gly-Ala-Gly (see Wyatt *et al.*, 1995),
$(Gly)_2$-Arg-$(Gly)_2$-Ser (see Bellamy-McIntyre *et al.*, 2007),
$(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_{n=3\sim4}$ (see Arai *et al.*, 2006), and
Ser-$(Gly)_2$-Ser-Gly (see Bahrami *et al.*, 2007).

A preferred sequence is the sequence Gly-$(Ser)_2$-Gly disclosed herein. It will be understood that selection of suitable polypeptide linker sequences is a matter of routine experimentation for a person skilled in this field, and the modified HCV E2 glycoproteins of the present invention are not limited to the particular linker sequences disclosed herein. Without wishing to be bound by any theory, by replacing HVR1, HVR2 and igVR with short flexible linkers, disulfide-bond formation between Cys-569 to Cys-581 and between Cys-459 to Cys-486, for example, and the intrinsic folding of conserved E2 core domain, is substantially retained in the modified glycoproteins.

References in this specification to "deletion" of at least part of one of the variable regions of the HCV E2 receptor-binding domain are to be understood as references to deletion or removal of at least part of the sequence of the variable region and optional insertion of a flexible linker sequence to replace the deleted sequence.

The modified HCV E2 glycoproteins of the present invention may be prepared by any suitable method, including in particular preparation of the modified glycoproteins in the form of recombinant products by expression of appropriate DNA deletion constructs as described in the Examples herein.

Preferably at least one of the second and third variable regions of the core E2 receptor-binding domain, HVR2 and igVR, is modified by removal of at least a portion of the residue within the region and inserting a flexible linker sequence. It has been found that deletion of at least part of the HVR2 region, in combination with other variable region deletions, substantially reduces E1E2 heterodimerisation.

In a further embodiment, all three of the variable regions are modified by removal of at least a portion of the residues within these regions and inserting a linker sequence.

Deletion of intergenotypic variable region (igVR) from the E2 glycoprotein that already contains deletions of HVR1 and HVR2 improves binding to CD81 relative to simultaneous deletions of HVR1 and HVR2 alone.

It has been found by the inventors that HVR1, HVR2, and igVR are all required for E1E2-pp mediated viral entry into Huh7 cells. E1E2-pp containing deletions of at least each of HVR1, HVR2, and igVR have been found to retain wild-type levels of recombinant CD81 binding. This represents a significant improvement in the ability to (a) mimic cell binding of the HCV virion, and (b) initiate an immune response without exposing a patient to highly variable immunodominant regions that may be immune decoys .

In a preferred embodiment, all three variable regions can be deleted and retain the core E2 folding domain which is required to assemble at least three discontinuous binding elements involved in the CD81-binding site. A further advantage of various combined deletions of HVR1, HVR2, and igVR from the E2 receptor-binding domain is that a soluble form of the E2 core domain is obtainable, which is suitable for use as an immunogen.

The present invention also provides a composition comprising a modified HCV E2 glycoprotein as broadly described above, together with a pharmaceutically acceptable carrier or diluent.

Such a composition may be formulated as a vaccine composition, preferably including an adjuvant.

Conventional pharmaceutically acceptable carriers, excipients, buffers or diluents, may be included in vaccine compositions of this invention. Generally, a vaccine composition in accordance with the present invention will comprise an immunologically effective amount of the modified HCV E2 glycoprotein, and optionally an adjuvant, in conjunction with one or more conventional pharmaceutically acceptable carriers and/or diluents. An extensive

though not exhaustive list of adjuvants can be found in Cox and Coulter, "Advances in Adjuvant Technology and Application", in *Animal Parasite Control Utilizing Biotechnology*, Chapter 4, Ed. Young, W.K., CRC Press 1992, and in Cox and Coulter, "Adjuvants – A Classification and Review of Their Modes of Action", *Vaccine* **15(3)**, 248-256, 1997. As used herein "pharmaceutically acceptable carriers and/or diluents" include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and is described by way of example in *Remington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Company, Pennsylvania, U.S.A.

In yet another aspect, the present invention also provides a method of eliciting an immune response in a patient, which comprises administration to the patient of an effective amount of a modified HCV E2 glycoprotein as broadly described above.

In this aspect, the invention includes a method for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises administration to the patient of an effective amount of a modified HCV E2 glycoprotein as broadly described above.

Reference herein to "treatment" is to be understood in its broadest context. Accordingly, the term "prophylactic treatment" includes treatment to protect the patient against infection or to reduce the likelihood of infection. Similarly, the term "therapeutic treatment" of infection does not necessarily imply that the patient is treated until total recovery from infection, and includes amelioration of the symptoms of infection as well as reducing the severity of, or eliminating, the infection.

The modified HCV E2 glycoprotein of this invention is administered in an effective amount. An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of the infection. The amount varies depending upon the health and physical condition of the individual to be treated, the racial background of the individual to be treated, the degree of protection desired, the formulation of the composition, the

assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. If necessary, the administration of an effective amount may be repeated one or several times. The actual amount administered will be determined both by the nature of the infection which is being treated and by the rate at which the active immunogen is being administered.

Preferably, the patient is a human, however the present invention extends to treatment and/or prophylaxis of other mammalian patients including primates and laboratory test animals (e.g. mice, rabbits, rats, guinea pigs).

In accordance with the present invention, the modified HCV E2 glycoprotein is preferably administered to a patient by a parenteral route of administration. Parenteral administration includes any route of administration that is not through the alimentary canal (that is, not enteral), including administration by injection, infusion and the like. Administration by injection includes, by way of example, into a vein (intravenous), an artery (intraarterial), a muscle (intramuscular) and under the skin (subcutaneous). The modified HCV E2 glycoprotein may also be administered in a depot or slow release formulation, for example, subcutaneously, intradermally or intramuscularly, in a dosage which is sufficient to obtain the desired pharmacological effect.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active component which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in a polyethylene glycol and lactic acid. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, suitable carbohydrates (e.g. sucrose, maltose, trehalose, glucose) and isotonic sodium chloride solution. In addition, sterile, fixed oils are conveniently employed as a solvent or suspending medium. For this purpose, any bland

fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

In a further aspect, this invention provides the use of a modified HCV E2 glycoprotein as broadly described above in, or in the manufacture of a medicament for, eliciting an immune response in a patient.

In a further aspect, this invention includes the use of a modified HCV E2 glycoprotein as broadly described above in, or in the manufacture of a medicament for, prophylactic or therapeutic treatment of HCV infection in a patient.

In this further aspect, this invention provides an agent for eliciting an immune response in a patient, which comprises a modified HCV E2 glycoprotein as broadly described above.

In this further aspect, the invention includes an agent for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises a modified HCV E2 glycoprotein as broadly described above.

In one embodiment of the invention, it has been found that recombinant E2 glycoprotein in which at least region igVR is subject to deletion of residues in the region and replacement with a flexible linker sequence improves recognition by conformational-dependent antibodies. In addition, it has been found that, in a comparative study with 'wild-type' E2 glycoprotein, modification of the region igVR alone and in various combinations with HVR1 and HVR2 show binding to full-length CD81-LEL (large extracellular loop of human tetraspanin CD81) approaching that of the wild-type.

It has been shown by the inventors that alignment of diverse E2 glycoprotein sequences across the six major genotypes identified a novel third variable region, "igVR," between residues 560 and 581. It is suggested that "igVR" is not strictly a hypervariable region as it exhibits its greatest variability across different genotypes, whereas it is relatively conserved within a genotype and is thus unlikely to be under immune selection pressure. Furthermore, it consists of a series of insertions or deletions that shifts, but always

maintains, the glycosylation site located within this region. The inventors have also observed that both variable regions HVR2 and "igVR" are flanked by conserved cysteine residues and proposed that these regions may form solvent-exposed loops stabilised by disulfide-bonding between these residues.

The inventors have proposed that the hypervariable regions form flexible solvent-exposed subdomains that enable the E2 glycoprotein to move between 'open' and 'closed' conformations, the former being more competent for CD81 binding by exposing the conserved CD81-binding determinants located within the core E2 domain as delineated within this study. Indeed, conformational changes within the E2 glycoprotein have been previously observed upon CD81-binding. In addition, binding of non-neutralizing antibodies to the E2 glycoprotein has been demonstrated to reduce its susceptibility to neutralizing antibodies and is consistent with a model where the non-neutralizing antibodies inhibit the flexibility of these surface-exposed hypervariable regions thus blocking access to the conserved epitopes located within the E2 core domain.

Therefore, this suggests that the modified E2 core domain represents a promising vaccine candidate for eliciting neutralizing antibodies to conserved epitopes within the E2 glycoprotein, including the CD81-binding determinants, that are otherwise occluded by these surface-exposed variable regions that may act as immunological decoys at the surface of the glycoprotein complex during HCV replication.

The present invention also provides an isolated antibody raised against a modified HCV E2 glycoprotein as broadly described above.

The term "antibody" is used broadly herein to include both monoclonal and polyclonal antibodies that specifically bind to the modified HCV E2 glycoprotein, as well as antigen binding fragments of such antibodies including, for example, Fab, $F(ab^1)_2$, Fd and Fv fragments of an antibody that retain specific binding activity for the modified HCV E2 glycoprotein.

Antibodies having the desired specificity, both monoclonal and polyclonal, can be obtained using methods which are well known in the art (see, for example, Harlow and Lane, "Antibodies, A laboratory manual", Cold Spring Harbor Laboratory Press, 1988). Methods for preparing antibodies, and antigen binding fragments thereof, are also described in International Patent Publication WO 02/22155.

The invention also provides a method for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises administration to the patient of an effective amount of an antibody as described above. Accordingly, this method provides passive immunotherapy of the patient.

As previously described, the invention also provides the use of an antibody as described above in, or in the manufacture of a medicament for, prophylactic or therapeutic treatment of HCV infection in a patient.

In this aspect also, the invention provides an agent for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises an antibody as described above.

Further, the invention provides the use of an antibody as broadly described above in diagnosis of HCV infection. In this aspect, the invention provides a method of detecting HCV infection in a patient, comprising contacting a biological sample from the patient with an antibody as described above under conditions which allow formation of an antibody-antigen complex, and detecting said complex, wherein formation of said complex is indicative of the presence of HCV in the sample.

Preferably, the antibody is detectably labelled. Suitable labels are well known in the art and include, for example, enzymes, radioisotopes, fluorescent compounds, colloidal metals, and chemiluminescent, phosphorescent and bioluminescent compounds. Preferably, the biological sample is a sample of a body fluid from the patient, such as a blood sample.

The modified HCV E2 glycoproteins of the present invention may also be used in drug discovery techniques, including for example small molecule screening techniques.

The present invention is further illustrated by the following non-limiting Examples.

Example 1

Materials and methods used in this Example are described below.

1. Role of the individual variable regions in E1E2 glycoprotein structure and function.

The results for the various variable deletion mutants are summarized in Table 1.

Intracellular E1E2 precursor folding and heterodimerisation

To examine the role of the individual variable regions in intracellular E1E2 biosynthesis, metabolically-labelled cell lysates of 293T cells transfected with the single variable region deletion constructs were immunoprecipitated with antibodies reactive to E1 and/or E2 epitopes. These preparations were analysed under both non-reducing and reducing conditions to characterise non-covalently associated E1E2 heterodimers existing within the large intracellular population of covalently-linked aggregate as previously observed.

Immunoprecipitation with the polyclonal anti-E1E2 antibody (779) detected the total intracellular population of E1 (~ 30 kDa) and E2 (~ 70 kDa) glycoproteins to show efficient expression and cleavage from the polyprotein occurring for each single variable region deletion construct (see Figure 1A). The mutant E2 glycoproteins exhibited variably lower molecular weights than wild-type: HVR1con < HVR2 and "igVR" < HVR1 < WT. This is consistent with the loss of two glycosylation sites predicted to reside within the HVR1con deletion, one within each of the HVR2 and "igVR" deletions and none within the HVR1 deletion (Figure 2). However, deglycosylation of these E2 glycoproteins is required to confirm their backbone molecular weights as predicted in Table 2. The band migrating to ~100 kDa represents the E1E2 polyprotein that has not been cleaved by host signal peptidases and is more pronounced in both the single HVR2 and "igVR" deletion constructs (Figure 1A). This is reflected in the reduced levels of total intracellular E1 and

E2 observed for the HVR2 and "igVR" mutants suggesting that these deletions may be effecting polyprotein processing.

The non-conformation-dependent anti-E1 monoclonal antibody (A4) precipitated an E1 doublet at ~30 kDa and ~27 kDa for both the mutant and the wild-type constructs under reducing and non-reducing conditions (Figure 1B). This doublet represents either alternative E1 glycosylation states or messenger RNA splicing isoforms as previously observed. Furthermore, E1 co-precipitated low levels of the wild-type E2 glycoprotein under non-reducing conditions, whereas it efficiently co-precipitated both wild-type and mutant E2 glycoproteins under reducing conditions. This indicates that A4 recognises an E1 epitope that is more exposed within covalently-linked intracellular E1E2 complexes.

The non-conformation dependent anti-E2 monoclonal antibody (A11) detected intracellular E2 glycoprotein expression for all the individual variable region deletion constructs (Figure 1C). However, only the mutant E2 glycoproteins containing the HVR1con or HVR1 deletions co-precipitated a detectable amount of E1 under non-reducing and reducing conditions. This indicates that the individual HVR2 and "igVR" deletions are either directly or indirectly disrupting the formation of the intracellular E1E2 heterodimer. Notably, this reduced heterodimerisation is not due to the lower total intracellular E1 and E2 observed for the HVR2 and "igVR" deletions as E2 glycoprotein expression was detected at wild-type levels by A11 in both these mutants. In addition, E2 co-precipitated generally low levels of E1, particularly under reducing conditions, suggesting that the A11 epitope is partly occluded in intracellular E1E2 heterodimer complexes.

The conformation-dependent anti-E2 monoclonal antibody (H53) has been shown to recognise native E2 on the surface of HCV virions and thus the amount of E1 co-precipitating with this E2 species is a good indicator of the proportion of functional non-covalently associated E1E2 heterodimers forming. Immunoprecipitation with H53 detected intracellular E2 glycoprotein expression for all individual variable region deletion constructs (Figure 1D). This indicates that the inserted Gly-Ser-Ser-Gly linker motif provides sufficient flexibility within these E2 glycoproteins to retain the intrinsic folding

of the native E2 glycoprotein required to present this conformation-dependent epitope. However, the intracellular E2 glycoproteins containing HVR2 or "igVR" deletions failed to co-precipitate E1, suggesting that these regions are directly or indirectly required in the intracellular assembly of the non-covalently associated E1E2 heterodimer.

E1E2 glycoprotein maturation and incorporation into pseudotyped HIV-1 particles

To examine the role of the individual variable regions in E1E2 glycoprotein maturation, the small amount of E1 and E2 escaping from the ER to transit through the secretory pathway was enriched for by the incorporation of these complexes into pseudotyped HIV-1 particles as previously described. These metabolically-labelled E1E2-pseudotyped HIV-1 particles were lysed prior to immunoprecipitation with the conformation-dependent anti-E2 monoclonal antibody (H53) and IgG from an HIV-1 infected individual (IgG14).

Immunoprecipitation with IgG14 demonstrated that none of the single variable region deletion constructs had affected processing and virion incorporation of the HIV-1 structural proteins $Pr55^{Gag}$, $p17^{MA}$, $p24^{CA}$ or $p66^{RT}$ (Figure 3B). Immunoprecipitation with H53 detected the virion-incorporated E2 glycoprotein as a diffuse molecular weight band (~ 70-90 kDa) typical of the mature glycoprotein containing various complex- and hybrid-type carbohydrate modifications as previously described (Figure 3A). The different mutant E2 glycoproteins exhibited further variation in their relative molecular weights (HVR1con < HVR2 and "igVR" < HVR1 < WT) reflecting the loss of glycosylation sites located within these deleted regions as described for the intracellular data. All mutant E2 glycoproteins were incorporated into E1E2-pseudotyped HIV-1 particles at approximately wild-type levels, except for the HVR1con deletion that showed a significantly reduced E2 band. This suggests that the conserved region adjacent to HVR1 is required for the completion of E2 folding, maturation and incorporation into pseudotyped HIV-1 particles.

Immunoprecipitation with H53 also detected a protein species migrating at ~ 33 kDa and another lower molecular weight band at ~ 31kDa initially suggesting that the virion-incorporated E1 glycoprotein also migrates as a doublet (Figure 3A). However, across two independent experiments, the lower molecular weight band co-migrated with a non-specific band in the negative control (empty) and thus the higher molecular weight band

alone is likely to represent E1. The virion-incorporated E1 glycoprotein (~33 kDa) also migrates more slowly than the intracellular species (~30 kDa) reflecting additional carbohydrate modifications at one or two E1 glycosylation sites acquired during transit through the secretory pathway as previously observed. Furthermore, the virion-incorporated E2 glycoproteins containing the individual HVR2 and "igVR" deletions could not co-precipitate E1 at detectable levels indicating that these variable regions are directly or indirectly required in the formation of the functional E1E2 heterodimer.

E1E2-mediated entry into Huh7 cells

The formation of the functional non-covalently associated E1E2 heterodimer has been shown to be critical for E1E2-mediated entry into Huh-7 cells. In order to investigate the role of the individual variable regions in viral entry, the single deletion constructs were used to generate E1E2-pseudotyped HIV-1 particles to infect Huh7 cells as previously described. As expected, the HVR1con deletion conferred a total loss of entry due to its retarded incorporation of the E2 glycoprotein into pseudotyped HIV-1 virions as observed above (Figure 4). Similarly, the HVR2 and "igVR" deletions were not entry competent due to the disruption of the functional E1E2 heterodimer complex. However, the HVR1 deletion also conferred a total loss of entry despite retaining both wild-type heterodimerisation levels and incorporation of E1E2 glycoproteins into HIV-1 virions suggesting that this region may have a direct role in E1E2-mediated viral entry.

CD81-LEL binding

It has been previously demonstrated that the E2 glycoprotein alone is sufficient to mediate binding to the CD81 receptor and therefore, despite exhibiting a loss of heterodimerisation and/or viral entry, both intracellular and virion-incorporated mutant E2 glycoproteins were examined for their ability to bind to the large-extracellular loop (LEL residues 113-201) of CD81. This involved applying both cell and viral lysates to a solid-phase CD81 MBP-LEL (residues 113-201) binding assay as previously described. This data was normalized for the amount of monomeric E2 expressed from each vector as precipitated with the conformation-dependent anti-E2 monoclonal antibody H53 and observed by SDS-PAGE under non-reducing conditions; Figures 1D (intracellular) and 3A (virion-incorporated).

All intracellular E2 glycoprotein precursors containing the single variable region deletions exhibited CD81-LEL binding at wild-type levels, except for HVR1con that demonstrated a total loss of binding (Figure 5A). This indicates that the inserted Gly-Ser-Ser-Gly linker motifs are providing sufficient flexibility within the HVR1, HVR2 and "igVR" deleted E2 glycoproteins to form the E2 CD81-binding site and suggests that these individual variable regions are not required in this function. It also indicates that the intracellular E2 glycoprotein precursor lacking the conserved region adjacent to HVR1 (HVR1con) cannot form the CD81-LEL binding site despite maintaining the conformation-dependent epitope recognised by H53. Intracellular forms of both the HVR2 and "igVR" deletions displayed an additional enhancement of maximum CD81-LEL binding compared to wild-type (Figure 5A) although they did not exhibit a significant shift in the overall binding curve and thus additional independent experiments are required to determine whether these variable regions are modulating CD81-LEL binding.

As expected, the virion-incorporated E2 glycoprotein containing the HVR1con deletion could not mediate CD81-LEL binding, reflecting both its reduced incorporation into E1E2-pseudotyped HIV-1 particles as well as the intracellular data above (Figure 5B). In contrast, the virion-incorporated E2 glycoproteins containing the HVR1, HVR2 and "igVR" deletions all retained at least wild-type levels of CD81-LEL binding indicating that these mutant E2 glycoproteins retain the CD81-binding site even after maturation. Interestingly, the virion-incorporated E2 glycoprotein containing the HVR1 deletion demonstrated an additional enhancement of maximum CD81-LEL binding compared to wild-type and exhibited an approximately 4-fold increase in the overall binding curve (Figure 5B). This suggests that HVR1 is negatively modulating CD81-LEL binding within the virion-incorporated E2 glycoprotein despite not being required for this function. The observed absence of this effect in the intracellular E2 glycoprotein precursor containing the same deletion (Figure 5A) reflects changes in E1E2 folding and the CD81-binding site that occur during glycoprotein maturation through the secretory pathway as previously observed.

2. Effect of the extended linker on E1E2 glycoprotein structure and function

Intracellular E1E2 precursor folding and heterodimerisation

In order to enhance the folding of the E2 glycoproteins containing the individual variable region deletions, the Gly-Ser-Ser-Gly linker was extended by reintroducing several conserved cysteine-proximal residues deleted from the original constructs. Notably, this series of modified deletion constructs does not have a HVR1 counterpart due to the highly variable nature of this region. These constructs were analysed for E1 and E2 glycoprotein expression and heterodimerisation within 293T cells by pulse-chase metabolic-labelling and immunoprecipitation.

Immunoprecipitation with the anti-E1E2 polyclonal antibody (779) detected intracellular expression and cleavage of the E1 (~30 kDa) and E2 (~70 kDa) glycoproteins from the polyprotein occurring for each extended linker deletion construct (Figure 6A). Polyprotein cleavage appeared to be slightly less efficient in the HVR2link and "igVR link" constructs suggesting that these deletions may be affecting polyprotein processing. The extended linkers did not restore the glycosylation sites deleted from the original constructs and thus the mutant E2 glycoproteins again exhibited variably lower molecular weights (kDa) than the wild-type conferred by the absence of these glycans: HVR1conlink < HVR2link and "igVR link" < WT. However, deglycosylation of these E2 glycoproteins would be required to verify their backbone molecular weights as predicted in Table 2.

The anti-E1 monoclonal antibody (A4) detected the presence of the E1 doublet (~30 kDa and ~27 kDa) for all extended linker constructs (Figure 6B). E1 failed to co-precipitate wild-type E2 glycoprotein under non-reducing conditions, whereas it co-precipitated both mutant and wild-type E2 under reducing conditions again reflecting the low sensitivity of the A4 antibody for non-covalently associated E1. Precipitation with the non-conformation dependent anti-E2 monoclonal antibody (A11) detected wild-type E2 glycoprotein expression for each extended linker construct although only the HVR1conlink deletion co-precipitated detectable amounts of E1 (Figure 6C). The conformation-dependent monoclonal antibody (H53) also precipitated wild-type levels of the intracellular E2 glycoprotein for each extended linker construct indicating that these mutants retain the

intrinsic folding properties of the native E2 glycoprotein required to present this conformation-dependent epitope (Figure 6D). Furthermore, the extended "igVR" linker recovered co-precipitation with E1 at wild-type levels suggesting that this additional linker region is required, directly or indirectly, for the intracellular assembly of the non-covalently associated E1E2 heterodimer. Notably, however, this effect was not observed for the extended HVR2 linker construct.

E1E2 glycoprotein maturation and incorporation into pseudotyped HIV-1 particles

To examine whether the extended linkers altered E1E2 glycoprotein maturation and virion incorporation, the modified single deletions were introduced into E1E2-pseudotyped HIV-1 particles prior to lysis and immunoprecipitation with the conformation-dependent anti-E2 monoclonal antibody (H53) and IgG from an HIV-1 infected individual (IgG14) as previously described.

Immunoprecipitation with IgG14 indicated that none of the modified single deletion constructs affected processing and virion incorporation of the HIV-1 structural proteins (Figure 7B). Precipitation with H53 again detected a diffuse molecular weight band (~70-90 kDa) typical of the mature virion-incorporated E2 glycoprotein (Figure 7A). The virion-incorporated mutant E2 glycoproteins also exhibited marginally lower molecular weights (kDa) than the wild-type species conferred by the loss of one or two glycosylation sites as described for the intracellular data. Precipitation with H53 again demonstrated reduced virion-incorporation of the E2 glycoprotein containing the HVR1conlink deletion despite the reintroduction of four conserved cysteine-proximal residues absent in the original HVR1con deletion construct. The HVR2link deletion demonstrated wild-type levels of E2 glycoprotein incorporated into pseudotyped HIV-1 particles, yet still failed to co-precipitate a detectable amount of E1 reflective of the intracellular data. In contrast, the virion-incorporated E2 glycoprotein containing the extended "igVR" linker co-precipitated wild-type levels of E1 suggesting that this region is directly or indirectly required in the formation of the functional E1E2 heterodimer.

E1E2-mediated entry into Huh7 cells

To examine whether these extended linkers conferred E1E2-mediated entry into Huh-7 cells, these modified single deletion constructs were used to generate E1E2-pseudotyped HIV-1 particles to infect Huh7 cells as previously described. As expected, the extended linkers in the HVR1conlink and HVR2link deletion constructs did not recover any entry activity consistent with the observed failure of these mutant E2 glycoproteins to be incorporated into virions or heterodimerise, respectively (Figure 8). However, the virion-incorporated E2 glycoprotein containing the extended "igVR" linker demonstrated a significant return of entry activity (~ 8-fold) compared to the negative control (empty) consistent with its recovered heterodimerisation with E1. Notably, however, "igVR link" still exhibited reduced entry activity compared to wild-type (~10-fold).

CD81-LEL binding

To examine whether the intracellular or virion-incorporated E2 glycoproteins containing these extended linkers altered CD81-LEL binding, both cell and viral lysates were applied to a solid phase CD81 MBP-LEL (residues 113-201) binding assay as previously described. This data was normalized for the amount of monomeric E2 expressed from each vector as precipitated with H53 and observed by SDS-PAGE under non-reducing conditions; Figures 6D (intracellular) and 7A (virion incorporated). The extended linkers did not appear to alter the CD81-LEL binding ability of the intracellular E2 glycoproteins containing the HVR2link or "igVR link" deletions as they still demonstrated wild-type levels, or even slightly enhanced, binding as exhibited by the original HVR2 and "igVR" deletion constructs (Figure 9A). Notably, the intracellular E2 glycoprotein containing the HVR1conlink deletion demonstrated a marginal recovery in CD81-LEL binding compared to the negative control (empty) suggesting that the conserved cysteine-proximal residues reintroduced into this construct may contribute directly or indirectly to the formation of the CD81-binding site. The virion-incorporated E2 glycoprotein containing HVR1conlink deletion, however, lost even this reduced CD81-LEL binding ability (Figure 9B) consistent with its retarded incorporation into HIV-1 virions. The virion-incorporated E2 glycoproteins containing the HVR2link and "igVR link" deletion constructs maintained CD81-LEL binding in viral lysates, but generated inconsistent results in regard to their

relative CD81-LEL binding ability across two independent assays (Figures 9B and 9C) and thus a third independent assay is required to verify these results.

3. Effect of multiple variable region deletions on E1E2 glycoprotein structure and function

Intracellular E1E2 precursor folding and heterodimerisation

Despite a loss of heterodimerisation with E1 and/or viral entry, the above findings indicate that HVR1, HVR2 and "igVR" can be individually deleted from both the intracellular and virion-incorporated E2 glycoprotein without disrupting its intrinsic folding properties as recognised by the conformation-dependent monoclonal antibody H53 and binding to the large extracellular loop (LEL) of the CD81 receptor. In order to further characterise these variable regions and delineate a conserved E2 core domain, multiple deletions were introduced into the E1E2 polyprotein and analysed within 293T cells by pulse-chase metabolic-labelling and immunoprecipitation.. Precipitation with the anti-E1E2 polyclonal antibody (779) showed efficient expression and cleavage of E1 (~30 kDa) and E2 (~70 kDa) glycoproteins from the polyprotein occurring for each of the multiple variable region deletion constructs (Figure 10 A). These mutant E2 glycoproteins also migrated faster than the wild-type species corresponding to the loss of a glycosylation site within each HVR2 and/or "igVR" deletion: HVR1+2+igVR and HVR2+igVR < HVR1+2 and HVR1+igVR < WT. However, deglycosylation of these glycoproteins is required to confirm their backbone molecular weights as predicted in Table 3. The anti-E1 monoclonal antibody (A4) again detected an E1 doublet (~30 kDa and ~27 kDa) present for each of the variable region deletion constructs (Figure 10B). E1 co-precipitated with both wild-type and mutant E2 glycoproteins under reducing conditions demonstrating the formation of covalently-linked E1E2 heterodimers. The failure to detect the wild-type E1E2 heterodimer under non-reducing conditions again reflects the low sensitivity of A4 for the non-covalently associated E1 glycoprotein.

In contrast, the non-conformation dependent anti-E2 monoclonal antibody (A11) did not detect the E2 glycoproteins containing these multiple variable deletions coprecipitating E1 under reducing or non-reducing conditions despite demonstrating wild-type levels of

intracellular E2 glycoprotein expression for each of these deletion constructs (Figure 10C). The conformation-dependent anti-E2 monoclonal antibody H53 also precipitated wild-type levels of the mutant E2 glycoproteins (Figure 10D). This indicates that the inserted Gly-Ser-Ser-Gly linker motifs provide sufficient flexibility within these mutant E2 glycoproteins to present this conformation-dependent epitope despite the introduction of multiple variable region deletions (Figure 10D). These mutant E2 glycoproteins also demonstrated a loss of heterodimerisation with E1 consistent with the absence of HVR2 and/or "igVR" required in the assembly of the E1E2 heterodimer as observed above.

E1E2 glycoprotein maturation and incorporation into pseudotyped HIV-1 particles

To examine whether the multiple variable region deletions altered E1E2 glycoprotein maturation, these deletions were introduced into E1E2-pseudotyped HIV-1 particles and lysed prior to immunoprecipitation with the conformation-dependent anti-E2 monoclonal antibody H53 and IgG from an HIV-1 infected individual (IgG14) as previously described.

