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(54) Title: VECTORS ENCODING HCMV GLYCOPROTEIN AND EXPRESSION PRODUCTS (57) Abstract <p>A recombinant expression vector is provided which comprises DNA derived from the genome of human cytomegalovirus (HCMV), such as HXLF 1-5, wherein said DNA encodes a polypeptide which represents at least a portion of the gclI HCMV glycoprotein complex.</p>		

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VECTORS ENCODING HCMV GLYCOPROTEIN AND EXPRESSION PRODUCTS

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Field of the Invention

This invention relates to a family of antigenically related glycoprotein complexes contained within the envelope of human cytomegalovirus (HCMV), and in particular to a multi-gene family encoding a principal glycoprotein component of these complexes, gp47-52. This glycoprotein has been shown to elicit humoral and cell-mediated immune responses in humans, and is useful for the diagnosis and treatment of HCMV infection.

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Background of the Invention

Human cytomegalovirus is the most common cause of congenital infection leading to mental retardation, deafness and other neurological handicaps in the United States. Moreover, this virus is frequently associated with life-threatening opportunistic infection in organ and bone marrow transplant recipients, patients with congenital and acquired immunodeficiency diseases, and patients receiving immunosuppressive therapy for cancer, autoimmune diseases and the like. Because HCMV is ubiquitous in nature, measures to limit exposure of individuals at risk of serious infection have met with limited success. Furthermore, antiviral chemotherapy is of little value to infants who have already suffered

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the devastating consequences of intrauterine HCMV infection, and thus far has shown little promise in ameliorating the progression of systemic HCMV infection and HCMV-associated interstitial pneumonia in the
5 immunocompromised host.

Therefore, a safe, effective HCMV vaccine is urgently needed to protect against congenital and acquired HCMV infection. Moreover, improved diagnostic tests can play an important role in detection of sub-
10 clinical HCMV infection, thereby facilitating early therapeutic intervention to decrease the incidence or severity of clinical disease.

HCMV is a member of the family herpesviridae. HCMV virions consist of a linear double stranded DNA
15 molecule enclosed within an icosahedral nucleocapsid, surrounded by a tegument and a lipid-containing envelope containing a number of viral glycoproteins and glycoprotein complexes. Three major antigenically distinct sets of HCMV envelope glycoprotein complexes
20 have been described thus far, each made up of two or more glycoproteins associated by disulfide bonds. The first set of complexes, designated gC1, contains glycoproteins with molecular weights of 55,000 (gp55), 93,000 (gp93) and 130,000 (gp130). The gene encoding
25 gp55 has been identified, and exhibits extensive homology with the glycoprotein B gene of herpes simplex virus (HSV) type 1. M. Mach et al., J. Gen. Virol., 67, 1461 (1986).

A second set of antigenically related complexes, designated gCII, appears to represent multi-
30 plexes, designated gCII, appears to represent multimeric forms of one or more gene product(s). The isolation and characterization of the principal glycoprotein contained within this family of complexes, gp47-52, has been described in detail in U.S. patent application
35 Serial No. 933,789, wherein it is referred to as GLP-B,

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and in U.S. patent application Serial No. 158,389, filed February 22, 1988, wherein it is referred to as gp52(II). The disclosure of these applications are incorporated by reference herein.

5 Glycoproteins with molecular weights of 86,000 (gp86) and 145,000 (gp145) have been isolated from a third complex, designated gcIII. The gene coding for gp86 has been identified and exhibits homology with gH of HSV-1.

10 A single monoclonal antibody (mcAb) reactive with the gcII family of complexes, 9E10 (IVI-10118), has been extensively characterized, and is described in detail in U.S. patent application Serial No. 933,789. This monoclonal antibody exhibits cross-reactivity
15 among several strains of HCMV, adenovirus type 2, and herpes simplex virus types 1 and 2. Moreover, this monoclonal neutralizes Towne strain HCMV without complement, suggesting the possibility that gp47-52 plays an integral role in the biological events required for
20 replication of the virus.

Therefore, a need exists to identify and characterize HCMV glycoprotein complexes and their constituent glycoproteins and polypeptide backbones, particularly those which can elicit protective humoral and
25 cellular immune responses.

Summary of the Invention

The present invention provides a recombinant expression vector, such as a plasmid, comprising DNA
30 derived from the HCMV genome. This HCMV DNA can code a polypeptide which reacts with gcII-specific monoclonal antibody 9E10 (IVI-10118), and corresponds to a portion of gp47-52, an immunogenic glycoprotein present in the major HCMV envelope glycoprotein complex gcII.

35 Weston and Barrell, J. Mol. Biol., 192, 177 (1986), have recently sequenced the short unique (U_S)

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region of the HCMV genome (AD169 strain), revealing at least 38 open reading frames (ORFs) that potentially code for (glyco)proteins of HCMV. One gene family, designated HXLF (Hind III X left reading frame), consists of 5 ORFs that lie in tandem with varying degrees of homology. The products of this gene family were predicted to have potential glycosylation sites for both N-linked and O-linked oligosaccharides; signal sequences for membrane insertion; and C-terminal hydrophobic domains for anchoring in cell membranes. Of particular interest, clusters of serine/threonine were predicted by the sequences of HXLF 1 and 2, which in turn suggested that the gene product might contain a high amount of O-linked oligosaccharides, which is atypical of most viral glycoproteins.