IgG14 precipitation indicated that none of the multiple variable deletions had affected processing and virion incorporation of the HIV-1 structural proteins (Figure 11 B). Immunoprecipitation with H53 again detected E2 as a diffuse band (~70-90 kDa) typical of the mature virion-incorporated E2 glycoprotein (Figure 11A) and demonstrated the E2 glycoproteins containing multiple variable region deletions to be incorporated into HIV-1 virions at wild-type levels. These mutant E2 glycoproteins also migrated marginally faster than the wild-type species reflecting the loss of one or two glycosylation sites within the HVR2 and/or "igVR" deletions as described for the intracellular data. This suggests that the virion-incorporated E2 glycoproteins containing multiple variable region deletions retain the conformation-dependent H53 epitope even after maturation. Again, none of these mutant E2 glycoproteins co-precipitated detectable amounts of E1 as observed in the intracellular data.

E1E2-mediated entry into Huh7 cells

As expected, E1E2-pseudotyped HIV-1 particles containing multiple variable region deletions were not competent for entry into Huh7 cells (Figure 12).

CD81-LEL binding

Despite a loss of heterodimerisation and E1E2-mediated viral entry, both the intracellular and virion-incorporated E2 glycoproteins containing multiple variable region deletions were examined for their ability to bind CD81-LEL by applying both cell and viral lysates to a solid-phase CD81 MBP-LEL (residues 113-201) binding assay as previously described. This data was normalized for the amount of monomeric E2 expressed from each vector as precipitated with H53 and observed by SDS-PAGE under non-reducing conditions; Figures 10D (intracellular) and 11A (virion-derived).

The intracellular E2 glycoproteins containing multiple variable region deletions all demonstrated wild-type levels of CD81-LEL binding (Figure 13A). This indicates that the Gly-Ser-Ser-Gly linker motifs provide sufficient flexibility within these mutant E2 glycoproteins to form the precursor CD81 receptor-binding site. In addition, the E2 glycoprotein containing the HVR2 and "igVR" double deletion (pE1E2 Δ23) demonstrated a further enhancement of maximum CD81-binding across two independent experiments. This is consistent with this same effect observed in the individual HVR2 and "igVR" mutants although, similarly, the overall binding curve was not increased and thus additional independent assays are required to determine the statistical significance of this effect.

The virion-incorporated E2 glycoproteins containing multiple variable region deletions all maintained CD81-LEL binding (Figure 13B) again indicating that the inserted linker motifs provide sufficient flexibility within these glycoproteins to present the CD81-binding site even after maturation. This again suggests that HVR1, HVR2 and "igVR" are not required for this function reflecting both the intracellular data and that obtained for the individual variable region deletions. Importantly, this demonstrates that the E2 glycoprotein containing the triple variable deletion encompasses all the structural and functional determinants required in CD81-LEL binding and thus constitutes a minimal E2 core domain. These virion-derived E2 glycoproteins containing double and triple variable region deletions also demonstrated slightly reduced or enhanced maximum CD81-LEL binding activity compared to wild-type, respectively, although again none of these mutants exhibited a significant increase in the overall binding curve.

4. Role of the variable regions in E2 receptor-binding domain (E2 RBD_{661myc}) structure and function

Folding and secretion

The above results demonstrate that the simultaneous deletion of all three variable regions does not disrupt the intrinsic folding of the E2 glycoprotein as recognised by the conformation-dependent monoclonal antibody H53 or binding to the CD81-LEL strongly suggesting that this construct constitutes an E2 core domain. However, in the context of the full-length E2 glycoprotein, the presence of both the transmembrane and membrane-proximal regions would make it difficult to crystallize this core E2 structure. Therefore, both single and multiple variable region deletions were introduced into E2 receptor-binding domain (E2 RBD_{661myc}) that is both soluble and amenable to high level expression. The E2 RBD_{661myc} glycoproteins containing single and multiple variable region deletions were metabolically labelled within 293T cells and both the intracellular and secreted protein analysed by immunoprecipitation using the conformation-dependent anti-E2 monoclonal antibody (H53).

H53 precipitated wild-type levels of intracellular E2 RBD_{661myc} (~ 50 kDa) for all single and multiple deletion constructs indicating that each of these mutant glycoproteins is efficiently expressed (Figure 14A). These intracellular E2 RBD_{661myc} glycoproteins also exhibited variably lower molecular weights than wild-type consistent with the loss of glycosylation sites located within the deleted regions as observed in the context of the full-length E1E2 heterodimer. However, deglycosylation of these truncated glycoproteins is required to confirm their molecular weights as predicted in Table 4. Furthermore, H53 precipitated a diffuse molecular weight band (~55-65 kDa) corresponding to the secreted form of the E2 RBD_{661myc} (Figure 14B) reflecting the various complex- and hybrid-type carbohydrate modifications acquired during transit through the secretory pathway. All E2 RBD_{661myc} glycoproteins containing single and multiple variable region deletions were shown to be efficiently secreted and again displayed variable molecular weights (kDa) as described for the intracellular data. Together these results confirm that the variable regions can be deleted – individually and in combination – from the E2 RBD_{661myc} and retain the

intrinsic folding of both the intracellular and secreted receptor-binding domain as detected by the conformation-dependent anti-E2 monoclonal antibody H53.

CD81-LEL binding

To determine whether the E2 receptor-binding domain containing single and multiple variable region deletions bind to CD81-LEL, both intracellular and secreted E2 RBD_{661myc} lysates were applied to a solid-phase CD81 MBP-LEL (residues 113-201) binding assay as previously described. This data was normalized for the amount of monomeric E2 expressed from each vector as precipitated with H53 and observed by SDS-PAGE; Figures 14 A (intracellular) and 14B (secreted).

Both intracellular and secreted forms of the E2 RBD_{661myc} containing the single variable region deletions demonstrated CD81-LEL binding although, as observed in the context of the full-length E2 glycoprotein, the HVR1con deletion conferred a total loss of CD81-LEL binding (Figure 15A and 15B). The secreted form of the E2 glycoprotein containing the single HVR1 deletion also exhibited an additional enhancement of CD81-LEL binding compared to wild-type consistent with the data obtained for the mature virion-derived forms of the full-length E2 glycoprotein. Importantly, both intracellular and secreted forms of the E2 RBD_{661myc} containing the triple variable region deletion (HVR1+2+igVR) retained CD81-LEL binding at wild-type levels (Figure 16A and 16B) confirming that this glycoprotein species encompasses all the structural and functional CD81-binding determinants to constitute a minimal or near-minimal E2 core receptor-binding domain.

5. Binding of E2 RBD_{661} bearing simultaneous HVR deletions to cell surface expressed full-length CD81.

The abilities of secreted E2 RBD_{661} proteins with multiple HVR deletions to bind cell surface expressed full length CD81 were determined. CHO-K1 cells were transfected with a vector encoding human CD81. Forty-eight hours after transfection, cells were placed on ice, and equivalent amounts of E2 added to cells. Four hours later, bound E2 was detected with radioiodinated MAb 9E10 and the amount of bound E2 quantitated. The results (Figure 17) show that wild type and ΔHVR2+igVR-deleted E2 RBD_{661} bound to full

length CD81 at similar levels. By contrast, the binding of ΔHVR1+2-E2 RBD_{661} to CD81 was significantly decreased (p=0.038); further deletion of igVR in construct E2 Δ123-myc restored wild type CD81 binding activity (p=0.11). These data indicate that the 3 variable regions can be deleted simultaneously without disruption to the CD81 binding ability of E2. Interestingly, simultaneous deletion of HVR1 and HVR2 from E2 RBD_{661} led to decreased CD81 binding however this defect was rectified by further deletion of igVR.

6. Ability of E2 RBD_{661} bearing simultaneous HVR deletions to be recognised by human conformation sensitive monoclonal antibodies.

Wildtype E2- myc and E2 Δ123-myc was radiolabelled with ^{35}S-Met/Cys and immunoprecipitated with a panel of human conformation sensitive monoclonal antibodies (Keck et al., 2004) and analysed by non-reducing SDS-PAGE. This panel of conformation-dependent MAbs are specific to conformational epitopes representing three distinct immunogenic domains of E2 - A, B and C (Keck et al., 2004). The E2 Δ123-myc protein demonstrated a similar immunogenic profile to the wild-type E2-myc protein (Fig. 18) indicating that the E2-myc, lacking all three variable regions, retains the conformational epitopes specific to these three distinct structural and functional domains of E2. This data is consistent with previously published data by Keck et al. that also demonstrated a lack of HVR1 involvement in these domains (Keck et al., 2004). This data extends these observations to suggest that HVR2 and igVR also do not participate in these domains.

7. Discussion

In the course of this study, the two recognized E2 variable regions, HVR1 and HVR2, and the novel variable region "igVR" have been individually deleted to further investigate the role of these regions in E1E2 biosynthesis, heterodimerisation, CD81-binding and viral entry. All E2 glycoproteins containing these variable region deletions were shown to retain the intrinsic folding properties of the wild-type glycoprotein as recognized by the conformation-dependent monoclonal antibody H53. HVR2 and "igVR" were further shown to be required for heterodimerisation and all three variable regions were required in pre- or post-CD81-binding stages in viral entry. It has also been demonstrated that none of the individual variable regions are required for CD81-binding and that all three can be

simultaneously deleted from the E2 glycoprotein with the retention of CD81-binding properties.

The envelope glycoproteins E1 and E2 exhibit the greatest genetic heterogeneity in the HCV genome, especially in hypervariable region 1 (HVR1) – a highly variable ~27 amino-acid sequence located at the N-terminus of the E2 glycoprotein. Here, it has been demonstrated that deletion of HVR1 (polyprotein residues 387-408) results in a loss of E1E2-mediated entry despite retaining both wild-type levels of heterodimerisation and incorporation of E1E2 into pseudotyped HIV-1 particles suggesting that this region has a direct role in viral entry. However, it has also been shown that HVR1 is not required for CD81-LEL binding in the context of either the functional E1E2 heterodimer or the E2 receptor-binding domain (E2 RBD_{661myc}) indicating that HVR1 is involved in another pre- or post-CD81-binding stage in viral entry. This is consistent with a previous study that has reported the deletion of HVR1 (polyprotein residues 384-410) to ablate E2 binding to the SR-B1 receptor suggesting that this region may be required for essential SR-B1 contacts in viral entry.

However, the present findings contradict several other studies that have demonstrated HCV pseudotyped particles (HCVpp) lacking HVR1 to mediate entry into Huh7 cells, although at reduced levels, inconsistent with an essential role for this region in viral entry. It has also been reported that the enhancement of HCVpp infectivity observed in the presence of the high density lipoprotein (HDL), a natural ligand of SR-B1, is lost upon the deletion of HVR1. This suggests that HVR1 facilitates infectivity rather than being essential for this function, although whether this effect is due to the association of HCV particles with HDL remains undetermined. In addition, a recent study has found that the substitution of all the conserved basic residues within HVR1 significantly reduces HCVpp entry although these residues were not found to be involved in either CD81-LEL binding or to correlate with an HDL enhancement of SR-B1 binding. Therefore the exact role of HVR1 in viral entry remains unclear.

It has also been shown that the deletion of HVR1 significantly enhances CD81-binding (approximately 4-fold) in agreement with previous studies. This suggests that HVR1

negatively modulates the accessibility of the conserved CD81-binding sites within the E2 glycoprotein despite not being required in this function. This is consistent with the recent identification of a conserved CD81-binding determinant G^{436}WLAGLFY located between HVR1 and HVR2. In addition, the broadly neutralizing antibody AP33 has been demonstrated to recognise a conserved epitope directly adjacent to HVR1 (polyprotein residues 412-423) and to inhibit interactions between CD81 and a range of presentations of the E2 glycoprotein. The extension of the HVR1 deletion to include the conserved I^{411} residue has also been shown to confer a reduction, rather than an enhancement, in CD81-binding further implicating this region in this function. Similarly, it has been demonstrated that the extension of the HVR1 deletion to include this adjacent conserved region (HVR1con, polyprotein residues 387-428) disrupts E2 glycoprotein folding and maturation critical in the formation of the CD81-binding site. Furthermore it has been demonstrated that residue W^{420} located in this conserved region is also involved in CD81 binding

The location of these highly conserved regions adjacent to HVR1, suggests that this highly variable region performs the dual function of both mediating viral entry and modulating the accessibility of these conserved regions to the host immune system to escape recognition by broadly neutralizing antibodies such as AP33. Indeed, a previous study has found that deletion of HVR1 results in an increased sensitivity of the envelope glycoprotein complex to neutralizing antibodies and sera. Therefore, it has been proposed that HVR1 forms a solvent-exposed subdomain external to a conserved core domain of the E2 glycoprotein consistent with both its modulating role in receptor-binding and its elicitation of an immunodominant response. In order to further characterise this proposed conserved core domain, the remaining E2 variable regions, HVR2 and "igVR"" have also been deleted. As observed for the HVR1 deletion, both E2 glycoproteins lacking the individual HVR2 and "igVR" regions retained the intrinsic folding properties of the wild-type glycoprotein as detected by the conformation-dependent monoclonal antibody H53.

Interestingly, however, both HVR2 and "igVR" were shown to be required for heterodimerisation and, accordingly, the deletion of these regions demonstrated a total loss of E1E2-mediated entry in to Huh7 cells. This suggests that these regions within the E2 glycoprotein are involved in direct heterodimer contacts or, alternatively, modulate

allosteric effects that are indirectly required for this function. Therefore to further investigate the role of these regions, several conserved cysteine-proximal residues were reintroduced into the HVR2 and "igVR" deletion constructs affecting to extend their linker motifs and thus enhance E2 glycoprotein folding. The extension of the "igVR" linker was found to recover wild-type levels of heterodimerisation with E1 and a significant level of E1E2-mediated entry (~8-fold) although this effect was not observed for HVR2. This finding may reflect the relatively short length of the "igVR" deletion where two of the five glycine residues lost from this region are further compensated for by the introduction of the Gly-Ser-Ser-Gly linker motif resulting in only a five amino-acid total deletion within this region. The role of the "igVR" linker in heterodimerisation is also consistent with its observed conservation within genotypes. Notably, the extended "igVR" linker construct still exhibited a reduction in entry activity compared to wild-type (~10-fold) and is perhaps due to absence of a conserved N-linked glycosylation site within this region that has been previously observed to reduce HCVpp entry.

Furthermore, it has been demonstrated that the E2 glycoprotein containing either the HVR2 or "igVR" deletions retains wild-type levels of CD81-binding indicating that these regions, as observed in HVR1, are not required in this function. Previous studies have shown that monoclonal antibodies targeting HVR2 inhibit E2 glycoprotein binding to CD81 and proposed that this region forms a CD81-binding determinant although, based on the present findings, it is more likely that these antibodies are creating a steric effect that occludes the G^{436}WLAGLFY CD81-binding determinant adjacent to this region. Therefore, despite a loss of heterodimerisation and/or viral entry, HVR1, HVR2 and "igVR" can all be individually deleted from the E2 glycoprotein without disrupting its intrinsic folding properties and, indeed, it has been further shown that all three variable regions can be simultaneous deleted with a retention of CD81-binding. This strongly supports the hypothesis that these variable regions form solvent-exposed subdomains external to an E2 core domain encompassing the conserved structural and functional determinants required for binding to the confirmed HCV receptor, CD81.

Interestingly, it has also been observed that the E2 glycoproteins containing the HVR1 deletion in combination with either HVR2 or "igVR" deletions did not exhibit the dramatic

enhancement in CD81-LEL binding observed for the individual HVR1 deletion. A previous study has demonstrated that the substitution of HVR1 and HVR2 with corresponding sequences from a different subtype in combination achieves either increased or decreased CD81 binding (depending on the introduced and backbone strains) that is not observed for either of the individual HVR1 or HVR2 substitutions. Together this data suggests that intramolecular interactions are occurring between these regions to modulate CD81-binding and that they are likely to adopt intrinsically flexible structures to entertain these contacts. This is further supported by both the recent identification of a conserved CD81-binding determinant $G^{436}WLAGLFY^{443}$ located directly between HVR1 and HVR2 as well as the immunodominant responses elicited by both HVR1, and to a lesser extent, HVR2 consistent with their position as surface-exposed subdomains that may perform modulating functions in cell attachment. This same effect observed for the HVR1 and "igVR" double deletion suggests that the "igVR" region may also contribute to these cooperative interactions.

One hypothesis to explain this data, proposes that the variable regions form flexible solvent-exposed subdomains that enable the E2 glycoprotein to move between 'open' and 'closed' conformations, the former being more competent for CD81 binding by exposing the conserved CD81-binding determinants located within the core E2 domain as delineated within this study. Indeed, conformational changes within the E2 glycoprotein have been previously observed upon CD81-binding. In addition, binding of non-neutralizing antibodies to the E2 glycoprotein has been demonstrated to reduce its susceptibility to neutralizing antibodies and is consistent with a model where the non-neutralizing antibodies inhibit the flexibility of these surface-exposed variable regions thus blocking access to the conserved epitopes located within the E2 core domain. Therefore, this suggests that the modified E2 core domain represents a promising vaccine candidate for eliciting neutralizing antibodies to conserved epitopes within the E2 glycoprotein, including the CD81-binding determinants, that are otherwise occluded by these surface-exposed variable regions that may act as immunological decoys at the surface of the glycoprotein complex during HCV replication.

The observed loss of heterodimerisation in the E2 glycoproteins containing HVR2 and "igVR" deletions suggests that conformational changes are occurring within these glycoproteins that cannot be recognised by the single conformation-dependent monoclonal antibody H53. Therefore, a panel of conformation-dependent antibodies that have been previously used to identify the three major immunogenic regions within the E2 glycoprotein were obtained to examine more subtle alterations in glycoprotein structure and function mediated by these variable regions. The results show that in the context of E2 RBD $_{661myc}$ monoclonal antibodies to domain A, B and C retain wild-type levels of reactivities to the RBD containing simultaneous deletion of all three variable regions. This further suggests that the E2 Δ123-mycΔ123RBD construct retains the intrinsic core domain folding properties of the native E2 RBD.

8. Materials and Methods

Construction of the variable region deletion mutants

Vectors

In order to introduce the E2 variable region deletions into the E1E2 heterodimer, the HCV-based expression vector pE1E2H77c was obtained (Drummer et al., 2003). The pE1E2H77c vector contains a DNA sequence from the full length pCV-H77c (genotype 1a) infectious clone (Yanagi et al., 1997) encoding the E1E2 polyprotein residues 165-746 as described (Drummer et al., 2003). To ensure efficient ER targeting and glycoprotein cleavage, this sequence includes the E1 signal peptide located at the C-terminus of the immature core protein and both the full-length E1 and E2 polyprotein sequences (Figure 19). This DNA fragment was cloned into the backbone of the pCDNA4HisMax vector (Invitrogen, Carlsbad, CA, USA) that contains both a translational enhancer sequence and a cytomegalovirus (CMV) promoter for expresssion in mammalian systems. It also contains an ampicillin resistance gene for selection in bacterial cells.

The CD81 open reading frame was amplified by PCR from pcDM8-TAPA-1 (Levy et al., 1998)30) using the primers, 5'-CCG**AAGCTT**CCACCATGGGAGTGGAGGGCTGC-3' and 5'-GGC**TCTAGA**TTAGTACACGGAGCTGTTCCG-3'. The PCR product was

cloned into pcDNA3 using *Hind*III and *Xba*I (shown in bold type) to generate the plasmid pcDNA3-CD81.

To enable characterisation of the variable regions within the soluble E2 receptor-binding domain (E2 RBD_{661}), the HCV-based expression vector pE2661 was also obtained (Drummer et al., 2002). The pE2661 vector contains a pCV-H77c DNA sequence encoding HCV polyprotein residues 384-661 as described (Drummer et al., 2006)2002). This sequence encodes an independently folding subdomain of the E2 glycoprotein that has been shown to retain both CD81 and SR-B1 receptor-binding (Pileri et al., 1998, Scarselli et al., 2002, Pileri et al., 1998). This DNA product was cloned into a pCDNA3 vector (Invitrogen) backbone at the C-terminus of a tissue plasminogen-activator (tpa) leader sequence designed to ensure efficient ER targeting and signal cleavage of the E2 RBD_{661} in the absence of E1 (E2-myc). This vector also contains a CMV promoter for expression in mammalian cells and an ampicillin resistance gene to facilitate selection in bacterial cells.

Overlap Extension PCR

Overlap extension PCR is a two step strategy that facilitates the deletion of large segments of DNA (Figure 20) (Horton et al., 1989). In round one, the pE1E2H77c vector was used as a template to introduce individual variable region deletions into the full-length E1E2 polyprotein. This step required two oligonucleotide pairs (Geneworks, Ann Arbor, MI, USA); each pair containing one external and one internal primer responsible for amplifying the sequence either upstream (5') or downstream (3') of the variable region. The internal oligonucleotide primers were designed to introduce the Gly-Ser-Ser-Gly linker as well as a short overlap sequence complementary to the corresponding 5' or 3' fragment (Table 5). Once generated, these first round 5' and 3' PCR products were isolated by agarose-gel electrophoresis and purified prior to being added to the second round PCR reaction. Round two allowed annealing of the 5' and 3' fragments via their overlapping, complementary sequences to form a template for extension and amplification by the relevant external primers. The external primers also contained unique EcoR1/XbaI restriction endonuclease sites to facilitate cloning back into the template vector.

E1E2 variable region deletion constructs

The overlap extension PCR strategy was used to generate the single variable region deletions as summarized in Table 5 and represented in Figure 20. Notably, two alternative HVR1 deletion constructs, HVR1 and HVR1con, were designed to further investigate this region. The HVR1 deletion lacks the highly variable segment between polyprotein residues 387 and 408, whereas HVR1con extends this deletion to encompass the adjacent region up to the first conserved cysteine residue in E2 (polyprotein residues 387-428) proposed to anchor this N-terminal region to the rest of the glycoprotein. The first three amino-acids ($E^{384}TH$) of HVR1 were also retained in these constructs to ensure efficient cleavage between E1 and E2 during glycoprotein biosynthesis. HVR2 and "igVR" deletions encompassed polyprotein residues 460-485 and 570-580, respectively.

To enhance E2 glycoprotein folding, a second series of modified single variable region deletion constructs were generated using overlap extension PCR (Figure 21). This strategy utilized a set of modified internal oligonucleotide primers to extend the Gly-Ser-Ser-Gly linker motif by reintroducing several conserved cysteine-proximal residues deleted from the original constructs as summarized in Table 2 and represented in Figure 21. Notably, this modified ΔHVRlink E1E2 series lacks a HVR1 deletion construct due to the lack of any conserved residues located within this region.

In order to delineate a minimal E2 core domain, multiple HVR1, HVR2 and "igVR" deletions were introduced into the context of the E1E2 polyprotein as represented in Figure 19. These multiple deletion constructs were again generated using overlap extension PCR, but utilized the relevant single or double variable region deletion constructs a template instead of the wild-type pE1E2H77c vector as outlined in Table 3.

E2 RBD_{661myc} variable region deletion constructs (E2-myc)

In order to characterise the single and multiple variable region deletions in the context of the soluble E2 receptor-binding domain (E2 RBD_{661myc}), standard PCR was used to amplify polyprotein residues 384 to 661 from the full-length E1E2 constructs containing the single and multiple variable region deletions as outlined in Table 4 and represented in

Figure 22. The oligonucleotide primers were designed to introduce a C-terminal myc epitope tag and unique NheI/XbaI restriction endonuclease sites to facilitate the cloning of these PCR products into the pE2661vector.

All PCR reactions were carried out in the Biometra Thermocycler (Biometra, Goettingen, Germany) using the Expand High Fidelity PCR system (Invitrogen) under the conditions summarized in Table 6.

Agarose-gel Electrophoresis

DNA products were isolated by agarose-gel electrophoresis using Bio-Rad gel tanks (Bio-Rad Laboratories, Hercules, CA, USA) containing TAE (0.001M EDTA and 0.04M Tris-Acetate). 1% (w/v) DNA-grade agarose in TAE was made up with ethidium bromide (0.5µg/mL) to enable DNA visualization under UV light (Sambrook and Russel, 2001). DNA samples were made up at 20% (v/v) with Orange G gel-loading dye (1% (w/v) Orange G (Sigma, St Louis, MO, USA), 50% (v/v) glycerol). Agarose gels were run at 100V, 23mA for 30-40min and DNA fragment size was confirmed and quantified using the GelDOC system (Bio-Rad) in reference to a 1kb Plus DNA Marker (Invitrogen). Confirmed DNA products were subsequently purified from PCR reactions using the Mo Bio PCR Clean-up kit (Mo Bio, Carlsbad, CA, USA) or from agarose gels with the Mo Bio Gel-Spin kit (Mo Bio) according to the manufacturer's instructions.

Restriction endonuclease digestion of DNA

All DNA was digested with New England Biolabs restriction endonucleases according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Any digested vectors were de-phosphorylated with Shrimp Alkaline-phosphatase (Invitrogen) for 30min at 37°C to reduce self-annealing.

Ligation and transformation of plasmid constructs

Digested insert and vector DNA were ligated together at approximately a 4:1 ratio using the T4 DNA-ligase system (Invitrogen) and incubated at 4°C for 16hrs. DNA ligation products were isolated using the sodium-acetate precipitation method (3M Na-Acetate pH 5 and 100% Ethanol) (Sambrook and Russel, 2001) and resuspended for 1hr in sterile

distilled water. Ligation products were transformed into 20uL of electroporation-competent DH10B E.coli cells (New England Biolabs) using 1mm electroporation cuvettes (BTX, Holliston, MA, USA) and a Gene Pulser Electroporator (BioRad) set at 2.0V, 200Ω and 25μF. The electro-treated DH10B cells were then transferred into Luria Bertani Medium (LB) (1% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 0.5% (w/v) NaCl) to recover for 1hr at 37°C. The cells were then spread-plated out onto LB-Agar plates (LB, 5% (w/v) Agar) containing 50ug/mL ampicillin and incubated at 37°C for 16hrs to select for transformed cells (Sambrook and Russel, 2001).

Colony PCR

In order to rapidly screen for transformed colonies containing the desired DNA insert, approximately 20 colonies were selected from each LB-Agar plate for colony PCR (Sambrook and Russel, 2001). Colony PCR was performed using the Taq polymerase system (Invitrogen) and the appropriate external primers in the Biometra Thermocycler (Biometra) under the conditions specified in Table 6. The colonies containing positive inserts were identified by agarose-gel electrophoresis as described above.

Small-scale preparation of plasmid DNA

Small-scale preparations of plasmid DNA from positive colonies were then generated using the Mo Bio Mini-prep kit (Mo Bio) using the alkaline lysis method in accordance with the manufacturer's specifications. Inserts were confirmed by restriction endonuclease digest and agarose-gel electrophoresis as described above.

DNA Sequencing

The sequences of all plasmid DNA inserts were confirmed using the relevant sequencing primers and the PRISM BigDye Terminator Mix (version 3.1) (Applied Biosystems, Foster City, CA, USA) according to the Micromon Reaction Set-Up Protocol (Micromon, Victoria, Australia). All sequencing reactions were run in a Biometra Thermocycler (Biometra) under the conditions outlined in Table 6 and the resulting DNA prepared according to the Micromon Reaction Clean-Up Protocol (Micromon). Sequence analysis was performed in a 3730S Genetic Analyser (Applied Biosystems) at the Micromon sequencing facility.

Large -scale preparation and quantification of plasmid DNA

Plasmid DNA from confirmed clones was then isolated on a larger scale using the Qiagen Midi-prep Kit (Qiagen, Hilden, Germany) based on the alkaline lysis method in accordance with the manufacturer's specifications. These large scale plasmid DNA preparations were purified using phenol-chloroform (1:1) and centrifuged at 10 000 xg for 3 min to obtain an upper phase containing the DNA and repeated with chloroform (50% (v/v)). Purified DNA was concentrated using sodium-acetate (10% (v/v) and 2.5 volumes of 100% ethanol at 70°C for 30 min. DNA was pelleted at 10 000 xg for 15 mins prior to washing twice in 70% precipitation method and resuspended in 100uL of TE. All DNA concentrations were determined using a Biophotometer at 260nm (Eppendorf, Hamburg, Germany).

During this study, all bacterial work utilised an orbital-shaker (Ratek Instruments, Victoria, Australia) or an incubator (Memmet, Schwabach, Germany) set at 37°C.

Biochemical and Functional Assays

Cell Culture

293T human embryo kidney cells (HEK 293T) from a human fibroblast cell line was used in this study to ensure high transfection efficiency and to achieve good levels of cellular protein expression. The human liver hepatocyte is the primary target cell for HCV and thus the human hepatocellular carcinoma cell line, Huh7, was used as a model liver cell system in this study. Futhermore, the Huh7 cell line has been demonstrated to support subgenomic HCV replicons, is highly permissive to HCVpp entry and can support cell culture grown HCV (HCVcc) suggesting that it contains all the cellular factors required for HCV tropism in vivo (ZhongBartosch et al., 2005, Wakita et al., 20052003a, Lindenbach et al., 2005b, Lohmann et al., 1999, LindenbachWakita et al., 2005a, Zhong et al., 2005, Bartosch et al., 2003a).

All cells were maintained in DMF10: Dulbecco's minimal essential medium (Invitrogen), 10% (v/v) heat-inactivated foetal bovine serum (Invitrogen), 2mM L-glutamine (GE Healthcare, Bukinghamshire, UK), 1M HEPES buffer solution (Invitrogen), Gentamycin

(GE Healthcare) and 2ug/mL minocycline-hydrochloride salt (Sigma). The cells were subcultured every 3-4 days in 75cm^2 or 150cm^2 Falcon flasks (Becton Dickenson, Franklin Lakes, NJ, USA) using 0.025% (v/v) Trypsin in PBS-EDTA (phosphate buffered saline (PBS) – ethylenediaminetetraacetic acid (EDTA)) to detach the monolayer. All cells were incubated in a Thermo Direct Heat CO_2 incubator at 37°C with 5% CO_2 (Thermo, Waltham, MA, USA). During this study, all vectors were transfected into HEK 293T cells seeded in 6-well culture dishes (Nalge Nunc, Rochester, NY, USA) using the FuGene6 transfection reagent (Roche) as previously described (Drummer et al., 2003).

Antibodies

The non-conformational anti-E1 monoclonal antibody A4 and non-conformational anti-E2 monoclonal antibody A11 were gifts from Drs. Jean Dubuisson and Harry Greenberg (Dubuisson et al., 1994). The anti-E2 conformation-dependent monoclonal antibody, H53, was also a gift from Dr. Jean Dubuisson (Deleersnyder et al., 1997). The anti-E1E2 polyclonal antibody, 779, purified from the plasma of an HCV genotype 1a-infected individual was obtained (Drummer and Poumbourios, unpublished). Immunoglobulin G from an HIV-1 infected individual (IgG14) and the anti-myc monoclonal antibody 9E10 were also obtained (Drummer et al., 2002, Drummer et al., 2003). A panel of conformation-dependent human MAbs, specific to conformational epitopes representing three distinct immunogenic domains of E2 A, B and C, were obtained from Dr. Steven Foung (Keck et al., 2004) .

E1E2 pseudotyped HIV-1 particle entry assay

The incorporation and display of functional HCV envelope glycoproteins by heterologous retro- or lentiviral core particles, called pseudotyping, provides a relatively rapid and simple method for characterising mutant E1E2 glycoproteins without introducing these mutations into the full-length HCV genome (Drummer et al., 2003, Bartosch et al., 2003b)2003a, Drummer et al., 2003). This strategy involves the co-transfection of both an E1E2 expression vector and a retroviral or lentiviral expression vector lacking its native envelope gene and containing a reporter construct (Figure 23). As the viral core proteins assemble within the cell they acquire an envelope by budding from the plasma membrane and incorporate the HCV envelope glycoproteins present at the cell surface in place of

their native envelope complex. These E1E2-pseudotyped particles (HCVpp) can undergo a single round of E1E2-mediated infection and replication that can be quantified by measuring the activity of the reporter gene within the infected cell. During this study, the human immunodeficiency virus (HIV-1) fire-fly luciferase vector, HIV-1 NL4-3.LUC.R$^-$E$^-$ (He and Landauet al., 1995) was used to generate E1E2-pseudotyped virus for infection of Huh-7 cells as previously described (Drummer et al., 2003).

293T cells were seeded at 350 000 cells/well in 6-well culture dishes (Nalge Nunc) and co-transfected with 1ug of NL4-3.LUC.R$^-$E$^-$ and 1ug of either pE1E2H77c (wild-type), pΔHVR E1E2 or the empty pCDNA4HisMax vector (negative control). After three days incubation, the tissue culture fluid containing E1E2-pseudotyped HIV-1 particles was collected and filtered using Minisart 0.45um sterile syringe filters (Sartorius, Goettingen Germany). The filtered product was then used to infect Huh-7 cells seeded at 30 000 cells/well in 48-well culture plates (Nalge Nunc) in triplicate. Following 4hr incubation at 37°C, the innoculum was removed and the cells cultured in DMF10 for a further 3 days before being lysed with cell culture lysis reagent (Promega, Madison, WI, USA). Cell lysates were clarified of cellular debris prior to being transferred into white 96-well plates (BMG Labtech, Offenburg, Germany) to be analysed for luciferase activity using the Steady-Glo luciferase reagent system (Promega) and a Fluostar (BMG Labtech) fitted with luminescence optics. The average luciferase activity (relative light units) was calculated from triplicate infections and the standard deviation calculated accordingly.

Radioimmunoprecipitation (RIP)

Radiolabelling

In order to analyse the role of the variable regions in intracellular E1E2 biosynthesis, 293T cells seeded at 500 000 cells/well in 6-well culture dishes (Nalge Nunc) were transfected with 2ug of either pE1E2 (wild-type), pE1E2 containing one or more variable region deletions or empty pCDNA4 vector (negative control). 24 hours post-transfection, cells were pulse-chase metabolically labelled for 30 min at 37°C with 150μCi Trans-^{35}S-label/well (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in L-cysteine and L-methionine deficient DMF10: DMEM (MP Biomedicals), 10% (v/v) heat-inactivated fetal

bovine serum (Invitrogen) and 2mM L-glutamine (GE Healthcare). The cells were then chased for 4hrs in DMF10 at 37°C prior to being washed in PBS and lysed in RIP lysis buffer (0.6 M KCl, 0.05 M Tris pH 7.4, 1mM EDTA, 0.02% sodium azide, 1 % Triton X-100). The cell lysates were clarified of any remaining cell debris by centrifugation for 10min at 4°C in a refrigerated bench-top centrifuge (Heraeus, Hanau, Germany).

To enrich for mature E1E2 heterodimers as incorporated into pseudotyped HIV-1 particles, 293T cells seeded at 350 000 cells/well in 6 well-culture dishes were transfected with 1ug of pNL4-3.LUC.R-E- plus 1ug of either pE1E2 (wild-type), pE1E2 containing one or more variable region deletions or empty pCDNA4 vector (negative control). 24hrs hours post-transfection, the cells were metabolically labelled with 75μCi Trans-^{35}S label/well (Santa Cruz Biotechnology) in L-cysteine and L-methionine deficient DMF10 for 16 hrs at 37°C. The tissue culture fluid was then collected and filtered through a Minisart 0.2um syringe filter (Sartorius) prior to being enriched for virions through a sucrose gradient (25% sucrose (v/v) in PBS) using the Beckman L-90 ultracentrifuge (SW41 rotor, Beckman Coulter, Fullerton, CA, USA) at 25 000 xg for 2hrs at 4°C. The supernatant was removed prior to lysis of the virions in RIP lysis buffer.

To characterise the role of the variable regions in the context of the E2 receptor-binding domain (E2 RBD_{661myc}), metabolically labelled 293T cells seeded at 350 000 cells/well in 6-well culture dishes (Nalge Nunc) were transfected with 2ug of either the E2-myc (wild-type), E2-myc containing one or more variable region deletions por empty pCDNA3 vector (negative control). 6hrs post-transfection, cells were pulse-chase metabolically labelled with 75μCi Trans-^{35}S-label/well (Santa Cruz Biotechnology) for 1hr at 37°C and chased in OptiMEM serum reduced media (Invitrogen) for 16hrs to accumulate secreted protein. This tissue culture fluid was then collected and lysed in RIP lysis buffer prior to clarification by centrifugation at 10,000 xg at 4°C. The cell monolayer was also washed in PBS, lysed in RIP lysis buffer and clarified as described above.

Immunoprecipitation

Radioimmunoprecipitation was used as a conformational-sensitive strategy to analyse protein expression. All protein preparations above were pre-cleared with Sepharose (GE

Healthcare) coupled to BSA (Sigma) in the presence of the relevant antibody for 16hrs at 4°C. The BSA-Sepharose was pelleted at 8 000 xg for 10min to remove any non-specific protein species. The supernatant containing antibody-bound proteins was then precipitated using 30% (v/v) Protein-G Sepharose (GE Healthcare) in RIP lysis buffer for 1hr at room temperature and isolated by centrifugation as above prior to washing three times in RIP wash buffer (0.5 M NaCl, 0.05 M Tris pH 7.4, 1mM EDTA, 0.02% sodium azide and 1% Triton-X 100) and once in PBS.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) Protein Separation and Analysis

All immunoprecipitates were resuspended in sample loading buffer (0.5 M Tris pH 6.8, 5% (v/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue). Intracellular lysates were run under either reducing (+ 3% (v/v) β-mercaptoethanol) or non-reducing conditions (no β-mercaptoethanol) to identify non-covalently associated E1E2 species within the large amounts of intracellular covalently-linked E1E2 aggregate as previously observed (Dubuisson et al., 1994). Metabolically-labelled virion-incorporated E1E2 in viral lysates were run under non-reducing conditions, while the HIV-1 structural proteins were run under reducing conditions. All samples were denatured at 100°C for 5min prior to separation on 10-15% SDS-PAGE polyacrylamide gradient gels (except IgG14 samples that were separated on a 7.5-15% gradient) using the Miniprotean II SDS-PAGE system (BioRad) in reference to a pre-stained broad-range protein marker (BioRad). Electrophoresis was conducted at 100V, 23mA for 1.5 hrs in 1x electrode buffer (0.2M Tris-HCl and 2M Glycine). The SDS-PAGE gels were then submerged in 10% (v/v) acetic acid and 10% (v/v) methanol to fix the samples prior to drying at 80°C using a vacuum slab-gel dryer (Hoefer Scientific, San Francisco, CA, USA). The broad-range marker positions were marked with ^{35}S-Trans label (Santa Cruz Biotechnology) prior to protein analysis and quantification using the FLA-2000 Phosphoimager and Software (Fuji Film, Tokyo, Japan).

Solid-phase CD81-LEL Binding Assay

The large extracellular loop of CD81 has been demonstrated to be sufficient to mediate E2 glycoprotein binding (Pileri et al., 1998, Petracca et al., 2000, Pileri et al., 1998) and contains all the residues that form the E2 interaction site and contains all the residues that

form the E2 interaction site (Drummer et al., 2002). Therefore, to rapidly screen for the CD81-LEL binding ability in the variable region deletion mutants, we used a solid-phase binding assay constructed using a chimera composed of the maltose-binding protein (MBP) linked to the CD81 large-extracellular loop residues 113-201 (MBP-LEL) as previously described (Drummer et al., 2002).

Briefly, 96-well maxisorb enzyme linked immunosorbant plates (Nalge Nunc) were coated with 5ug/mL of dimeric CD81 MBP-LEL in PBS and incubated for 16hrs at 4°C. The MBP-LEL was removed prior to blocking with $BSA_{10}PBS$ (10mg/mL BSA (Sigma) in PBS) for 2hrs at 37°C to reduce non-specific binding. The plates were then washed four times in PBST (0.05% Tween-20 (Sigma) in PBS) prior to the addition of all the protein lysate preparations at twelve two-fold serial dilutions in BSA_5PBST (5mg/mL BSA in PBST). Plates were incubated for 2hrs at room temperature, washed again, and probed for bound E2 with the conformation-dependent anti-E2 monoclonal antibody H53 for 1hr (1:1000 dilution in BSA_5PBST). After further washing in PBST, the antibody-bound E2 complexes were detected using a rabbit anti-mouse immunoglobulin–horseradish peroxidise conjugate (DAKO) (1:1000 dilution in BSA_5PBS/Tween) and developed with a tetra-methylbenzidine substrate (Sigma) according to the manufacturer's instructions. The resulting absorbance values (optical density) were read at 450 nm-620 nm (background) on the Fluostar (BMG technologies). This data was then normalized against monomeric E2 as detected by conformation-dependent antibody H53, visualized and quantified in the previous sections.

E2-myc-CD81 cell surface-binding assays. CHO-K1 cells were seeded in 12-well culture plates at 1.25 x 10^5 cells/well and transfected with 2 μg pcDNA3-CD81 24 h later. At 48-h post transfection the CD81 transfected CHO-K1 cells were chilled on ice and incubated with serial dilutions of wild-type or E2-myc protein containing variable region deletions in $BSA_{10}PBS$ for 4 h on ice. The cells then were washed twice in $BSA_{10}PBS$ prior to a 1 h incubation with ^{125}I-MAb 9E10 (10^6 cpm) that had been precleared with 10^7 CHO-K1 cells for 2 h on ice. After 4 further washes with $BSA_{10}PBS$, the cells were lysed in 1% SDS in PBS and counted in a Packard Auto-Gamma counter.

Table 1. Summary of results obtained for variable region deletion constructs in both the context of the E1E2 polyprotein and the E2 receptor-binding domain constructs containing a myc epitope tag (E2 -myc).

Polyprotein Mutant	Deletion	Heterodimerization [1]	H53 Recognition	CD81-LEL Binding [2]	Heterodimerization [1]	H53 Recognition	Viral Entry [3]	CD81-LEL binding [2]
		Intracellular E1E2 precursors			Mature virion-incorporated E1E2			
pE1E2	Wild-type	+	+	+	+	+	+	+
pE1E2 Δ1	HVR1	+	+	++	+	+	-	++
pE1E2 Δ1con	HVR1 +conserved region	+	+	-	-	reduced	-	-
pE1E2 Δ2	HVR2	-	+	++	-	+	-	+
pE1E2 Δ3	igVR	-	+	++	-	+	-	+
pE1E2 Δ1 conlink	HVR1 + conserved region with extended linker	+	+	+/-	-	reduced	-	-
pE1E2 Δ2 link	HVR2 with extended linker	-	+	++	-	+	-	+
pE1E2 Δ3 link	igVR with extended linker	+	+	++	+	+	+/-	+
pE1E2 Δ12	HVR1 and 2	-	+	+	-	+	-	+
pE1E2 Δ13	HVR1 and igVR	-	+	+	-	+	-	+
pE1E2 Δ23	HVR2 + igVR	-	+	++	-	+	-	+
pE1E2 Δ123	HVR1, HVR2 +igVR	-	+	+	-	+	-	++

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E2 RBD Mutant		Intracellular E2 RBD_{661myc}			Secreted E2 RBD_{661myc}		
E2-myc	Wild-type	+	+	+	+		+
E2 Δ1-myc	HVR1	+	++		+		++
E2 Δ1con-myc	HVR1 + conserved region	+	-		+		-
E2 Δ2-myc	HVR2	+	+/-		+		+
E2 Δ3-myc	igVR	+	+/-		+		+
E2 Δ12-myc	HVR1 and 2	+	+/-		+		-
E2 Δ13-myc	HVR1 and igVR	+	+		+		++
E2 Δ23-myc	HVR2 + igVR	+	+		+		+
E2 Δ123-myc	HVR1, HVR2 +igVR	+	++		+		+

1 + represents heterodimerization levels similar to wild-type E1E2. – represents no heterodimerization detected. +/- represents reduced heterodimerization relative to wild-type E1E2.

2 + represents CD81-LEL binding levels similar to wild-type E1E2. – represents no CD81-LEL binding detected. +/- represents reduced CD81-LEL binding relative to wild-type E1E2. ++ enhanced binding relative to wild-type.

3. + represents viral entry at levels similar to wild-type E1E2. – represents no detectable viral entry. +/- represents partially entry competent.

Table 2. Summary of the overlap extension PCR strategy used to introduce modified single variable region deletions with linker sequences into the HCV E1E2 polyprotein.

pE1E2 construct	Template Vector	Round 1 PCR Product	External Primer	Internal Primer	RE Site	Epitope Tag	PCR product (bp)	Insert size (bp)	MW (Da)
pE1E2 Δ1conlink	pE1E2H77c	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gct ttc att gca att caa ggc cgt GCC GCT ACT ACC gtg ggt ttc cgc gtc gac 3'	EcoR I	none	716	1563	36 482
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC acg gcc ttg aat tgc aat gaa agc ctt aac acc 3'	XbaI	none	993		
pE1E2 Δ2 link	pE1E2H77c	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gta gtg cca gca gta ggg GCC GCT ACT ACC aag gcg tcg gca gct ggc ca acct ctc 3'	EcoR I	none	938	1614	38 071
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC ccc tac tgc tgg cac tac cct cca aga cct tgt ggc 3'	XbaI	none	816		
pE1E2 Δ3 link	pE1E2H77c	3'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gca atc agt ggg gca gag caa GCC GCT ACT ACC gat gac aca agg ggg cgc tcc gca cac 3'	XbaI	none	1268	1656	39 693
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC ttg ctc tgc ccc act gat tgc ttc cgc 3'	XbaI	none	531		

Capitalised and underlined primer sequences represent the introduced Gly-Ser-Ser-Gly linker motif and restriction endonuclease sites, respectively.

Table 3. Summary of the overlap extension PCR strategy used to introduce multiple variable region deletions into the HCV E1E2 polyprotein.

pE1E2 construct	Template Vector	Round1 PCR Product	External Primer	Internal Primer	RE Site	Epitope Tag	PCR product (bp)	Insert size (bp)	MW (Da)
pE1E2 Δ12	pE1E2 Δ2	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' ttg gat gtt ctg GCC GCT ACT ACC gtg ggt ttc cgc gtc gac 3'	EcoRI	none	704	1545	35 697
	pE1E2 Δ2	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC cag aac atc caa ctg atc aac acc 3'	XbaI	none	981		
pE1E2 Δ13	pE1E2 Δ3	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' ttg gat gtt ctg GCC GCT ACT ACC gtg ggt ttc cgc gtc gac 3'	EcoRI	none	704	1590	37 566
	pE1E2 Δ3	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC cag aac atc caa ctg atc aac acc 3'	XbaI	none	1041		
pE1E2 Δ23	pE1E2 Δ3	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gta gtg cca gca GCC GCT ACT ACC gca gct ggc caa cct ctc 3'	EcoRI	none	932	1578	36 651
	pE1E2 Δ3	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC tgc tgg cac tac cct cca aga cct tgt ggc 3'	XbaI	none	810		
pE1E2 Δ123	pE1E2 Δ12	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gca atc agt ggg gca GCC GCT ACT ACC aca agg ggg cgc tcc gca cac 3'	EcoRI	none	1256	1524	34 962
	pE1E2 Δ12	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC tgc ccc act gat tgc ttc cgc 3'	XbaI	none	525		

Capitalized and underlined primer sequences represent the introduced Gly-Ser-Ser-Gly linker motif and restriction endonuclease sites, respectively.

Table 4. Summary of the standard PCR strategy used to construct single and multiple variable region deletions into the E2 receptor-binding domain (residues 384-661).

E2-myc construct	Template Vector	External sense primer	External anti-sense primer	RE Sites	Epitope Tag	Insert Size (bp)	MW (Da)
E2 Δ1con-myc	pE1E2 Δ1con	5' caa gct agc gaa acc cac ggt agt agc ggc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	783	29,385
E2 Δ1-myc	pE1E2 Δ1	5' caa gct agc gaa acc cac ggt agt agc ggc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	843	31,623
E2 Δ2-myc	pE1E2 Δ2	5' cca gct agc gaa acc cac gtc acc ggg gga aat gc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	831	31,282
E2 Δ3-myc	pE1E2 Δ3	5' cca gct agc gaa acc cac gtc acc ggg gga aat gc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	876	33,139
E2 Δ12-myc	pE1E2 Δ12	5' caa gct agc gaa acc cac ggt agt agc ggc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	777	28,865
E2 Δ13-myc	pE1E2 Δ13	5' caa gct agc gaa acc cac ggt agt agc ggc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	822	30,624
E2 Δ23-myc	pE1E2 Δ23	5' cca gct agc gaa acc cac gtc acc ggg gga aat gc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	811	30,381
E2 Δ123-myc	pE1E2 Δ123	5' caa gct agc gaa acc cac ggt agt agc ggc 3'	5' ccg tct aga cta att cag atc ctc ttc tga gat gag ttt ttg ttc agt act ctc gga cct gtc cct gtc 3'	NheI/XbaI	myc	756	27,964

Underlined primer sequences represent introduced restriction endonuclease sites.

Table 5. Summary of the overlap extension PCR strategy used to introduce single variable region deletions into the HCV E1E2 polyprotein.

pE1E2 construct	Template Vector	Round 1 PCR Product	External Primer	Internal Primer	RE Site	Epitope Tag	PCR product (bp)	Insert size (bp)	MW (Da)
pE1E2 Δ1con	pE1E2H77c	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gct ttc att gca GCC GCT ACT ACC gtg ggt ttc cgc gtc gac 3'	EcoRI	none	704	1551	36 083
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC tgc aat gaa agc ctt aac acc 3'	XbaI	none	981		
pE1E2 Δ1	pE1E2H77c	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' ttg gat gtt ctg GCC GCT ACT ACC gtg ggt ttc cgc gtc gac 3'	EcoRI	none	704	1611	38 301
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC cag aac atc caa ctg atc aac acc 3'	XbaI	none	1041		
pE1E2 Δ2	pE1E2H77c	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gta gtg cca gca GCC GCT ACT ACC gca gct ggc caa cct ctc 3'	EcoRI	none	932	1599	37 386
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC tgc tgg cac tac cct cca aga cct tgt ggc 3'	XbaI	none	810		
pE1E2 Δ3	pE1E2H77c	5'	5' ggt gga att ctg gca aca ggg aac ctt cct gg 3'	5' gca atc agt ggg gca GCC GCT ACT ACC aca agg ggg cgc tcc gca cac 3'	EcoRI	none	1256	1644	39 255
	pE1E2H77c	3'	5' ccg tct aga tta cgc ctc cgc ttg gga tat gag 3'	5' GGT AGT AGC GGC tgc ccc act gat tgc ttc cgc 3'	XbaI	none	525		

Capitalized and underlined oligonucleotide sequences represent the introduced Gly-Ser-Ser-Gly linker motif and restriction endonuclease sites, respectively.

Table 6. Polymerase chain reaction (PCR) amplification reaction conditions.

Overlapping: Round 1		
95°C	2min	
92°C 65°C 72°C	30sec 30sec 2min	X 29
72°C	10min	

Overlapping: Round 2		
95°C	2min	
92°C 55°C 72°C	30sec 30sec 4min	X 29
72°C	10min	

Standard		
95°C	2min	
92°C 55°C 72°C	30sec 30sec 2min	X 29
72°C	10min	

Colony		
94°C	2min	
94°C 55°C 72°C	30sec 30sec 1min	X 29
72°C	10min	

Sequencing		
96°C	1min	
96°C 50°C 60°C	20sec 15sec 4min	X 29
4°C	10min	

Example 2

1. Antigenic structure of E2-myc and E2 Δ123-myc probed with a panel of sera obtained from HCV-infected individuals.

A panel of sera obtained from HCV-infected individuals was used to compare the global antigenic profiles of biosynthetically labeled E2-myc and E2 Δ123-myc by immunoprecipitation. The sera were screened for the presence of neutralizing antibodies towards H77c E1E2 pseudotyped retroviral particles as described previously (Grollo et al., 2006), with 50% neutralization titers ranging from 0 to 1,600 observed (Figure. 24, lower panel). The antibody reactivity pattern of E2-myc (Figure 24, upper panel) was almost identical to that of E2 Δ123-myc (Figure 24, middle panel), indicating that the gross antigenic structure of the two proteins is similar.

2. CD81 binding properties of E2-myc wild type and variant proteins.

The abilities of the secreted E2-myc proteins to interact with the large extracellular loop (LEL) of the HCV cellular receptor CD81 was examined. In this assay, CD81-E2-myc binding was detected using a conformation-dependent E2 monoclonal antibody (H53) in an ELISA employing solid-phase maltose binding protein fused to the large extracellular loop of CD81 (residues 113-201; "CD81-LEL"). E2-myc proteins containing one or more deletions of the variable regions displayed wild-type levels of CD81-LEL binding, again indicating that the E2 global fold was not detectably affected by the deletions (Figure 25A,B). By contrast, binding between the E2 L441M-myc protein, containing an LEL binding site mutation (L441M), and CD81-LEL was not observed, confirming the specificity of the binding assay (Figure 25A).

3. Abilities of E2-myc wild type and variant proteins to interact with full-length CD81 expressed in CHO-K1 cells.

The abilities of E2-myc proteins to interact with full-length CD81 receptor were determined by a cell surface binding assay using CHO-K1 cells transfected with a full-length CD81 expression vector as previously described (Drummer et al., 2002). The secreted E2-myc wild type and variant proteins were serially diluted and incubated with

CD81-transfected CHO-K1 cells on ice. After washing, the bound E2-myc proteins were detected by using ^{125}I-labeled monoclonal antibody 9E10, directed to the C-terminal c-myc epitope tag. The specificity of the assay was confirmed by the lack of binding by E2 L441M-myc, containing the L441M CD81 binding site mutation, to wild-type-CD81-transfected CHO-K1 cells (Figure 26A).

The results shown in Figures 26A and B indicate that E2-myc, E2 Δ23-myc, E2 Δ13-myc and E2 Δ123-myc have similar CD81 binding properties, whereas the deletion of HVR1 plus HVR2 (E2 Δ12-myc) caused an approximately 50% reduction in CD81 binding compared to binding by E2 Δ23-myc ($p < 0.035$). By contrast, the CD81 binding abilities of E2 Δ13-myc (which exhibited a binding curve identical to that of E2-myc) and E2 Δ123-myc were not significantly different ($p = 0.62$), indicating that the presence of igVR compromises the CD81 binding function when HVR1 and HVR2 are absent in E2 Δ12-myc. Although HVR1, HVR2, and igVR are not required for the core folding properties of E2 RBD_{661}, these data point to a functional interaction between igVR and one or both of HVR1 and HVR2 such that the CD81 binding site is properly formed or becomes fully accessible to the receptor.

Although variations in binding to recombinant CD81-LEL for singly and multiply deleted E2-myc constructs were not detected in Figures 25A and 25B, differences in binding to surface-expressed CD81 were observed in Figures 26A and 26B, perhaps reflecting subtle differences in LEL structure when it is expressed in isolation versus in native tetraspanin.

4. Materials and Methods.

Neutralization assays

The abilities of immune and control human sera to neutralize a single cycle of infection by HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses were determined as follows. HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses were prepared by cotransfection of HEK-293T monolayers (350,000 cells per well of 6-well culture dishes) with pE1E2 and pNL4.3LUCR-E- plasmids (Drummer et al., 2003). After 3 days incubation at 37 °C in a humidified atmosphere containing 5 % CO_2, the culture supernatants were filtered through 0.45 μm sterile syringe filters (Sartorius). Serial

dilutions of heat-inactivated immune and control human sera were preincubated with HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses (1 h) and then added to quadruplicate Huh7 cell monolayers in 48-well tissue culture plates. Following a 4 h incubation (37 °C, 5 % CO_2) the cells were washed with PBS and fresh medium replaced. After an additional 3-day incubation (37 °C in 5 % CO_2), the cells were lysed, the lysates clarified by centrifugation and then assayed for luciferase activity (Promega) in a Fluostar (BMG) fitted with luminescence optics. The neutralization titres of individual sera were determined as the serum dilution giving 50 % neutralization compared to HCV glycoprotein-pseudotyped HIV-1 luciferase reporter virus preincubated with medium alone.

Radioimmunoprecipitation.

293T cells seeded at 500,000 cells/well in 6-well culture dishes were transfected with E2-myc expression vectors using Fugene 6 (Roche). At 24 hours post-transfection, the cells were labelled with 150 μCi Trans-^{35}S-label/well (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in L-cysteine and L-methionine deficient DMF10 (DMEM [MP Biomedicals], 10% (v/v) heat-inactivated fetal bovine serum [Invitrogen] and 2mM L-glutamine [GE Healthcare]). After harvesting the cell supernatent, the labelled secreted proteins were adjusted to 0.6 M KCl, 0.05 M Tris pH 7.4, 1mM EDTA, 0.02% sodium azide, 1 % Triton X-100 and pre-cleared with CNBr-activated Sepharose (GE Healthcare) coupled to BSA (Sigma) in the presence of the relevant antibody for 16 h at 4°C. Antibody-antigen complexes within the clarified supernatants were then immunoprecipitated using 30% (v/v) Protein-G Sepharose (GE Healthcare) prior to washing three times in RIP wash buffer (0.5 M NaCl, 0.05 M Tris pH 7.4, 1mM EDTA, 0.02% sodium azide and 1% Triton-X 100) and once in PBS. The immunoprecipitated proteins were subjected to SDS-PAGE in 10-15 % polyacrylamide gradient gels under nonreducing conditions and visualized by scanning in a phosphorimager.

Transient expression of E2-myc proteins

HEK 293T cells (350,000 cells per well of 6-well culture plates) were transfected with E2-myc expression vectors using Fugene 6 (Roche). At 8-h post transfection, the transfection medium was replaced with Optimem (Invitrogen) and the cells incubated for 3 days at

37°C in a humidified atmosphere containing 5 % CO_2. The tissue culture fluid was clarified through 0.45-μm-pore-size filters and then concentrated by approximately 10-fold in Centricon YM30 concentrators (Amersham).

Recombinant CD81 large extracellular loop (CD81-LEL) binding properties.

The abilities of the E2-myc proteins to interact with the HCV cellular receptor CD81 were examined using a solid phase enzyme immunoassay. Enzyme immunoassay plates (Nunc Maxisorb®) were coated with maltose binding protein fused to the recombinant large extracellular loop of CD81 (residues 113-201) at 5 μg/ml in PBS overnight at 4°C. Coating solution was removed and unoccupied sites blocked with bovine serum albumin (10mg/ml) in PBS ($BSA_{10}PBS$) for 1 h at room temperature. Plates were washed 4 times with PBS containing 0.05% Tween 20 (PBST). The secreted E2-myc proteins were serially diluted in a 50 μl PBS containing 5mg/ml bovine serum albumin (BSA_5PBST) and incubated for 2 h. Bound E2-myc proteins were detected using an E2 specific monoclonal antibody followed by rabbit anti-mouse immunoglobulins coupled to horseradish peroxidase (Dako). Plates were developed using tetramethylbenzidine hydrochloride substrate and stopped by the addition of 1M HCl. Absorbance values were measured at 450nm and the background at 620nm subtracted in a Fluostar plate reader (BMG technologies).

CD81 binding properties.

CHO-K1 cells were seeded in 12-well culture plates at 1.25×10^5 cells/well and transfected with 2 μg pcDNA3-CD81 24 h later. At 48-h post transfection the CD81 transfected CHO-K1 cells were chilled on ice and incubated with serial dilutions of wild-type E2-myc or E2-myc proteins containing variable region deletions in $BSA_{10}PBS$ for 4 h on ice. The cells then were washed twice in $BSA_{10}PBS$ prior to a 1-h incubation with ^{125}I-MAb 9E10 (10^6 cpm) that had been precleared with 10^7 CHO-K1 cells for 2 h on ice. After 4 further washes with $BSA_{10}PBS$, the cells were lysed in 1% SDS in PBS and counted in a Packard Auto-Gamma counter.

Example 3.

1. SDS-PAGE analysis of purified E2-his wild type and variant proteins.

The purity of E2-his wild type and variant proteins was assessed by SDS-PAGE under reducing conditions. Figure 27 indicates a single band for each purified protein species. The migration of each species was consistent with the number of variable regions deleted. For example, E2 Δ123-his, lacking 3 variable regions, exhibited the fastest migration. The diffuse nature of the protein bands is consistent with glycosylation of E2.

2. Blue-Native PAGE of purified E2-his proteins.

The oligomerization status of the purified E2-his wild type and variant proteins was analysed using blue native polycrylamide gel electrophoresis. Figure 28 shows two major bands for each E2-his protein corresponding in molecular weight to monomer and dimer.

3. Immunodetection of E2-his proteins.

The purified E2-his wild type and variant proteins were detected by a non-conformation dependent E2 specific monoclonal antibody (H52) in a western blot. A major protein species migrating at the expected molecular mass was revealed for each E2-his protein variant (Figure 29).