The present invention is based upon our discovery that the gcII glycoprotein, gp47-52, is encoded by one or more of the genes encompassed by the HXLF gene family. Therefore, the present invention is also directed to the isolated gene family, e.g., to a substantially pure composition of matter consisting essentially of at least one of the members of the HXLF gene family of HCMV, e.g., HXLF1, HXLF2, HXLF3, HXLF4 and HXLF5. As used within the context of the present invention, the term "DNA derived from the HCMV genome" refers to both DNA which is isolated from HCMV virions, such as Towne strain virions, and to DNA which is chemically synthesized.

The term "a portion of gp47-52" refers to either fully-, partially-, or non-glycosylated polypeptide segments of gp47-52 which comprise epitopes which are reactive with 9E10 or with patient sera comprising antibodies reactive with gp47-52. Non-glycosylated segments of gp47-52 are also referred to as parts of the "polypeptide backbone" of gp47-52.

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The present recombinant expression vectors can be employed to produce immunogenic polypeptides which formally correspond to a portion of gp47-52. For example, a plasmid containing all or a portion of the HXLF gene family can be linearized, mRNA synthesized in vitro, and then translated under conditions whereby the corresponding polypeptides are either glycosylated, or nonglycosylated. The translation products can be either analyzed by SDS-PAGE and extracted therefrom, or solubilized for immunoprecipitation as described in the examples hereinbelow.

Therefore, the present invention also provides a number of new gp47-52-related polypeptides (p20, p21-25) and glycoproteins (gp30, gp25) which comprise epitopes which react with 9E10, as well as with antibodies in the HCMV-positive sera of human patients.

The role of HCMV gp47-52 in virus infection is unknown, as is the role of its parent glycoprotein complex, gcII. However, gcII is a major component of the virion envelope and is a target for antibody-mediated neutralization. Taken together, these data strongly support the inclusion of gp47-52 and/or the present "subunit proteins" of gp47-52 into HCMV vaccines, which may also include other classes of HCMV envelope glycoproteins, such as gcI.

The gp47-52-related polypeptides and glycoproteins can be readily prepared in quantity by transformation or transfection of suitable host cells with the present vectors; or by known chemical methods of polypeptide synthesis. After isolation and purification of the polypeptide, an immunologically-effective amount thereof is combined with a pharmaceutically-acceptable vehicle, such as a sterile liquid carrier, and parenterally administered to a mammal, in order to raise the titer of anti-HCMV antibodies in the blood of the recipient.

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For a description of a putative vaccine employing "glycoprotein A" of HCMV, see L. Pereira (U.S. Patent No. 4,689,225), the disclosure of which is incorporated by reference herein.

5 Apart from their use in vaccines, the present compositions are useful in the production of monoclonal antibodies, which in turn can be used either directly or indirectly to diagnose HCMV, or to treat HCMV infections. The present immunogenic compositions can also
10 be used as diagnostic reagents, e.g., to detect anti-gcII antibodies in physiological fluids, e.g., by RIA or ELISA procedures. The present compositions are also useful to produce clonal populations of antigen-specific T-helper lymphocytes, which in turn can be
15 used for HCMV therapy.

 The present invention also provides RNA probes, a number of which are exemplified hereinbelow, which are complementary to sequences in the HXLF gene family. These probes can be used to detect HCMV in
20 clinical specimens using hybridization methods known in the art.

 In a preferred embodiment of the present invention, the five HXLF genes were cloned into an SP6 expression vector in both the sense and antisense
25 orientations (Fig. 1C). An abundant 1.62 kb bicistronic mRNA, predicted to originate from HXLF1 and HXLF2, was detected in the cytoplasm of infected human fibroblast cells by Northern blot analysis. Less abundant RNAs of 1.0 and 0.8 kb, predicted to originate from the
30 HXLF5 and HXLF2 genes, respectively, were also detected.

 Monocistronic, bicistronic, and polycistronic RNAs synthesized in vitro using SP6 polymerase were translated in rabbit reticulocyte lysates with or
35 without canine pancreatic microsomal membranes. The

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HXLF1 or the HXLF1 plus HXLF2 translation products were detected when using the above mRNAs. However, the HXLF3, 4 and 5 gene products were not detected by in vitro translation of the SP6 derived polycistronic mRNA.

Nonglycosylated or glycosylated HXLF1 and HXLF2 gene products were immunoprecipitated by monoclonal antibody 9E10 which is specific for a virion envelope glycoprotein complex (gc) designated gcII. In addition, the monoclonal antibody 9E10 immunoprecipitated a diffuse glycoprotein (gp) band designated gp47-52 from HCMV-infected cell lysates. The amino acid composition of gp47-52 purified from virion envelopes has the highest similarity to the predicted amino acid composition of the product of the HXLF1 plus HXLF2 open reading frames, but it is more similar to that of HXLF2 than to that of HXLF1. The Northern blot results imply that gp47-52 is synthesized predominantly from the abundant 1.62 kb bicistronic mRNA encoded by the HXLF1 and HXLF2 genes. However, the glycoprotein could also be synthesized by the monocistronic 0.8 kb mRNA encoded by the HXLF2 gene as well as the mRNAs predicted from the other HXLF genes.