4. CD81 binding properties of E2-his wild type and variant proteins.

The ability of the purified E2-his proteins to interact with the large extracellular loop (LEL) of the HCV cellular receptor CD81 was examined. In this assay, CD81-E2-his binding was detected using a conformation-dependent E2 monoclonal antibody (H53) in an ELISA employing solid-phase maltose binding protein fused to the large extracellular loop of CD81 (residues 113-201; "CD81-LEL"). E2-his proteins containing one or more deletions of the variable regions displayed wild-type levels of CD81-LEL binding, indicating that the E2 global fold was not detectably affected by the deletions (Figure 30A). By contrast, binding between the E2-his proteins and CD81-LEL containing an E2 binding site mutation (F186S) was not observed, confirming the specificity of the binding assay (Figure 30B).

5. Immunoreactivity towards homologous E2-his antigen of mouse sera obtained after 2 immunizations with E2-his protein variants.

The immunoreactivity of mouse sera obtained after 2 immunizations with the E2-his wild type and variant proteins towards solid-phase "homologous antigen" (i.e. that antigen used to immunize a particular mouse group) was examined by ELISA. Substantial antibody binding activity towards the homologous solid-phase antigen was demonstrated in each of the vaccinated animals (Figure 31). By contrast, antibody reactivity towards solid-phase E2-his proteins was not detected in the mice vaccinated with adjuvant alone, nor in two representative control sera obtained prior to immunization (prebleed) from each vaccination group (data not shown), confirming that the animals receiving E2-his protein elicited antibodies specific to the immunogen.

6. Immunoreactivity towards E2-his and E2Δ123-his antigens of mouse sera obtained after 2 immunizations with E2-his protein variants.

The ability of the antibodies in immune sera from each vaccination group to bind to solid-phase E2-his (containing the three variable regions) was compared to the antibody reactivity towards solid-phase E2 Δ123-his (lacking the three variable regions) in ELISA. Substantial binding titres against solid phase E2-his (Figure 32A) and E2 Δ123-his (Figure 32B) were observed for all immunization groups (except adjuvant alone; data not shown).

Pair-wise statistical analyses of the antibody binding titres obtained for the various immunization groups were performed in order to determine whether apparent differences in binding titre were significant. Table 7 indicates that the antibody titres against solid-phase E2-his calculated for the E2-his-immunization group were not significantly different to those of the other immunization groups ($p > 0.06$). However, statistically significant differences in antibody reactivity against solid-phase E2-his were observed between the E2 Δ1-his immunization group versus E2 Δ13-his, E2 Δ23-his and E2 Δ123-his ($p \leq 0.04$).

A pair-wise statistical analysis of antibody binding titres against solid-phase E2 Δ123-his for the various immunization groups revealed a highly significant increase in antibody reactivity for the E2 Δ123-his immunization group compared with the E2-his

immunization group ($p = 0.003$, Table 8). Statistically significant increases in antibody reactivity for the E2 Δ1-his, E2 Δ3-his, and E2 Δ12-his immunization groups versus E2-his were observed, and also for E2 Δ1-his relative to E2 Δ2-his immunization groups ($p \leq 0.04$).

7. Immunoreactivity towards homologous E2-his antigen of mouse sera obtained after 3 immunizations with E2-his protein variants.

The immunoreactivity of mouse sera obtained after 3 immunizations with the E2-his wild type and variant proteins towards solid-phase "homologous antigen" (i.e. that antigen used to immunize a particular mouse group) was examined by ELISA. Substantial antibody binding activity towards the homologous solid-phase antigen was demonstrated in each of the vaccinated animals (Figure 33), but not in two representative control sera obtained prior to immunization (prebleed) from each vaccination group (data not shown). Furthermore, antibody reactivity towards solid-phase E2-his proteins was not detected in the mice vaccinated with adjuvant alone (data not shown), confirming that the animals receiving E2-his protein elicited antibodies specific to the immunogen.

8. Immunoreactivity towards E2-his and E2Δ123-his antigens of mouse sera obtained after 3 immunizations with E2-his protein variants.

The ability of the antibodies in immune sera from each vaccination group to bind to solid-phase E2-his (containing the three variable regions) was compared to the antibody reactivity towards solid-phase E2 Δ123-his (lacking the three variable regions) in ELISA. Substantial binding titres against solid phase E2-his (Figure 34A) and E2 Δ123-his (Figure 34B) were observed for all immunization groups (except adjuvant alone, data not shown).

Pair-wise statistical comparisons of the antibody binding titres obtained for the various immunization groups were performed in order to determine whether apparent differences in binding titre were significant. In contrast to the data obtained after the second immunization, Table 9 indicates that the antibody titres against solid-phase E2-his calculated for the E2-his-immunization group were significantly higher than those of 6 of 7 other immunization groups($p = 0.003$ to 0.04), excepting the E2 Δ123-his ($p = 0.11$).

A pair-wise statistical analysis of antibody binding titres against solid-phase E2 Δ123-his for the various immunization groups revealed a highly significant increase in antibody reactivity for the E2 Δ123-his immunization group compared with the E2-his immunization group ($p = 0.0007$, Table 10). Statistically significant differences were also observed between the E2-his versus E2 Δ3-his, E2 Δ12-his, and E2 Δ23-his immunization groups, and also between the E2 Δ1-his versus E2 Δ3-his, E2 Δ12-his, E2 Δ23-his and E2 Δ123-his immunization groups ($p = 0.009$ to 0.022).

Finally, the mean binding titres obtained for each immunization group against solid-phase wild type E2-his antigen (i.e. containing HVR1, HVR2 and IgVR) were compared with the binding titres towards solid-phase E2 Δ123-his antigen (i.e. lacking HVR1, HVR2 and igVR and representing the conserved glycoprotein core). This analysis was performed in order to gauge the relative abilities of the immunogens to elicit antibodies reactive with the conserved core of the $E2_{RBD}$. Table 11 shows significantly lower antibody titres to solid-phase E2 Δ123-his antigen relative to solid-phase E2-his antigen for antisera elicited by E2-his immunogen (~ 4.4-fold reduction; $p = 0.0009$) and E2 Δ1-his immunogen (~ 2-fold reduction; $p = 0.005$).

9. Immunoreactivity towards solid-phase Con1 $E2_{RBD}$-his and JFH1 $E2_{RBD}$-myc antigens of mouse sera obtained after 3 immunizations with E2-his protein variants.

The abilities of the E2-his wild type and variant proteins, which are derived from the genotype 1a H77c isolate, to elicit cross-genotype reactive antibodies was determined by comparing the immunoreactivity of immune sera with solid-phase Con1 $E2_{RBD}$-his (genotype 1b) and JFH1 $E2_{RBD}$-myc (genotype 2a) antigens in ELISA. Substantial binding titres against solid phase Con1 $E2_{RBD}$-his (Figure 35A) and JFH1 $E2_{RBD}$-myc (Figure 35B) were observed for all immunization groups (except adjuvant alone, data not shown).

A pair-wise statistical analysis of antibody binding titres against solid-phase Con1 $E2_{RBD}$-his for the various immunization groups revealed significantly higher binding titres for E2 Δ2-his, E2 Δ3-his, E2 Δ12-his, and E2 Δ123-his immunization groups compared with the E2-his immunization group ($p < 0.05$, Table 12). The Con1 $E2_{RBD}$-his-binding titre

elicited in the E2 Δ123-his immunization group was also significantly higher than the Con1 $E2_{RBD}$-his binding titres elicited by E2 Δ1-his and E2 Δ23-his ($p < 0.05$).

By contrast, a similar pair-wise statistical analysis of antibody binding titres against solid-phase JFH1 $E2_{RBD}$-myc for the various immunization groups did not reveal significantly different binding titres between groups (Table 13). Despite this lack of significance, the trends observed above were also apparent in these analyses.

10. Neutralization.

The abilities of immune and control mouse sera to neutralize a single cycle of infection by homologous H77c strain E1E2-pseudotyped HIV-1 luciferase reporter viruses were determined. Serial 5-fold dilutions of heat-inactivated immune and control mouse sera were preincubated with E1E2-pseudotyped HIV-1 luciferase reporter viruses (1 h) and then incubated for 4 h with Huh7 cell monolayers. The cells were washed with PBS, fresh culture medium added, and after an additional 3-day incubation (37 °C in 5 % CO_2), the cells were lysed and assayed for luciferase activity. Figure 36 shows the 80 %-neutralization titres of individual sera, which were determined as the serum dilution giving 80 % neutralization compared to HCV glycoprotein-pseudotyped HIV-1 luciferase reporter virus preincubated with medium alone. At least 80 % of mice immunised with E2-his, E2 Δ123-his and E2 Δ23-his produced neutralizing antibodies against the E1E2-HIV-1 pseudotypes with mean 80 % neutralization titres of 908, 413 and 140, respectively. By contrast, at least 80 % of mice immunized with E2 Δ1-his or adjuvant alone lacked 80 %-neutralization activity with mean 80 % neutralization titres of 7 and 26, respectively (Table 14).

11. Discussion

The E2-his proteins containing one or more deletions of the three variable regions were capable of eliciting antibody reactive towards homologous antigens. The specificity of the antibody response was examined by comparing the ability of the immune sera to react towards intact wild-type E2-his protein containing three variable regions to E2 Δ123-his protein which represents the core E2 folding unit lacking all three variable regions but

retaining CD81 binding. This analysis revealed that mice immunized with E2-his protein or E2 lacking HVR1 (E2 Δ1-his) elicited significantly less antibody reactive towards the core domain of E2. By contrast antibodies elicited in mice immunized with E2-his proteins lacking HVR1 and 2 (E2 Δ12-his), HVR1 and igVR (E2 Δ13-his), HVR2 and igVR (E2 Δ23-his) or lacking all three variable regions (E2 Δ123-his) reacted similarly towards wild-type E2-his and E2 Δ123-his antigens. This suggests that HVR2 and the igVR may hinder antibody access to epitopes present in the underlying conserved core domain of E2. To test this hypothesis, $E2_{RBD}$ constructs were synthesised representing heterologous isolates of HCV from genotype 1b (Con1) and genotype 2a (JF-H1). The immunoreactivity of mouse serum obtained after the third vaccination to these heterologous $E2_{RBD}$ constructs revealed that mice immunized with E2 Δ123-his, E2 Δ12-his, E2 Δ3-his, E2 Δ2-his elicited signficantly higher levels of cross-reactive antibody to epitopes present in the genotype 1b isolate Con1. Although cross-reactive antibody to the $E2_{RBD}$ of genotype 2a isolate JF-H1 were also elicited, the differences in binding titres between the immunogen groups were not statistically significant although trends similar to those above were observed. These data suggest that deletion of at least one variable region, preferably either HVR2 and/or igVR, may improve the ability of E2-his proteins to elicit cross-reactive antibodies with broader neutralization capacity.

The ability of the immune serum to neutralize homologous virus was examined using HCV E1E2 pseudotyped retroviral particles. The data showed that mice vaccinated with E2 Δ123-his or E2 Δ23-his possessed on average ~59 and 20 fold higher neutralizing antibody titres compared with E2-his lacking HVR1 alone ($p=0.11$ and 0.002, respectively). HVR1 has previously been shown to be an immunodominant region of the E1E2 glycoprotein complex. Deletion of HVR1 region alone may result in the removal of an important type-specific immunodominant epitope. However, the presence of HVR2 and/or the igVR regions in the HVR1 deleted construct may shield underlying cryptic neutralizing epitopes. It is therefore likely that the antibody response elicited by wild-type E2-his protein may be largely directed towards the HVR1 region and not the conserved core. This is in part reflected by (i) the lower level of antibody reactive to the E2 core domain (Figure 34B) and (ii) the lower levels of cross-reactive antibody (Figure 35A).

Together the data suggest that deletion of HVR2 and/or the igVR region is a significant improvement on the use of E2 RBD constructs lacking none of the variable regions or HVR1 alone. It is likely that E2 RBD proteins lacking at least HVR2 and/or igVR and possibly all three variable regions will elicit antibody capable of cross-neutralizing divergent HCV strains from within a genotype as well as cross-genotype neutralization. E2 RBD proteins lacking one or more variable regions may be useful tools for both therapeutic or prophylactic vaccination strategies for the prevention or treatment of HCV infection. In addition these variable region deleted E2 RBD constructs may also be useful to elicit novel specificities of antibodies directed to the conserved E2 core region that neutralize HCV for therapeutic and prophylactic use.

12. Materials and Methods

Construction of wild type and variant HCV E2-his cDNAs and secreted expression of the encoded proteins in mammalian cells.

Generation of cDNA Expression Plasmids Encoding HCV E2-his Variants

A synthetic gene encoding a wild type E2 protein fragment (residues 384-661; strain H77c) was constructed by Geneart AG (Regensburg, Germany). The human trypsinogen signal peptide (MNPLLILTFVAAALA) was appended in-frame to the N-terminus of the wild type E2 mature protein in order to facilitate secretion of the mature polypeptide into the expression medium. A Kozak sequence was introduced just before the N-terminus to increase translational initiation and a $(His)_6$ sequence was added in-frame to enable subsequent purification of the secreted proteins by immobilised metal affinity chromatography. Two stop codons were added after the His-tag at the C-terminus to ensure efficient translational termination. This construct is referred to as E2-his. The codon usage of the E2-his cDNA was adapted to the codon bias of *Homo sapiens* genes. An Nhe I restriction site at the 5' end of the cDNA and a Xho I restriction site was introduced at the 3' end in order to ligate the Geneart cDNA into Nhe I-Xho I digested pcDNA3.1 (Invitrogen).

The cDNAs encoding seven variants of the E2-his mature protein where one or more of the variable regions were substituted by linker sequences, were constructed using standard

PCR mutagenesis procedures with Accuprime *Pfx* DNA Polymerase (Invitrogen) according to the manufacturer's instructions and the primers shown in Table 15. The combinations of primers used to construct cDNAs of the E2-his are shown in Table 16.

The specific PCR parameters were as follows: 2 minutes of denaturation at 95°C was followed by 18 cycles of 95°C for 15 seconds, 64°C for 30 seconds and 68°C for 2 minutes with a final 2 minute incubation at 68°C.

The Geneart wild type E2-his cDNA was used as a PCR template for all the E2-his variant cDNAs. Each deletion variant was constructed using sequential overlap PCR of multiple fragments. Once each cDNA was complete, it was digested with Nhe I and Xho I and ligated into pcDNA3.1. Large scale preparations of plasmid DNA were carried out using a Qiagen Maxi Kit. The nucleotide sequences of all the plasmid constructs were verified by sequencing both strands using Big Dye Terminator v3.1 Cycle Sequencing and an Applied Biosystems Automated Sequencer.

The sequences of the mature wild type and variant E2 proteins are as follows:

E2-his mature protein

ETHVTGGNAGRTTAGLVGLLTPGAKQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERL
ASCRRLTDFAQGWGPISYANGSGLDERPYCWHYPPRPCGIVPAKSVCGPVYCFTPSPVVVGTTDRSGAPTYSW
GANDTDVFVLNNTRPPLGNWFGCTWMNSTGFTKVCGAPPCVIGGVGNNTLLCPTDCFRKHPEATYSRCGSGPW
ITPRCMVDYPYRLWHYPCTINYTIFKVRMYVGGVEHRLEAACNWTRGERCDLEDRDRSE

E2Δ1-his mature protein

ETHQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERLASCRRLTDFAQGWGPISYANGS
GLDERPYCWHYPPRPCGIVPAKSVCGPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFG
CTWMNSTGFTKVCGAPPCVIGGVGNNTLLCPTDCFRKHPEATYSRCGSGPWITPRCMVDYPYRLWHYPCTINY
TIFKVRMYVGGVEHRLEAACNWTRGERCDLEDRDRSE

E2Δ2-his mature protein

ETHVTGGNAGRTTAGLVGLLTPGAKQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERL
ASCGSSGCWHYPPRPCGIVPAKSVCGPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFG
CTWMNSTGFTKVCGAPPCVIGGVGNNTLLCPTDCFRKHPEATYSRCGSGPWITPRCMVDYPYRLWHYPCTINY
TIFKVRMYVGGVEHRLEAACNWTRGERCDLEDRDRSE

E2Δ3-his mature protein

ETHVTGGNAGRTTAGLVGLLTPGAKQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERL
ASCRRLTDFAQGWGPISYANGSGLDERPYCWHYPPRPCGIVPAKSVCGPVYCFTPSPVVVGTTDRSGAPTYSW
GANDTDVFVLNNTRPPLGNWFGCTWMNSTGFTKVCGAPPCGSSGCPTDCFRKHPEATYSRCGSGPWITPRCMV
DYPYRLWHYPCTINYTIFKVRMYVGGVEHRLEAACNWTRGERCDLEDRDRSE

E2Δ12-his mature protein

ETHQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERLASCGSSGCWHYPPRPCGIVPAK
SVCGPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFGCTWMNSTGFTKVCGAPPCVIGG

VGNNTLLCPTDCFRKHPEATYSRCGSGPWITPRCMVDYPYRLWHYPCTINYTIFKVRMYVGGVEHRLEAACNW
TRGERCDLEDRDRSE

E2Δ13-his mature protein

ETHQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERLASCRRLTDFAQGWGPISYANGS
GLDERPYCWHYPPRPCGIVPAKSVCGPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFG
CTWMNSTGFTKVCGAPPCGSSGCPTDCFRKHPEATYSRCGSGPWITPRCMVDYPYRLWHYPCTINYTIFKVRM
YVGGVEHRLEAACNWTRGERCDLEDRDRSE

E2Δ23-his mature protein

ETHVTGGNAGRTTAGLVGLLTPGAKQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERL
ASCGSSGCWHYPPRPCGIVPAKSVCGPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFG
CTWMNSTGFTKVCGAPPCGSSGCPTDCFRKHPEATYSRCGSGPWITPRCMVDYPYRLWHYPCTINYTIFKVRM
YVGGVEHRLEAACNWTRGERCDLEDRDRSE

E2Δ123-his mature protein

ETHQNIQLINTNGSWHINSTALNCNESLNTGWLAGLFYQHKFNSSGCPERLASCGSSGCWHYPPRPCGIVPAK
SVCGPVYCFTPSPVVVGTTDRSGAPTYSWGANDTDVFVLNNTRPPLGNWFGCTWMNSTGFTKVCGAPPCGSSG
CPTDCFRKHPEATYSRCGSGPWITPRCMVDYPYRLWHYPCTINYTIFKVRMYVGGVEHRLEAACNWTRGERCD
LEDRDRSE

Cell Culture

FreeStyle™ 293-F cells (Invitrogen) were cultured in FreeStyle™ Expression Medium (Invitrogen) supplemented with penicillin/streptomycin/fungizone (Invitrogen). All cells were maintained at 37°C in humidified incubators with an atmosphere of 8% CO_2.

Transient protein expression

Transient expression of each of the E2-his proteins was carried out in FreeStyle™ 293-F cells by transfection with the pcDNA3.1-based expression plasmids and 293fectin transfection reagent (Invitrogen) according to the manufacturer's instructions. The cells in a total volume of 180 ml were transfected at a final concentration of 1 x 10^6 viable cells/ml and incubated in a sterile shaker flask (Corning) for 5 days on an orbital shaker (IKA) rotating at 150 rpm in a 37°C humidified incubator with an atmosphere of 8% CO_2. Twenty-four hours after transfection the cell cultures were supplemented with Tryptone N1 (Organotechnie, France) to a final concentration of 0.5 % v/v. Typically the cell cultures were harvested 5 days after transfection. Protein expression was examined by electrophoresis of a sample of cell culture supernatant using 4-20% Tris-Glycine SDS polyacrylamide gel and the proteins visualised by staining with Coomassie Blue reagent. For protein purification, cell culture supernatants were harvested by centrifugation at 2500 rpm and then passed through a 0.45μM filter (Nalgene) prior to chromatography.

Purification of expressed wild type and variant HCV E2-his proteins.

Following filtration the cell culture supernatants were subjected to immobilised metal affinity chromatography (IMAC) using Nickel sepharose to purify the wild type and variant E2-his proteins.

The purification procedure is described below:

1. Buffers:

Ni-MAC Buffer A
50 mM NaH_2PO_4 pH 8.0
300 mM NaCl
10 mM imidazole

Ni-MAC Buffer B (Elution)
50 mM NaH_2PO_4 pH 8.0
300 mM NaCl
500 mM imidazole

2. Protocol:

1. Procedures 2 to 6 were carried out at 4-8°C
2. 1 ml Ni Sepharose 6 Fast Flow resins (GE Healthcare) in a 10 ml Poly-Prep columns (Bio-Rad) were washed with 5 volumes of ddH_2O.
3. The columns were equilibrated with 10 ml Ni-MAC Buffer A.
4. Samples were loaded onto the columns, and break-through (B/T) collected.
5. The columns were washed with 10 ml Ni-MAC Buffer A.
6. The proteins were eluted with 5 ml Ni-MAC Buffer B (Elution) and 1 ml fractions collected.
7. Peak fractions were identified using 96-well plate format Bradford Assay and Coomassie-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels.
8. Peak fractions were pooled and dialysed in 1X PBS overnight at 4°C.
9. Following dialysis protein concentrations were determined using 96-well plate format Bradford Assay and 1 mg/ml BSA for a standard curve.

Construction of Con1 $E2_{RBD}$-his and JFH1 $E2_{RBD}$-myc cDNAs and secreted expression of the encoded proteins in mammalian cells.

Generation of cDNA Expression Plasmids Encoding Con1 $E2_{RBD}$-his and JFH1 $E2_{RBD}$-myc.

cDNAs encoding Con1 E2 (HCV Con1 polyprotein residues 384 to 661; Genbank accession number AJ238799) and JFH1 E2 (HCV JFH1 polyprotein residues 384 to 665; Genbank accession number AB047369), were prepared by PCR using Expand-Hifi polymerase (Roche) and the primers indicated in Table 17. The forward primers encoded an NheI restriction site for incorporation in-frame at the 5' ends of the cDNAs. The reverse primers encoded (His)6 and c-myc epitope tags for addition in-frame to the 3' ends of Con1 and JFH1 cDNAs, respectively, and for both cases were followed by a TAG translation termination codon and an XbaI restriction site. The specific PCR parameters were as follows: 5 minutes of denaturation at 95°C was followed by 30 cycles of 92° C for 1 min, 55 °C for 1 min and 72 °C for 2 minutes with a final 10 minute incubation at 72 °C. The cDNAs were cloned downstream of a tissue plasminogen activator signal peptide in vector pcDNA3 to facilitate secretion of the expressed proteins. The nucleotide sequences of the cloned cDNAs were verified by using Big Dye Terminator v3.1 Cycle Sequencing and an Applied Biosystems Automated Sequencer.

Transient expression of Con1 $E2_{RBD}$-his and JFH1 $E2_{RBD}$-myc

HEK 293T cells (350,000 cells per well of 6-well culture plates) were transfected with Con1 $E2_{RBD}$-his and JFH1 $E2_{RBD}$-myc expression vectors using Fugene 6 (Roche). At 8-h post transfection, the transfection medium was replaced with Optimem (Invitrogen) and the cells incubated for 3 days at 37 °C in a humidified atmosphere containing 5 % CO_2. The tissue culture fluid was clarified by centrifugation at 1500 rpm for 10 min followed by filtration through 0.45-µm-pore-size syringe filters.

Biochemical and functional analysis of wild type and variant E2-his proteins.

Blue-native PAGE

The oligomerization status of the purified E2-his proteins was analysed using blue native polycrylamide gel electrophoresis (BN-PAGE). Ten µg of each protein was added to 10 µl

of solubilization buffer and 1.5 µl of sample buffer and loaded onto 5-15% gradient separating gels containing a 4 % stacking gel. The gels were electrophoresed at 4°C at 100 volts until the dye front entered the stacking gel. The voltage was then increased to 200 volts until the dye front migrated to the bottom of the gel. Electrophoresis was conducted for 1.5 h with 1 x cathode buffer containing 0.01% Serva G in the upper reservoir, followed by electrophoresis for 1-1.5 h in 1x cathode buffer without Serva G. The lower reservoir contained 1 x anode buffer. After electrophoresis, the gels were destained overnight and scanned in an Odyssey scanner at 680 nm.

The solutions used for BN-PAGE are as follows:

Polyacrylamide Gels:

	4% stacker	5% separating gel	15% separating gel
40% acrylamide	0.299ml	0.262ml	0.787ml
3x gel buffer	1ml	0.7ml	0.7ml
75% Glycerol		0.14ml	0.62ml
water	1.72ml	0.978ml	0
TEMED	6µl	2µl	2µl
5% ammonium persulphate	32µl	11µl	11µl

Other Solutions:

3x Gel Buffer. 150mM BisTris-HCl, .5M 6-amino caproic acid, pH 7.0

10x Cathode buffer. 0.5M Tricine, 150mM BisTris

5x Anode buffer. 0.25M BisTris-HCl, pH 7.0

2xBisTrisACA. 200mM BisTris-HCl, 1M 6-amino-caproic acid, pH 7.0.

Sample buffer. 50mg ServaG, 500 µl 2xBisTrisACA, 400µl 75% sucrose and 100µl water.

Solubilization buffer. 0.5M 6-amino-caproic acid, 20mM BisTris, 2mM EDTA, pH 7.0, 1% Triton X-100, 10% glycerol.

Destain. 10% acetic acid, 10% methanol, 80% water.

Immunodetection of E2-his proteins.

Samples of the purified E2-his proteins were subjected to reducing SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. E2-his proteins were detected with a non-conformation dependent E2 specific monoclonal antibody followed by goat anti-mouse immunoglobulin coupled to Alexa fluor 680nm (Invitrogen). Immunoblots were scanned in an Odyssey detection system.

CD81 binding properties.

The ability of the E2-his proteins to interact with the HCV cellular receptor CD81 was examined using a solid phase enzyme immunoassay. Enzyme immunoassay plates (Nunc Maxisorb®) were coated with maltose binding protein fused to the recombinant large extracellular loop of CD81 (residues 113-201) at 5 μg/ml in PBS overnight at 4°C. Coating solution was removed and unoccupied sites blocked with bovine serum albumin (10mg/ml) in PBS ($BSA_{10}PBS$) for 1 h at room temperature. Plates were washed 4 times with PBS containing 0.05% Tween 20 (PBST). 50 ng E2-his proteins were serially diluted in a 50 μl PBS containing 5mg/ml bovine serum albumin (BSA_5PBST) and incubated for 2 h. Bound E2-his protein was detected using an E2 specific monoclonal antibody followed by rabbit anti-mouse immunoglobulins coupled to horse radish peroxidase (Dako). Plates were developed using tetramethylbenzidine hydrochloride substrate and stopped by the addition of 1M HCl. Absorbance values were measured at 450nm and the background at 620nm subtracted in a Fluostar plate reader (BMG technologies).

Immunogenicity in mice of wild-type and variant E2-his proteins.

Immunization Protocol.

Groups of 10 7-8 week old female Balb/c mice were immunised with purified E2-his wild type and variant proteins formulated with ISCOMATRIX® adjuvant (Pearse and Drane, 2005) (Table 18). Each mouse dose contained 10 μg specific protein and 5 μg ISCOMATRIX® adjuvant in a 0.1ml volume. Mice were dosed subcutaneously three times at three-weekly intervals and bleeds taken 1 day prior to the first dose and at one week after both the second and third doses.

Enzyme-linked immunosorbent assay (ELISA)

The immune mouse sera were examined for the presence of anti-E2 antibodies in ELISA. The E2-his, Con1 $E2_{RBD}$-myc or JFH1 $E2_{RBD}$-myc proteins were captured on 96 well Maxisorb microtitre plates (Nunc) using Galanthis nivalis (GNA) lectin. Plates were coated with GNA lectin at 5 μg/ml in PBS overnight at 4°C. Unoccupied sites were blocked with 100 μl $BSA_{10}PBS$ for 1 h at room temperature. After washing the plates 4 times with PBST, the E2 proteins were applied in 50 μl BSA_5PBST and incubated for 1 h

at room temperature. After washing the plates 4 times with PBST, serial dilutions of mouse sera were applied in a 50 µl volume of BSA_5PBST and incubated for 1 h room temperature. Bound immunoglobulins were detected with rabbit anti-mouse immunoglobulin coupled to horseradish peroxidase and developed using tetramethylbenzidine hydrochloride substrate and stopped by the addition of 1M HCl. A Fluostar plate reader (BMG technologies) was used to measure the absorbance at 450 nm and the background at 620 nm. The background was subtracted from the absorbance to generate the binding curves.

Neutralization Assays

The abilities of immune and control mouse sera to neutralize a single cycle of infection by HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses were determined as follows. HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses were prepared by cotransfection of HEK-293T monolayers (350,000 cells per well of 6-well culture dishes) with pE1E2 and pNL4.3LUCR-E- plasmids (Drummer et al., 2003). After 3 days incubation at 37 °C in a humidified atmosphere containing 5 % CO_2, the culture supernatants were filtered through 0.45 µm sterile syringe filters (Sartorius). Serial 5-fold dilutions of heat-inactivated immune and control mouse sera were preincubated with HCV glycoprotein-pseudotyped HIV-1 luciferase reporter viruses (1 h) and then added to quadruplicate Huh7 cell monolayers in 48-well tissue culture plates. Following a 4 h incubation (37 °C, 5 % CO_2) the cells were washed with PBS and the medium replaced. After an additional 3-day incubation (37 °C in 5 % CO_2), the cells were lysed, the lysates clarified by centrifugation and then assayed for luciferase activity (Promega) in a Fluostar (BMG) fitted with luminescence optics. The neutralization titres of individual sera were determined as the serum dilution giving 80 % neutralization compared to HCV glycoprotein-pseudotyped HIV-1 luciferase reporter virus preincubated with medium alone.

Table 7. Pair-wise statistical comparison of antibody titres reactive to solid-phase E2-his antigen obtained after 2nd vaccination. *p* values calculated using student's *t* test.

	Immunogen Group						
	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his
E2-his	0.06	0.96	0.25	0.26	0.37	0.6	0.08
E2 Δ1-his		0.06	0.27	0.09	**0.034**	**0.014**	**0.04**

Table 8. Pair-wise statistical comparison of antibody titres reactive to solid phase E2 Δ123-his protein antigen obtained after 2nd vaccination. *p* values calculated using student's *t* test.

	Immunogen Group						
	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his
E2-his	**0.04**	0.96	**0.02**	**0.02**	0.09	0.077	**0.003**
E2 Δ1-his		**0.04**	0.09	0.12	0.06	0.06	0.17

Table 9. Pair-wise statistical comparison of antibody titres reactive to solid-phase E2-his protein antigen obtained after 3rd vaccination. *p* values calculated using student's *t* test assuming unequal variances.

	Immunogen Group						
	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his
E2-his	**0.02**	**0.003**	**0.002**	**0.04**	**0.002**	**0.02**	0.11
E2 Δ1-his		0.1	**0.021**	0.58	0.44	0.9	0.17

Table 10. Pair-wise statistical comparison of antibody titres reactive to solid-phase E2 Δ123-his protein antigen obtained after 3rd vaccination. *p* values calculated using student's *t* test assuming unequal variances.

	Immunogen						
	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his
E2-his	0.64	1	**0.016**	**0.004**	0.31	**0.003**	**0.0007**
E2 Δ1-his		0.62	**0.022**	**0.007**	0.44	**0.005**	**0.009**

Table 11. Statistical comparison of relative antibody titres obtained after 3rd vaccination against E2-his antigen and E2 Δ123-his antigen. *p* values calculated using student's *t* test assuming unequal variances.

	Immunogen							
	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his	E2-his
Mean antibody titre against E2-his ($x10^5$)	1.16	0.81	0.68	1.31	.71	1.19	1.54	2.26
Mean antibody titre against E2 Δ123-his ($x10^5$)	0.56	0.51	1.15	1.11	0.71	1.19	1.54	0.51
p value	**0.005**	**0.045**	0.06	0.46	1	1	1	**0.0009**

Table 12. Pair-wise statistical comparison of antibody titres reactive to solid-phase Con1 $E2_{RBD}$-his protein antigen obtained after 3rd vaccination. *p* values calculated using student's *t* test assuming unequal variances.

	E2-his	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his
E2-his								
E2 Δ1-his	0.526							
E2 Δ2-his	**0.0453**	0.072						
E2 Δ3-his	**0.043**	0.061	0.883					
E2 Δ12-his	**0.0123**	0.091	0.926	0.797				
E2 Δ13-his	0.185	0.343	0.847	0.756	0.888			
E2 Δ23-his	0.173	0.568	0.284	0.238	0.217	0.534		
E2 Δ123-his	**0.0123**	**0.028**	0.259	0.325	0.197	0.251	**0.0499**	

Table 13. Pair-wise statistical comparison of antibody titres reactive to solid-phase JFH1 $E2_{RBD}$-myc protein antigen obtained after 3rd vaccination. *p* values calculated using student's *t* test assuming unequal variances.

	E2-his	E2 Δ1-his	E2 Δ2-his	E2 Δ3-his	E2 Δ12-his	E2 Δ13-his	E2 Δ23-his	E2 Δ123-his
E2-his								
E2 Δ1-his	0.609							
E2 Δ2-his	0.148	0.511						
E2 Δ3-his	0.159	0.253	0.371					
E2 Δ12-his	0.305	0.549	0.861	0.48				
E2 Δ13-his	0.392	0.624	0.916	0.484	0.970			
E2 Δ23-his	0.123	0.287	0.492	0.652	0.705	0.702		
E2 Δ123-his	0.058	0.084	0.117	0.406	0.154	0.156	0.209	

Table 14. Mean 80% Neutralizing antibody titres and statistical comparison. *p* values were calculated using student's *t* test assuming unequal variances.

	Immunogen group				
	E2-his	E2 Δ1-his	E2 Δ23-his	E2 Δ123-his	No antigen
Mean neutralizing antibody titre	908	7	140	413	26
p value relative to E2-his		**0.044**	**0.08**	0.29	**0.048**
p value relative to E2 Δ1-his			**0.002**	0.11	0.31

Table 15. Primers used for preparation of E2-his protein variant DNA sequences.

Primer designation	Primer Sequence
HCV-1	5' TATAGCTAGCGCCACCATGAACCCCCTGC 3'
HCV-2	5' CTGGATGTTCTGGTGGGTCTCGGCCAG 3'
HCV-3	5' GCCGAGACCCACCAGAACATCCAGCTG 3'
HCV-4	5' CGCTGCTGCCGCAGGAGGCGAG 3'
HCV-4a	5' GTAGTGCCAGCAGCCGCTGCTGCCGCAGGAGGCGAGCCTCTCGGG 3'
HCV-5	5' GGCAGCAGCGGCTGCTGGCACTAC 3
HCV-5a	5' CTCGCCTCCTGCGGCAGCAGCGGCTGCTGGCACTACCCCCCCAGA 3'
HCV-6a	5' GTCGGTGGGGCAGCCGCTGCTGCCGCAGGGAGGGGCGCCACACA C 3'
HCV-7a	5' GCCCCTCCCTGCGGCAGCAGCGGCTGCCCCACCGACTGCTTTAGG 3'
HCV-8	5' TCTGCTCGAGTTATCAGTGGTGATGGTGGTGG 3'
HCV-11	5' CTCTCGTTGCAGTTCAGGGCGGTGCTG 3'
HCV-12	5' CAGCACCGCCCTGAACTGCAACGAGAG 3'

Table 16. Primer combinations used for preparation of E2-his protein variant DNA sequences.

E2 protein	Primer combination
E2Δ1-his	HCV-1 HCV-3 HCV-8
E2Δ2-his	HCV-1 HCV-11 HCV-12 HCV-8
E2Δ3-his	HCV-1 HCV-6a HCV-7a HCV-8
E2Δ12-his	HCV-1 HCV-2 HCV-3 HCV-4 HCV-5
E2Δ13-his	HCV-1 HCV-6a HCV-7a HCV-8
E2Δ23-his	HCV-1 HCV-5a HCV-4A HCV-6A HCV-7A HCV-8
E2Δ123-his	HCV-1 HCV-6a HCV-7a HCV-8

Table 17. Primers used for preparation of Con1 $E2_{RBD}$-his and JFH1 $E2_{RBD}$-myc DNA sequences

$E2_{RBD}$ construct		Primer Sequence	RE site	Epitope tag
Con1 $E2_{RBD}$-his	Forward primer	5'-CAAGCTAGCggaacctatgtgacaggg	NheI	
	Reverse primer	5'-CCCTCTAGATTAGTGGTGGTGGTGGTGGCCGCCCTCTGATCTGTCCCTGTC	XbaI	His_6
JFH1 $E2_{RBD}$-myc	Forward primer	5' CAAGCTAGCggcaccaccaccgttggaggc	NheI	
	Reverse primer	5'CCGTCTAGActaattcagatcctcttctgagatgagttttgttcagtactCTGACTCCTGTCCCTGTC	XbaI	myc

Table 18. Immunisation of mice with E2-his wild type and variant proteins formulated with ISCOMATRIX® adjuvant Antigens

Group	Protein	ISCOMATRIX® adjuvant
1	E2Δ1-his	+
2	E2Δ2-his	+
3	E2Δ3-his	+
4	E2Δ123-his	+
5	E2Δ12-his	+
6	E2Δ13-his	+
7	E2Δ23-his	+
8	E2-his	+
9	-	+

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CLAIMS:

1. A modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein in at least one of said variable regions at least a part of the variable region is replaced with a flexible linker sequence.

2. A modified glycoprotein according to claim 1, wherein in two of said variable regions at least a part of the variable region is replaced with a flexible linker sequence.

3. A modified glycoprotein according to claim 2, wherein said two variable regions are the HVR1 and HVR2 variable regions.

4. A modified glycoprotein according to claim 2, wherein said two variable regions are the HVR1 and igVR variable regions.

5. A modified glycoprotein according to claim 2, wherein said two variable regions are the HVR2 and igVR variable regions.

6. A modified glycoprotein according to claim 1, wherein in all three of said variable regions at least a part of the variable region is replaced with a flexible linker sequence.

7. A modified glycoprotein according to claim 1, wherein in at least one of the HVR2 and igVR variable regions at least a part of the variable region is replaced with a flexible linker sequence.

8. A modified glycoprotein according to claim 7, wherein in both of the HVR2 and igVR variable regions at least a part of the variable region is replaced with a flexible linker sequence.

9. A modified glycoprotein according to claim 7, wherein in the HVR2 variable

region at least a part of the variable region is replaced with a flexible linker sequence.

10. A modified glycoprotein according to claim 7, wherein in the igVR variable region at least a part of the variable region is replaced with a flexible linker sequence.

11. A modified glycoprotein according to any of claims 7 to 10 wherein at least a part of the HVR1 variable region is removed or is replaced with a flexible linker sequence.

12. A modified hepatitis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein at least a part of the HVR2 variable region is removed or is replaced with a flexible linker sequence.

13. A modified glycoprotein according to claim 12, wherein in at least one of the HVR1 and igVR variable regions at least a part of the variable region is replaced with a flexible linker sequence.

14. A modified heptatis C virus (HCV) E2 glycoprotein comprising the HCV-E2 receptor-binding domain (RBD) including the HVR1, HVR2 and igVR variable regions, wherein at least a part of the igVR variable region is removed or is replaced with a flexible linker sequence.

15. A modified glycoprotein according to claim 14, wherein in at least one of the HVR1 and HVR2 variable regions at least a part of the variable region is replaced with a flexible linker sequence.

16. A modified glycoprotein according to any of claims 1 to 15, wherein the or each linker sequence permits the glycoprotein to bind to the HCV receptor CD81.

17. A modified glycoprotein according to claim 16, wherein the or each linker sequence comprises a peptide sequence of up to 20 amino acid residues and comprises residues selected from the group consisting of Gly,Ser, Ala, Thr and Arg.

18. A modified glycoprotein according to claim 17, wherein the or each linker sequence comprises a Gly-Ser-Ser-Gly (GSSG) sequence.

19. A modified glycoprotein according to claim 17, wherein the sequence of the HVR1 variable region, if modified to include a linker sequence, comprises a Glu-Thr-His-Gly-Ser-Ser-Gly (ETHGSSG) sequence.

20. A composition comprising a modified HCV E2 glycoprotein according to any of claims 1 to 19, together with a pharmaceutically acceptable carrier or diluent.

21. A composition according to claim 20, further comprising an adjuvant.

22. A method of eliciting an immune response in a patient, which comprises administration to the patient of an effective amount of a modified HCV E2 glycoprotein according to any of claims 1 to 19.

23. A method for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises administration to the patient of an effective amount of a modified HCV E2 glycoprotein according to any of claims 1 to 19.

24. A method according to claim 22 or claim 23 wherein the patient is a human.

25. Use of a modified HCV E2 glycoprotein according to any of claims 1 to 19 in, or in the manufacture of a medicament for, eliciting an immune response in a patient.

26. Use of a modified HCV E2 glycoprotein according to any of claims 1 to 19 in, or in the manufacture of a medicament for, prophylactic or therapeutic treatment of HCV infection in a patient.

27. An agent for eliciting an immune response in a patient, which comprises a modified HCV E2 glycoprotein according to any of claims 1 to 19.

28. An agent for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises a modified HCV E2 glycoprotein according to any of claims 1 to 19.

29. An isolated antibody raised against a modified HCV E2 glycoprotein according to any of claims 1 to 19.

30. An isolated antibody according to claim 29 which is a polyclonal antibody.

31. An isolated antibody according to claim 29 which is a monoclonal antibody.

32. A method for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises administration to the patient of an effective amount of an antibody according to any of claims 29 to 31.

33. Use of an antibody according to any of claims 29 to 31 in, or in the manufacture of a medicament for, prophylactic or therapeutic treatment of HCV infection in a patient.

34. An agent for prophylactic or therapeutic treatment of HCV infection in a patient, which comprises an antibody according to any of claims 29 to 31.

35. A method of detecting HCV infection in a patient, comprising contacting a biological sample from the patient with an antibody according to any of claims 29 to 31 under conditions which allow formation of an antibody-antigen

complex, and detecting said complex, wherein formation of said complex is indicative of the presence of HCV in the sample.

36. A method according to claim 35, wherein the antibody is detectably labelled.

37. A method according to claim 35, wherein the biological sample is a sample of a body fluid, such as blood, from the patient.

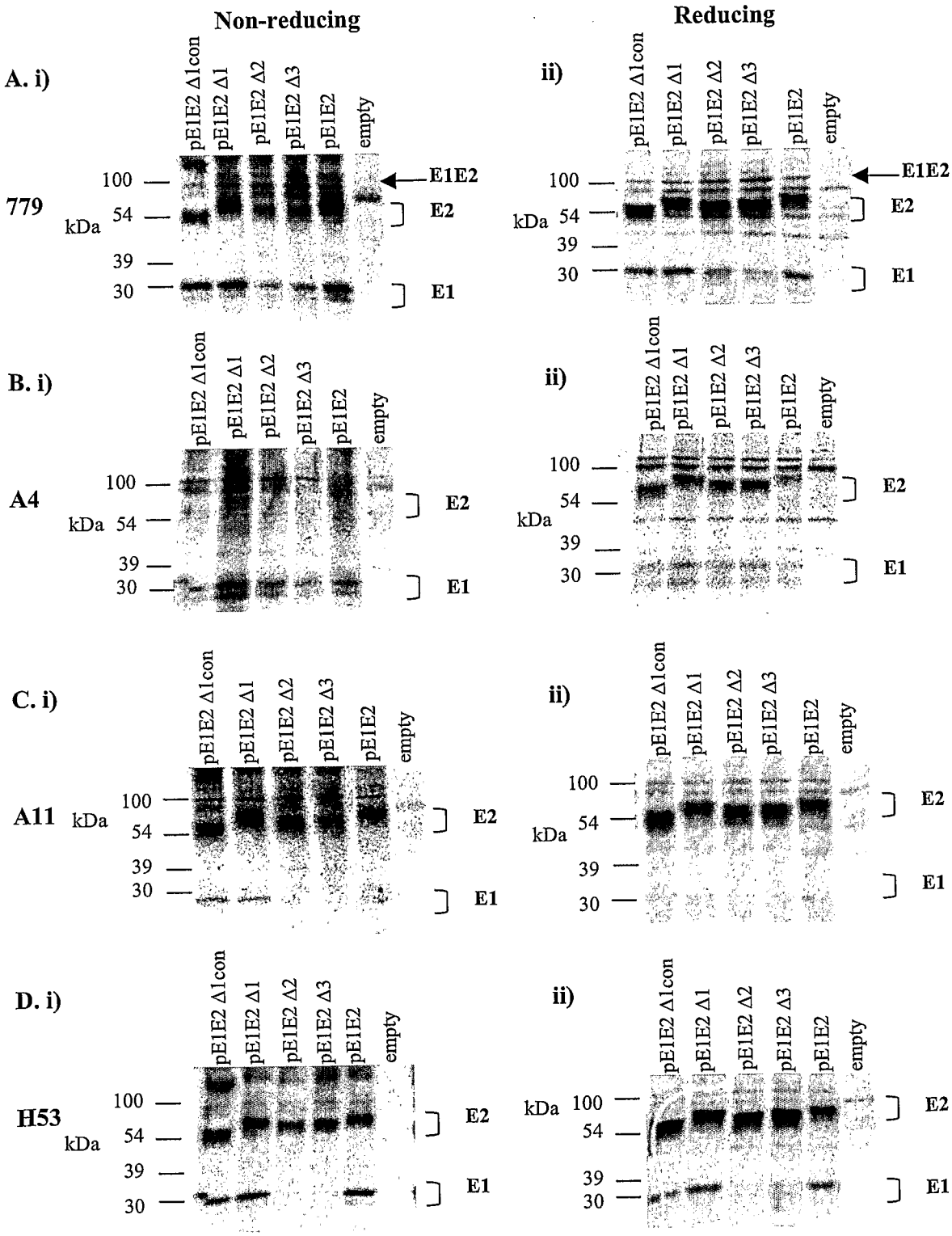
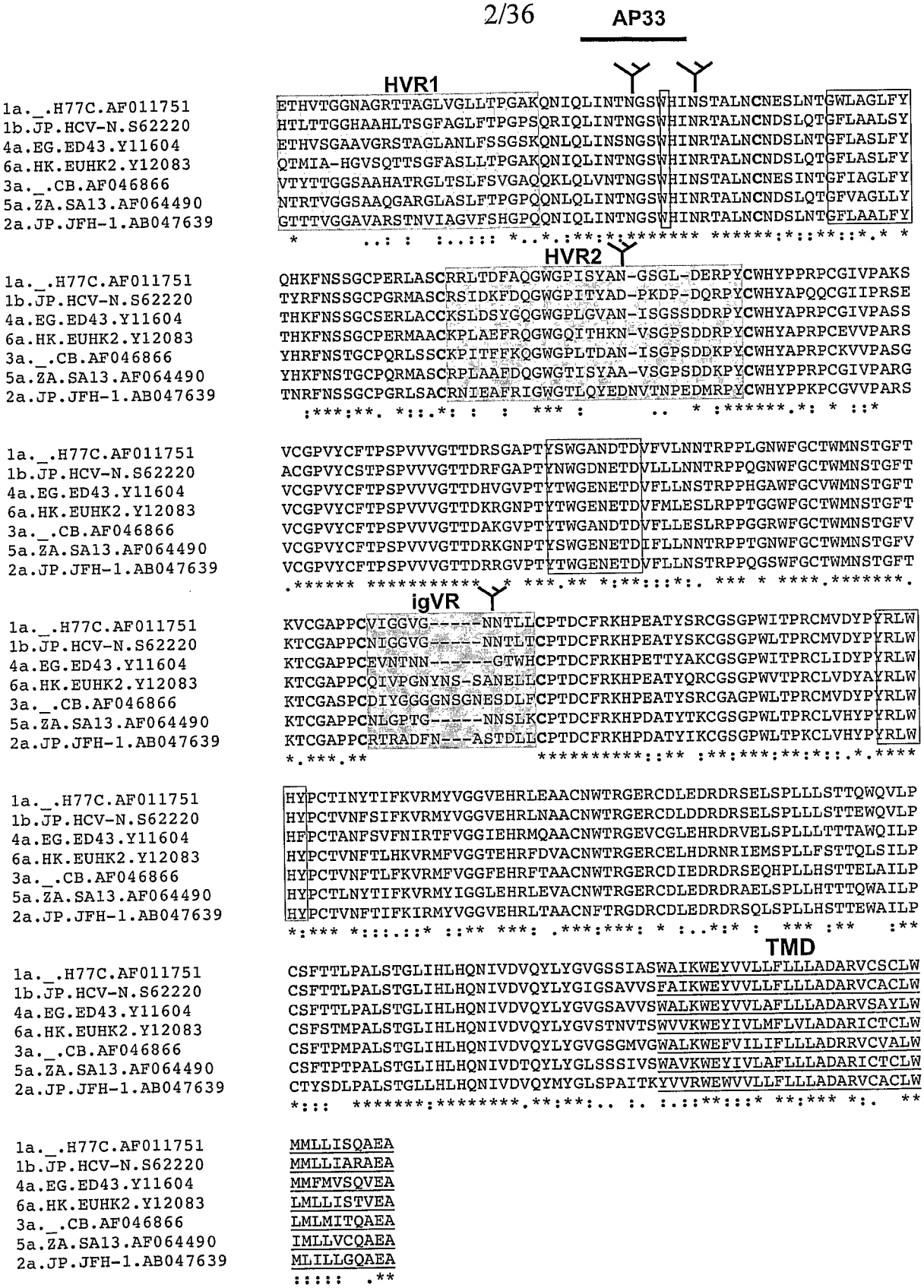

Non-reducing
Reducing
A. i)
ii)
779
B. i)
ii)
A4
C. i)
ii)
A11
D. i)
ii)
H53
pE1E2 Δ1con
pE1E2 Δ1
pE1E2 Δ2
pE1E2 Δ3
pE1E2
empty
kDa
100
54
39
30
E1E2
E2
E1

Figure 1.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HVR2
igVR
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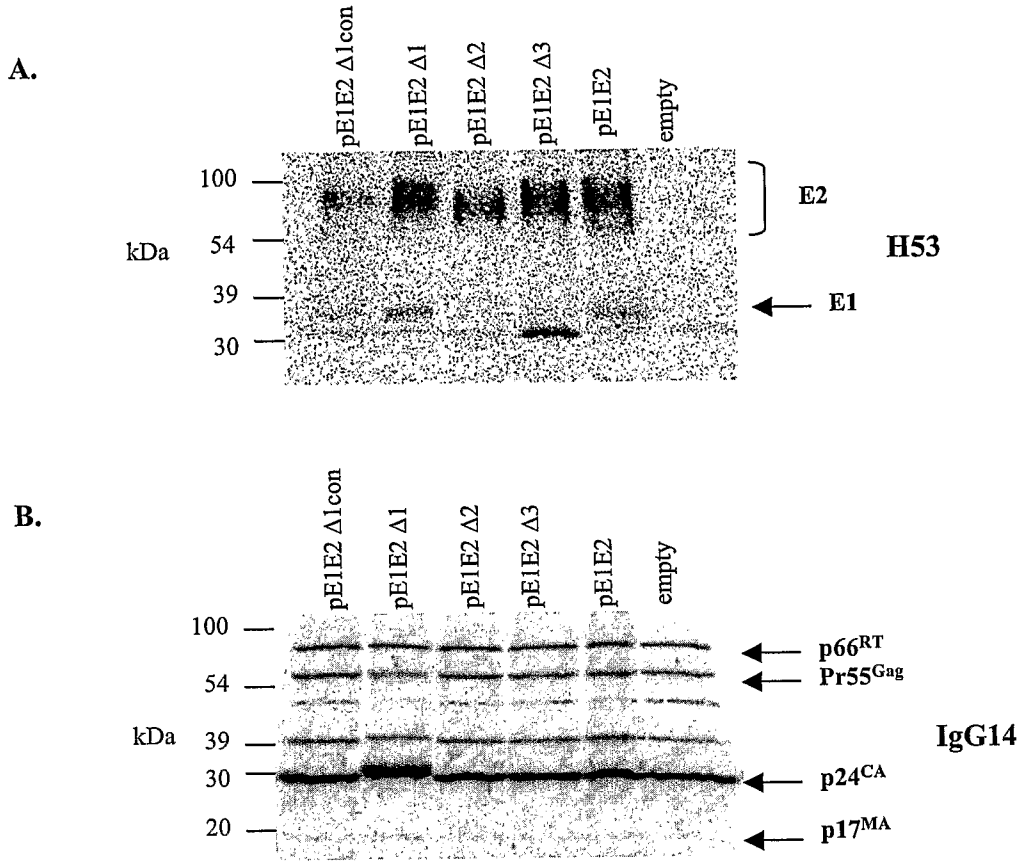
Figure 2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pE1E2 Δ1
pE1E2 Δ2
pE1E2 Δ3
pE1E2
empty
100
54
39
30
kDa
E2
E1
H53
B.
pE1E2 Δ1con
pE1E2 Δ1
pE1E2 Δ2
pE1E2 Δ3
pE1E2
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100
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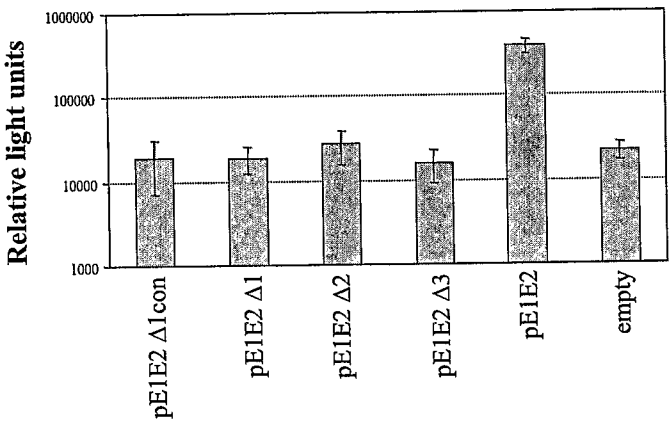
30
20
kDa
p66RT
Pr55Gag
p24CA
p17MA
IgG14

Figure 3.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100000
10000
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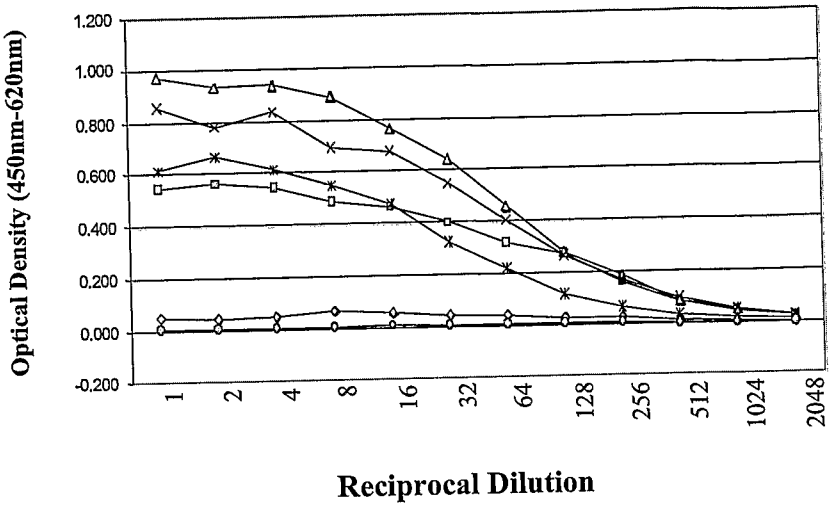
pE1E2 Δ1
pE1E2 Δ2
pE1E2 Δ3
pE1E2
empty

Figure 4.

A.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0.400
0.200
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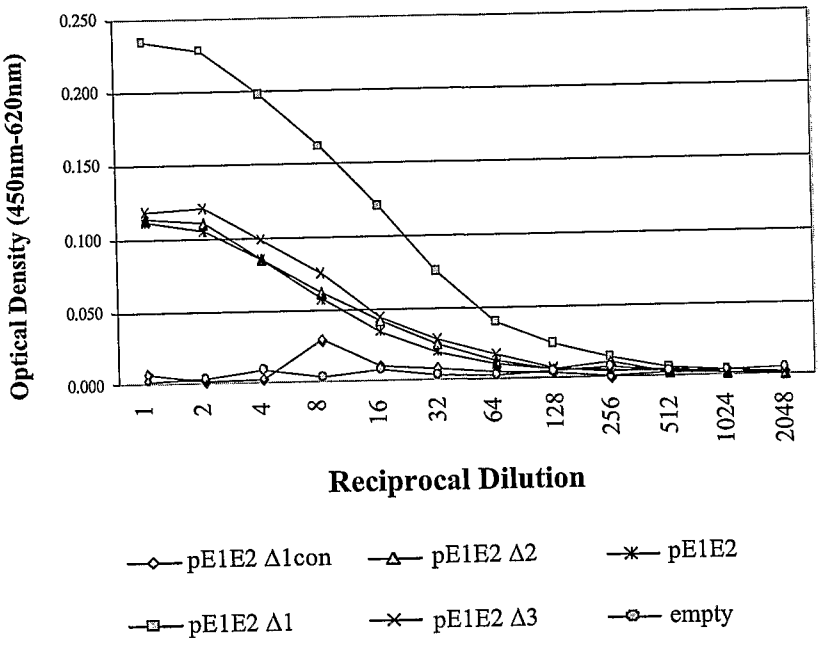
2
4
8
16
32
64
128
256
512
1024
2048
Reciprocal Dilution

B.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8
16
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1024
2048
Reciprocal Dilution
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pE1E2 Δ2
pE1E2
pE1E2 Δ1
pE1E2 Δ3
empty

Figure 5.