Brief Description of the Drawing

Figure 1 schematically depicts the location of the HXLF homologous gene family of human cytomegalovirus and its cloning into an SP6 expression vector. Panel (A) depicts the prototype arrangement of the human cytomegalovirus (HCMV) genome showing large unique (U_L), short unique (U_S), terminal repetitive (TR) and internal repetitive (IR) regions. Panel (B) depicts the HindIII-XbaI fragment derived from the U_S region and designates the map units on the viral genome. The HXLF family of homologous ORFs are

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depicted by closed bars labelled HXLF1 through HXLF5. Dashed arrows indicate predicted mRNA transcripts with approximate sizes in kb based on the locations of TATA boxes and polyadenylation signals (stars) relative to the ORFs. Beneath each bar representing an ORF is the molecular weight ($\times 10^3$) of the protein product predicted by the respective ORF. Restriction endonuclease sites are indicated by arrows. Panel (C) depicts the HXLF gene family cloned into an SP6 expression vector in both the sense (SP65IT) and antisense (SP64IT) orientations. Abbreviations: Bs (BstE II); HIII (HindIII); Xb (XbaI); Xh (XhoI); SP (SP6 promoter); Amp (ampicillin resistance gene).

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Deposit

Plasmid pSP65IT has been deposited at In Vitro International, 611(P) Hammond's Ferry Road, Linthicum, MD 21090, under the access code IVI-10168.

20

Detailed Description of the Invention

The invention will be further described by reference to the following detailed examples wherein the methodologies were as described hereinbelow. The disclosures of the cited publications are incorporated by reference herein.

Virus and cells. The culturing of human foreskin fibroblasts and the propagation of human cytomegalovirus Towne strain have been described previously by M. F. Stinski in J. Virol., 26, 686 (1978). The viral DNA polymerase inhibitor phosphonoacetic acid was used as described by M. W. Wathen et al., J. Virol., 41, 462 (1982).

Expression vectors. The pSP64 and pSP65 cloning vectors contain the Salmonella typhimurium phage SP6 promoter upstream of a polylinker sequence containing

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unique restriction sites for insertion of DNA sequences. The bacteriophage SP6 RNA polymerase initiates transcription exclusively at an SP6 promoter, and thus allows for synthesis of pure, full-length
5 single stranded RNA from a DNA template cloned downstream of the SP6 promoter. The polylinker is cloned in opposite directions relative to the SP6 promoter in pSP64 and pSP65 to allow cloning of the DNA sequences in sense and anti-sense orientations, respectively.
10 The SP6 expression system used in these studies was adapted from an original cloning vector derived by insertion of the SP6 promoter into pBR322.

A particular disadvantage of the original cloning vector was the presence of 250 base pairs
15 upstream of the restriction site used for cloning, resulting in transcription of extraneous sequences between the transcription start site and the restriction sites of the polylinker. pSP64 and pSP65 have only six bases and nine bases, respectively, between
20 the transcription start site and the restriction enzyme insertion site of the polylinkers. A particular advantage of these cloning vectors is that the major RNA transcripts are derived from transcription of the DNA template strand to the last and penultimate nucleotide.
25 Nicks in the DNA template do not serve as transcription initiation sites, nor does SP6 RNA polymerase terminate at nicks in the template and thus premature termination resulting in RNA transcripts shorter than full-length, is relatively rare.

30 The construction of the pSP64/65 expression system and its utility for in vitro synthesis of biologically-active RNA was first described in detail by D. A. Melton et al., "Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes
35 from plasmids containing a bacteriophage SP6 promoter."

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Nucleic Acids Research, 12, 7035-7056 (1984), the disclosure of which is incorporated by reference herein. The restriction maps and sequences for pSP64 and pSP65 are published in this manuscript (page 7041).

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In vitro transcription and translation. A 4.5 kb XbaI to HindIII fragment spanning 0.857 to 0.879 map units of the Towne strain genome was subcloned into SP6 expression vectors SP64 and SP65 (Amersham; Amersham Hts., IL) using the methodology described previously by D. R. Thomsen et al., Gene, 16, 207 (1981). RNA synthesis from linearized templates was accomplished as described by P. A. Krieg et al., Nucl. Acids Res., 12, 7057 (1984). SP6 polymerase was purchased from
15 Boehringer-Mannheim (Indianapolis, IN).

The in vitro translation of RNA in rabbit reticulocyte lysates (Promega; Madison, WI) was accomplished as described by M. Stinski et al., J. Virol., 46, 1 (1983). Translation products were radiolabelled
20 with ³⁵S-methionine (>1000 Ci/mmol; Amersham). For some experiments, translation was supplemented with canine pancreatic microsomal membranes as recommended by the manufacturer (Amersham).

Immunoprecipitation and SDS-PAGE. Infected or mock-infected cells were pulse-labelled at various times after infection with 40 µCi/ml ³H-glucosamine (20-40 Ci/mmol; Amersham) as described previously by M. F. Stinski et al., J. Virol., 26, 686 (1978). In certain
30 examples, cells were pretreated for 4 hr with 1.5 µM monensin (a gift from C. Grose, U. of Iowa) before pulsing in the presence of monensin. Antigens were solubilized in RIPA buffer (PBS, pH 7.4, containing 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 100 µg/ml
35 PMSF and 0.02% sodium azide) and immunoprecipitated

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with monoclonal or polyclonal antibodies plus protein A sepharose (Pharmacia; Uppsala, Sweden) as described by D. R. Gretch et al., Anal. Biochem., 163, 270 (1987). The analysis of radiolabelled antigens by SDS-poly-
5 acrylamide gel electrophoresis (SDS-PAGE) and autoradiography has been described by M. F. Stinski et al., J. Virol., 19, 594 (1976). ¹⁴C-labelled protein molecular weight standards were purchased from Bethesda Research Laboratories (Gaithersburg, MD).

10 The production and characterization of monoclonal antibodies 41C2 (IVI-10119) and 9E10 has been described by B. Kari et al., J. Virol., 60, 345 (1986) and monoclonal antibody 1G6 was a gift from L. Rasmussen, Stanford University. Human convalescent
15 serum (218) was a gift from L. Frankel, U. of Cincinnati.

Northern blot analysis. The purification of cytoplasmic RNA from HCMV-infected or mock-infected cells and
20 Northern blotting conditions were performed as described previously by M. W. Wathen et al., J. Virol., 41, 462 (1982). E. coli ribosomal RNA standards were purchased from Promega (Madison, WI). Antisense RNA probes were synthesized from linearized pSP64IT templates as directed by the supplier (Amersham) except
25 that 150 μ Ci of α^{32} P-UTP (800 Ci/mmol; Amersham) was supplemented with cold UTP to a final concentration of 50 μ M to ensure full length probe synthesis.

30 Amino acid composition analysis. gp47-52 was purified from virion and dense body envelopes using ion exchange and gel exclusion high performance liquid chromatography (HPLC) as described previously by B. Kari et al., cited above. After strong acid hydrolysis, amino
35 acid compositions were determined by reverse phase HPLC

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on a C-18 column (E. M. Science, Elmsford, NY) as described by H. Scholze, J. Chromat., 350, 453 (1985).

Example I.

5 Northern blot analysis of the HXLF steady state mRNA in
infected cells. The HXLF gene family, which consists
of five homologous ORFs in tandem, was cloned into the
SP6 expression vectors in sense and antisense orienta-
10 tions as outlined in Figure 1. Four different mRNA
transcripts were predicted from the HXLF gene family
based on the locations of TATA boxes and polyadenyla-
tion signals relative to the ORFs (see Figure 1B). A
1.6 kb bicistronic RNA was predicted from the HXLF1 and
HXLF2 genes while a 2.7 kb tricistronic RNA was pre-
15 dicted from the HXLF3 through HXLF5 genes. In addi-
tion, monocistronic transcripts of 0.8 and 1.0 kb genes
were predicted from HXLF2 and HXLF5 genes, respec-
tively.

Northern blot analysis was performed on steady
20 state cytoplasmic RNA harvested from mock- or HCMV-
infected cells at 24 and 72 hr p.i. In addition, RNA
was harvested from infected cells treated for 24 hr
with phosphonoacetic acid (PAA) which inhibits the
replication of HCMV DNA and, consequently, blocks the
25 early to late transition in the viral replicative
cycle. In addition, PAA treatment increases the steady
state level of viral RNA.

A 4.5 kb antisense RNA probe which covered all
five HXLF genes was synthesized from pSP64IT by linear-
30 izing the antisense template with restriction endonu-
clease XbaI (see Figure 1C). A shorter 3.1 kb probe,
which covered HXLF3, 4 and 5 genes only, was synthe-
sized from a BstE II-linearized template. Both the
full length probe and the truncated probe detected a
35 predominant 1.62 kb transcript at 24 hr p.i. and at 24

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hr p.i. when PAA was present (data not shown). Reduced levels of the 1.62 kb RNA were detected by both probes at 72 hr p.i. in the absence of PAA. The 1.62 kb transcript is presumably the bicistronic mRNA predicted from the HXLF1 and HXLF2 genes. Both probes would be expected to hybridize to the 1.62 kb mRNA given the relative homology between the various HXLF genes. The full length probe detected minor mRNAs of 0.8 and 1.0 kb which correspond to the monocistronic RNAs predicted from the HXLF2 and HXLF5 genes, respectively. Neither probe detected the 2.7 kb polycistronic transcript predicted from the HXLF3, HXLF4 and HXLF5 genes.

Thus, the predominant mRNA observed was a 1.62 kb mRNA predicted to be encoded by the HXLF1 and HXLF2 genes which accumulated in the cytoplasm of virus-infected cells at early times after infection. The level of this transcript relative to total cellular and viral RNA is decreased at late times after infection.

Example II.

In vitro synthesis of the HXLF gene products. To allow for the in vitro synthesis of mRNA from either the first ORF (i.e., HXLF1), the first two ORFs together (HXLF1 plus HXLF2), or all five ORFs (HXLF1 through HXLF5), the sense plasmid pSP65IT was completely linearized with either restriction endonuclease XhoI, BstEII or HindIII, respectively (see Figure 1C). The approximate sizes of the in vitro synthesized mRNAs were determined by agarose-formaldehyde gel electrophoresis (data not shown) and the RNAs were translated in rabbit reticulocyte lysates in the presence or absence of dog pancreas membranes (DPM). DPM allow for core glycosylation with N-acetylglucosamine and mannose sugars during glycoprotein translation in vitro. [J. E. Rothman et al., Nature, 269, 775 (1977)] The

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translation products were either analyzed by SDS-PAGE or solubilized for immunoprecipitation as described hereinbelow.