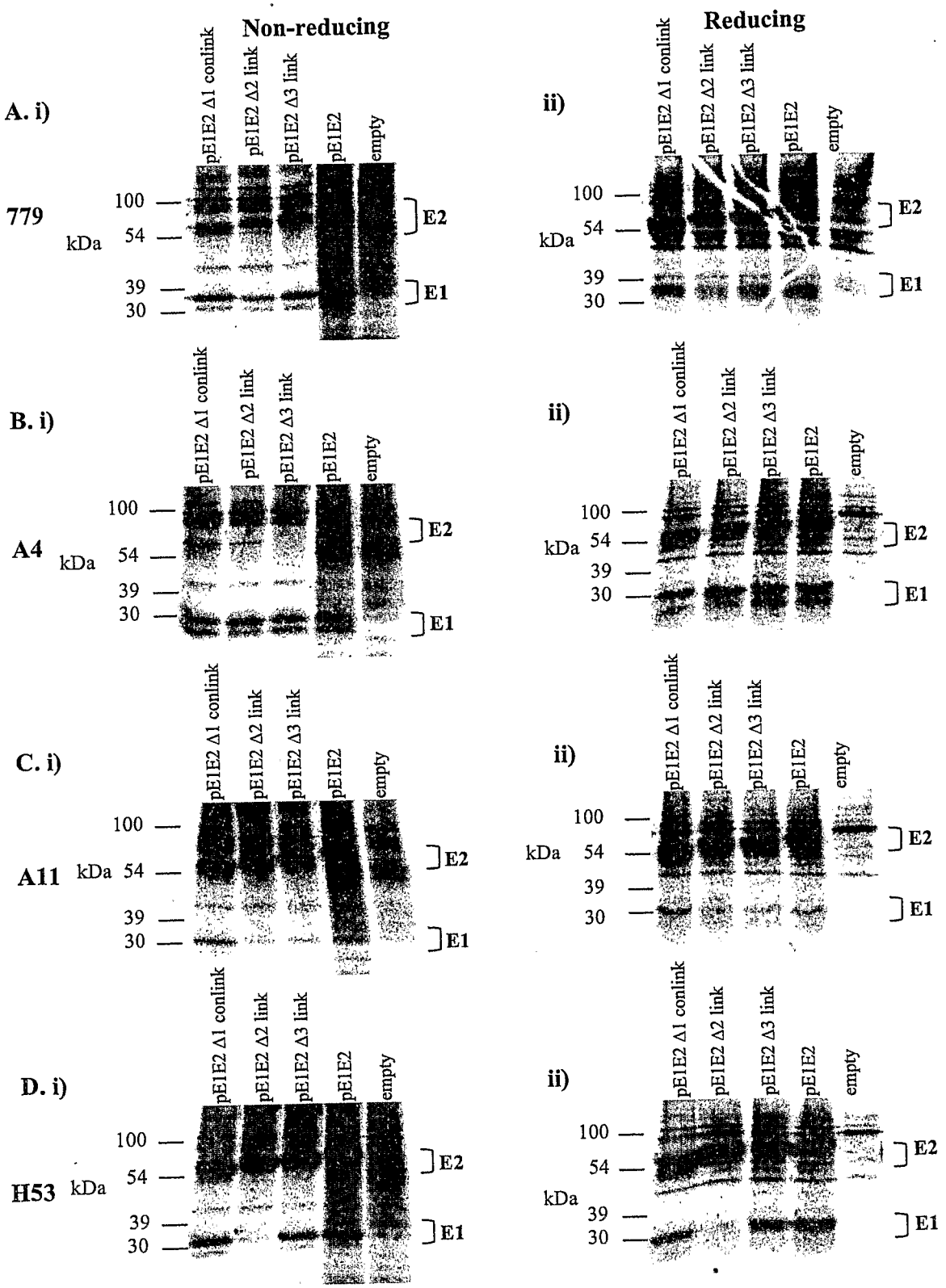
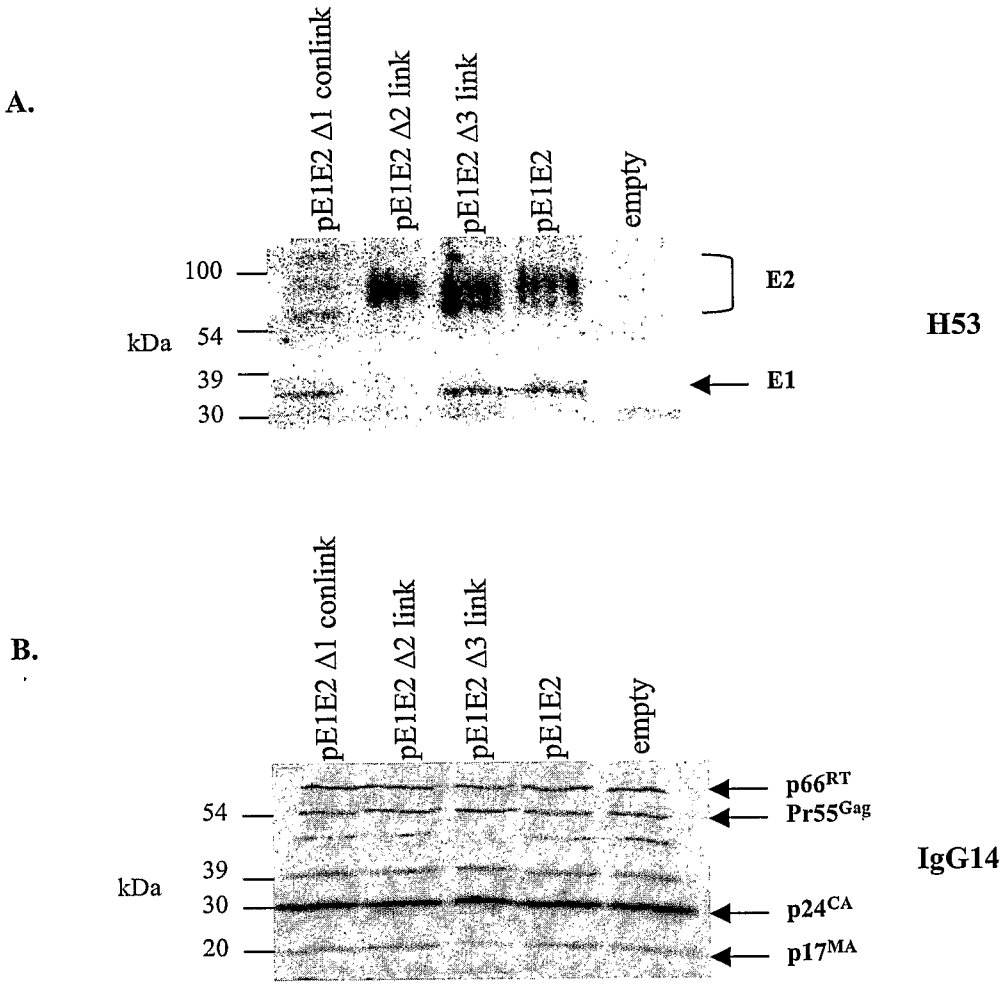

Non-reducing
Reducing
A. i)
ii)
779
B. i)
ii)
A4
C. i)
ii)
A11
D. i)
ii)
H53
pE1E2 Δ1 conlink
pE1E2 Δ2 link
pE1E2 Δ3 link
pE1E2
empty
kDa
100
54
39
30
E2
E1

Figure 6.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pE1E2 Δ2 link
pE1E2 Δ3 link
pE1E2
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100
54
39
30
kDa
E2
E1
H53
B.
pE1E2 Δ1 conlink
pE1E2 Δ2 link
pE1E2 Δ3 link
pE1E2
empty
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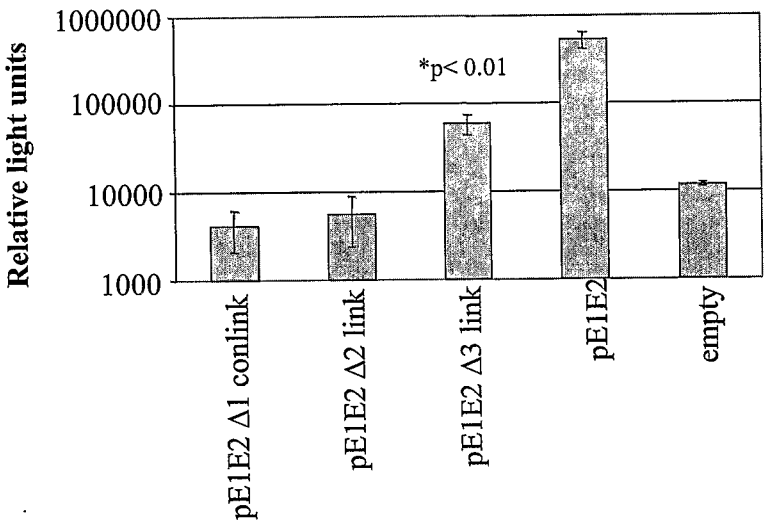
30
20
kDa
p66RT
Pr55Gag
p24CA
p17MA
IgG14

Figure 7.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10000
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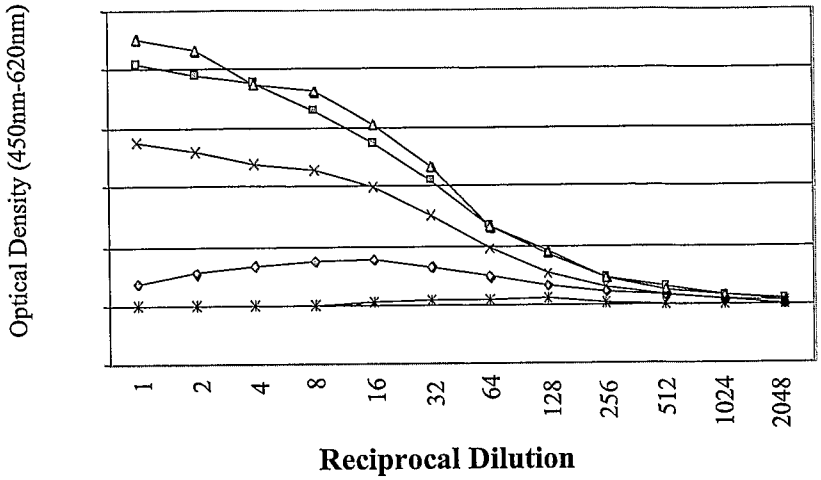
pE1E2 Δ2 link
pE1E2 Δ3 link
pE1E2
empty

Figure 8.

A.


Optical Density (450nm-620nm)
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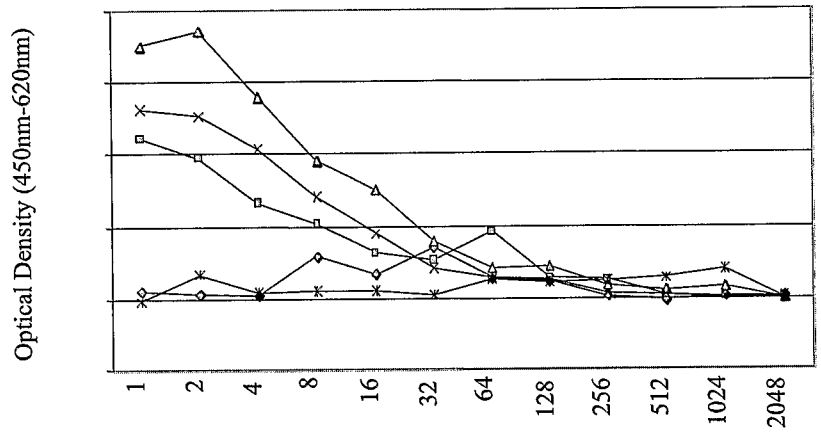
4
8
16
32
64
128
256
512
1024
2048
Reciprocal Dilution

B.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16
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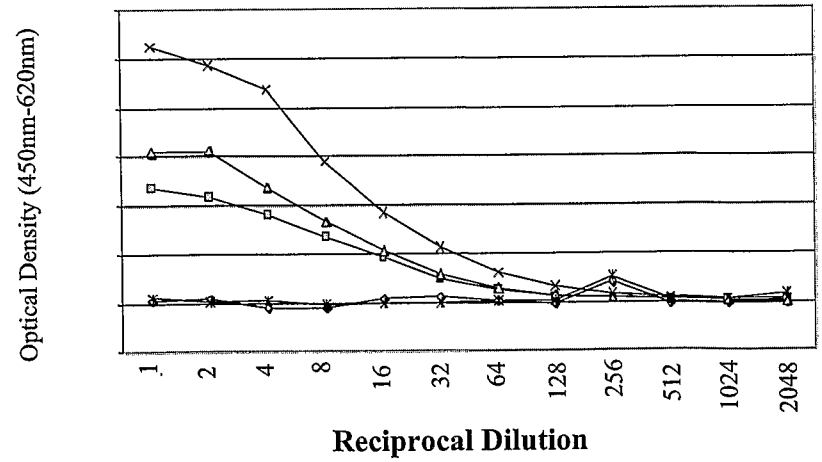
64
128
256
512
1024
2048
Reciprocal Dilution

C.


Optical Density (450nm-620nm)
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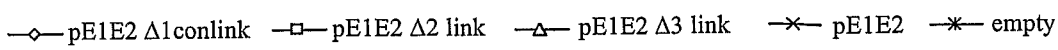
1024
2048
Reciprocal Dilution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pE1E2 Δ3 link
pE1E2
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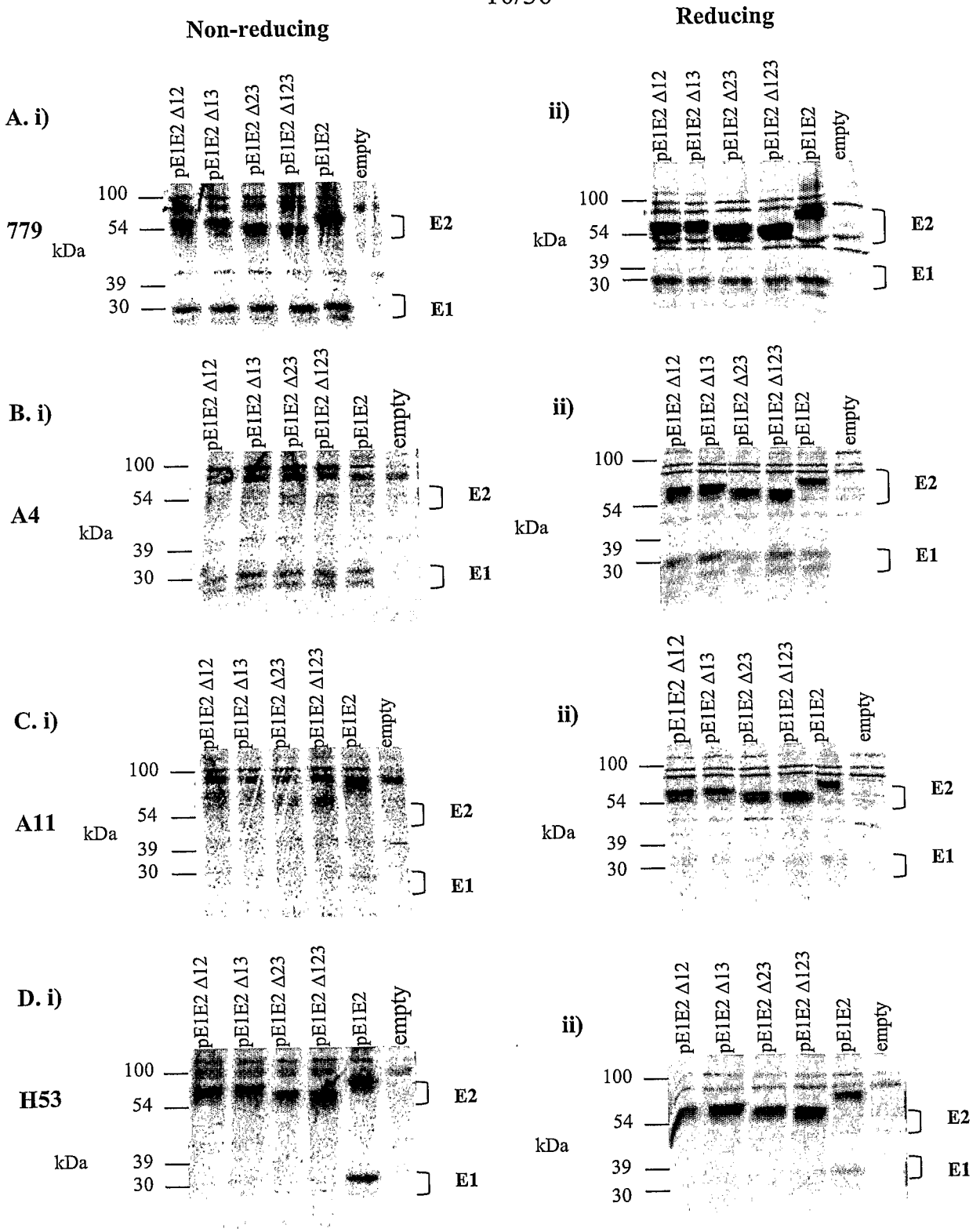
Figure 9.


Non-reducing
Reducing
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ii)
779
B. i)
ii)
A4
C. i)
ii)
A11
D. i)
ii)
H53
pE1E2 Δ12
pE1E2 Δ13
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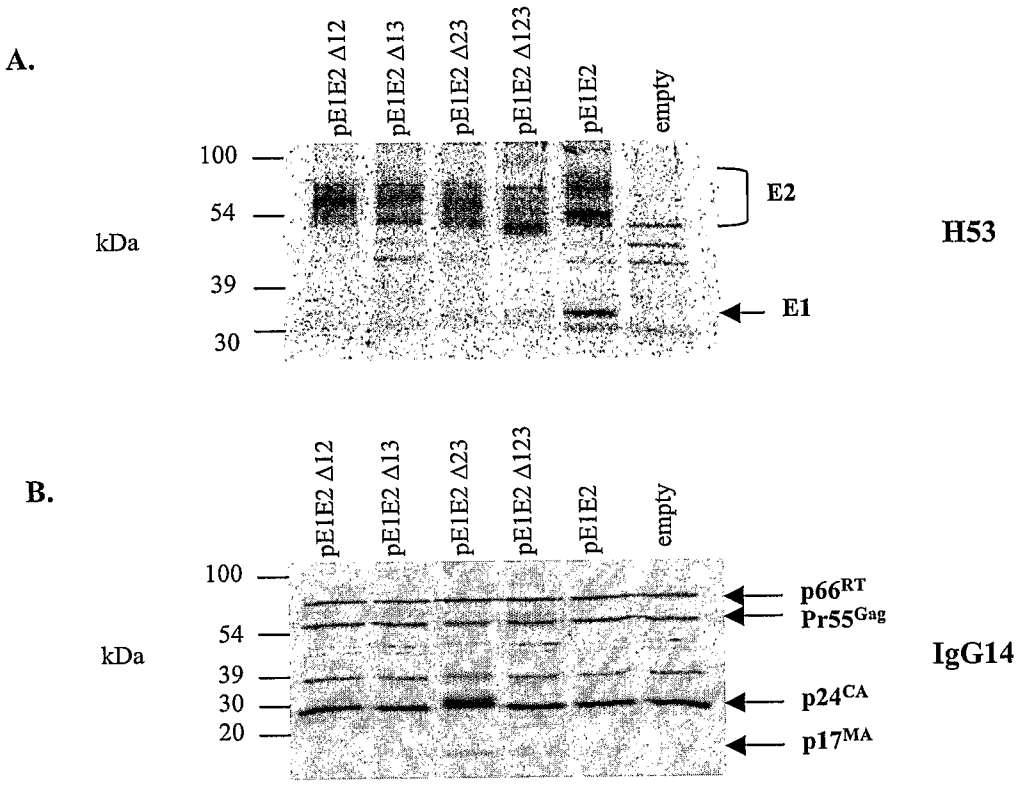
39
30
kDa
E2
E1

Figure 10.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pE1E2 Δ23
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kDa
E2
E1
H53
B.
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Figure 11.

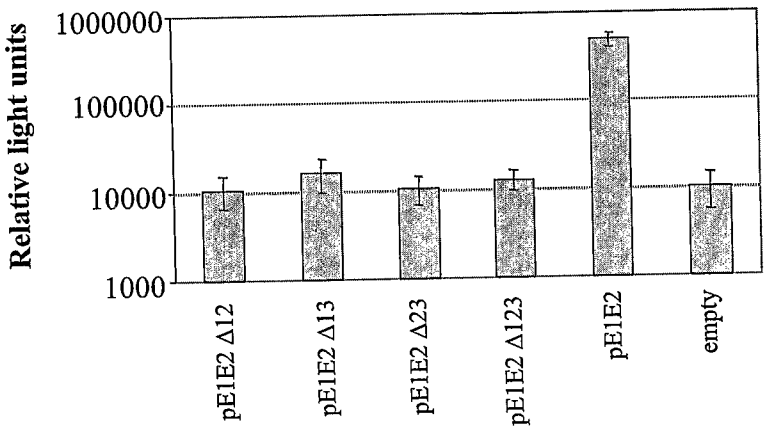
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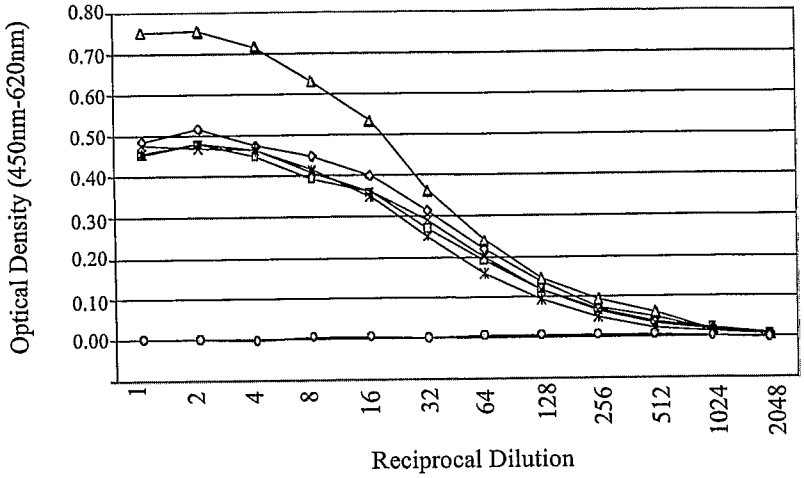
Figure 12.

A.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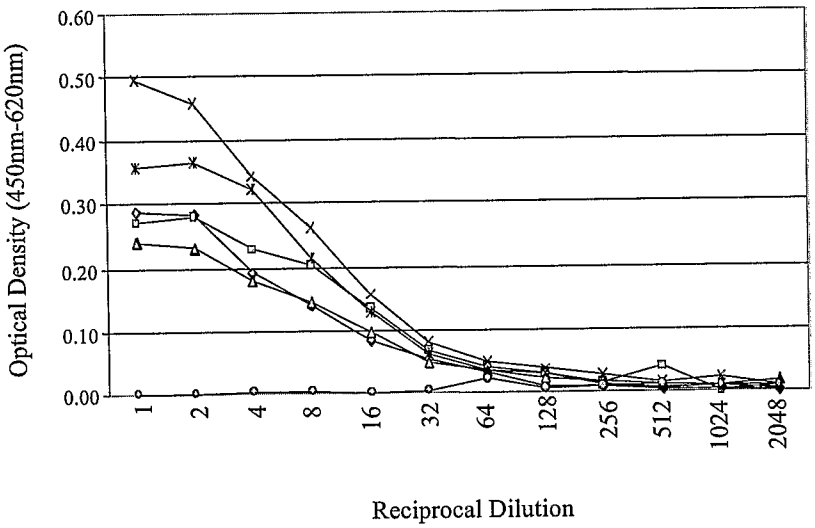
512
1024
2048
Reciprocal Dilution

B.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1024
2048
Reciprocal Dilution
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pE1E2 Δ123
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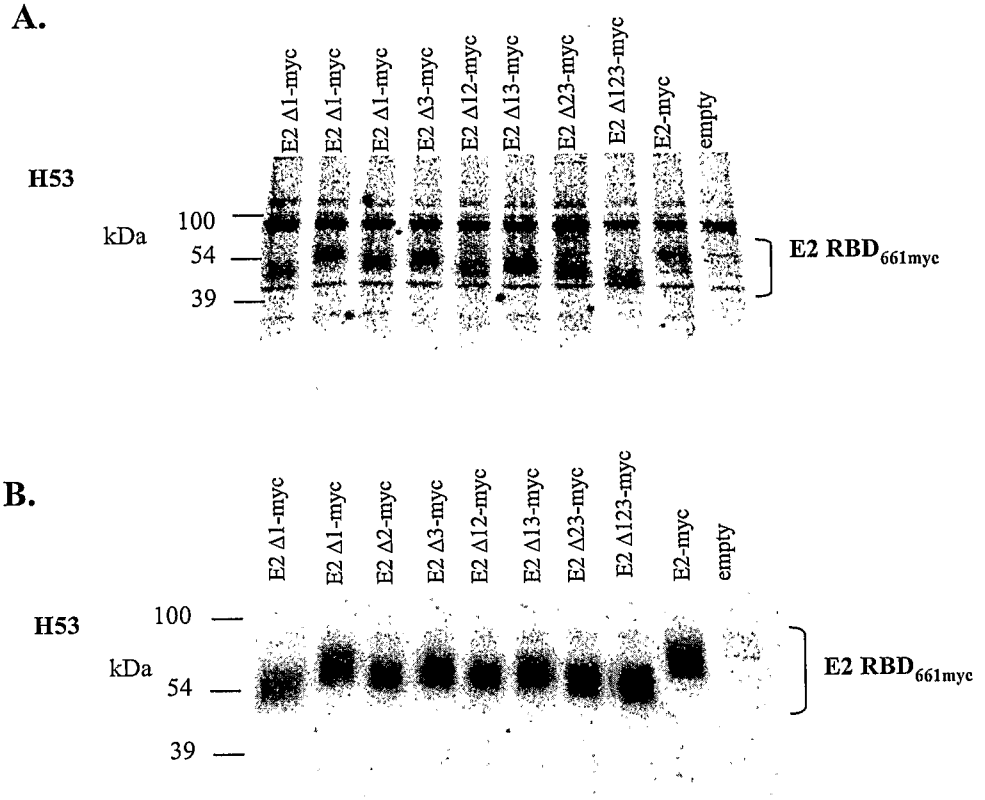
Figure 13.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E2 Δ23-myc
E2 Δ123-myc
E2-myc
empty
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B.
H53
kDa
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39
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E2 Δ1-myc
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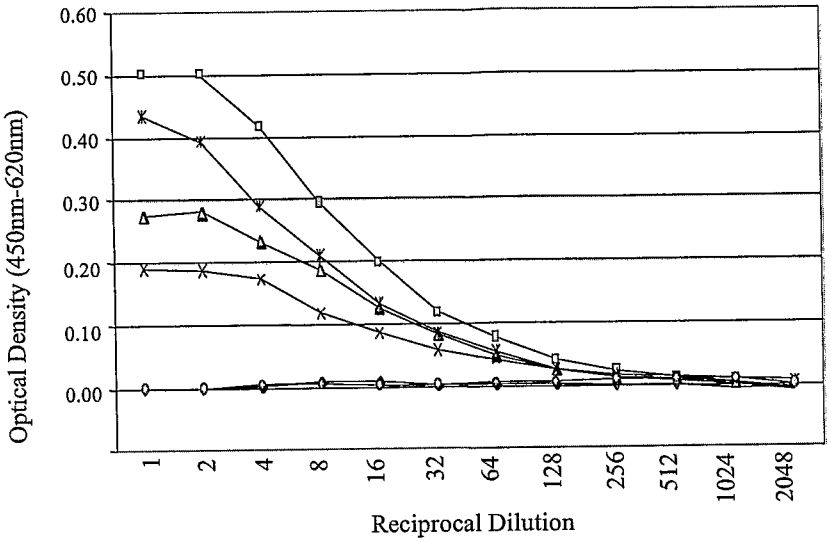
Figure 14.

A.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0.10
0.00
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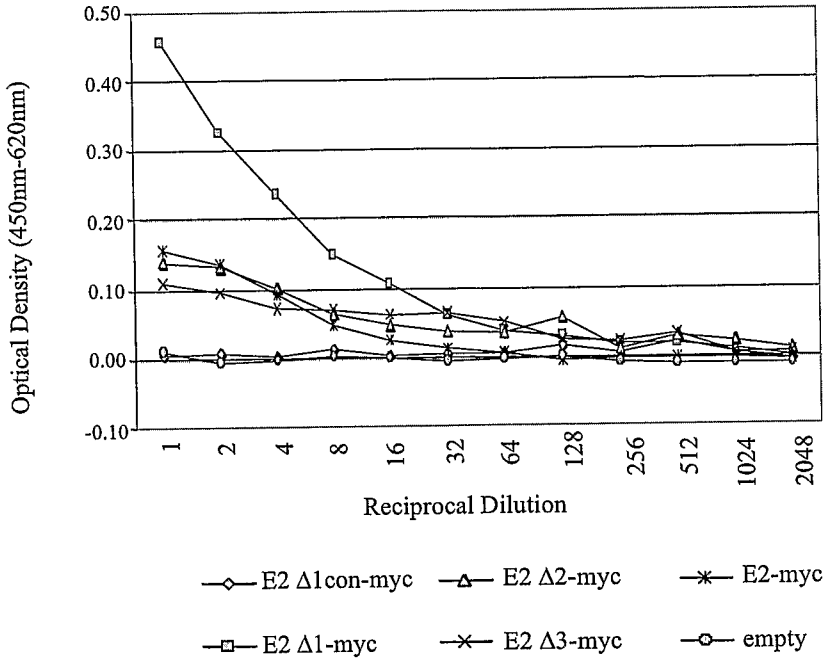
128
256
512
1024
2048
Reciprocal Dilution

B.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0.20
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0.00
-0.10
Optical Density (450nm-620nm)
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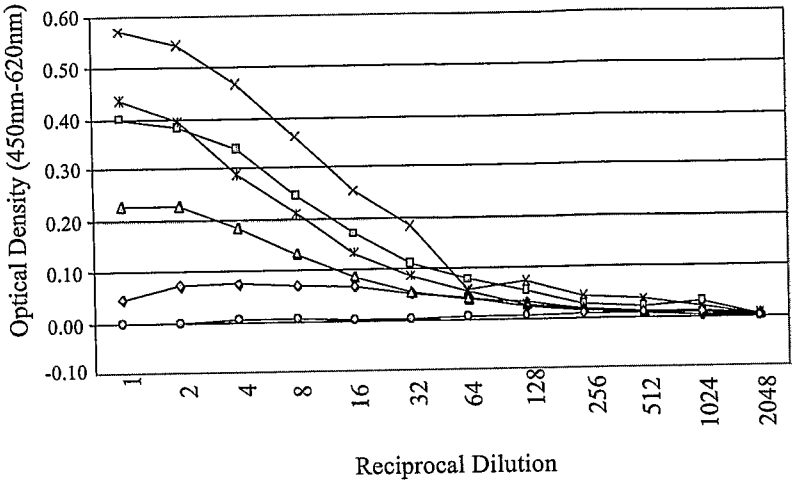
16
32
64
128
256
512
1024
2048
Reciprocal Dilution
E2 Δ1con-myc
E2 Δ2-myc
E2-myc
E2 Δ1-myc
E2 Δ3-myc
empty

Figure 15.

A.


Optical Density (450nm-620nm)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
1
2
4
8
16
32
64
128
256
512
1024
2048
Reciprocal Dilution

B.

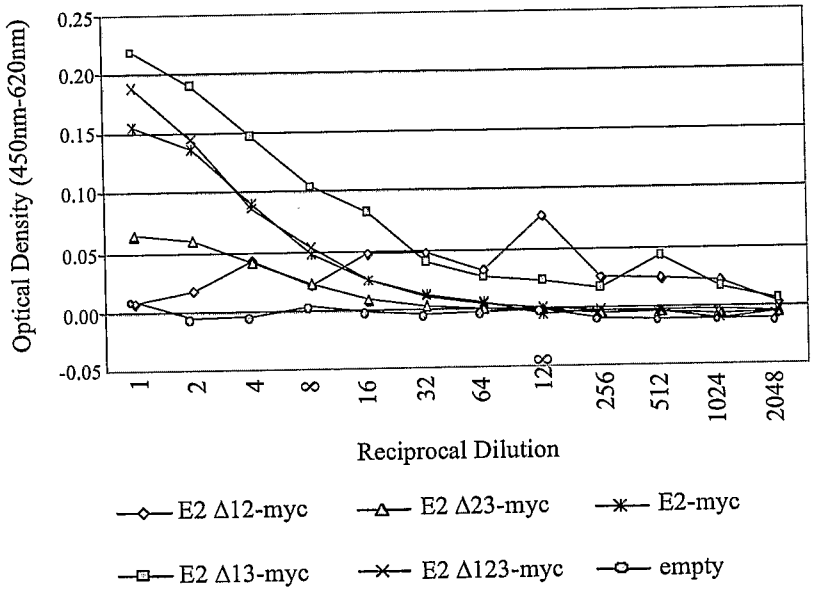

Optical Density (450nm-620nm)
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
1
2
4
8
16
32
64
128
256
512
1024
2048
Reciprocal Dilution
E2 Δ12-myc
E2 Δ23-myc
E2-myc
E2 Δ13-myc
E2 Δ123-myc
empty

Figure 16.