After translation of mRNA from the HXLF1 gene in the absence of DPM, a diffuse band ranging in molecular weight from 21 to 25 kDa (p21-25) was detected. After translation of mRNA from the HXLF1 plus HXLF2 genes in the absence of DPM, the diffuse p21-25 plus an additional non-diffuse 20 kDa polypeptide (p20) were detected. The apparent molecular weights of these in vitro translation products are similar to the predicted protein molecular weights from the HXLF1 and HXLF2 ORFs (see Fig. 1B).

The diffuse nature of p21-25 is characteristic of the HXLF1 gene product only and may be due to internal initiation or premature termination during translation of the HXLF1 mRNA. Alternatively, the diffuse nature of p21-25 may be due to the higher proline content predicted to be present in HXLF1 (see Table I). Proline rich regions can disrupt part of the protein's alpha helical structure leading to anomolous mobilities in denaturing polyacrylamide gels.

After translation of mRNA from all five ORFs, a protein profile identical to translation of mRNA from HXLF1 and HXLF2 was detected. The HXLF3, HXLF4 and HXLF5 ORFs were predicted to make proteins of 28, 27.5 and 26.2 kDa, respectively (see Figure 1B). Thus, the products of the downstream HXLF ORFs were apparently not expressed in vitro even though full-length RNA was detected by agarose-formaldehyde gel electrophoresis. It is unlikely that the protein products migrated in gels the same as the HXLF1 and HXLF2 products.

The HXLF ORFs were predicted by Weston et al., cited above, to have sites for N- as well as O-glycosylation. Therefore, the in vitro synthesized

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mRNAs were also translated in the presence of dog pancreas membranes (DPM) to allow for core glycosylation of the nascent polypeptides [J. E. Rothman et al., Nature, 269, 775 (1977)]. After translation of mRNA from the HXLF1 gene in the presence of DPM, a 30 kDa glycoprotein (gp30) was detected. When mRNA from the HXLF1 plus HXLF2 genes was translated in the presence of DPM, a major 30 kDa glycoprotein band was again detected. It is possible that the HXLF1 and HXLF2 proteins were glycosylated to approximately the same apparent molecular weight, the DPM suppressed translation of the HXLF2 mRNA, or the HXLF2 glycosylated protein is present in low amounts. A similar result was obtained when mRNA from all five genes (HXLF1 through HXLF5) was translated in the presence of DPM.

Example III.

Immunoprecipitation of the HXLF gene products with a HCMV-specific monoclonal antibody or with human convalescent serum.

Native polypeptide translation To determine whether or not the HXLF genes specified bonafide HCMV envelope glycoproteins, monoclonal antibodies that react with envelope glycoprotein complexes (gc)I (gp55, gp93-130), II (gp47-52) and III (gp86, gp145) were used for immunoprecipitation. Monoclonal antibody 9E10, which recognizes a major glycoprotein component of the gcII family, gp47-52, reacted strongly with the diffuse p21-25 translated from the HXLF1 mRNA.

In addition, monoclonal antibody 9E10 immunoprecipitated both the diffuse p21-25 and non-diffuse p20 specified by mRNA from HXLF1 and HXLF2. When mRNA from HXLF1 through HXLF5 was translated, monoclonal antibody 9E10 again immunoprecipitated p21-25 and p20. However, neither monoclonal 41C2, which reacts with the

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gcI family, nor monoclonal antibody 1G6, which reacts with the gcIII family, immunoprecipitated the HXLF mRNA translation products.

Thus, p21-25 from the HXLF1 ORF is gcII specific, since immunoprecipitation with 9E10 detects the entire diffuse band p21-25. The p20 product of the HXLF2 ORF is apparently also recognized by monoclonal antibody 9E10 even though there is only a low degree of homology between these two ORFs. The possibility that p20 was immunoprecipitated via disulfide-linkage with p21-25 is unlikely because immunoprecipitated p20 was also detected by non-reducing SDS-PAGE.

Example IV.

DPM-mediated translation. To further analyze the HXLF gene products, mRNA from all five ORFs was translated in the presence of DPM and the products were immunoprecipitated with either monoclonal antibody 9E10 or human convalescent serum 218. Both reacted with the 30 kDa product of the HXLF1 ORF. In addition, both immunoprecipitated a second 25 kDa species which may represent either unglycosylated HXLF1 ORF product or glycosylated product from the HXLF2 ORF. Monoclonal antibodies to gcI or gcIII, as well as HCMV negative human serum reacted weakly or failed to react with these translation products. The weak reactivity with monoclonal antibody 41C2 could be prevented by adding 1 mg/ml of ovalbumin (data not shown).

These results demonstrate that monoclonal antibody 9E10, which is specific for the envelope glycoprotein gp47-52, immunoprecipitates gene products from the HXLF1 and HXLF2 genes. In addition, human convalescent serum contains antibodies which react with the HXLF gene products.

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Example V.

Synthesis of gp47-52 in infected cells. The immunoprecipitation studies presented above suggested that gp47-52 is a product of at least the HXLF1 and HXLF2 genes. Gp47-52 resides in virion envelopes as a family of disulfide-linked glycoprotein complexes which are collectively designated gcII. Gp47-52 contains high levels of O-linked sugars, lower levels of N-linked sugars and multiple polypeptides. The synthesis of gp47-52 was investigated using infected human fibroblast cells in the presence or absence of the ionophore monensin, which inhibits golgi transport and allows the addition of high mannose N-linked but not O-linked oligosaccharides [D. C. Johnson et al., J. Virol., 43, 1102 (1982) and Cell, 32, 987 (1983)].

Infected cells were radiolabelled with ^3H -glucosamine at various times after infection and in the presence of monensin, and the resultant antigenic products were solubilized and immunoprecipitated with 9E10 as described hereinabove. A characteristically diffuse glycoprotein band (gp47-52) was detected at 48 and 72 hr p.i. but not at 24 hr p.i. or in uninfected cells.

In the presence of monensin, glycoproteins designated gp32 and gp25 were immunoprecipitated by 9E10. These glycoproteins are probably simple N-linked precursors of gp47-52 since monensin blocks the processing of simple N-linked sugars as well as the addition of O-linked oligosaccharide chains. It is unlikely that gp32 represents golgi-mediated processing of gp25 or vice versa since the concentration of monensin used completely blocked processing of the HCMV gB homolog precursor glycoprotein (data not shown). These results are consistent with the immunoprecipitation of

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30 and 25 kDa HXLf gene products from in vitro translations supplemented with DPM since DPM allows simple N-glycosylation in vitro. In addition, the identification of multiple precursors in the presence of monensin
5 implies that gp47-52 is encoded by multiple HXLf genes.

Example VI.

Comparison of the amino acid composition of gp47-52 with the predicted amino acid compositions of the HXLf gene products. To further demonstrate that gp47-52 is
10 a product of the HXLf genes, this glycoprotein was purified from virion envelopes by a combination of ion exchange and gel exclusion high performance liquid chromatography (HPLC) followed by immunoprecipitation
15 as described by B. Kari et al., J. Virology, 60, 345 (1986), the disclosure of which is incorporated by reference herein. The reduced and alkylated gp47-52 was subjected to strong acid hydrolysis and its amino acid composition was determined by reverse phase HPLC
20 as described by Scholze, in J. Chromatography, 350, 453 (1985). The observed amino acid composition of gp47-52 was then compared to the predicted amino acid compositions of the products coded by the HXLf genes as shown in Table I, below.

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Table I

Comparison of the observed amino acid composition
of gp47-52 with the predicted amino acid composition
of the HXLF1 and HXLF2 ORFs

Amino Acid Compositions ^{a/} <u>Predicted from ORF ^{b/}</u>						
	Amino Acid	HXLF1	HXLF2	Mean of HXLF1 and 2	Mean of HXLF1, 2, 3, 4 and 5	Mean of Observed gp47-52 ^{c/}
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10	Lys	0.2	0.3	0.2	0.4	0.2
	His	0.2	0.4	0.3	0.5	0.2
	Asp	1.0	0.3	0.5	0.6	0.4
15	Met	0.2	0.1	0.1	0.2	0.3
	Ile	0.2	0.3	0.2	0.4	0.4
	Gly	0.7	0.7	0.7	0.9	0.5
	Ala	1.0	1.0	1.0	1.0	1.0
	Pro	1.8	0.8	1.2	1.2	1.3
20	Glu	2.0	0.8	1.2	1.0	1.3
	Thr	1.2	1.2	1.2	1.0	1.0
	Val	2.3	0.8	1.4	1.7	1.2
	Leu	2.8	1.4	1.9	1.9	2.1
	Tyr	1.8	0.6	1.1	1.0	0.7
25	Arg	2.3	0.7	1.3	1.2	0.6
	Phe	0.5	0.3	0.4	0.5	0.9
	Ser	1.7	0.6	1.0	1.4	0.5

^{a/} Amino acid compositions given relative to Ala =
30 1.0 in all cases.

^{b/} Based on DNA sequence by Weston and Barrell, J.
Mol. Biol., 192, 177 (1986).

^{c/} Based on the average of two separate analyses of
purified gp47-52.

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The observed mean amino acid composition of gp47-52 is more similar to the predicted composition of the product coded by HXLF1 plus HXLF2 than to the compositions coded by either gene. However, the observed mean composition had more similarities to the predicted composition of HXLF2 than HXLF1, 3, 4 or 5.

Although some of the variance may reflect differences between the Towne strain used in this study and the sequenced AD169 strain, the presence of the HXLF3, HXLF4 and HXLF5 gene products, or experimental error, it is likely that gp47-52 is composed of multiple HXLF gene products. The heterogeneous nature of gp47-52 and the different mRNA size classes detected by Northern blot analysis support this possibility. Therefore, gp47-52, which is a major component of the virion complex gcII, is specified by the HXLF gene family between map units of 0.857 and 0.879 in the short unique component of the viral genome.