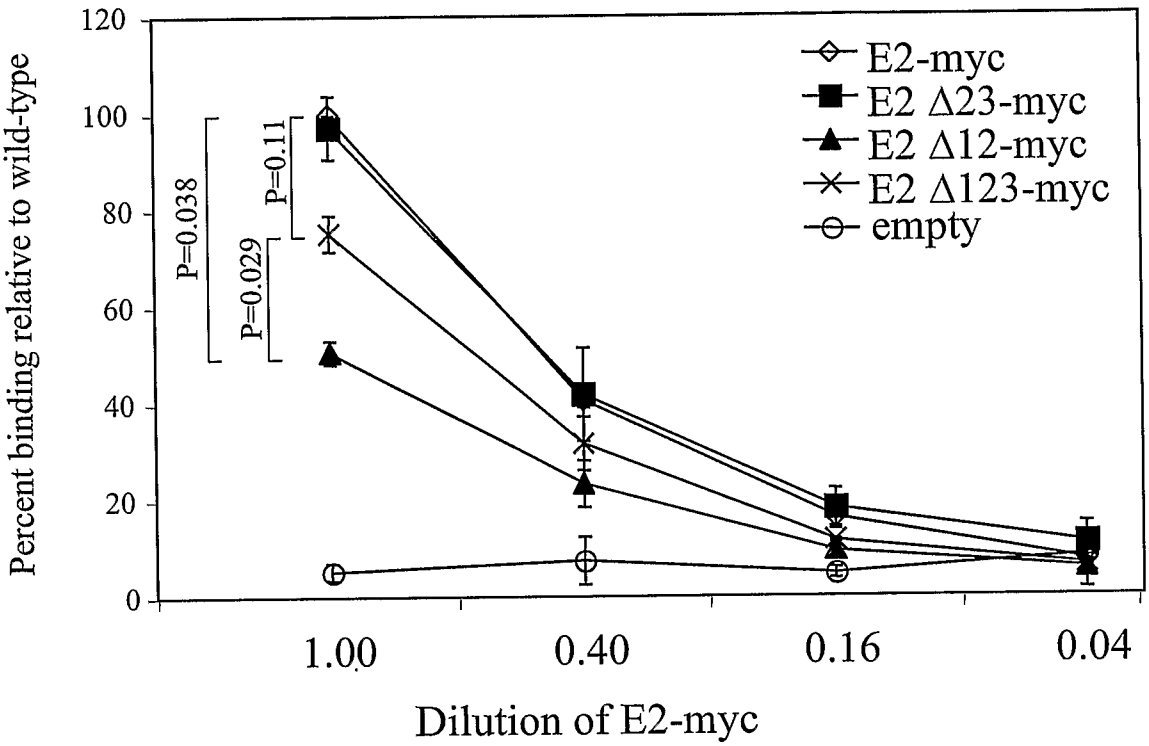

120
100
80
60
40
20
0
Percent binding relative to wild-type
P=0.038
P=0.11
P=0.029
E2-myc
E2 Δ23-myc
E2 Δ12-myc
E2 Δ123-myc
empty
1.00
0.40
0.16
0.04
Dilution of E2-myc

Figure 17.

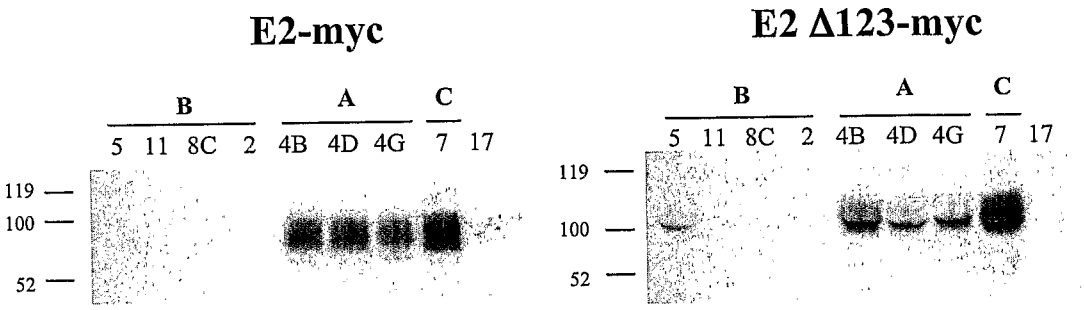

E2-myc
B
A
C
5 11 8C 2 4B 4D 4G 7 17
119
100
52
E2 Δ123-myc
B
A
C
5 11 8C 2 4B 4D 4G 7 17
119
100
52

Figure 18.

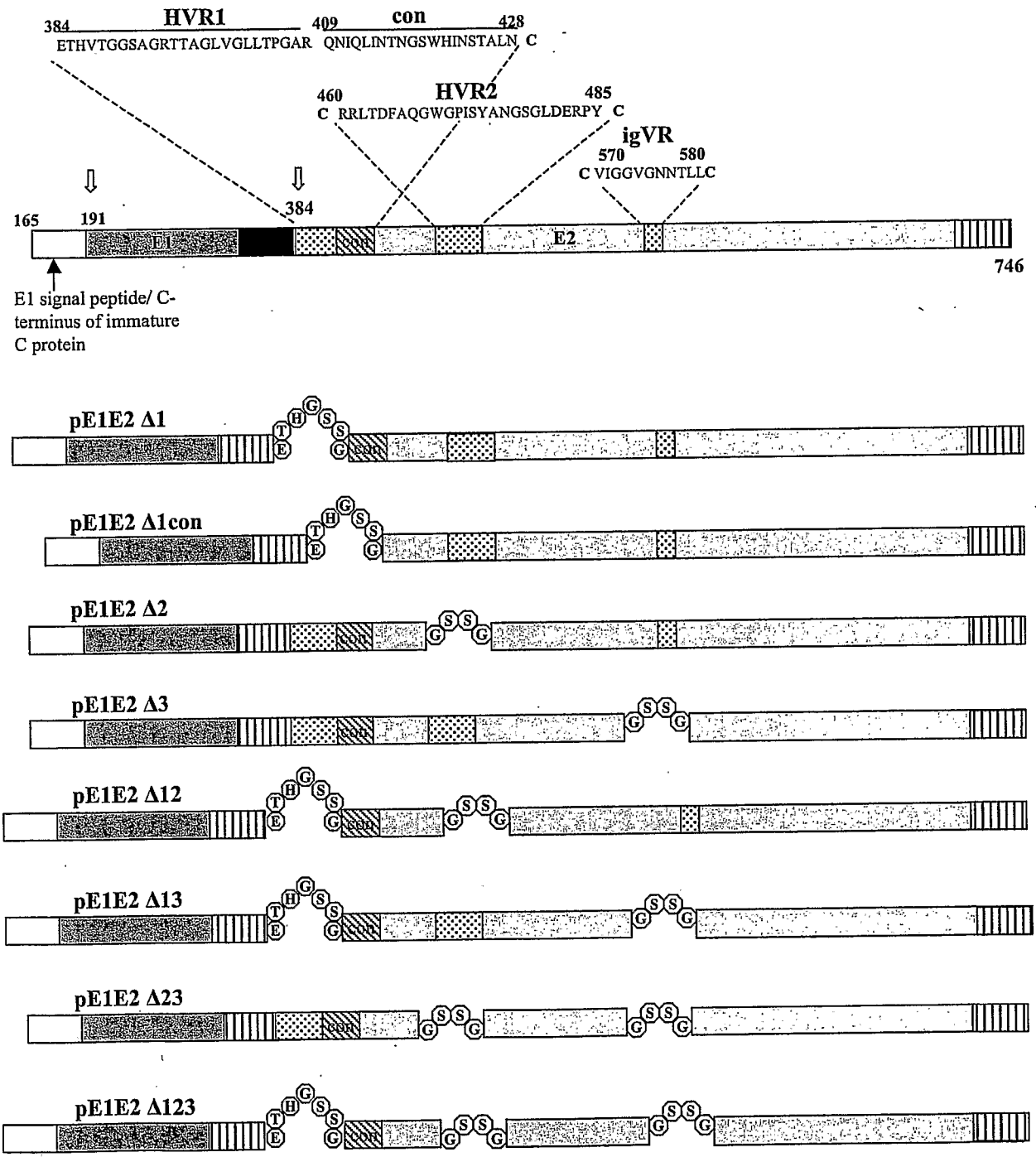

384
HVR1
409
con
428
ETHVTGGSAGRTTAGLVGLLTPGAR QNIQLINTNGSWHINSTALN C
460
HVR2
485
C RRLTDFAQGWGPISYANGSGLDERPY C
igVR
570
580
C VIGGVGNNTLLC
165
191
384
E1
E2
746
E1 signal peptide/ C-terminus of immature C protein
pE1E2 Δ1
pE1E2 Δ1con
pE1E2 Δ2
pE1E2 Δ3
pE1E2 Δ12
pE1E2 Δ13
pE1E2 Δ23
pE1E2 Δ123

Figure 19

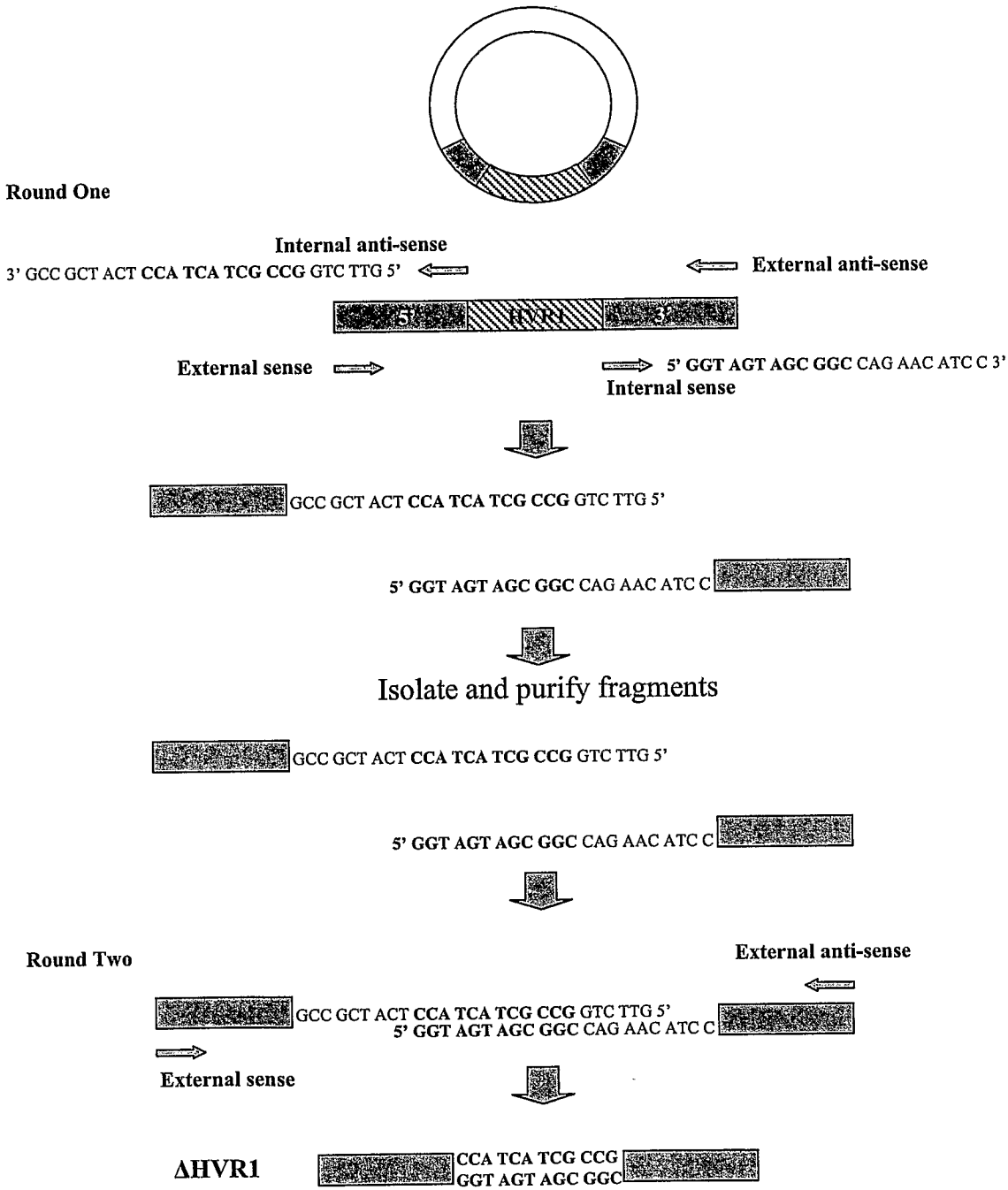

Round One
Internal anti-sense
3' GCC GCT ACT CCA TCA TCG CCG GTC TTG 5'
External anti-sense
5'
HVR1
3'
External sense
5' GGT AGT AGC GGC CAG AAC ATC C 3'
Internal sense
GCC GCT ACT CCA TCA TCG CCG GTC TTG 5'
5' GGT AGT AGC GGC CAG AAC ATC C
Isolate and purify fragments
GCC GCT ACT CCA TCA TCG CCG GTC TTG 5'
5' GGT AGT AGC GGC CAG AAC ATC C
Round Two
External anti-sense
GCC GCT ACT CCA TCA TCG CCG GTC TTG 5'
5' GGT AGT AGC GGC CAG AAC ATC C
External sense
ΔHVR1
CCA TCA TCG CCG
GGT AGT AGC GGC

Figure 20.

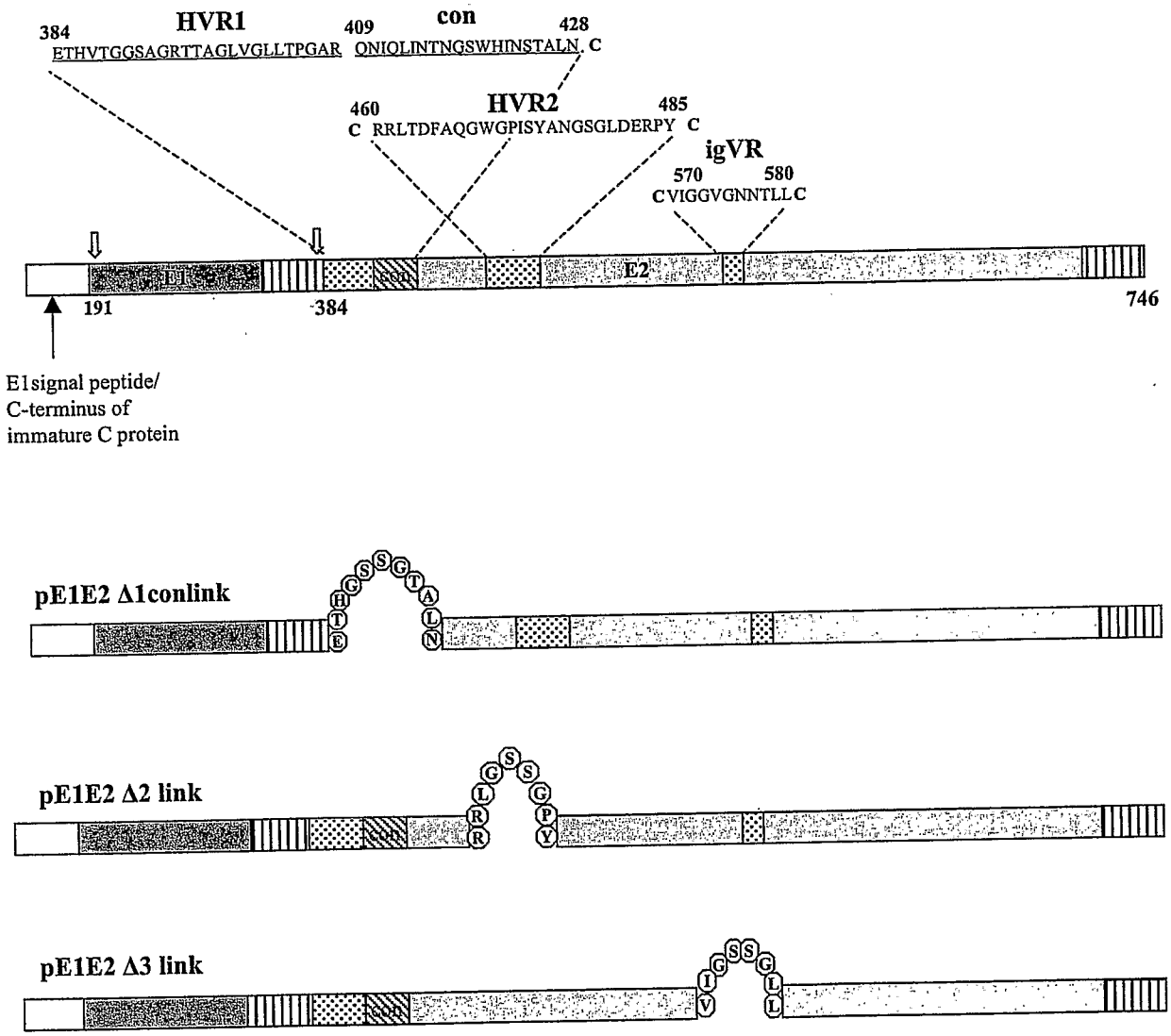
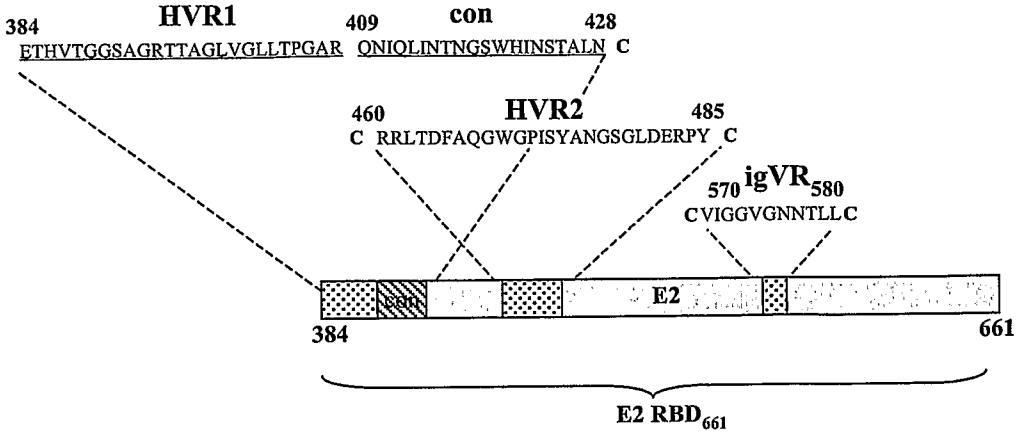

384
HVR1
409
con
428
ETHVTGGSAGRTTAGLVGLLTPGAR QNIQLINTNGSWHINSTALN C
460
HVR2
485
C RRLTDFAQGWGPISYANGSGLDERPY C
igVR
570
580
CVIGGVGNNTLLC
E2
191
384
746
E1signal peptide/
C-terminus of
immature C protein
pE1E2 Δ1conlink
pE1E2 Δ2 link
pE1E2 Δ3 link

Figure 21


HVR1
384
409
con
428
ETHVTGGSAGRTTAGLVGLLTPGAR QNIQLINTNGSWHINSTALN C
460
HVR2
485
C RRLTDFAQGWGPISYANGSGLDERPY C
570 igVR580
CVIGGVGNNTLLC
E2
384
661
E2 RBD661

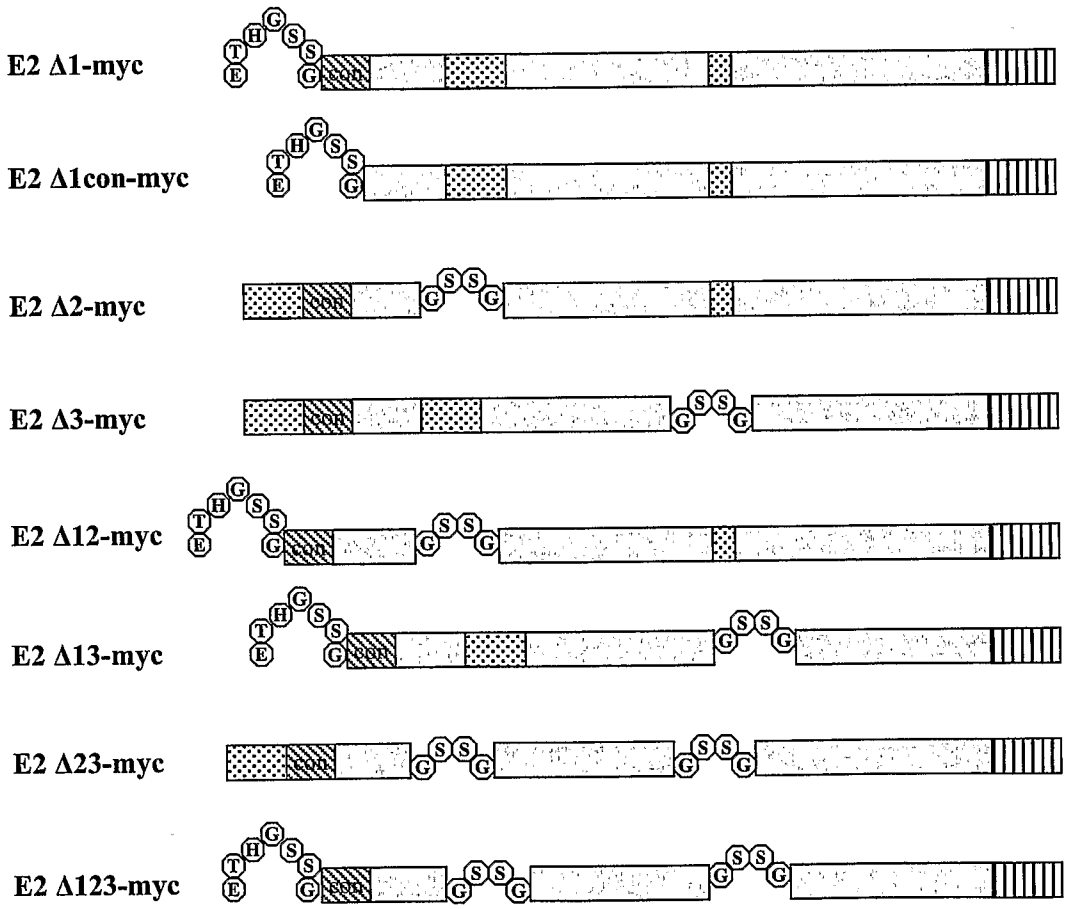

E2 Δ1-myc
E2 Δ1con-myc
E2 Δ2-myc
E2 Δ3-myc
E2 Δ12-myc
E2 Δ13-myc
E2 Δ23-myc
E2 Δ123-myc

Figure 22.

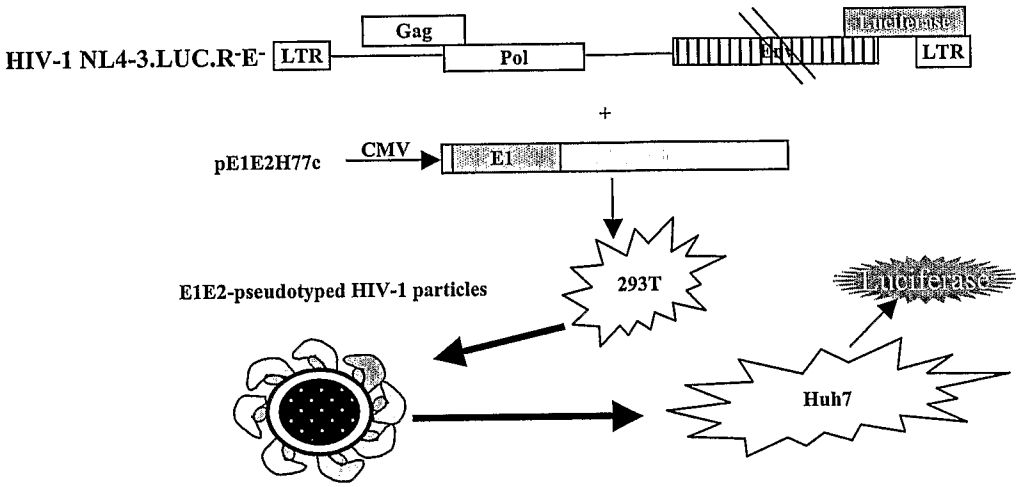

HIV-1 NL4-3.LUC.R-E-
LTR
Gag
Pol
Env
Luciferase
LTR
+
pE1E2H77c
CMV
E1
E1E2-pseudotyped HIV-1 particles
293T
Luciferase
Huh7

Figure 23.

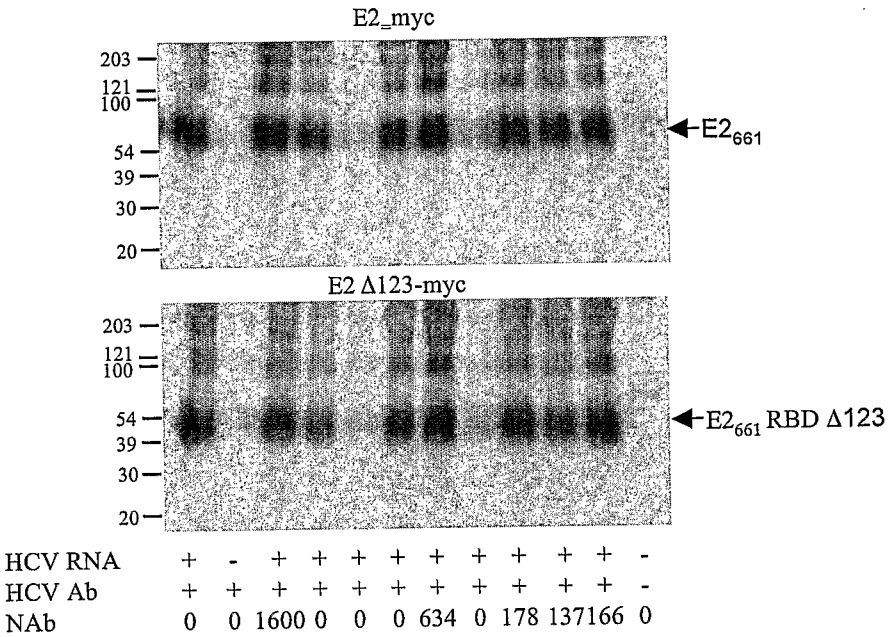

E2_myc
203
121
100
54
39
30
20
E2661
E2 Δ123-myc
203
121
100
54
39
30
20
E2661 RBD Δ123
HCV RNA + - + + + + + + + + + -
HCV Ab + + + + + + + + + + + -
NAb 0 0 1600 0 0 0 634 0 178 137166 0

Figure 24.