Discussion

The virion envelope glycoprotein gp47-52 is a product of the HXLF gene family of human cytomegalovirus. The evidence for this is as follows: (i) monoclonal antibody 9E10, which is specific for gp47-52, immunoprecipitated the in vitro synthesized HXLF1 and HXLF2 gene products; (ii) the mean amino acid composition of gp47-52 is most similar to the predicted composition of the HXLF1 plus HXLF2 genes; (iii) carbohydrate analysis of gp47-52 has revealed a high level of O-linked sugars and low amounts of N-linked sugars. This is consistent with the hydroxyamino acid content and number of N-glycosylation sites predicted for the HXLF gene products; and (iv) monoclonal antibody 9E10 immunoprecipitated two precursor glycoproteins of 25 and 32 kDa from monensin-treated infected cells, as

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well as two HXLF glycoprotein gene products of 25 and 30 kDa from in vitro translations supplemented with DPM. Taken together, these data indicate that gp47-52 is a product of the HXLF genes.

5 The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

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International Application No: PCT/

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MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page _____, line _____ of the description ¹**A. IDENTIFICATION OF DEPOSIT ²**Further deposits are identified on an additional sheet ☐ ³ pSP65IT (Plasmid)Name of depositary institution ⁴

IN VITRO INTERNATIONAL, INC.

Address of depositary institution (including postal code and country) ⁴611(P) Hammonds Ferry Road
Linthicum, Maryland 21090Date of deposit ⁵

April 26, 1988

Accession Number ⁶

IVI-10168

B. ADDITIONAL INDICATIONS ⁷ (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ⁸** (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS ⁹** (leave blank if not applicable)The indications listed below will be submitted to the International Bureau later ⁹ (Specify the general nature of the indications e.g., "Accession Number of Deposit")**E.** ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

Shirley Hamie☐ The date of receipt (from the applicant) by the International Bureau ¹⁰

was

(Authorized Officer)

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WHAT IS CLAIMED IS:

1. A recombinant expression vector comprising DNA derived from the HCMV genome, wherein said DNA encodes a polypeptide which reacts with monoclonal antibody IVI-10118, said polypeptide corresponding to a portion of gp47-52.
2. The expression vector of claim 1 which is a plasmid.
3. The expression vector of claim 1 which comprises the five homologous HCMV HXLF genes.
4. The expression vector of claim 1 which is essentially free of other DNA derived from the HCMV genome.
5. The expression vector of claim 1 which comprises the gene HXLF1.
6. The expression vector of claim 1 which comprises the genes HXLF1 and HXLF2.
7. A substantially pure polypeptide which is coded for by the expression vector of claims 5 and 6.
8. A substantially pure, nonglycosylated polypeptide having a molecular weight of about 21-25 kD which is coded by the expression vector of claim 5.
9. A substantially pure, nonglycosylated polypeptide having a molecular weight of about 20 kD which is coded by the expression vector of claim 6.

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10. A substantially pure, immunogenic glycoprotein having a molecular weight of about 30 kD, which is coded for by the expression vector of claim 5.
11. A substantially pure, immunogenic glycoprotein having a molecular weight of about 25 kD which is coded for by the expression vector of claim 5.
12. A vaccine against HCMV, comprising an immunologically effective amount of the polypeptide of claim 7 in combination with a pharmaceutically effective vehicle.
13. A method for raising the titer of an antibody against HCMV in the blood of a mammal, comprising administering to said mammal an immunogenically effective amount of the polypeptide of claim 7.
14. A method of detecting HCMV-specific antibody in a clinical sample, which comprises contacting the sample with the polypeptide of claim 7, and detecting the resultant polypeptide-antibody complex.
15. The method of claim 14 wherein the complex is detected by reacting it with a second antibody which incorporates a detectable label or a binding site for a detectable label.
16. The method of claim 14 wherein the polypeptide further incorporates a detectable label.
17. A substantially pure composition of matter consisting essentially of at least one member of the HXLF gene family of human cytomegalovirus (HCMV).

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18. The composition of matter of claim 17 wherein the member comprises the gene HXLF1 or HXLF2.

19. The composition of matter of claim 17 wherein the members comprise the genes HXLF1 and HXLF2.

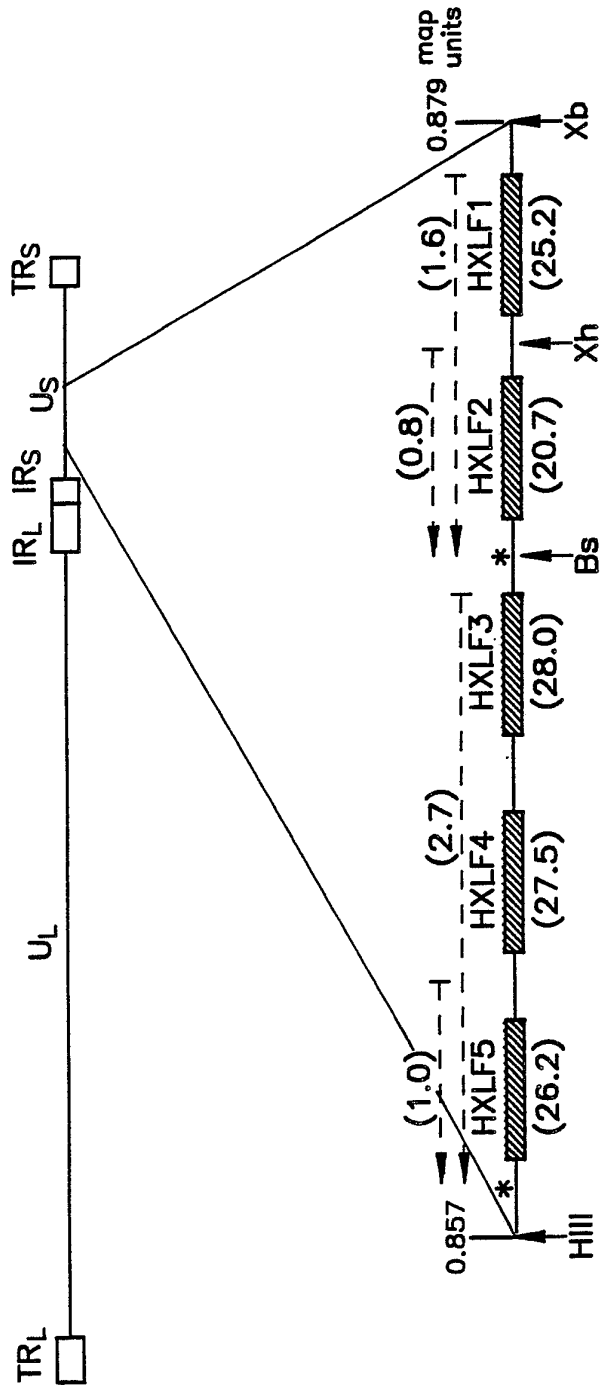


FIG. 1A

FIG. 1B

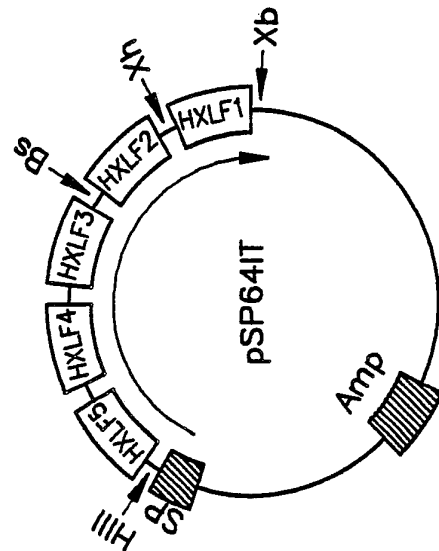
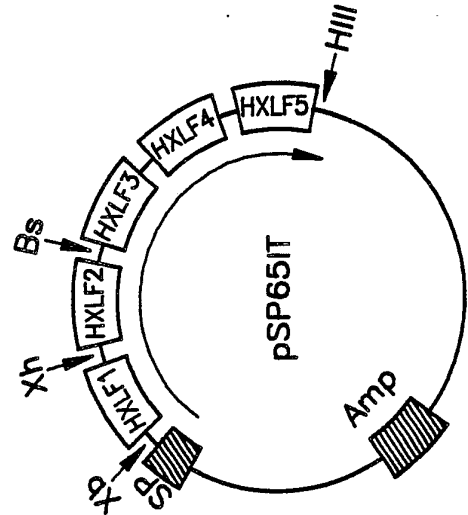



FIG. 1C

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/01850

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 12 N 15/00, A 61 K 39/245, G 01 N 33/569		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 12 N, A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	J. Mol. Biol., vol. 192, no. 2, 20 November 1986, Academic Press Inc. (London) Ltd, (London, GB), K. Weston et al.: "Sequence of the short unique region, short repeats, and part of the long repeats of human cytomegalovirus", pages 177-208, see page 182, column 1, line 6 - page 183, column 1, line 2; figure 5; page 189, column 2, line 46 - page 190 cited in the application --	1-6,17-19
Y	Biological Abstracts/RRM, no. BR35:92649, D.R. Gretch et al.: "In-vitro trans-lation of a bicistronic messenger RNA encoding a human cytomegalovirus envelope glycoprotein complex GCII", & Symposium on the molecular biology of RNA held at the 17th Annual UCLA (University of California-Los Angeles) Symposia on Molecular and Cellular Biology, Keystone, Colorado, USA, ./.	1-6,17-19
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"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</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"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.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
28th July 1989	26 SEP 1989	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 L. ROSSI	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	April 4-10, 1988; J Cell Biochem Suppl 0 (12 Part D). 1988, see title --	
X	Archives of Virology, vol. 98, 1988, Springer Verlag, B. Kari et al.: "Isolation and characterization of a human cytomegalo- virus glycoprotein containing a high content of O-linked oligosaccharides", pages 171-188, see paragraph "Discus- sion" --	7-12,14-16
A	Journal of Virology, vol. 62, no. 3, March 1988, American Society for Microbiology, D.R. Gretch et al.: "Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalo- virus", pages 875-881 --	
A	Journal of Virology, vol. 60, no. 2, November 1986, American Society for Microbiology, B. Kari et al.: "Characterization of monoclonal antibodies reactive to several biochemically distinct human cytomegalovirus glycoprotein complexes", pages 345-352 --	
A	J. Gen. Virol., vol. 67, 1986, SGM (GB), G.H. Farrar et al.: "Characterization of glycoprotein complexes present in human cytomegalovirus envelopes", pages 1469-1473 --	
P,X	Journal of Virology, vol. 62, no. 6, June 1988, American Society for Microbiology, D.R. Gretch et al.: "A multigene family encodes the human cytomegalo- virus glycoprotein complex gcII (gp47-52 complex)", pages 1956-1962, see the whole article -----	1-12,14-19

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 13..... because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.