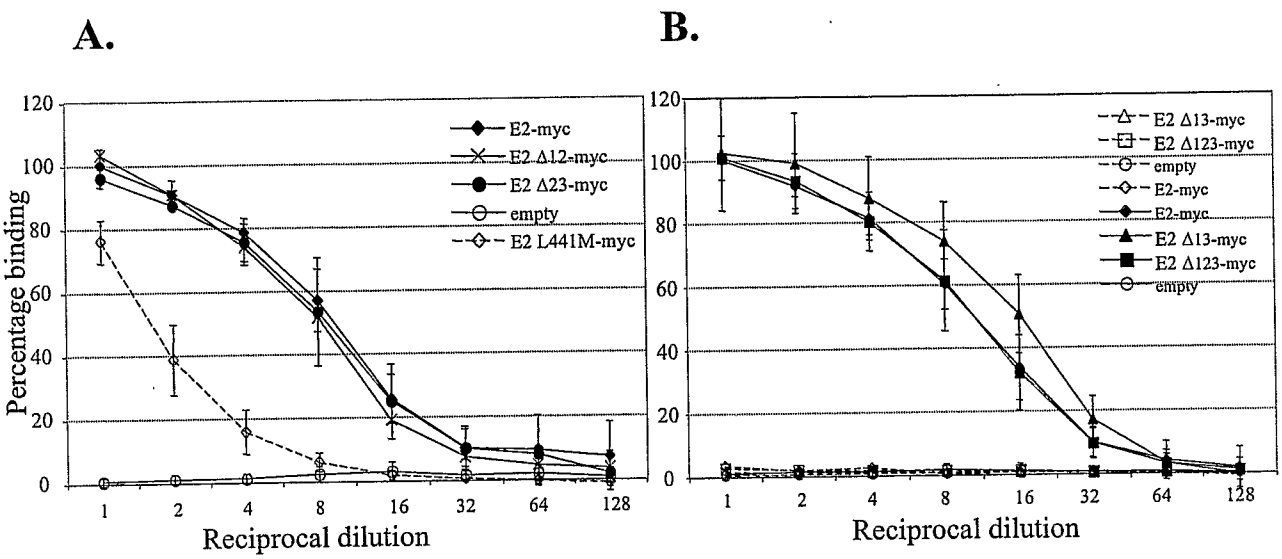

A.
B.
Percentage binding
120
100
80
60
40
20
0
1
2
4
8
16
32
64
128
Reciprocal dilution
E2-myc
E2 Δ12-myc
E2 Δ23-myc
empty
E2 L441M-myc
E2 Δ13-myc
E2 Δ123-myc
empty
E2-myc
E2-myc
E2 Δ13-myc
E2 Δ123-myc
empty
Reciprocal dilution

Figure 25

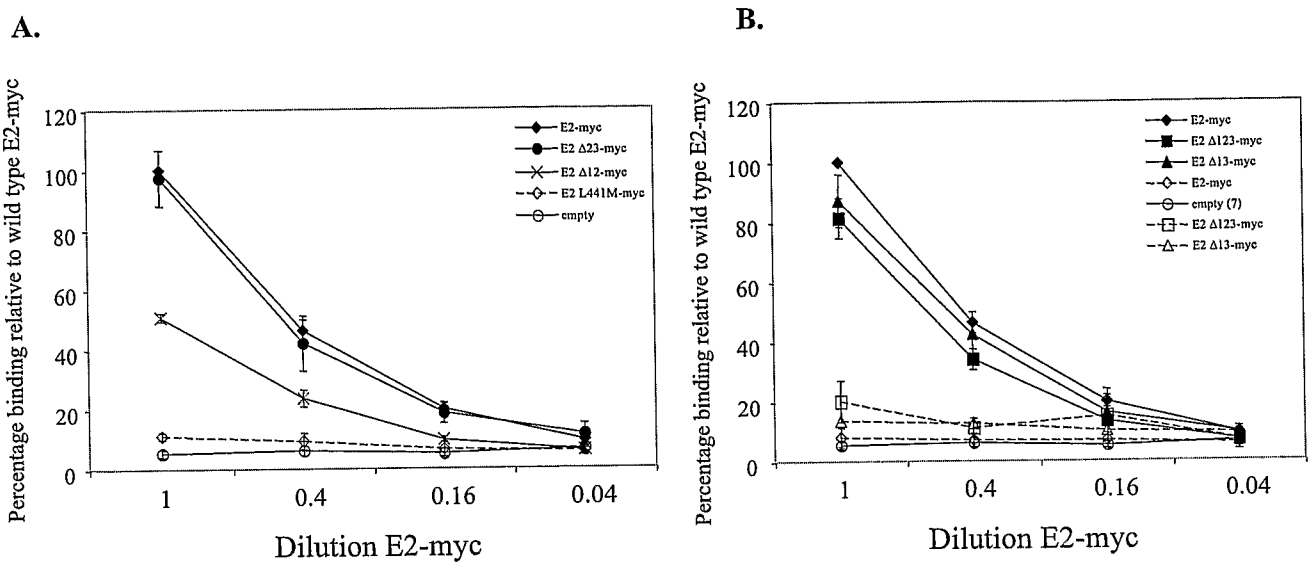
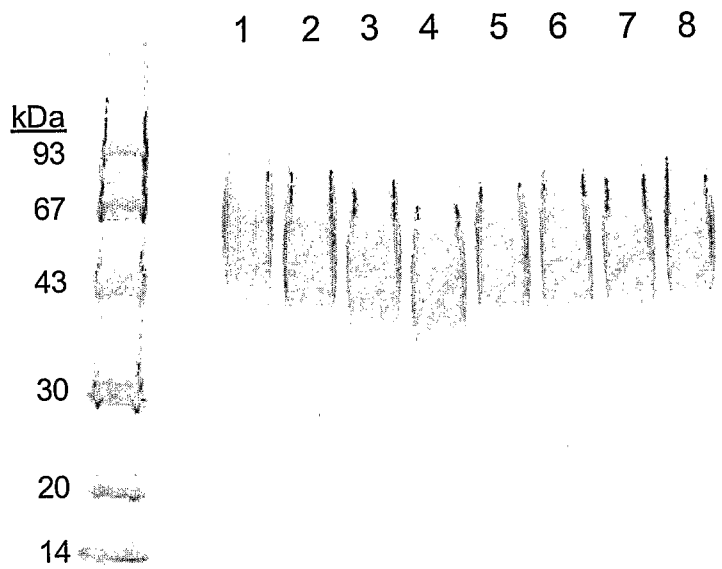

A.
B.
Percentage binding relative to wild type E2-myc
120
100
80
60
40
20
0
1
0.4
0.16
0.04
Dilution E2-myc
E2-myc
E2 Δ23-myc
E2 Δ12-myc
E2 L441M-myc
empty
E2 Δ123-myc
E2 Δ13-myc
empty (7)

Figure 26.



Lane:

Lane	Protein	Lane	Protein
1.	E2-his	5.	E2Δ13-his
2.	E2Δ1-his	6.	E2Δ2-his
3.	E2Δ12-his	7.	E2Δ23-his
4.	E2Δ123-his	8.	E2Δ3-his

Figure 27

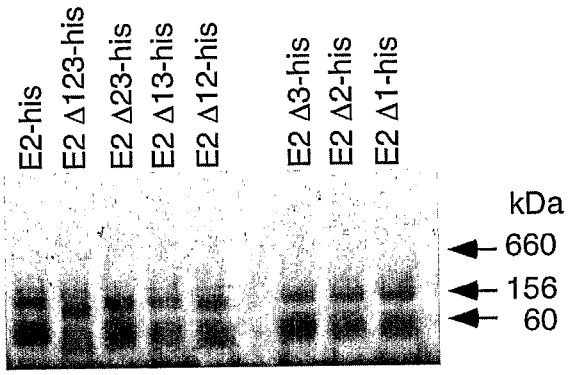

E2-his
E2 Δ123-his
E2 Δ23-his
E2 Δ13-his
E2 Δ12-his
E2 Δ3-his
E2 Δ2-his
E2 Δ1-his
kDa
660
156
60

Figure 28

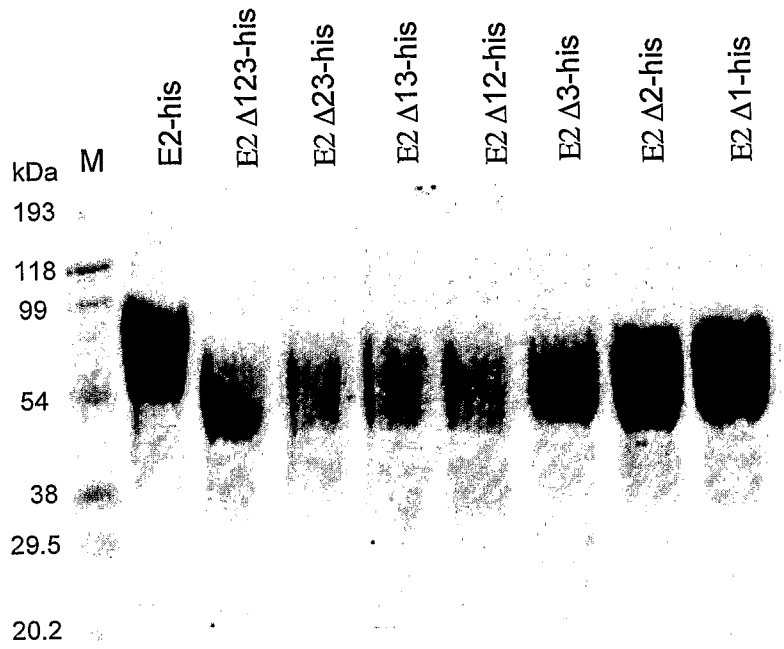
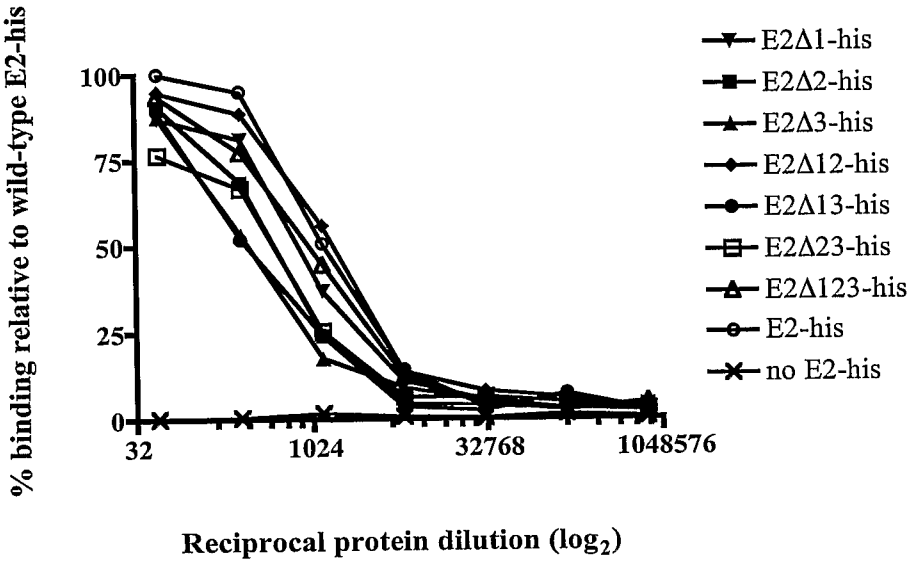

kDa
M
193
118
99
54
38
29.5
20.2
E2-his
E2 Δ123-his
E2 Δ23-his
E2 Δ13-his
E2 Δ12-his
E2 Δ3-his
E2 Δ2-his
E2 Δ1-his

Figure 29

A.


% binding relative to wild-type E2-his
100
75
50
25
0
32
1024
32768
1048576
Reciprocal protein dilution (log2)
E2Δ1-his
E2Δ2-his
E2Δ3-his
E2Δ12-his
E2Δ13-his
E2Δ23-his
E2Δ123-his
E2-his
no E2-his

B.

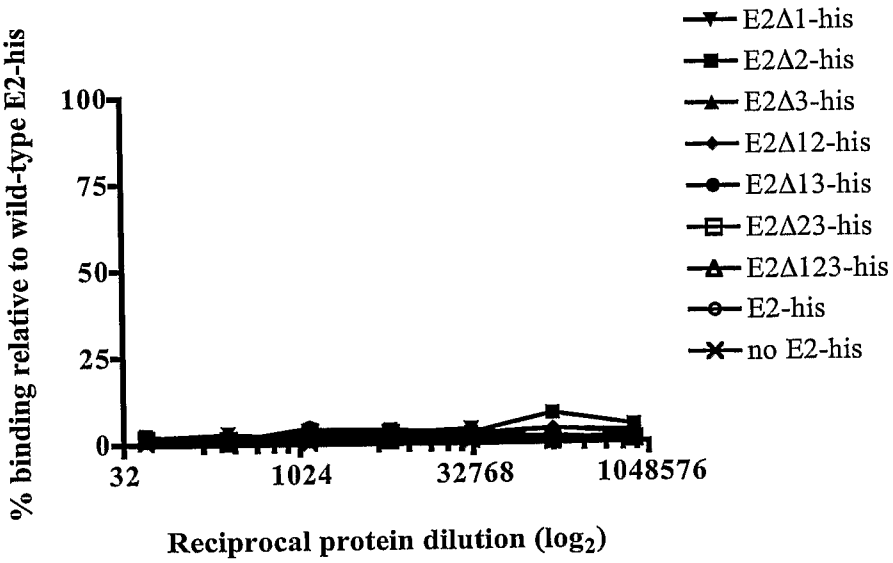

% binding relative to wild-type E2-his
100
75
50
25
0
32
1024
32768
1048576
Reciprocal protein dilution (log2)
E2Δ1-his
E2Δ2-his
E2Δ3-his
E2Δ12-his
E2Δ13-his
E2Δ23-his
E2Δ123-his
E2-his
no E2-his

Figure 30

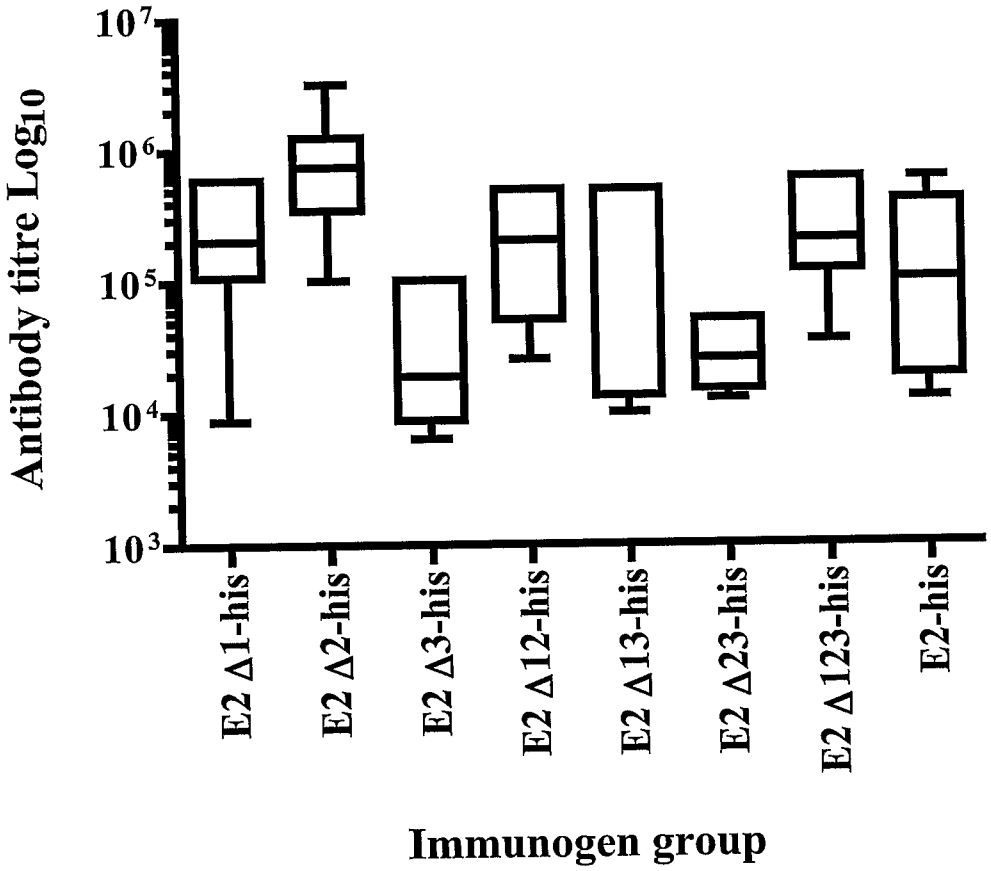

Antibody titre Log10
10^7
10^6
10^5
10^4
10^3
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
E2-his
Immunogen group

Figure 31

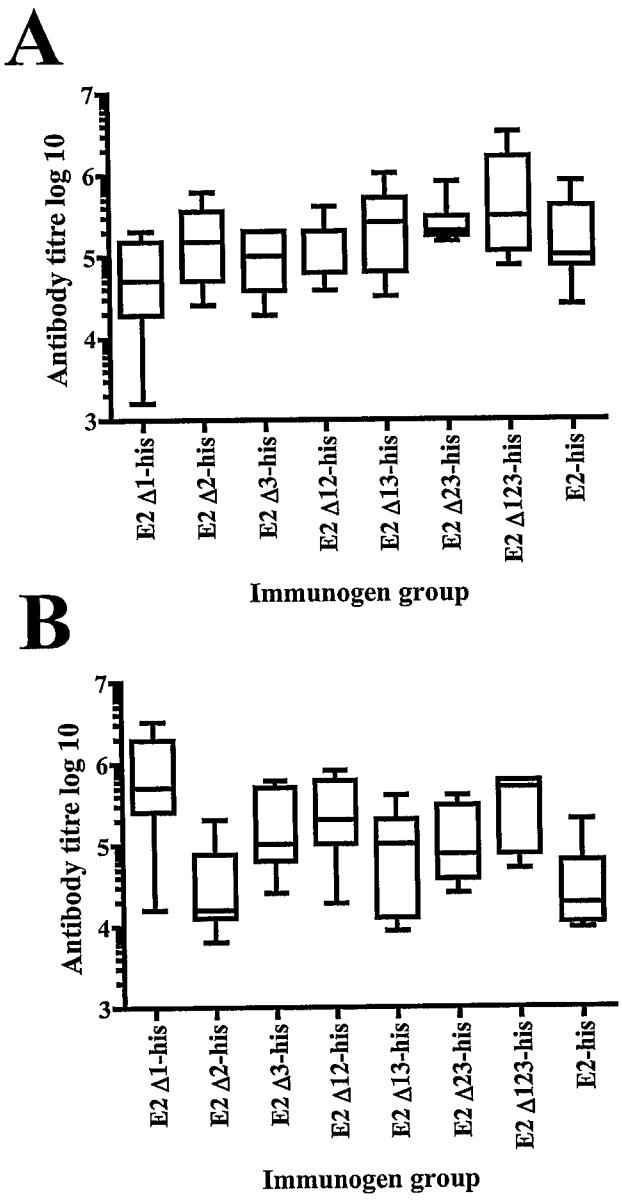

A
Antibody titre log 10
7
6
5
4
3
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
E2-his
Immunogen group
B
Antibody titre log 10
7
6
5
4
3
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
E2-his
Immunogen group

Figure 32

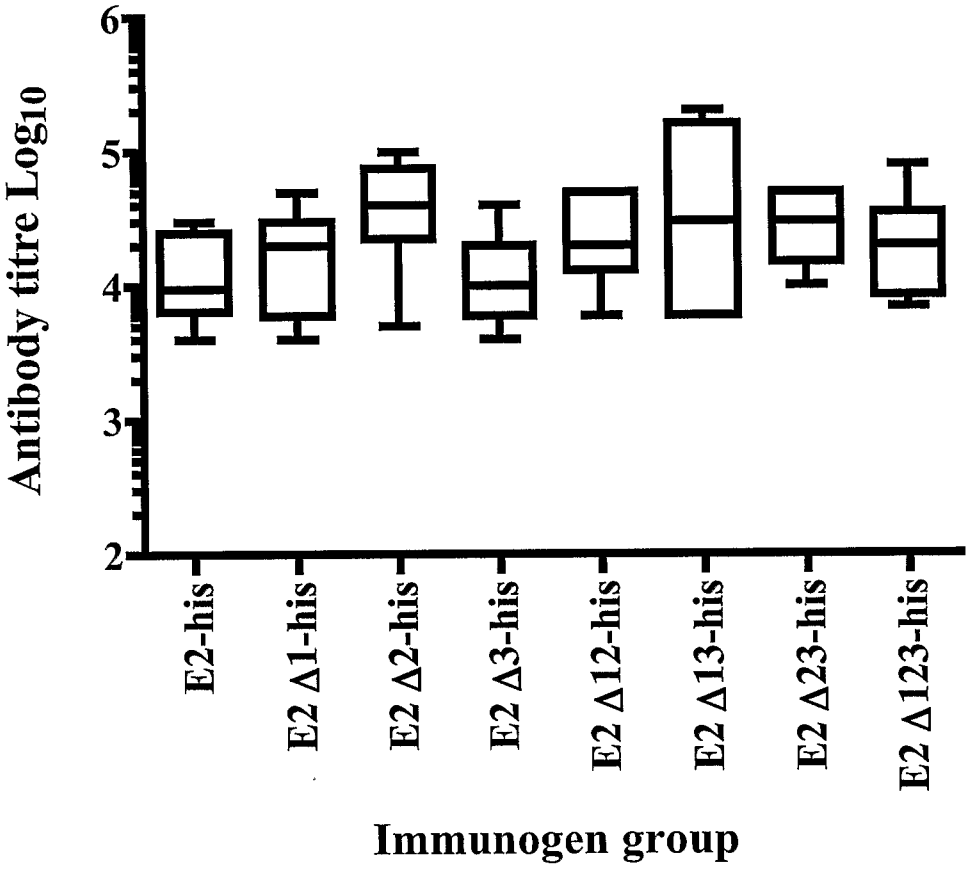

Antibody titre Log10
6
5
4
3
2
E2-his
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
Immunogen group

Figure 33

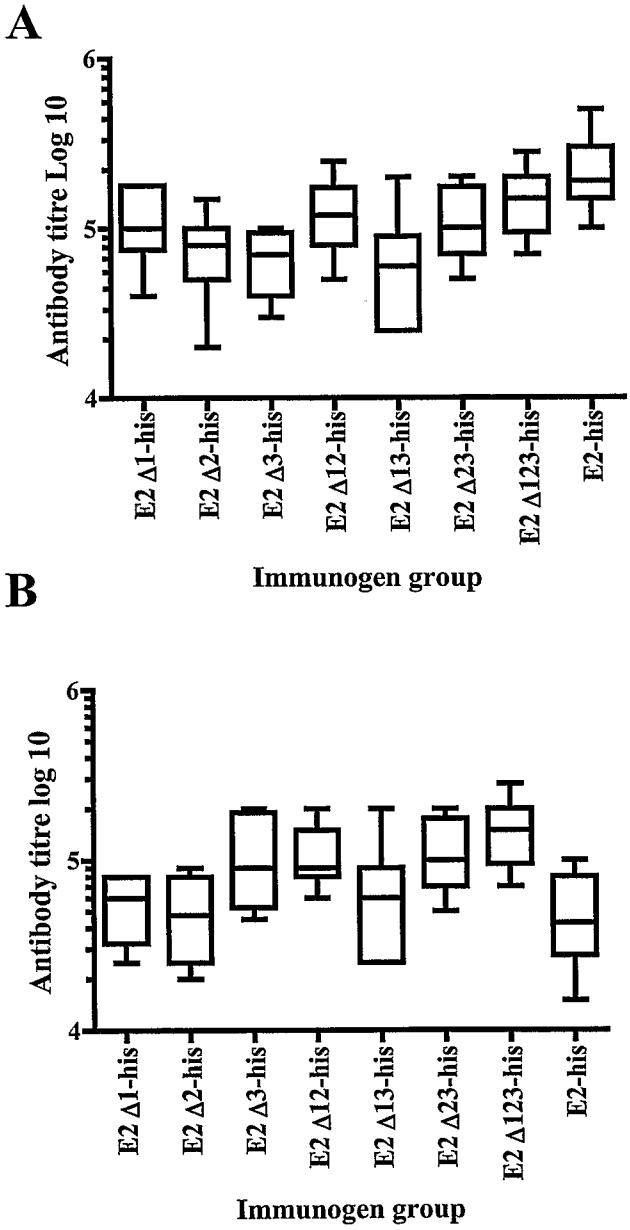
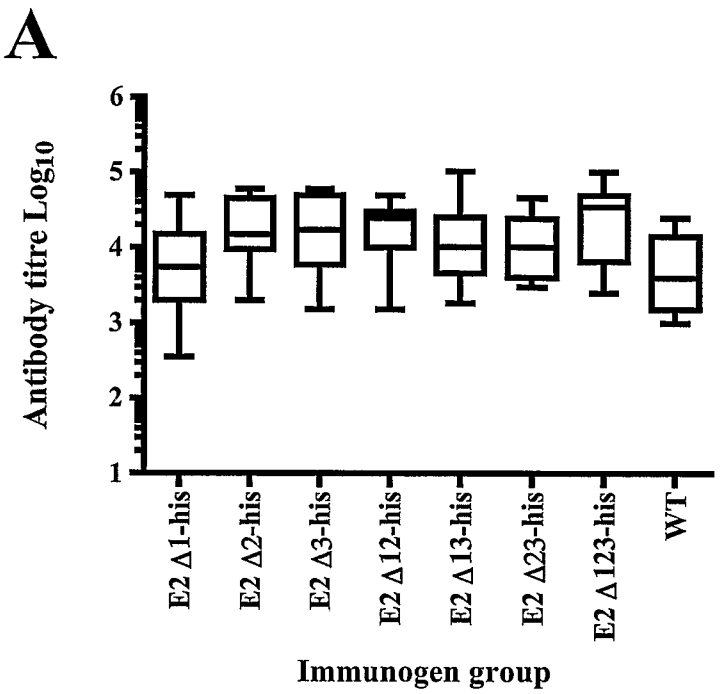

A
Antibody titre Log 10
6
5
4
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
E2-his
Immunogen group
B
Antibody titre log 10
6
5
4
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
E2-his
Immunogen group

Figure 34


A
Antibody titre Log10
6
5
4
3
2
1
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
WT
Immunogen group

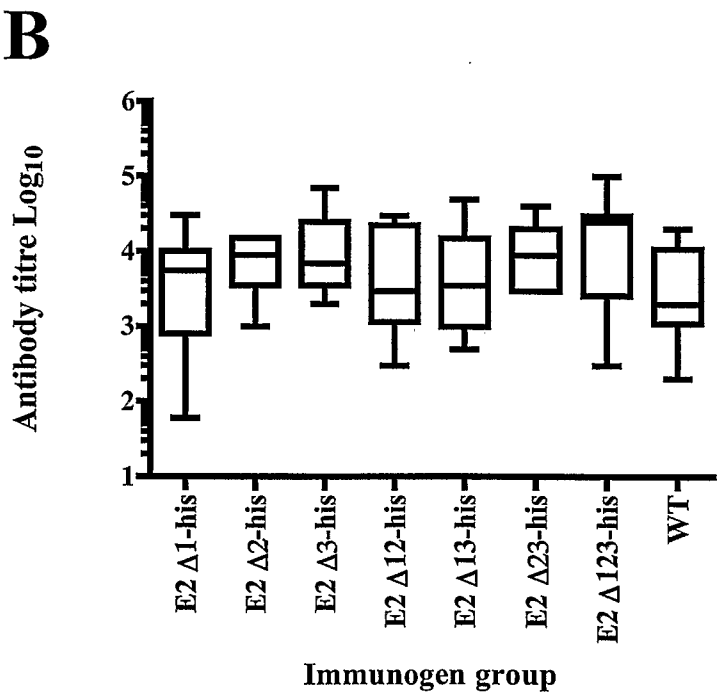

B
Antibody titre Log10
6
5
4
3
2
1
E2 Δ1-his
E2 Δ2-his
E2 Δ3-his
E2 Δ12-his
E2 Δ13-his
E2 Δ23-his
E2 Δ123-his
WT
Immunogen group

Figure 35

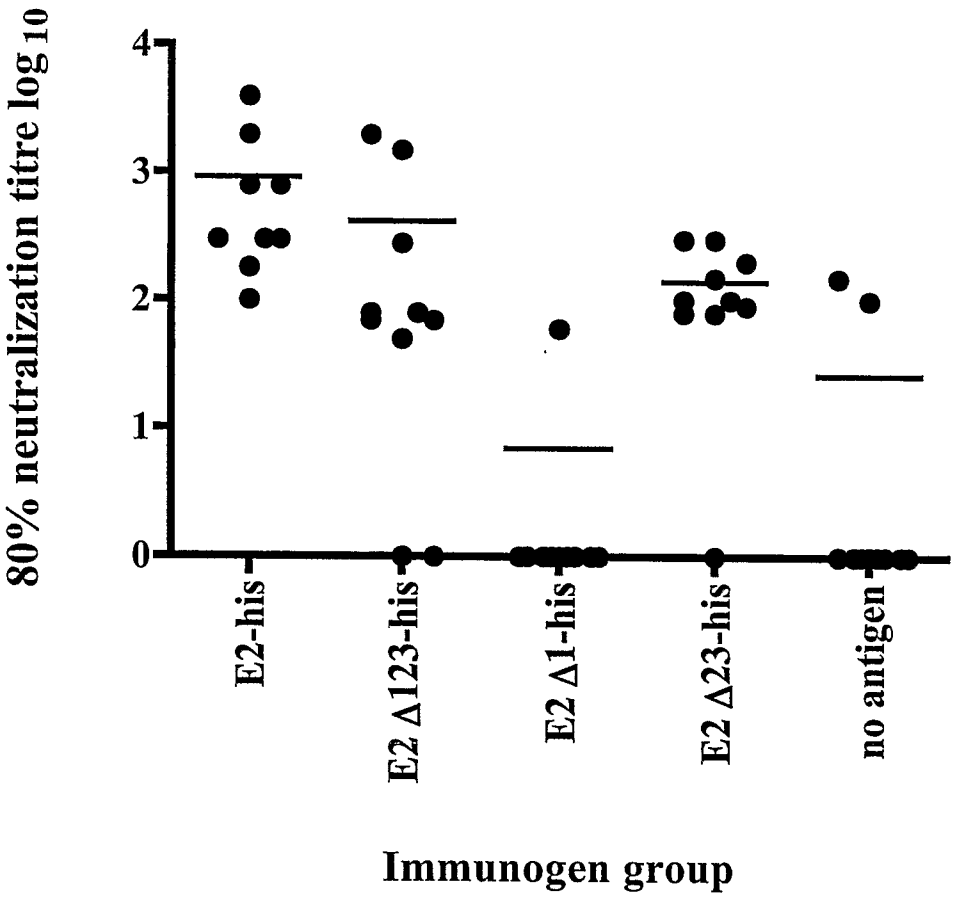

80% neutralization titre log 10
4
3
2
1
0
E2-his
E2 Δ123-his
E2 Δ1-his
E2 Δ23-his
no antigen
Immunogen group

Figure 36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001221

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 14/18 (2006.01) ***A61K 39/29*** (2006.01) ***C07K 1/36*** (2006.01)
A61K 35/76 (2006.01) ***A61K 39/42*** (2006.01) ***C07K 16/08*** (2006.01)
A61K 38/02 (2006.01) ***A61P 31/14*** (2006.01) ***C12P 21/08*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CA, WPIDS, BIOSIS & BIOTECHABS keywords: HCV, hepatitis C, variable region, hypervariable region, E2, E1, envelope, deletion, mutation, modified and similar terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	McCaffrey, K. et al, "Expression and Characterization of a Minimal Hepatitis C Virus Glycoprotein E2 Core Domain that Retains CD81 Binding" Journal of Virology, 2007, vol. 81, No. 17, pages 9584-9590 See whole document especially abstract and pp 9585	1-19 & 27-31
A	WO2001/21807 A1 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 29 March 2001 See whole document	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 October 2007	09 OCT 2007
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **Alanna Hurne** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : (02) 6222 3657

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001221

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Lechmann, M & Liang T.J., "Vaccine development for hepatitis C" Seminars in liver disease, 2000, vol. 20, No: 2, pp 211-26 See whole document especially page 216	
A	Forns, X. et al, "Characterization of modified hepatitis C virus E2 proteins expressed on the cell surface", Virology, 2000, vol. 274, No 1, pp 75-85 See whole document especially abstract and discussion	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2007/001221

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. type of material

      ☐ a sequence listing

      ☐ table(s) related to the sequence listing

   b. format of material

      ☐ on paper

      ☐ in electronic form

   c. time of filing/furnishing

      ☐ contained in the international application as filed

      ☐ filed together with the international application in electronic form

      ☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

There was a sequence listing originally filed but it was not used for the purpose of this search and opinion.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2007/001221

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
WO 0121807	AU 76028/00

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX