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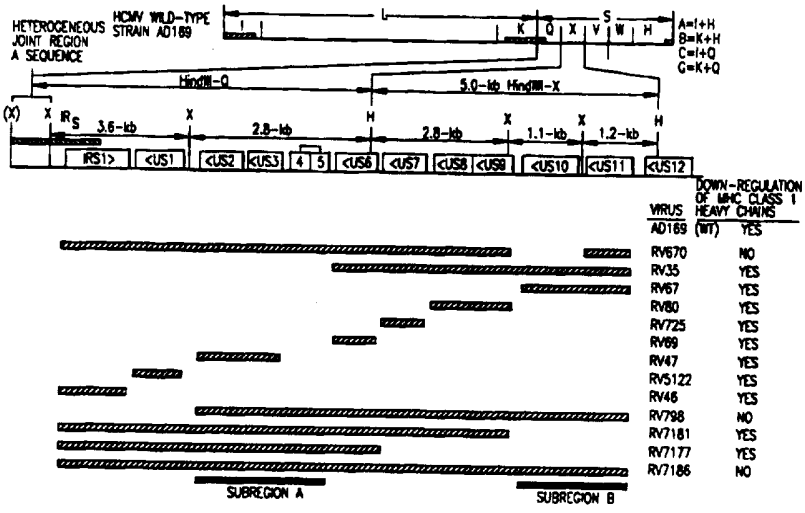

(57) Abstract

Infection of human fibroblast cells with human cytomegalovirus (HCMV) causes down-regulation of cell surface expression of MHC class I. The present invention is directed to a mutant with a 9-kb deletion in the S component of the HCMV genome (including open reading frames IRS1-US9 and US11) which fails to down-regulate class I heavy chains. By examining the phenotypes of mutants with smaller deletions with this portion of the HCMV genome, a 7-kb region containing at least 9 open reading frames was shown to contain the genes required for reduction in heavy chain expression. Furthermore, it was determined that two subregions (A and B) of the 7-kb region each contained genes which were sufficient to cause heavy chain down-regulation. In subregion B, the US11 gene product is involved. It encodes an endoglycosidase H-sensitive glycoprotein which is intracytoplasmic, similar to the adenovirus type 2 E3-19K glycoprotein which inhibits surface expression of class I heavy chains.

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IDENTIFICATION OF A HUMAN CYTOMEGALOVIRUS GENE REGION INVOLVED IN Down-REGULATION OF MHC CLASS I HEAVY CHAIN EXPRESSION

FIELD OF THE INVENTION

The present invention relates to recombinant mutant human cytomegalovirus (HCMV) which does not down-regulate expression of cellular MHC class I heavy chains upon infection.

BACKGROUND OF THE INVENTION

Human cytomegalovirus (HCMV) is a betaherpesvirus which causes clinically serious disease in immunocompromised and immunosuppressed adults, as well as in some infants infected in *in utero* or perinatally (Alford and Britt, 1990). The 230-kb dsDNA genome of HCMV was sequenced (Chee et al., 1990) and has at least 200 open reading frames (ORFs). For purposes of this application, open reading frame is defined as the portion of a gene which encodes a string of amino acids and hence may encode a protein. The function of some HCMV proteins are known or predicted due to their homology with other viral (esp. herpes simplex virus) and cellular proteins. However, for the majority of the HCMV ORFs, the function(s) of the proteins they encode is unknown.

In order to study HCMV gene function HCMV deletion mutants can be constructed in order to assess their *in vitro* growth properties (Jones et al., 1991; Jones and Muzithras, 1992). For purposes of this application, deletion mutants are defined as human cytomegalovirus mutants which lack regions of the wild-type viral genome. This strategy involves site-directed replacement mutagenesis

of selected HCMV gene(s) by a prokaryotic reporter gene, usually β-glucuronidase, although guanosine phosphoribosyltransferase can also be used. In this fashion, the recombinant virus can be isolated only if the replaced viral gene(s) is nonessential.

Several investigators have shown that infection by HCMV results in the down-regulation of cellular MHC class I heavy chains (Browne et al., 1990; Beersma et al., 1993; Yamashita et al., 1993). For purposes of this application, down-regulation is defined as reduction in either synthesis, stability or surface expression of MHC class I heavy chains. Such a phenomenon has been reported for some other DNA viruses, including adenovirus, murine cytomegalovirus, and herpes simplex virus (Anderson et al., 1985; Burget and Kvist, 1985; del Val et al., 1989; Campbell et al., 1992; Campbell and Slater, 1994; York et al., 1994). In the adenovirus and herpes simplex virus systems, the product of a viral gene which is dispensable for replication *in vitro* is sufficient to cause down-regulation of MHC class I heavy chains (Anderson et al., 1985; Burget and Kvist, 1985). The gene(s) involved in class I heavy chain down-regulation by murine cytomegalovirus have not yet been identified.

SUMMARY OF THE INVENTION

The present invention provides recombinant mutant human cytomegaloviruses which do not down-regulate expression of cellular MHC class I heavy chains upon infection. Deletions of gene sequences are made in the region of the genome of the recombinant cytomegalovirus (HCMV) mutant containing open reading frames IRS-1 - US11. Two such mutants, RV 798 and RV 799, both deleted of open reading frames US2-US11, lose the ability to down-regulate MHC class I heavy chains.

The present invention also provides a method of controlling down-regulation of major histocompatibility complex (MHC) class I expression in a cytomegalovirus infected cell which utilizes the

recombinant mutant human cytomegaloviruses.

The present invention also provides a vaccine which utilizes the recombinant mutant human cytomegalovirus, as well as a method of preventing or reducing susceptibility to acute cytomegalovirus in an individual by administering an immunogenic amount of the recombinant mutant human cytomegalovirus. A live attenuated HCMV vaccine lacking gene sequences in the region of the genome of the recombinant cytomegalovirus (HCMV) mutant containing open reading frames IRS-1 - US11 will elicit a better immune response than one containing this gene region, based on the lack of class I down-regulation by the former. Therefore a virus lacking the region is a superior immunogen.

The present invention also provides gene therapy vectors in which the HCMV gene involved in the MHC class I heavy chain down-regulation can be incorporated into adenovirus vectors or similar virus based gene therapy vectors to minimize the immune response. This will allow the use of the recombinant adenovirus or similar virus based gene therapy vectors to be used in gene therapy.

The invention may be more fully understood by reference to the following drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the detection of cell surface MHC class I by immunofluorescence-flow cytometry in HCMV-infected cells. Human foreskin fibroblast (HFF) cells were infected with the indicated virus at a multiplicity of infection of 5 PFU/cell for 72 h. At that time, cells were fixed in 1% paraformaldehyde and stained with primary antibody specific for HLA-A, B, C (W6/32) or control mouse IgG (isotype matched) followed by secondary FITC-conjugated goat anti-mouse IgG. Percent positive cells ($5x10^3$ total) and mean fluorescent intensity (MFI) were calculated on the basis of forward angle light scatter versus log-integrated 90° light scatter using the Immuno

Program, Coulter MDADS I.

FIG. 2 shows expression of MHC class I heavy chains in HCMV wild-type strain AD169-infected cells. FIG. 2A is a Western blot analysis. HFF cells were uninfected (U) or infected at a multiplicity of infection of 5 PFU/cell. At 24, 48, and 72 h postinfection, total cellular proteins were harvested, electrophoresed through a 15% SDS-polyacrylamide gel, electroblotted to nitrocellulose, and probed with TP25.99 murine monoclonal antibody (specific for a non-conformational epitope on MHC class I heavy chains) using an ECL chemiluminescent detection kit (Amersham). FIGS. 2B and C are immunoprecipitation analyses. HFF cells were uninfected or infected (as above), either in the absence or presence (+PFA) of phosphonoformate and radiolabeled either for 4 h at late times postinfection (69-73 h) (FIG. 2B) or for 2 h at the indicated time postinfection (FIG. 2C). Proteins were harvested immediately after radiolabeling and class I heavy chains were immunoprecipitated using TP25.99 murine monoclonal antibody.

FIG. 3 shows organization of recombinant virus genomes. FIG. 3A, the first line, is a schematic of the overall organization of the HCMV wild-type genome. Unique region sequences are shown by a line, while repeated region sequences are indicated by shaded boxes. Relevant *Hin*dIII fragments, within the L and S components, are indicated by letter designation (Oram et al., 1982). The second line is an expansion of the wild-type *Hin*dIII-Q, -X, and -V regions of the S component. The significant open reading frames, and their orientation, are shown as open boxes (Chee et al., 1990). The position of the IR_S repeated sequences is indicated by the shaded rectangle. The locations of *Hin*dIII (H) and *Xho*I (X) restriction endonuclease sites are shown. FIGS. 3B-I show the genomic organization of the indicated HCMV mutant. In each case, the first line is the organization of the AD169 wild-type genome, the second

line represents the organization of relevant sequences of the linearized plasmid used to make the recombinant virus. The slanted lines indicate the boundaries of the viral flanking sequences which may be involved in homologous recombination to create the desired mutation. The region deleted is indicated by a shaded box below the first line. FIG. 3J shows the derivation and organization of RV799. The first two lines are the same representations as FIGS. 3B-I, with the third line representing the organization of the relevant sequences of the linearized plasmid used to make RV799 from the RV134 parent (second line).

FIGS. 4A-C show the analysis of heavy chain expression in cells infected with HCMV mutants. HFF cells were uninfected (U) or infected with the indicated virus (multiplicity of infection of 5 PFU/cell) and radiolabeled for 4 h at late times postinfection (69-73 h). Proteins were harvested immediately after radiolabeling. FIG. 4A is a radiograph of class I heavy chains which were immunoprecipitated using TP25.99 murine monoclonal antibody. FIG. 4B is a radiograph of total radiolabeled proteins to verify approximately equivalent radiolabeling efficiency. FIG. 4C is a radiograph to verify equal progression through the viral replicative cycle. UL80 proteins were immunoprecipitated using anti-assembly protein rabbit polyclonal antiserum.

FIGS. 5A-C shows immunoprecipitation of class I heavy chains from RV798-, RV799-, RV134-, or AD169 wild-type-infected cells. HFF cells were uninfected (U) or infected with the indicated virus (multiplicity of infection of 5 PFU/cell) and radiolabeled for 2 h at late times postinfection (71-73 h). Proteins were harvested immediately after radiolabeling. FIG. 5A is a radiograph of class I heavy chains which were immunoprecipitated using TP25.99 murine monoclonal antibody. Equivalent radiolabeling efficiency (FIG. 5B) and progression through the viral replicative cycle (FIG. 5C) were verified

as described for FIG. 4B and C.

FIG. 6 is a radiograph showing the endoglycosidase H sensitivity of class I heavy chains synthesized in RV798-infected cells. HFF cells were infected with RV798 (multiplicity of infection of 5 PFU/cell) and radiolabeled for 2 h at early times (6-8 h) or late times (80-82 h) postinfection. For comparison purposes, uninfected cells were radiolabeled for 2 h. Proteins were harvested either immediately after radiolabeling (pulse) or after a 2 h chase (chase) in complete unlabeled media. Class I heavy chains were immunoprecipitated using TP25.99 murine monoclonal antibody. Immunoprecipitated protein were incubated for 6 h either in the presence (+) or absence (-) of 1.5mU of endoglycosidase H, prior to SDS-polyacrylamide gel electrophoresis and fluorography.

FIG. 7A-C show the immunoprecipitation of class I heavy chains from RV798-, RV7181-, RV7177-, or AD169 wild-type-infected cells. HFF cells were uninfected (U) or infected with the indicated virus (multiplicity of infection of 5 PFU/cell) and radiolabeled for 2 h at late times postinfection (65-67 h). Proteins were harvested immediately after radiolabeling. FIG. 7A is a radiograph of class I heavy chains which were immunoprecipitated using TP25.99 murine monoclonal antibody. Equivalent radiolabeling efficiency (FIG. 7B) and progression through the viral replicative cycle (FIG. 7C) were verified as described for FIG. 4B-C.

FIG. 8A-D are photographs which show localization of US11 gene product (gpUS11) in infected cells by immunofluorescence. HFF cells were uninfected or infected with either AD169 wild-type or RV699 (deleted of the US11 gene) at a multiplicity of infection of 5 PFU/cell. After 8 h, uninfected and infected cells were fixed with 4% paraformaldehyde. Some cells were then permeabilized with 0.2% Triton X-100. The primary antibody was rabbit polyclonal antisera raised against a US11 fusion protein

(Jones and Muzithras, 1991). Fluorescence was visualized through a Zeiss microscope.

FIG. 9A-D show analysis of heavy chain expression in cells infected with HCMV mutants at early times postinfection. HFF cells were uninfected (U) or infected with the indicated virus (multiplicity of infection of 5 PFU/cell) and radiolabeled for 4 h from 6-10 h postinfection. Proteins were harvested immediately after radiolabeling. FIG. 9A is a radiograph of class I heavy chains which were immunoprecipitated using TP25.99 murine monoclonal antibody. FIG. 9B is a radiograph in which, to verify approximately equal infection, the 72-kDa IE1 immediate-early protein was immunoprecipitated using the murine monoclonal antibody 9221. FIG. 9C is a radiograph of the immunoprecipitation of the cellular transferrin receptor with murine monoclonal antibody Ber-T9 to verify approximately equal expression of this glycoprotein. FIG. 9D is a radiograph of total radiolabeled proteins to verify approximately equivalent radiolabeling efficiency.

FIG. 10 provides a summary of MHC class I heavy chain expression data from HFF cells infected with wild-type and mutant HCMV. The first line is the overall organization of the HCMV wild-type genome, and the second line is an expansion of the wild-type HindIII-Q and -X regions of the S component. The ORFs are indicated by an unshaded rectangle; the unlabeled ORF overlapping US4 and US5 is US4.5. The deletions within the various HCMV mutants are indicated by the shaded rectangle. RV670 is deleted of IRS1-US9 and US11; RV35 is deleted of US6-US11; RV67 is deleted of US10-US11; RV80 is deleted of US8-US9; RV725 is deleted of US7; RV69 is deleted of US6; RV47 is deleted of US2-US3; RV5122 is deleted of US1; RV46 is deleted of IRS1; RV798 is deleted of US2-US11; RV7181 is deleted of IRS1-US9; RV7177 is deleted of IRS1-US6; and RV7186 is deleted of IRS1-US11. MHC class I heavy chain down-

regulation results are from immunoprecipitation experiments (using the heavy chain conformation-independent monoclonal antibody, TP25.99) in which HCMV-infected HFF cells were radiolabeled at late times postinfection. The last line shows the location of the two subregions which contain gene(s) which are sufficient for MHC class I heavy chain down-regulation. Subregion A contains ORFs US2-US5 (bases 193119-195607) and subregion B contains ORFs US10 and US11 (bases 199083-200360).

FIG. 11A-B are Western Blots of cell lines expressing the HCMV US11 gene. Uninfected human U373-MG astrocytoma cells stably transformed with a US11 expression plasmid were analyzed by Western Blot analysis for MHC class I heavy chain expression (FIG. 11A) and for US11 expression (FIG. 11B) using the TP25.99 monoclonal antibody and the US11 polyclonal antisera, respectively.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A recombinant HCMV mutant called RV670 has been constructed which expresses a marker gene (β-glucuronidase) in place of a group of viral genes. Upon infection of human fibroblast cells with this mutant, expression of the major histocompatibility complex (MHC) class I heavy chains is not reduced as it is when wild-type HCMV infects these cells.

Unlike wild-type HCMV, the present invention's virus does not result in the down-regulation of cellular MHC class I heavy chain protein expression. A 7kb region of the HCMV genome which contains genes which are required for down-regulation of heavy chain expression is utilized in the invention.

One skilled in the art will appreciate that efficient antigen processing and presentation is required to activate and expand cytotoxic T-Lymphocyte precursors for an efficient cell mediated immune response. Efficient viral antigen presentation requires the continued expression of MHC class I proteins throughout infection.

Infection of cells with RV670 results in continued expression of class I heavy chains.

One skilled in the art will appreciate that the virus (RV670) or another human cytomegalovirus with a deletion of similar genes, can be utilized to produce an effective live vaccine because class I heavy chains are still expressed in RV670-infected cells, as they are in uninfected cells, and therefore viral antigen presentation for the purpose of initiating a cytotoxic T cell response occurs.

In the present invention, flow cytometry and immunofluorescence experiments confirmed that cell surface expression of class I heavy chains are greatly reduced at late times postinfection in HCMV wild-type strain 8169 infected HFF cells. Radiolabeling-immunoprecipitation experiments indicated that down-regulation of newly synthesized MHC class I heavy chains occurred throughout the course of infection, beginning at very early times (3 h) postinfection (FIG. 2C). This reduction has been reported to be at the post-translational level: class I heavy chains have a higher turnover rate in HCMV-infected cells than in uninfected cells (Beersma et al., 1993). Such instability of class I heavy chains results in a reduced cell mediated immune response to HCMV infection since viral peptides will be inefficiently presented. Thus, the reduction in class I heavy chain expression is important in terms of evasion of a host's immune system in the establishment of persistent or latent infections by HCMV (Gooding, 1992).

We screened our bank of HCMV mutants, which represent 18 ORFs which are dispensable for viral replication in tissue culture, for their ability to cause down-regulation of MHC class I heavy chains. A 7-kb region of the S component of the HCMV genome, containing ORFs US2-US11 (bases 193119-200360), was clearly shown to contain genes which are required for this phenotype (data summarized in FIG. 10). Within this region, there are two subregions,

each of which contain genes sufficient for heavy chain down-regulation.

Subregion A contains ORFs US2-US5 (bases 193119-195607). It has been proposed that US2 and US3 encode membrane glycoproteins (Chee et al., 1990). US3 is a differentially spliced gene which is expressed throughout the viral replicative cycle and encodes a protein with transcriptional transactivating function (Tenney and Colberg-Poley, 1991; Colberg-Poley et al., 1992; Tenney et al., 1993; Weston, 1988). Several smaller ORFs are also present in this subregion (between the ORFs US3 and US5), but their expression characteristics or functions have not been reported. Gretch and Stinski (1990) reported that there is a 1.0-kb early mRNA transcribed from this region of the HCMV genome, but it was not fine-mapped. It is not yet known which of these genes are involved in heavy chain down-regulation.

Subregion B, which is also sufficient for MHC class I heavy chain reduction, contains the US10 and US11 genes (FIG. 10), bases 199083-200360. However, based on data using HCMV mutant RV670 which expresses wild-type levels of the US10 gene product, US10 expression is not sufficient for down-regulation of heavy chain expression (FIG. 2B). The genetic data implicated the US11 gene product as being required. We have demonstrated that US11 expression is sufficient to cause MHC class I heavy chain down-regulation in stably transformed uninfected cells in the absence of other MCNV proteins (FIG. 11). RNA and protein expression from both of these ORFs begins early and proceeds throughout the course of infection (Jones and Muzithras, 1991); US10 and US11 encode glycoproteins of 22-kDa (gpUS10) and 32-kDa, (gpUS11) respectively; both glycoproteins have N-linked sugar residues which are completely endoglycosidase H sensitive. These glycoproteins are retained in the endoplasmic reticulum or cis golgi. Consistent with this conclusion is

the immunofluorescence data in which gpUS11 was not detected on the cell surface, but was detected in the cytoplasm of HCMV-infected cells (FIG. 8). The characteristics of HCMV gpUS11 (as well as gpUS10) are similar to the 25-kDa glycoprotein (E3-19K) encoded from the E3 region of adenovirus type 2. Ad E3-19K is nonessential for viral replication. It has been shown to contain endoglycosidase H-sensitive N-linked sugar residues, be retained in the endoplasmic reticulum, and bind MHC class I heavy chains, thereby preventing their transport to the cell surface 9 (Anderson et al., 1985; Burgert and Kvist, 1985). In contrast to Ad E3-19K, a direct association between gpUS11 (or gpUS10) and class I heavy chains (i.e., by coimmunoprecipitation) was not detected (data not shown).

The identification of US2-US11 gene region as the region of the HCMV genome required for down-regulation of MHC class I heavy chains is significant in several respects. As mentioned above, expression from this region of the genome throughout the course of infection acts to interfere with an effective cell mediated immune response. Surface expression of MHC class I molecules is required for antigen presentation to activate and expand cytotoxic T lymphocyte (CTL) precursors populations (Schwartz, 1985). In addition, they are further required for target recognition by the activated CTLs (Zinkernagel and Doherty, 1980). In MCMV, CTLs against the major immediate-early protein are protective against lethal infection by this virus (Jonjic et al., 1988). However, in HCMV infected individuals, the frequency of CTLs against the analogous HCMV immediate-early protein, IE1, are reported to be extremely rare (Gilbert et al., 1993). Recent studies have shown that IE peptides are more efficiently presented by interferon-γ-treated HCMV-infected cells, than by untreated infected cells (Gilbert et al., 1993). Interferon μ causes increased surface expression of MHC class I proteins. Thus, increasing the expression of class I heavy chains in HCMV-infected

cells may be important in the efficient generation of IE-specific CTLs, or CTLs against other important HCMV antigens. A HCMV mutant deleted of the US2-US11 gene region would have this effect since the class I heavy chains are not down-regulated when cells are infected with this mutant. Therefore, a deletion of this region of the viral genome is important in the development of a live HCMV vaccine to induce an effective anti-HCMV immune response.

Several years ago it was reported that the HCMV UL18 ORF encoded a protein which resembled MHC class I heavy chains (Beck and Barrell, 1988). It was hypothesized that the down-regulation of heavy chains in HCMV-infected cells was due to competition of the UL18 gene product for β2-microglobulin, which effectively prevented the normal association of class I heavy chains and β2-microglobulin (Browne et al., 1990). This hypothesis was essentially dispelled when a HCMV mutant deleted of UL18 retained its ability to down-regulate heavy chain expression (Browne et al., 1992). It remained possible that the UL18 gene product was only one of several HCMV genes whose expression is sufficient for this phenotype. However, the present invention data indicates that only genes within the US2-US11 region are sufficient for class I heavy chain down-regulation.

The existence of two independent mechanisms which result in down-regulation of MHC class I expression emphasizes the importance of this phenotype for successful infection and persistence in the host. One mechanism may serve as a backup system for the other, but it is also plausible that there is cell type specificity for each system. In the case of the HFF cell system, both mechanisms are functional. However, in U373-MG cells, down-regulation of heavy chain expression is more dependent on the presence of the subregion A. In that case, there may be qualitative or quantitative differences in cellular proteins which interact with subregion B gene products. A

similar situation exists in the herpes simplex virus system. It was recently reported that the 88 amino acid US12 gene product (ICP47) is sufficient for class I heavy chain sequestering in the endoplasmic reticulum (York et al., 1994). However, expression of heavy chains is not affected in herpes simplex virus-infected mouse cells, although ICP47 is expressed in those cells and murine heavy chains are down-regulated when expressed in an HSV-infected human fibroblast system (York et al., 1994).

A pharmaceutical composition may be prepared containing the recombinant HCMV mutant of the present invention in which the genome is devoid of a gene sequence capable of down-regulating MHC Class I expression in infected cells. A stabilizer or other appropriate vehicle may be utilized in the pharmaceutical composition.

As discussed earlier, the recombinant HCMV mutant of the present invention which is devoid of the gene sequence capable of down-regulating MHC Class I expression may be used in a vaccine for the prevention of cytomegalovirus infections. The vaccine comprises an effective amount of the recombinant HCMV mutant in a pharmaceutically acceptable vehicle. An adjuvant may be optionally added to the vaccine.

A method of immunizing an individual against cytomegalovirus may be carried out by administering to the individual an immunogenic amount of the recombinant HCMV mutant of the present invention which is devoid of the gene sequence capable of down-regulating MHC class I expression.

A method of preventing or reducing susceptibility in an individual to acute cytomegalovirus may be carried out by administering to the individual an immunogenic amount of the recombinant HCMV mutant of the present invention which is devoid of the gene sequence capable of down-regulating MHC class I

expression.

Down-regulation of MHC class I expression in a cytomegalovirus infected cell may be controlled by a method having the steps of identifying a gene sequence capable of down-regulating the major histocompatibility complex and deleting the identified gene sequence from the cytomegalovirus genome.

As discussed earlier, the gene sequence involved in the MHC class I heavy chain down-regulation can be incorporated into adenovirus vectors or similar virus based gene therapy vectors to minimize the immune response and allow the use of the vectors in gene therapy. One virus based gene therapy vector comprises the gene sequence of the open reading frame of US11. Another virus based gene therapy vector comprises the gene sequences of subregions A and B (open reading frames US2-US5 and US10-US11, respectively).

EXAMPLE 1

Virus and Cells

HCMV strain AD169 is obtained from the American Type Culture Collection and propagated according to standard protocols known by those skilled in the art. Human foreskin fibroblast (HFF) cells were isolated in this laboratory and used below passage twenty (Jones and Muzithras, 1991). They were grown in Dulbeccos modified Eagle medium (DMEM) containing 10% fetal bovine serum and 25mM HEPES.

DNA Sequence

The numbering system of Chee et al. (1990) of the HCMV strain AD169 DNA sequence (Genbank accession number X17403) is used in the present invention.

Plasmids

Plasmids used for creation of HCMV mutants were constructed using the method described previously (Jones et al.,

1991; Jones and Muzithras; 1992). Generally, the β-glucuronidase reporter gene is surrounded on each side by 1.5-kb of HCMV sequences which flank the gene(s) to be deleted from the virus. In each case, the plasmid DNA is linearized with a restriction enzyme which cuts within the prokaryotic backbone prior to transfection. The HCMV strain AD169 genomic DNA fragments are derived from either pHind-G, pHind-X, or pXba-P which contain the *Hin*dIII-G (bases 176844 to 195837), -X (bases 195837 to 200856), and *Xba*I-P (bases 200391 to 206314) DNA fragments, respectively (Oram et al., 1982; Jones et al., 1991). pUS7/US3 contained the 1.7-kb *Pst*I-*Pst*I HCMV fragment (bases 196447 to 194741 in pIBI30 vector [International Biotechnologies, Inc.]) derived from pHind-G and pHind-X.

To replace HCMV ORFs US11 through IRS1 by β-glucuronidase (i.e., RV7186; FIG. 3), pBgdUS11/IRS1 was constructed. Sequentially, this plasmid contained the 1.8-kb fragment *Pst*I-*Xba*I fragment (bases 202207 to 200391, containing US13, US12, and US11 promoter sequences, from pXba-P), β-glucuronidase, a 288-b SV40 fragment containing the early and late polyadenylation signals (from pRcCMV [Invitrogen]), and the 1.7-kb *Nco*I-*Nco*I fragment (bases 189763 to 188062, containing J1I to IRL1 sequences, from pHind-G).

To replace HCMV ORFs US11 through US2 by β-glucuronidase (i.e., RV798; FIG. 3), pBgdUS11/US2 was constructed. Sequentially, this plasmid contained the 1.8-kb fragment *Pst*I-*Xba*I fragment (bases 202207 to 200391, containing US13, US12, and US11 promoter sequences, from pXba-P), β-glucuronidase, a 255-b fragment containing the US10 polyadenylation signal (bases 199276 to 199021, from pHind-X), and the 1.3-kb *Nhe*I-*Apa*I fragment (bases 193360 to 192033, containing C-terminal US2 to IRS1 sequences, from pHind-G).

To replace HCMV ORFs US11 through US6 by β-glucuronidase (i.e., RV35; FIG. 3), pBgdUS11/US6 was constructed. Sequentially, this plasmid contained the 1.8-kb *Pst*I-*Xba*I fragment (bases 202207 to 200391, containing US13, US12, and US11 promoter sequences, from pXba-P), β-glucuronidase, and the 1.5-kb *Hpa*I-*Sst*II fragment (bases 195589 to 194062, containing C-terminal US6 to US3 sequences, from pHind-G). Replacement of HCMV ORFs US11-US10, or ORF US11 (singly), by β-glucuronidase (i.e. RV67 and RV699, respectively) were described previously (Jones et al., 1991).

To replace HCMV ORFs US9 through IRS1 by β-glucuronidase (i.e. RV7181; Fig. 3), pBgdUS9/IRS1 was constructed. Sequentially, this plasmid contained the 1.1-kb *Sal*I-*Apa*I fragment (bases 200171 to 199021), the 351-b SV40 early promoter (from pRcCMV), β-glucuronidase, the 288-b SV40 polyadenylation signal fragment, and the 1.7-kb *Nco*I-*Nco*I fragment (bases 189763 to 188062, containing J1I to IRL1 sequences, from pHind-G).

To replace HCMV ORFs US6 through IRS1 by β-glucuronidase (i.e., RV7177; FIG. 3), pBgdUS6/IRS1 was constructed. Sequentially, this plasmid contained the 1.7-kb *Nco*I-*Nco*I fragment (bases 188062 to 189763, containing IRL1, J1I, and IRS1 promoter sequences, from pHind-G), β-glucuronidase, the 255-b fragment containing the US10 polyadenylation signal (bases 199276 to 199021, from pHind-X), and the 1.8-kb *Bsm*I-*Sau*I fragment (bases 196222 to 198030, containing US7 to C-terminal US9 sequences, from pHind-X).

To replace HCMV ORFs US3 and US2 by β-glucuronidase (i.e., RV47; FIG. 3), pBgdUS3/US2 was constructed. Sequentially, this plasmid contained the 1.7-kb *Pst*I-*Pst*I fragment (bases 196447 to 194741), a 180-b *Sma*I-*Hae*III fragment containing the HSV-1 gH promoter (McKnight, 1980), β-glucuronidase, the 255-b US10 polyadenylation signal fragment, and the 1.3-kb *Nhe*I-*Apa*I fragment

(bases 193360 to 192033, containing C-terminal US2 to IRS1 sequences, from pHind-G).

To replace HCMV ORF US1 by β-glucuronidase (i.e., RV5122; FIG. 3), pBgdUS1 was constructed. Sequentially, this plasmid contained the 1.8-kb *Aat*II-*Sst*I fragment (bases 190884 to 192648, containing IRS1 and US1 C-terminal sequences, from pHind-G), a 180-b *Sma*I-*Hae*III fragment containing the HSV-1 gH promoter (McKnight, 1980), β-glucuronidase, the 255-b US10 polyadenylation signal fragment, and the 1.6-kb *Sph*I-*Sph*I fragment (bases 192934 to 194544, containing US2 and C-terminal US3 sequences, from pHind-G).

To replace HCMV ORF IRS1 by β-glucuronidase (i.e., RV46; FIG. 3), pBgdIRS1 was constructed. Sequentially, this plasmid contained the 1.7-kb *Nco*I-*Nco*I fragment (bases 188062 to 189763, containing IRL1, J1I, and IRS1 promoter sequences, from pHind-G), β-glucuronidase, the 255-b fragment containing the US10 polyadenylation signal (bases 199276 to 199021, from pHind-X), and the 1.2-kb *Nar*I-*Xho*I fragment (bases 191830 to 193003, containing C-terminal IRS1 and US1 sequences, from pHind-G). To delete HCMV ORFs US11 through US2 without insertion of a reporter gene (i.e., RV799; FIG. 3), pdUS11/US2 was constructed. Sequentially, this plasmid contained the 1.8-kb fragment *Pst*I-*Xba*I fragment (bases 202207 to 200391, containing US13, US12, and US11 promoter sequences, from pXba-P), β-glucuronidase, 65-b *Nru*I-*Apa*I fragment containing the US10 polyadenylation signal (bases 199086 to 199021, from pHind-X), and the 1.3-kb *Nhe*I-*Apa*I fragment (bases 193360 to 192033, containing C-terminal US2 to IRS1 sequences, from pHind-G).

Isolation of Recombinant Mutant HCMV

Creation and isolation of recombinant mutant HCMV was done as described previously (Jones et al., 1991; Jones and

Muzithras, 1992). HFF cells were split so that they were 70-80% confluent on the day of transfection. The cells were trypsinized and suspended to $5.6x10^6$ cells per ml in DMEM/10% FCS/25mM HEPES. The DNA was transfected using a modified calcium phosphate co-precipitation technique. 1.5 μg of infectious HCMV DNA and 2.5 μg of linearized plasmid DNA were mixed in the calcium chloride solution (300 μl containing 10 mM Tris pH 7.0/250 mM calcium chloride) and chilled on ice. To initiate the co-precipitation, the DNA was removed from the ice and 300 μl 2X HeBS pH 6.95 (at room temperature; 1X HeBS was 19.2 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.8 mM sodium phosphate, 0.1% dextrose) was added dropwise with gentle mixing. After 1.5 minutes, the precipitate was placed on ice (to prevent further precipitate from forming). The precipitate was mixed with $3x10^6$ cells (in suspension) and placed in a 82mm tissue culture plate. After 6 h at 37°C, the media was removed and the cells were shocked with 20% DMSO in 1X HeBS for 2 minutes. The cells were washed twice with PBS and growth media was added. The media was changed every 4-7 days. After 14 days, viral plaques were observed and the cells were overlaid with 0.5% agarose in DMEM containing 150 μg/ml X-gluc (5-bromo 4-chloro 3-indol 1-glucuronide; Biosynth). Blue plaques (i.e., β-glucuronidase-positive mutant virus plaques) were picked several days after adding the overlay. Recombinant viruses were plaque purified three times. HCMV mutant RV799 was β-glucuronidase-negative and was isolated using a modification of the above procedure. In this case, β-glucuronidase-positive HCMV mutant RV134 was the parent virus (Jones et al., 1991). Thus, RV134 genomic DNA was used instead of wild-type strain AD169 DNA in the transfections. Primary plaques appearing on the primary transfection plates were picked at random and replated on HFF cells. After 10 days, the media was removed and the infected cells were overlaid with X-gluc-containing agarose as described above.

In this case, white plaques (β-glucuronidase-negative mutant virus plaques) were picked 4 days later and plaque purified. The proper genomic organization of each of the HCMV mutants was verified by DNA blot hybridization analysis as described previously (Jones et al., 1991).

Antibodies

Rabbit polyclonal antisera reactive with HCMV US11 proteins and HCMV UL80 proteins are described previously (Jones et al., 1991; 1994). Murine monoclonal antibodies W6/32, specific for a conformation-dependent epitope on the heavy chain of human MHC class I proteins, and Ber-T9, specific for the human transferrin receptor, were purchased. Murine monoclonal antibody TP25.99 (D'Urso et al., 1991), specific for a conformation-independent epitope on the heavy chain of human MHC class I proteins, was obtained from Dr. S. Ferrone (Department of Microbiology, New York Medical College, Valhalla, NY). Murine monoclonal antibody 9221, specific for the HCMV IE1 protein, was purchased from Dupont.

Radiolabeling and Immunoprecipitation of Infected Cell Proteins

Pulse-chase radiolabeling was done according to standard protocol (Sambrook et al., 1989). HCMV-infected HFF cells (multiplicity of infection equalled five) was pulse-labeled with 200μCi of [^{35}S] methionine and [^{35}S]cysteine (NEN-DuPont) per ml in methionine/cysteine-free Dulbecco's modified Eagle medium (DMEM) at the indicated time period postinfection. The radioactive media was removed, the cells washed twice in complete DMEM, and chases were done for the indicated time in complete DMEM. Proteins were extracted using triple detergent lysis buffer (Sambrook et al., 1989). The cleared protein extracts (supernatant after centrifugation for 5 minutes at 15000 x g and 4°C) were retained for immunoprecipitation according to standard protocol (Sambrook et al., 1989). Proteins

binding to antibodies were pelleted using protein A sepharose (Pharmacia). For immunoprecipitations of the human transferrin receptor, rabbit anti-mouse IgG (Pierce) were added prior to protein A sepharose. The washed immunoprecipitates were boiled in the presence of 2-mercaptoethanol and electrophoresed in denaturing polyacrylamide gels. The gels were fixed and soaked in 1M sodium salicylate fluor (Sambrook et al.,1989) prior to drying and autoradiography.

Immunofluorescence

Immunofluorescence assays were done according to standard protocol (Harlow, 1989). All procedures were done in 60mm tissue culture plates. Briefly, infected or uninfected HFF cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 (where indicated). After adding 3% bovine serum albumin in phosphate-buffered saline, the cells were held overnight at 4°C. The cells were treated sequentially with the following antisera, each for 30 minutes at room temperature: 10% HCMV-negative human serum (to block any Fc receptors); the indicated primary antibody; and FITC-conjugated anti-mouse or anti-rabbit IgG, as appropriate.

EXAMPLE 2

Class I Down-Regulation in HCMV Wild-Type-Infected Human Fibroblasts

We sought to ascertain the timing and nature of MHC class I heavy chain down-regulation in the present invention's human foreskin fibroblast (HFF) cell culture system. By flow cytometry, HCMV strain AD169 wild-type-infected HFF cells were significantly reduced in the expression of class I heavy chains on their cell surface at late times postinfection (i.e., 72 h) using the conformation-dependent class I monoclonal antibody W6/32 (FIG. 1). In Western analyses using the conformation-independent class I monoclonal antibody (TP25.99), it was demonstrated that the steady state level of

class I protein was also reduced at late times postinfection (FIG. 2A). Because viral peptides are presented at the cell surface by class I complexes assembled after infection, we sought to assess the status of class I proteins synthesized at various times postinfection by immunoprecipitation of metabolically radiolabeled proteins. As shown in FIG. 2B, reduction in expression of class I heavy chains was detected both in the presence and absence of the viral DNA synthesis inhibitor, phosphonoformate. This indicated that viral immediate-early or early gene functions are sufficient for heavy chain reduction. In addition, it was demonstrated that heavy chain down-regulation was detected at very early times postinfection: 3 h (FIG. 2C). Since this effect was observed using the conformation-independent antibody, the reduction reflects overall levels of newly synthesized heavy chains.

Screening of HCMV Mutants for the Loss of MHC Class I Down-Regulation

Several previously constructed HCMV deletion mutants, representing 18 nonessential ORFs (UL33, UL81, IRS1, US1-US13, US27-US28, and TRS1), were screened for heavy chain expression by flow cytometry and immunoprecipitation analyses. Only RV670, a mutant deleted of a 9-kb region within the S component of the HCMV genome (Jones and Muzithras, 1992), did not retain the wild-type down-regulation phenotype (FIG. 4A). This mutant was deleted of at least 11 ORFs, IRS1 through US11 (except for US10), which includes the US6 family of genes (US6-US11) which putatively encode glycoproteins (Chee et al., 1990). To confirm this observation, two additional independently derived mutants which had the same deletion as RV670 and a new mutant, RV7186, deleted of the entire IRS1-US11 region (FIG. 3) were tested. Each was phenotypically identical to RV670 and stably expressed class I heavy chains. Previously, we constructed HCMV mutants deleted of US6 family ORFs, either individually or in groups (Jones and Muzithras, 1992), and similar

deletion mutants within the adjacent IRS1-US3 region. By immunoprecipitation using the conformation-independent antibody, all of these mutants were shown to retain the ability to down-regulate class I heavy chains (FIG. 4A) at late times postinfection in HFF cells. Control experiments indicated that radiolabeling was equivalent between the different infected cell cultures (FIG. 4B) and that infection proceeded to late times equally, as judged by pp65 (FIG. 4B) and UL80 protein (FIG. 4C) expression. These data indicate: (i) that more than one viral gene is sufficient for the reduction in class I heavy chains; or (ii) gene(s) between US3 and US6, deleted in RV670 and RV7186 but not the other mutants, is required for the phenotype.

Identification of a 7-kb Region of the HCMV Genome Required for MHC Class I Down-Regulation

To further localize the region containing gene(s) involved in MHC class I heavy chain down-regulation, additional HCMV replacement mutants containing deletions of multiple genes within the IRS1-US11 gene region were created (FIG. 3). One of these mutants, RV798, was deleted of genes from US2-US11. In HFF cells infected by RV798 and analyzed at late times postinfection, MHC class I heavy chains were not down-regulated as they are in wild-type strain AD169-infected cells (FIG. 4A); in fact, a slight stimulation is observed. Several independently-derived deletion mutants identical to RV798 were examined similarly: all lacked the ability to down-regulate class I heavy chains. To further confirm that the 7-kb HCMV US2-US11 region contained the gene(s) required for heavy chain down-regulation, mutant RV799 was constructed which had the identical US2-US11 deletion as RV798, but was created by a different strategy. RV798 was derived from wild-type strain AD169 by inserting a β-glucuronidase marker gene in the place of US2-US11. In contrast, the parent of RV799 was RV134, a mutant which was β-glucuronidase-positive since it had a β-glucuronidase expression cassette inserted

within the US9-US10 intergenic region (Jones et al., 1991). To create RV799, a plasmid was designed which upon recombination with the RV134 genome would simultaneously delete US2-US11 and the β-glucuronidase expression cassette (FIG. 3). The proper RV799 HCMV mutant was isolated as a white plaque in the presence of the β-glucuronidase substrate, since it was β-glucuronidase-negative. RV799, but not the RV134 parent, was phenotypically identical to RV798 (FIG. 5) Thus, since RV798 and RV799 were created by different strategies using parents which retained the ability to down-regulate MHC class I heavy chains, this confirms that the gene(s) required for the phenotype are located within the 7-kb US2-US11 region (bases 193119-200360).

To determine whether the proper surface expression of class I heavy chains occurred at late times postinfection with either RV798 or RV799, immunofluorescence assays were done. Using either the conformation-dependent (W6/32) or conformation-independent (TP25.99) monoclonal antibodies, surface expression of MHC class I heavy chains was detected in uninfected and RV798- and RV799-infected HFF cells, but not wild-type AD169-infected HFF cells. Proper maturation of class I heavy chains in uninfected cells yielded endoglycosidase H resistant molecules. In contrast, class I heavy chains synthesized in AD169-infected cells were reported to be entirely endoglycosidase H sensitive (Beersma et al., 1993). As shown in FIG. 6, class I heavy chains synthesized in RV798-infected HFF cells, either at early or late times postinfection, were converted to the mature endoglycosidase H-resistant form at a rate similar to those synthesized in uninfected cells. Taken together, these data indicate that MHC class I synthesis, processing, and surface expression are not impaired in cells infected with these HCMV mutants. Furthermore, the results indicate that the 7-kb region containing US2-US11 genes contain one or more genes required for heavy chain down-regulation

by HCMV.

Two Subregions Within the US2-US11 Gene Region Contain Genes Which are Involved in Class I Heavy Chain Down-Regulation

The region of the HCMV genome deleted in RV35 was from US6-US11, and US2-US11 in RV798 (FIG. 3). In RV35-infected HFF cells, MHC class I heavy chains were down-regulated, but in RV798-infected cells they were not (Fig. 4A). This data indicates that one or more genes involved in heavy chain down-regulation maps within the 2-kb subregion from ORF US2 through US5 (subregion A; bases 193119-195607). To determine if this 2-kb subregion is required for class I heavy chain down-regulation, HCMV replacement mutants RV7181 and RV7177 were examined. HCMV ORFs IRS1-US9 and IRS1-US6 are deleted, respectively, in these mutants; hence, subregion A is absent from both mutants. Experiments in infected HFF cells at late times postinfection indicated that both mutants retained the ability to efficiently down-regulate class I heavy gene expression (FIG. 7). Therefore, when present in the HCMV genome, gene(s) within subregion A are sufficient for reduction of MHC expression (e.g., RV35), although their presence is not required for the phenotype. Furthermore, the cumulative data indicate that there are no HCMV genes within the identified 7-kb US2-US11 region (i.e., the region deleted in RV798) which are absolutely required for efficient heavy chain down-regulation in infected HFF cells, suggesting that gene(s) from another portion of the US2-US11 gene region are also sufficient for the phenotype at late times postinfection.

Evidence Indicating That the US11 Gene Product is Involved in MHC Class I Heavy Chain Down-Regulation

In HFF cells infected with mutant RV7181, deleted from IRS1-US9 (FIG. 3), MHC class I heavy chain expression was down-

regulated, in contrast to RV798-infected HFF cells (FIG. 7). This data suggests that a second subregion (subregion B), comprised of the US10 and US11 genes (bases 199083-200360), is involved in reduction of heavy chain expression. However, the expression of US10 from the context of the HCMV genome is not sufficient for heavy chain down-regulation. HCMV mutant RV670 expressed US10 at steady-state levels similar to wild-type and was deleted of all of the other ORFs in the 7-kb US2-US11 gene region, but it did not cause down-regulation of MHC class I heavy chains in infected HFF cells (FIGS. 2B and 4A).

US11 encodes a 32-kDa glycoprotein (gpUS11) containing N-linked, but not O-linked, carbohydrates which are completely sensitive to endoglycosidase H, indicating that the sugars are in the high mannose form. gpUS11 was detected throughout infection, beginning at very early times (i.e. 3 h) and continuing through late times postinfection. However, levels of gpUS11 in the infected cell are most abundant at approximately 8 h postinfection. To determine its location in the infected cell, rabbit polyclonal antisera (Jones and Muzithras, 1991) was used in immunofluorescence assays of wild-type strain AD169-infected cells. Uninfected and RV699-infected HFF cells were used as negative controls. RV699 is an HCMV mutant which is isogeneic with AD169, except for a deletion of the US11 ORF (Jones et al., 1991). In cells fixed and permeabilized at 8 h postinfection, cytoplasmic fluorescence which obscured definition of the nucleus was observed in AD169-infected HFF cells, but not in either negative control cells (FIG. 8). In general, the specific fluorescence was more intense in the perinuclear area. There was no specific fluorescence detected in non-permeabilized cells (FIG. 8). The fluorescence and endoglycosidase-H sensitivity data indicate that gpUS11 is not a cell surface glycoprotein. From the translated DNA sequence, gpUS11 is predicted to have hydrophobic

domains near its N- and C-termini (Weston and Barrell, 1986) which are putative signal sequence and transmembrane domains, respectively. Thus, gpUS11 is associated with intracytoplasmic membranes, possibly the endoplasmic reticulum.

Down-Regulation of MHC Class I Expression at Early Times Postinfection by HCMV Mutants

MHC class I expression in wild-type strain AD169-infected cells are shown to begin at very early times postinfection (FIG. 2C). To determine if any of the mutants are deficient for this early down-regulation, immunoprecipitation experiments were performed using extracts from infected HFF cells radiolabeled from 6-10 h postinfection. The level of class I heavy chains were reduced during this early period postinfection in HFF cells with each of the mutants, except for RV798, the mutant deleted of the entire 7-kb US2-US11 region (FIG. 9A). Control experiments demonstrated that the different mutant-infected cells were equally infected and radiolabeled (FIG. 9B and D). Expression of another cellular glycoprotein, the transferrin receptor, was not differentially affected by the various mutants (FIG. 9C). Thus, genes required for heavy chain down-regulation at early times postinfection are the same as those necessary for reduction at late times postinfection. Moreover, expression of gene(s) from either subregion identified to be involved in down-regulation of heavy chain expression at late times postinfection are sufficient for reduction at very early times postinfection.

EXAMPLE 3

Recombinant HCMV (RV798) Vaccine Preparation

HCMV vaccines were prepared using a method described previously (Elek and Stern, 1974). HCMV mutant RV798 was grown on MRC-5 human diploid lung fibroblasts (CCL171 [American Type Culture Collection]) or human foreskin fibroblasts (MRHF [BioWhittaker]). Cells were infected at a multiplicity of infection equal

to one in Dulbecco's modified Eagle medium (DMEM) containing 5% calf serum and 5% fetal calf serum. After 24 h, the medium was removed and the cells washed three times with either Hank's balanced salt solution or Dulbecco's phosphate-buffered saline. Fresh DMEM medium without serum was added; the infected cells were incubated 4 days after the appearance of late viral cytopathic effect (usually 7 days postinfection). After a preclearing centrifugation step (6,000 x g for 20 minutes at 18°C), cell-free virus was pelleted by centrifugation at 15,500 x g for one hour at 18°C. The pelleted virus was resuspended in Dulbecco's phosphate-buffered saline containing 25% sorbitol and stored in aliquots at -70°C. The titer of RV798 vaccine stock is determined using standard procedures on human foreskin fibroblasts (Wentwork and French, 1970). The vaccine is administered by subcutaneous inoculation of approximately 10^3-10^7 plaque forming units into the deltoid region of the upper arm, as described previously (Elek and Stern, 1974; Gehrz et al., 1980; Starr et al., 1981).

EXAMPLE 4

gpUS11 is Sufficient for Down-Regulation of MHC Class I Heavy Chains

To determine if the US11 gene product, in the absence of any other viral gene products, is capable of causing heavy chain down-regulation, the US11 coding region (bases 200360-199716 [Chee et al., 1990]) and some non-coding flanking sequences, encompassing bases 200391-199683, were cloned into a eukaryotic expression plasmid under the transcriptional control of the constitutive HCMV major immediate-early promoter-enhancer. Human U373-MG astrocytoma cells (HTB 17 [American Type Culture Collection]) were transfected with this plasmid (Sambrook et al, 1989) and stably transformed cells were selected in the presence of 0.375 μg/ml of puromycin, since the plasmid also encodes for the prokaryotic

puromycin resistance gene. Clones were picked and expanded into cell lines. Those expressing gpUS11 were identified by Western Blot analysis; different cell lines expressed varying amounts of US11. MHC class I heavy chain expression in these cell lines was analyzed in a similar fashion. As shown in FIG. 11, expression of US11 was inversely correlated with the expression of class I heavy chains. These data prove that expression of HCMV US11 is sufficient for the down-regulation of MHC class I heavy chain expression in the absence of any other viral gene products.

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and D.C. Johnson. 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell 77:525-535.

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WHAT IS CLAIMED IS:

1. A recombinant cytomegalovirus (HCMV) mutant comprising a genome from which a gene sequence capable of down-regulating major histocompatibility complex (MHC) class I expression has been deleted, wherein the deleted gene sequence comprises a region containing open reading frames IRS-1 - US11.

2. The recombinant HCMV mutant of claim 1, wherein the region of the deleted gene sequence comprises open reading frames IRS-1 - US9 and US11.

3. The recombinant HCMV mutant of claim 1, wherein the region of the deleted gene sequence comprises the open reading frames US2 - US11.

4. The recombinant HCMV mutant of claim 1, wherein the region of the deleted gene sequence comprises the open reading frame US11.

5. The recombinant HCMV mutant of claim 1, wherein the region of the deleted gene sequence comprises subregion A, wherein subregion A comprises open reading frames US2 - US5, and subregion B, wherein subregion B comprises open reading frames US10 - US11.

6. The recombinant HCMV mutant of claim 5, wherein the subregion B of the deleted gene sequence consists of the open reading frame US11.

7. A method of controlling down-regulation of major histocompatibility complex (MHC) class I expression in a cytomegalovirus infected cell comprising the steps of:

(a) identifying a gene sequence in the region of the cytomegalovirus genome containing open reading frames IRS-1 - US11

capable of down-regulating the MHC class I expression; and

(b) deleting the identified gene sequence from the cytomegalovirus genome.

8. The method of claim 7, wherein the identified gene sequence is from the region of the cytomegalovirus genome containing open reading frames IRS-1 - US9 and US11.

9. The method of claim 7, wherein the identified gene sequence is from the region of the cytomegalovirus genome containing open reading frames US2 - US11.

10. The method of claim 7, wherein the identified gene sequence is from the region of the cytomegalovirus genome containing open reading frame US11.

11. The method of claim 7, wherein the identified gene sequence is from the region of the cytomegalovirus genome containing subregion A, wherein subregion A comprises open reading frames US2 - US5, and subregion B, wherein subregion B comprises open reading frames US10 - US11.

12. The method of claim 11, wherein the identified gene sequence from subregion B consists of the open reading frame US11.

13. A pharmaceutical composition comprising a recombinant cytomegalovirus (HCMV) mutant which comprises a genome from which a gene sequence capable of down-regulating major histocompatibility complex (MHC) class I expression has been deleted, wherein the deleted gene sequence comprises a region containing open reading frames IRS-1 - US11.

14. The pharmaceutical composition of claim 13, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises open reading frames IRS-1 - US9 and US11.

15. The pharmaceutical composition of claim 13, wherein the region of the deleted gene sequence of the recombinant

HCMV mutant comprises the open reading frames US2-US11.

16. The pharmaceutical composition of claim 13, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frame US11.

17. The pharmaceutical composition of claim 13, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises subregion A, wherein subregion A comprises open reading frames US2 - US5, and subregion B, wherein subregion B comprises open reading frames US10 - US11.

18. The pharmaceutical composition of claim 17, wherein the subregion B of the deleted gene sequence of the recombinant HCMV mutant consists of the open reading frame US11.

19. A vaccine composition for use in the prevention of cytomegalovirus infections which comprises an effective amount of a recombinant cytomegalovirus (HCMV) mutant comprising a genome from which a gene sequence capable of down-regulating major histocompatibility complex (MHC) class I expression is deleted, wherein the deleted gene sequence comprises a region containing open reading frames IRS-1 - US11, in a pharmaceutically acceptable vehicle.

20. The vaccine composition of claim 19, further comprising an adjuvant.

21. The vaccine composition of claim 19, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises open reading frames IRS-1 - US9 and US11.

22. The vaccine composition of claim 19, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frames US2-US11.

23. The vaccine composition of claim 19, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frame US11.

24. The vaccine composition of claim 19, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises subregion A, wherein subregion A comprises open readin frames US2 - US5, and subregion B, wherein subregion B comprises open reading frames US10 - US11.

25. The vaccine composition of claim 24, wherein the subregion B of the deleted gene sequence of the recombinant HCMV mutant consists of the open reading frame US11.

26. A method of immunizing an individual against cytomegalovirus comprising administering to the individual an immunogenic amount of a recombinant cytomegalovirus (HCMV) mutant comprising a genome from which a gene sequence capable of down-regulating major histocompatibility complex (MHC) class I expression has been deleted, wherein the deleted gene sequence comprises a region containing open reading frames IRS-1 - US11.

27. The method of claim 26, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises open reading frames IRS-1 - US9 and US11.

28. The method of claim 26, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frames US2-US11.

29. The method of claim 28, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frame US11.

30. The method of claim 26, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises subregion A, wherein subregion A comprises open reading frames US2 - US5, and subregion B, wherein subregion B comprises open reading frames US10 - US11.

31. The method of claim 30, wherein the subregion B of the deleted gene sequence of the recombinant HCMV mutant

consists of the open reading frame US11.

32. A method of preventing or reducing susceptibility to acute cytomegalovirus in an individual comprising administering to the individual an immunogenic amount of a recombinant cytomegalovirus (HCMV) mutant comprising a genome from which a gene sequence capable of down-regulating major histocompatibility complex (MHC) class I expression has been deleted, wherein the deleted gene sequence comprises a region containing open reading frames IRS-1 - US11.

33. The method of claim 32, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises open reading frames IRS-1 - US9 and US11.

34. The method of claim 32, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frames US2-US11.

35. The method of claim 32, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises the open reading frame US11.

36. The method of claim 32, wherein the region of the deleted gene sequence of the recombinant HCMV mutant comprises subregion A, wherein subregion A comprises open reading frames US2 - US5, and subregion B, wherein subregion B comprises open reading frames US10 - US11.

37. The method of claim 36, wherein the subregion B of the deleted gene sequence of the recombinant HCMV mutant consists essentially of the open reading frame US11.

38. A virus based gene therapy vector comprising a gene sequence from the open reading frame US11 of the human cytomegalovirus genome.

39. A virus based gene therapy vector comprising gene sequences from subregion A of the human cytomegalovirus genome,

wherein subregion A comprises open reading frames US2 - US5, and subregion B of the human cytomegalovirus genome, wherein subregion B comprises open reading frames US10 - US11.

40. A virus based gene therapy vector comprising gene sequences from subregion A of the human cytomegalovirus genome, wherein subregion A comprises open reading frames US2 - US5.

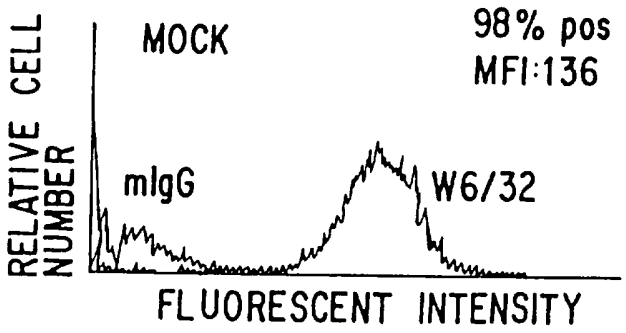


FIG. 1A

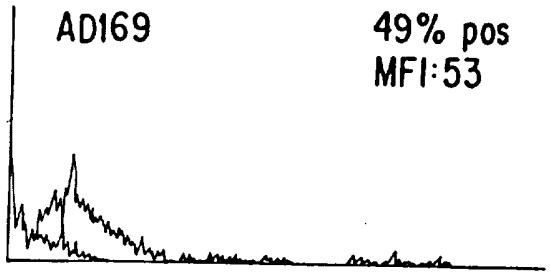


FIG.1B

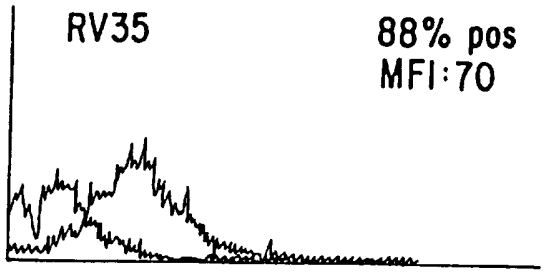


FIG. 1C

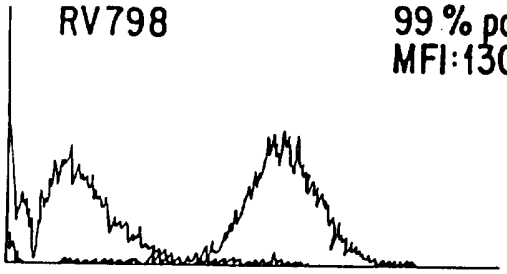


FIG.1D

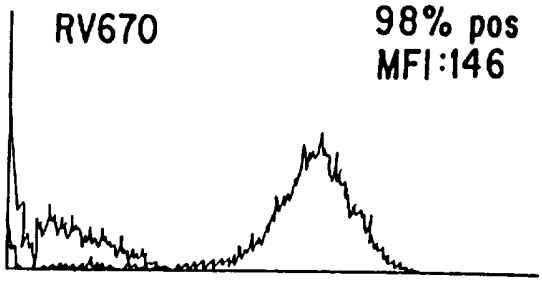


FIG. 1E

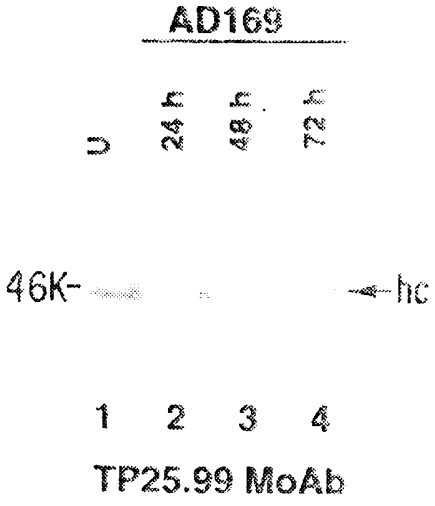
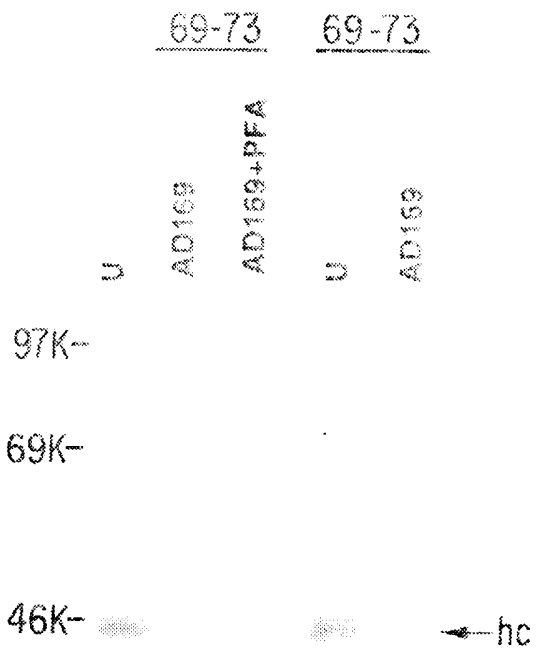


FIG. 2A



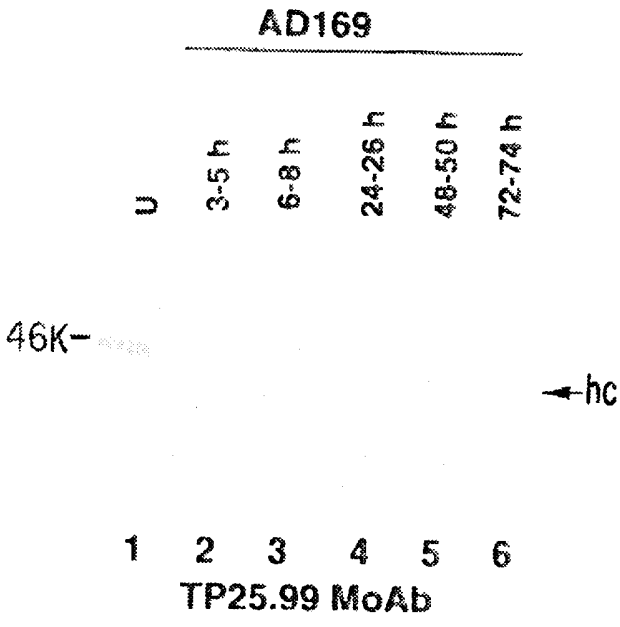


FIG. 2C

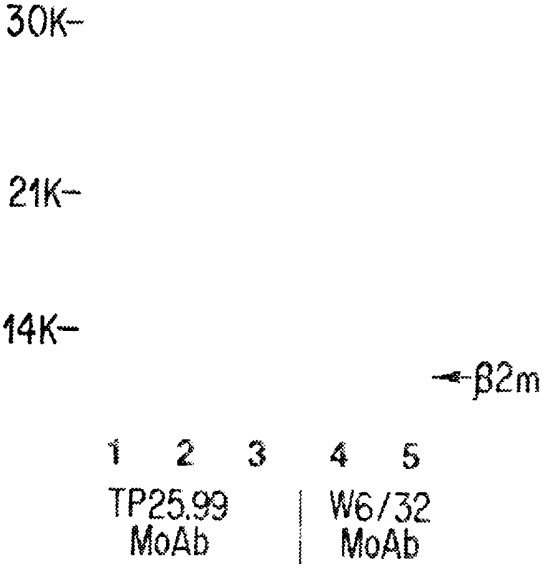
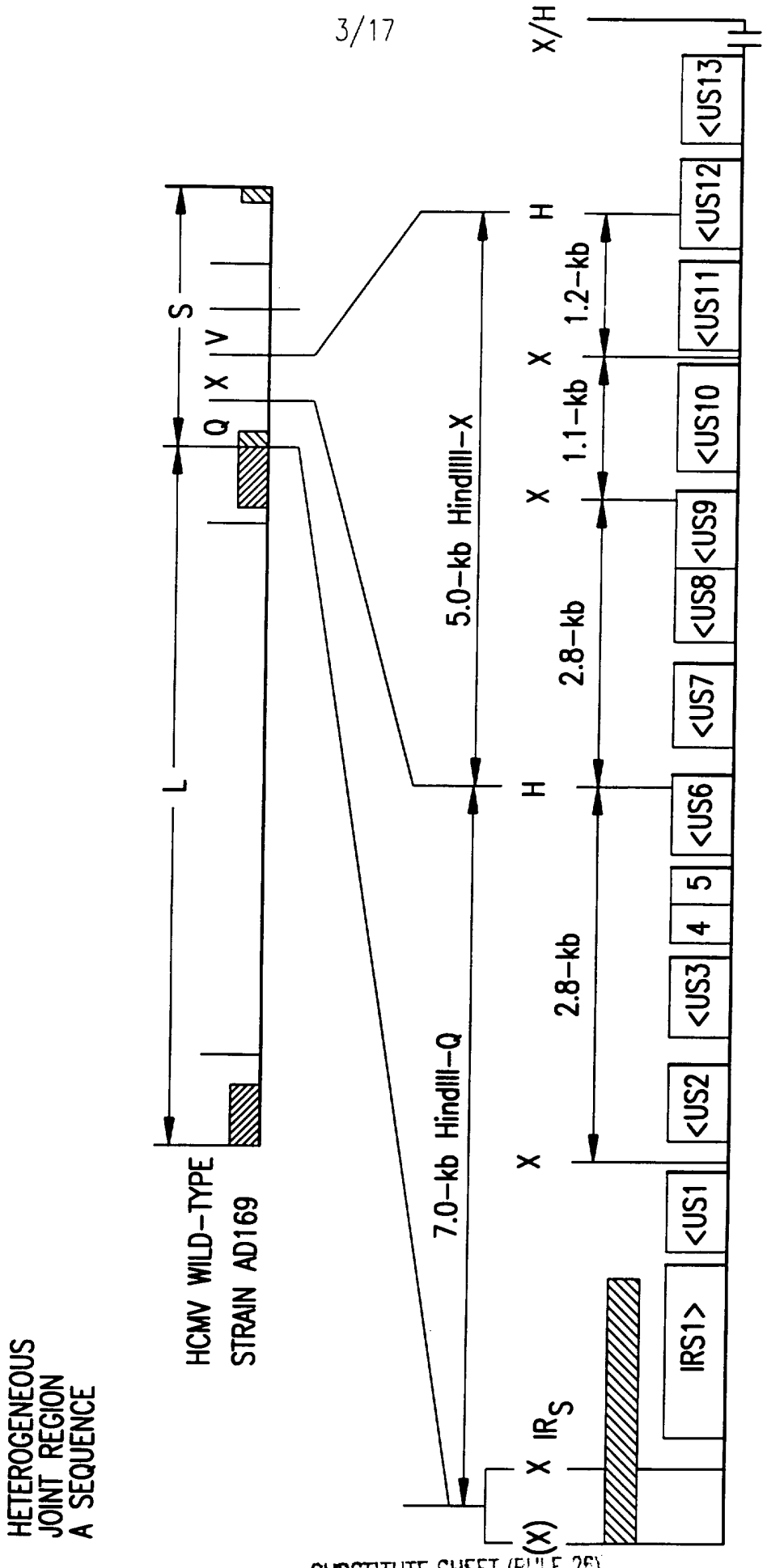


FIG. 2B


HETEROGENEOUS
JOINT REGION
A SEQUENCE
HCMV WILD-TYPE
STRAIN AD169
L
S
Q
X
V
IRS
X
(X)
IRS1>
<US1
X
7.0-kb HindIII-Q
X
2.8-kb
<US2
<US3
4
5
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H
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X
1.2-kb
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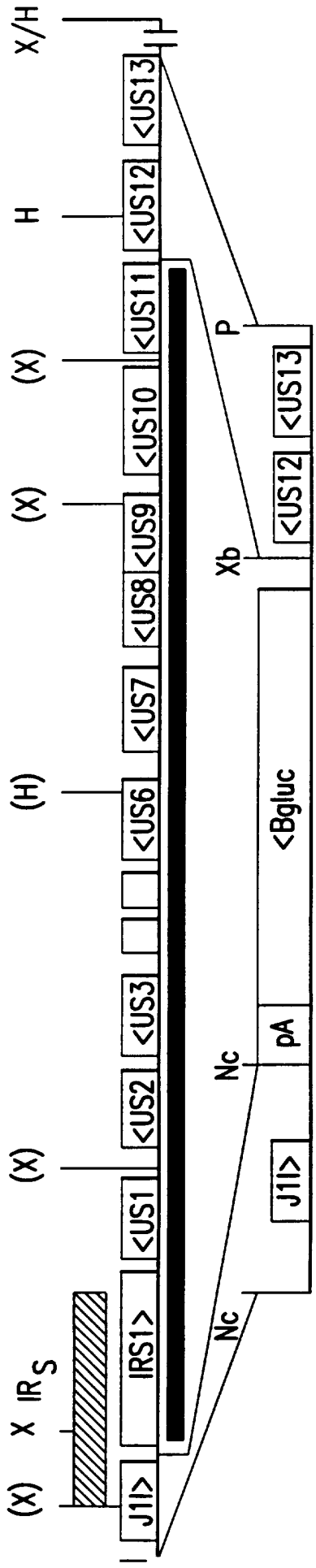
<US13
X/H

FIG. 3A



RV7186 (IRS1–US11 DELETED)

FIG. 3B

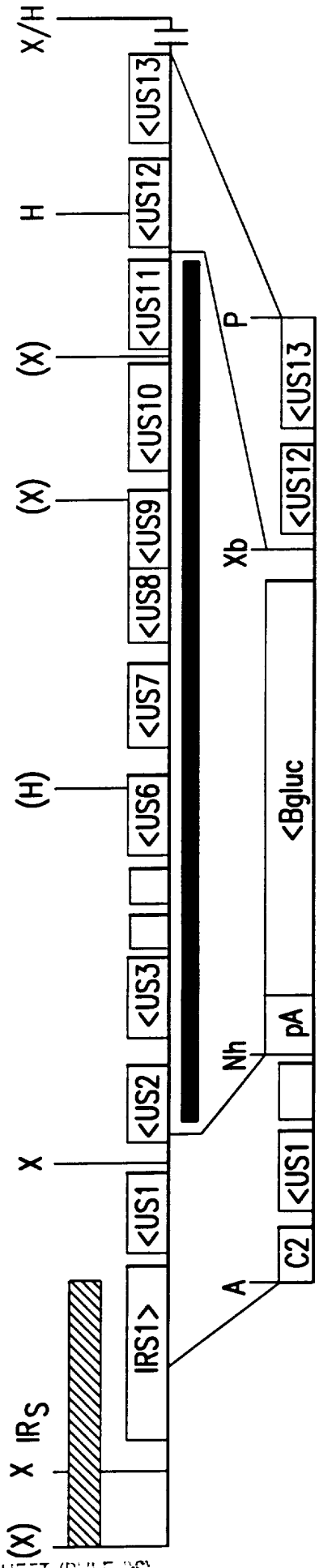
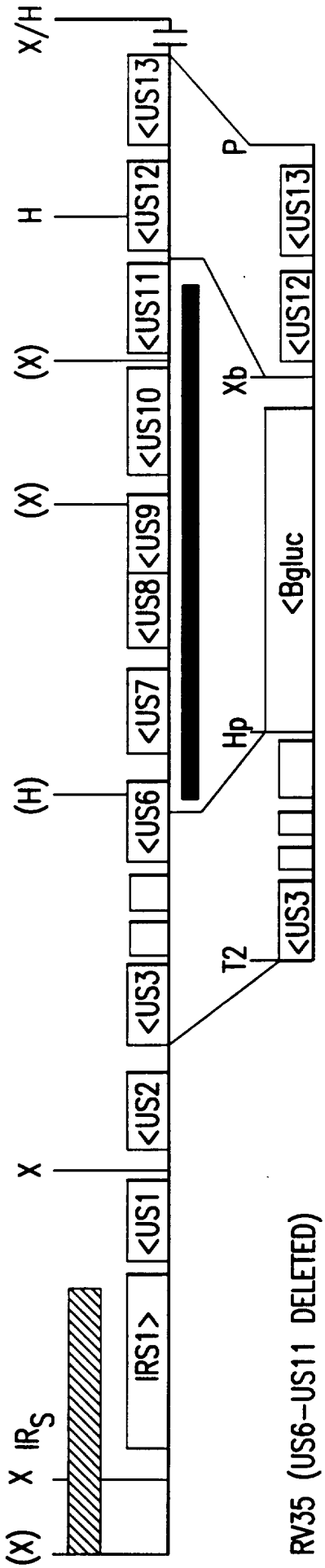


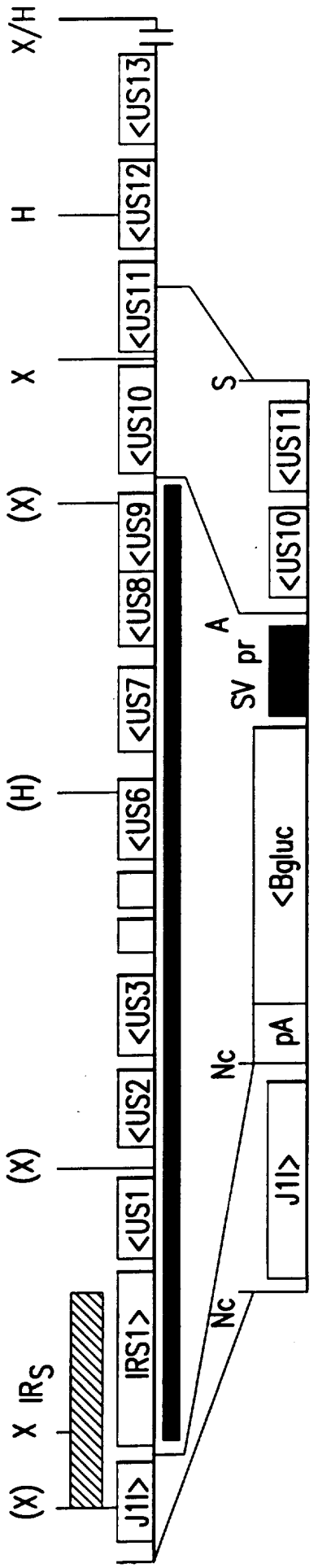
FIG. 3C

RV798 (US2–US11 DELETED)



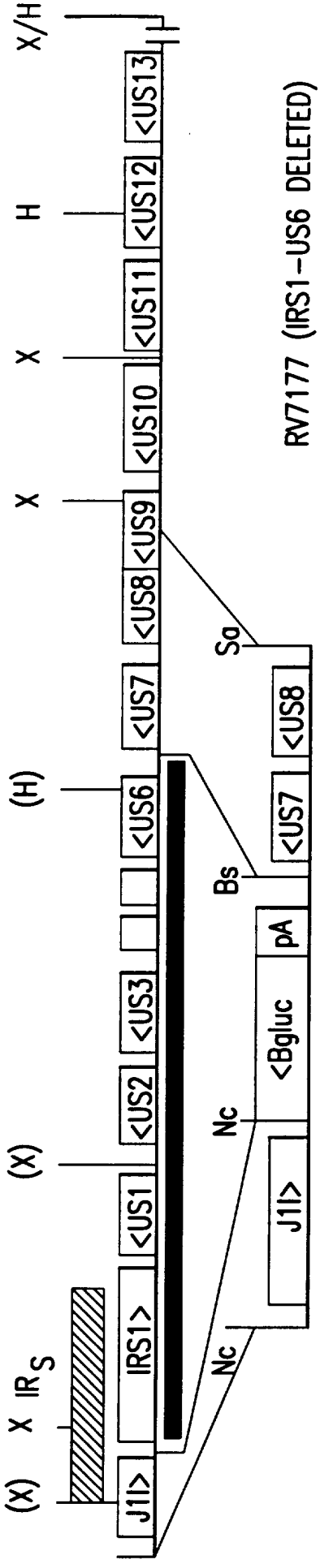
RV35 (US6–US11 DELETED)

FIG. 3D



RV7181 (IRS1–US9 DELETED)

FIG. 3E


RV7177 (IRS1–US6 DELETED)
X/H
H
X
X
(H)
(X)
(X) X IRS
J1I>
IRS1>
<US1
<US2
<US3
<US6
<US7
<US8
<US9
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<US11
<US12
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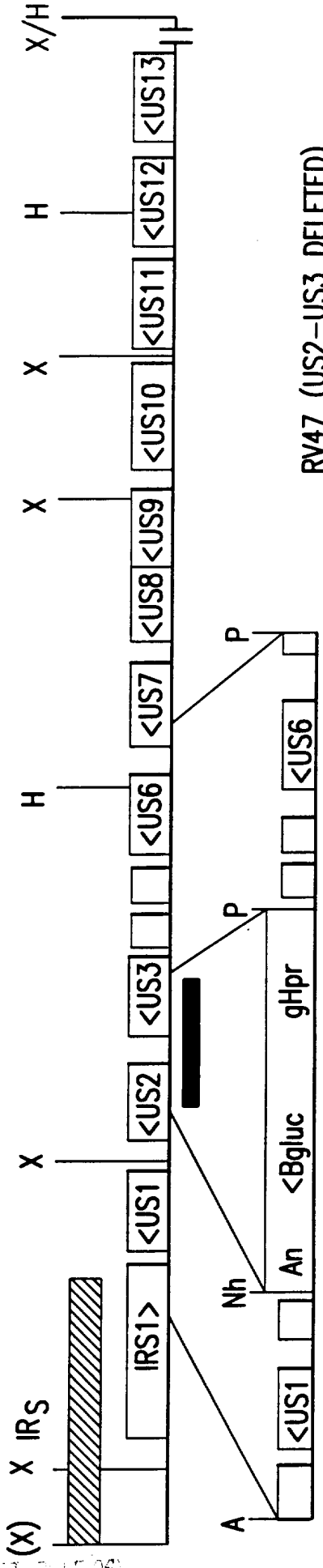
Nc
Nc
Bs
Sa
<Bgluc
pA
J1I>
<US7
<US8

FIG. 3F


RV47 (US2–US3 DELETED)
X/H
H
X
X
H
X
(X) X IRS
IRS1>
<US1
<US2
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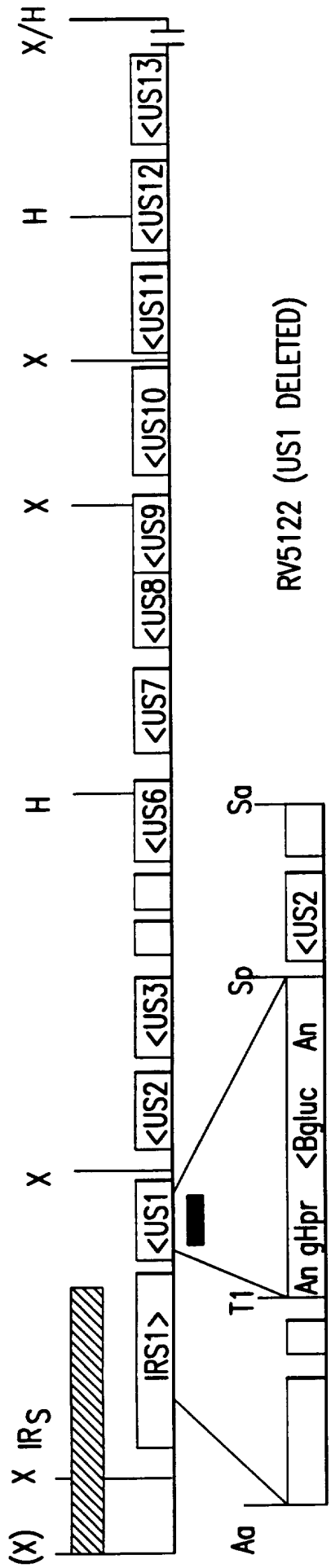
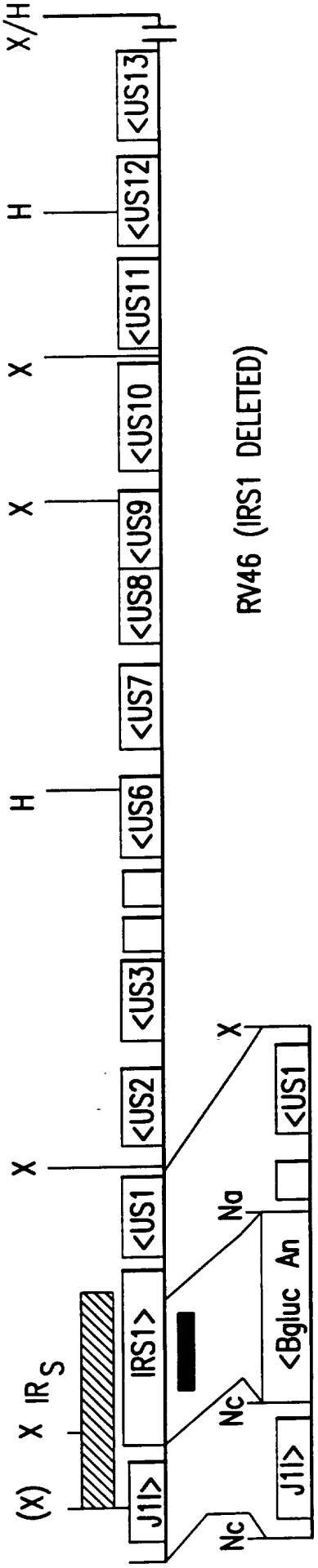
<US7
<US8
<US9
<US10
<US11
<US12
<US13
P
P
Nh
A
An
<Bgluc
gHpr
<US1
<US6

FIG. 3G



RV5122 (US1 DELETED)

FIG. 3H



RV46 (IRS1 DELETED)

FIG. 3I

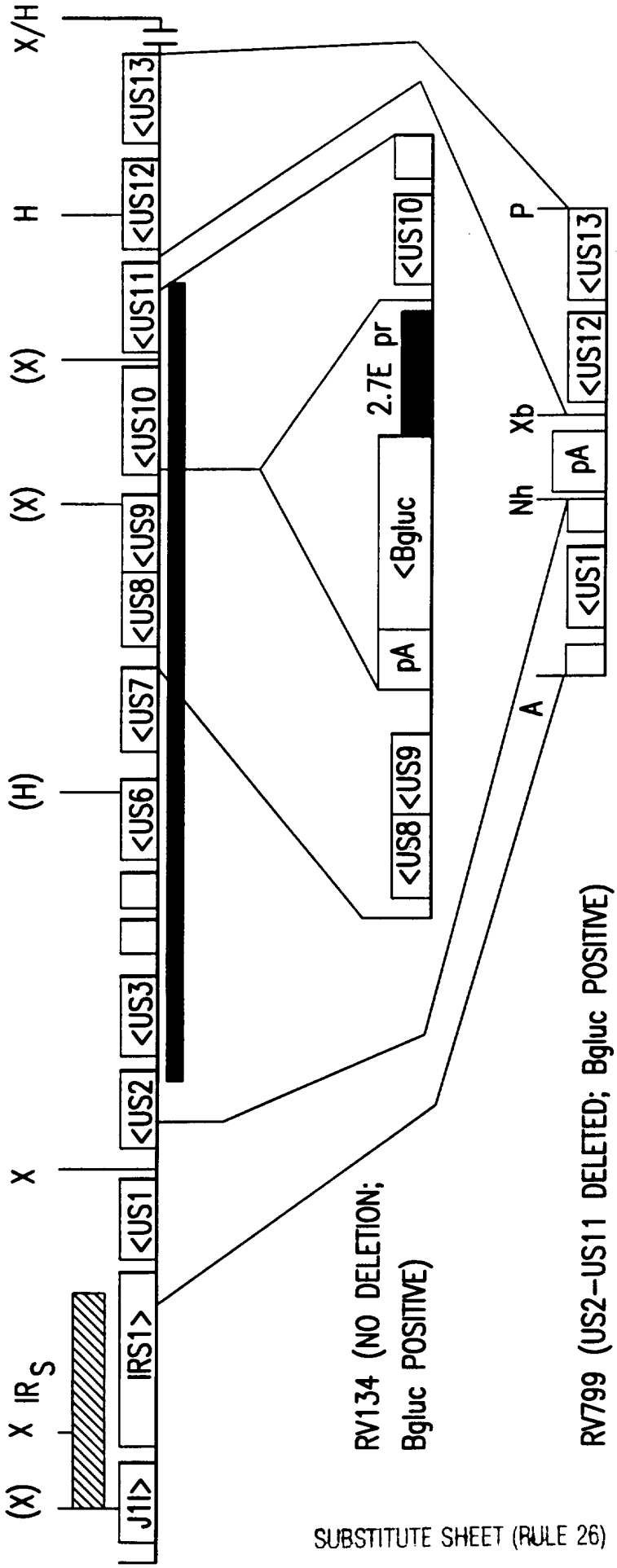

(X) X IRS
J1I>
IRS1>
<US1
X
<US2
<US3
(H)
<US6
<US7
(X)
<US8
<US9
(X)
<US10
<US11
H
<US12
<US13
X/H
RV134 (NO DELETION; Bgluc POSITIVE)
<US8
<US9
pA
<Bgluc
2.7E pr
<US10
A
Nh
<US1
Xb
pA
P
<US12
<US13
RV799 (US2–US11 DELETED; Bgluc POSITIVE)

FIG.3J

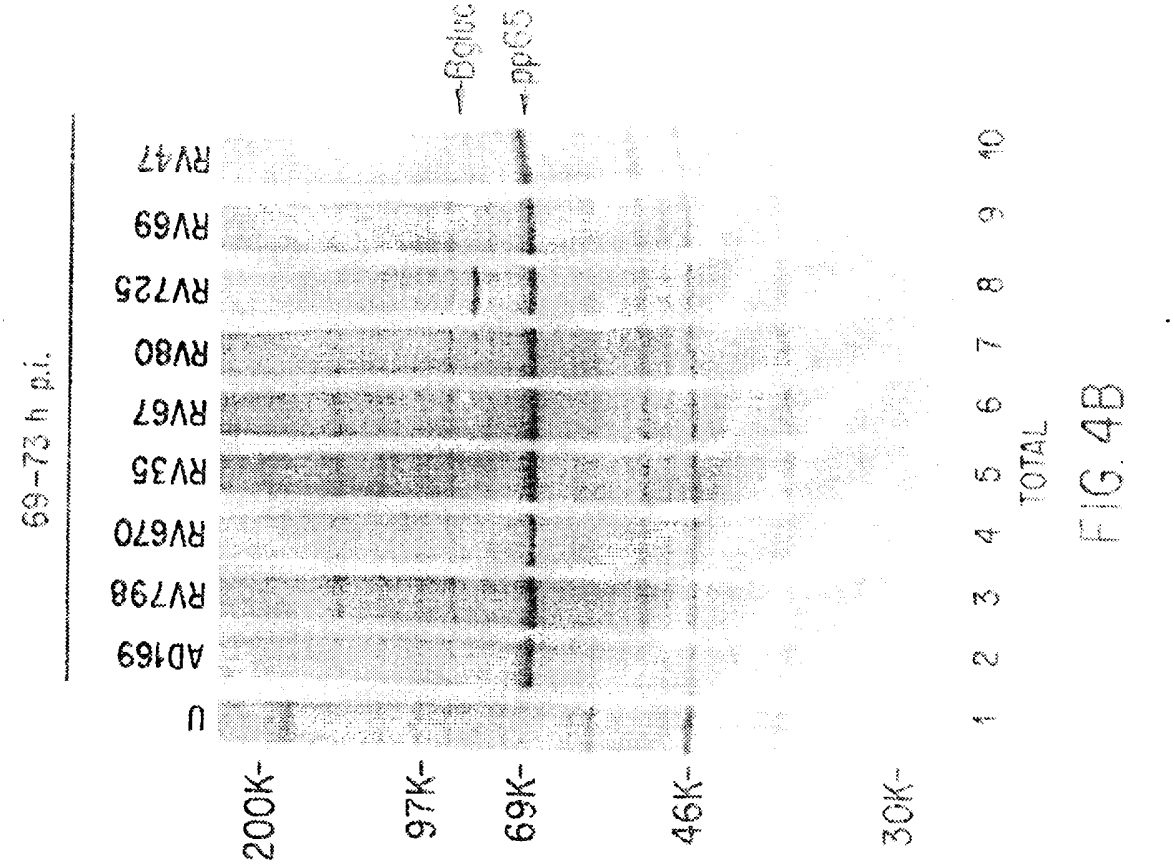


FIG. 4B

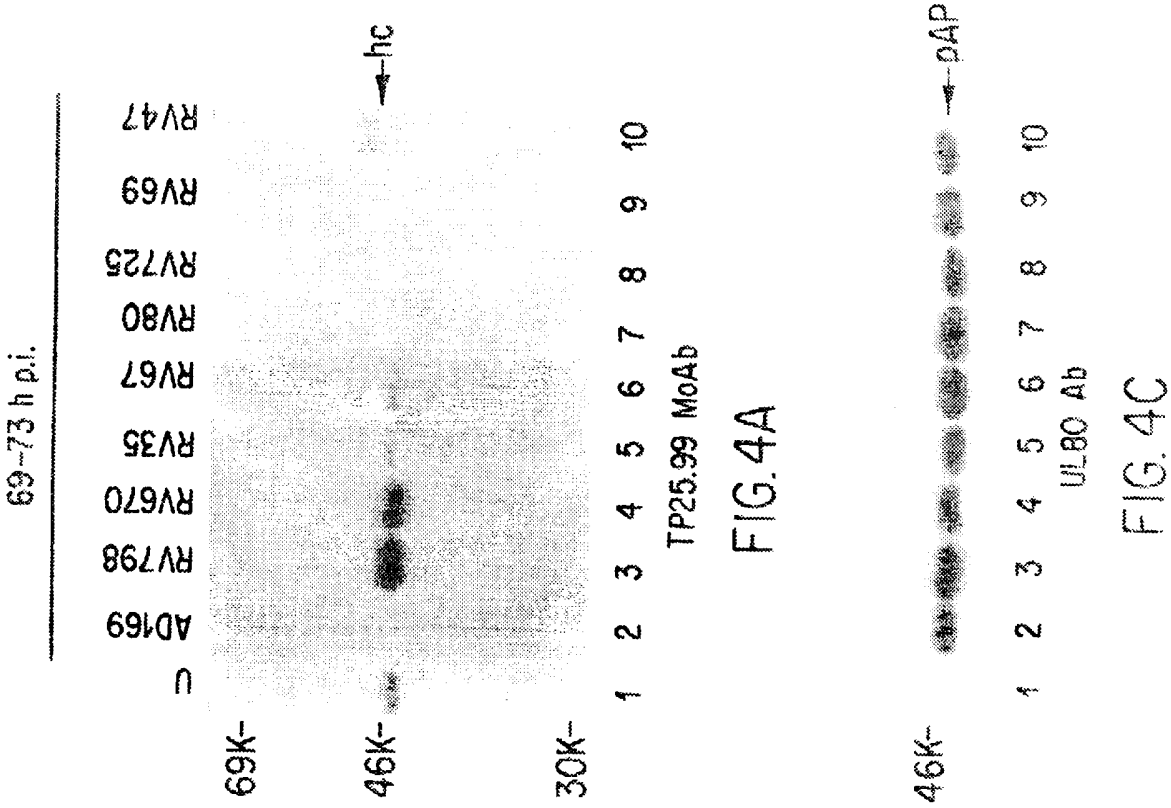


FIG. 4A

FIG. 4C

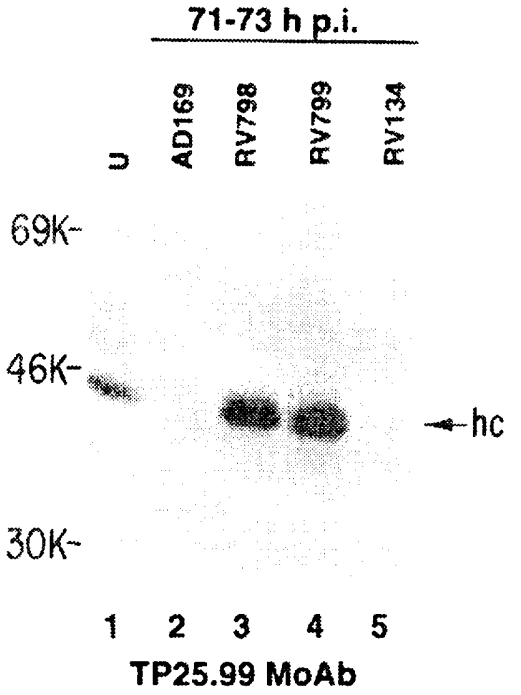


FIG. 5A

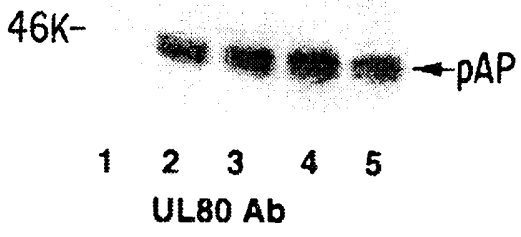


FIG. 5C

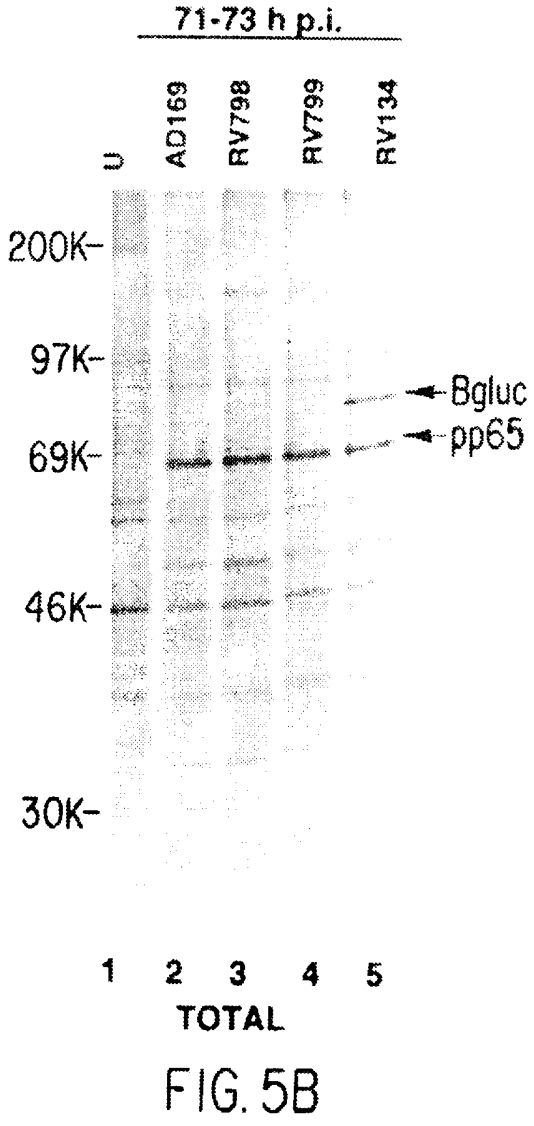


FIG. 5B

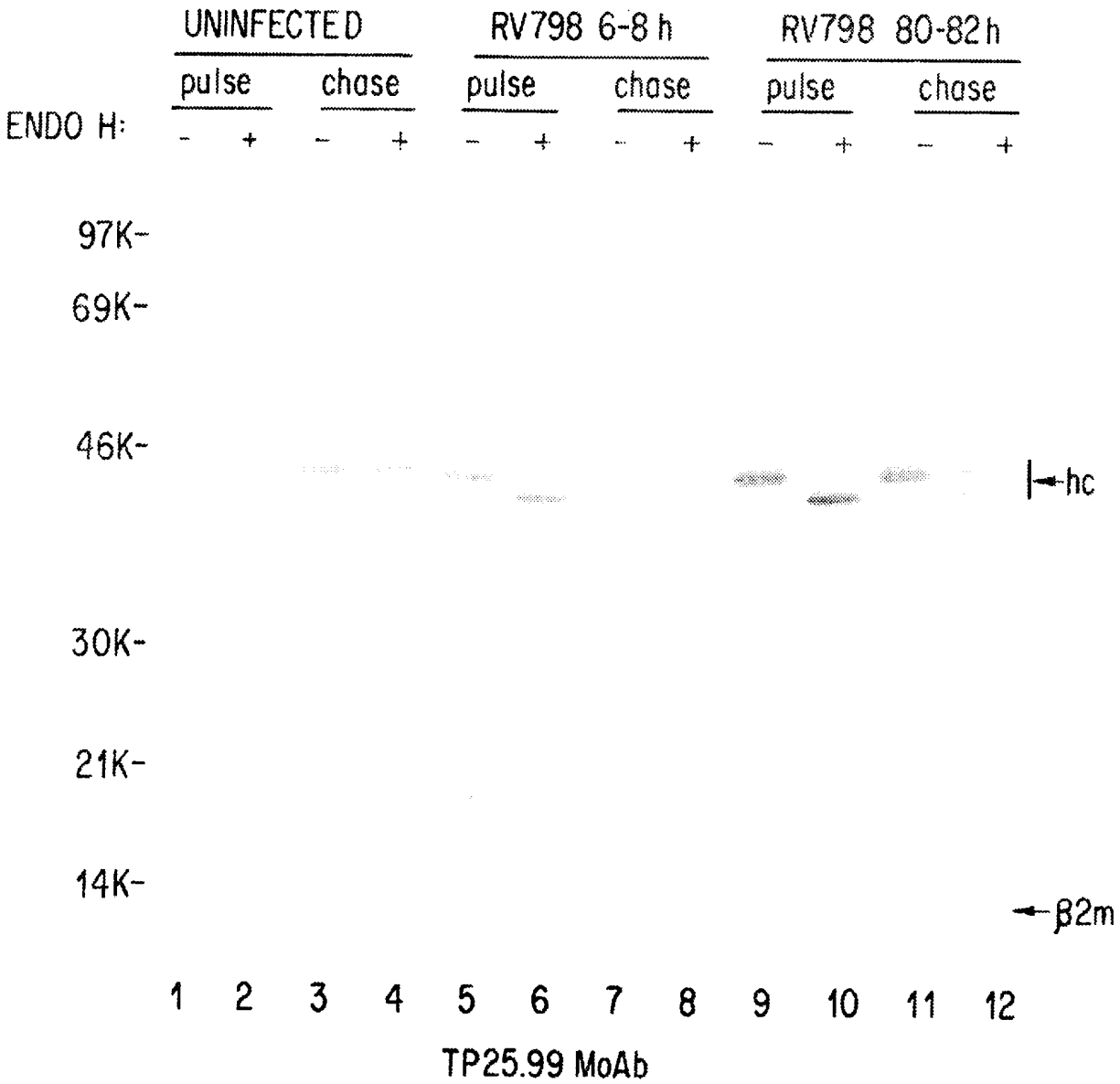

UNINFECTED
RV798 6-8 h
RV798 80-82 h
pulse
chase
pulse
chase
pulse
chase
ENDO H:
- + - + - + - + - + - +
97K-
69K-
46K-
30K-
21K-
14K-
hc
β2m
1 2 3 4 5 6 7 8 9 10 11 12
TP25.99 MoAb

FIG. 6

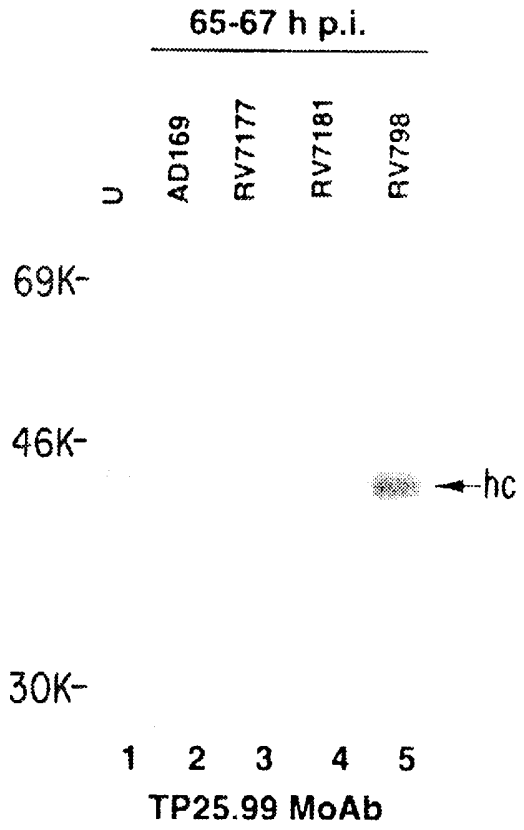


FIG. 7A

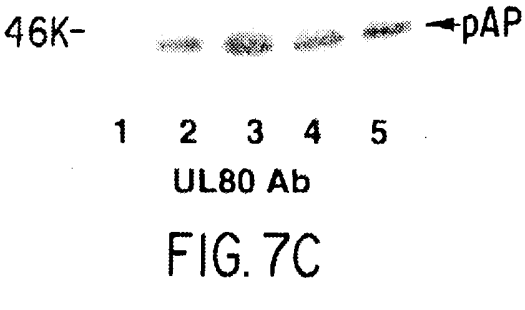


FIG. 7C

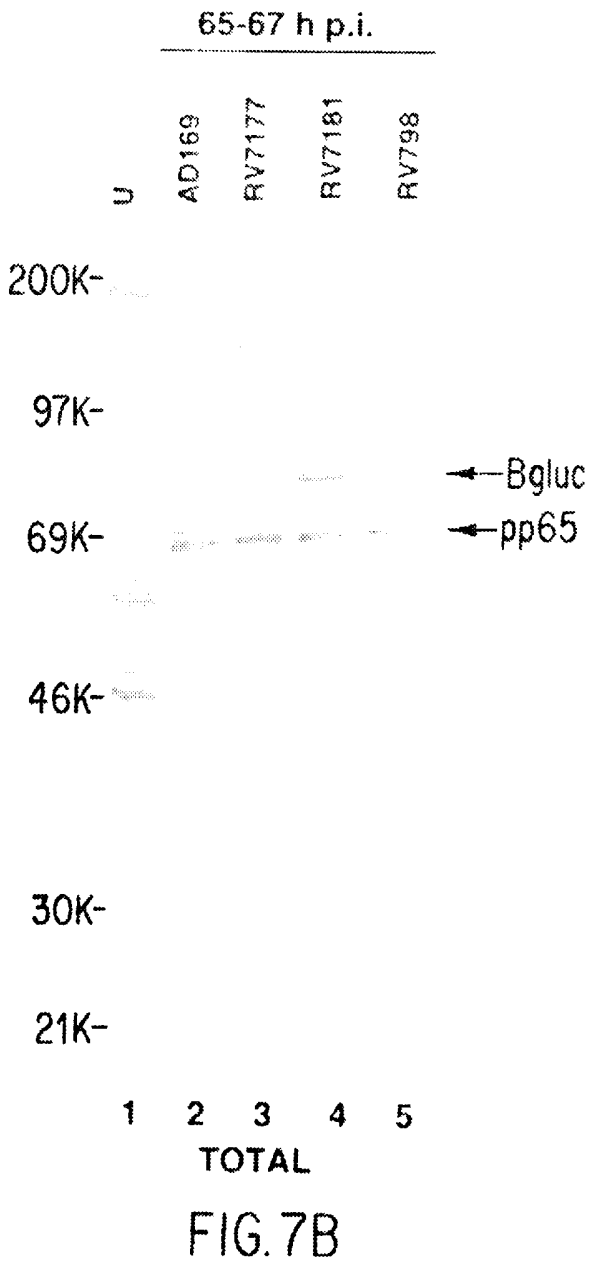


FIG. 7B

FIG.8A FIG.8B FIG.8C FIG.8D

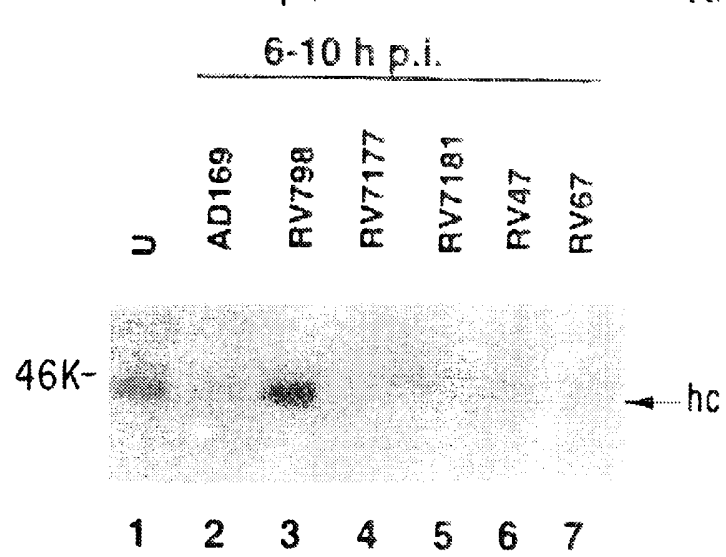


FIG. 9A

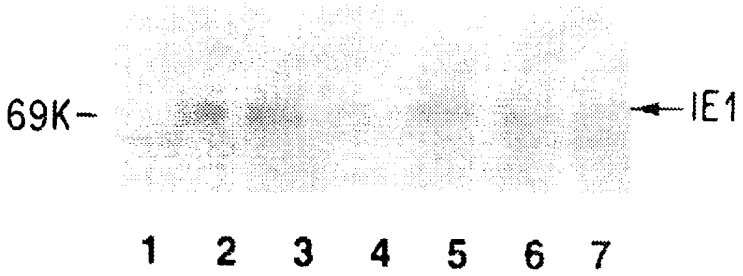


FIG. 9B

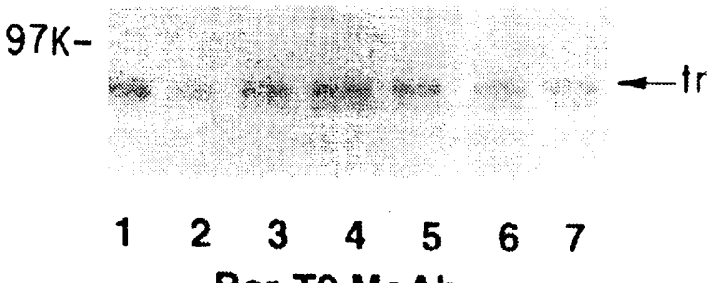


FIG. 9C

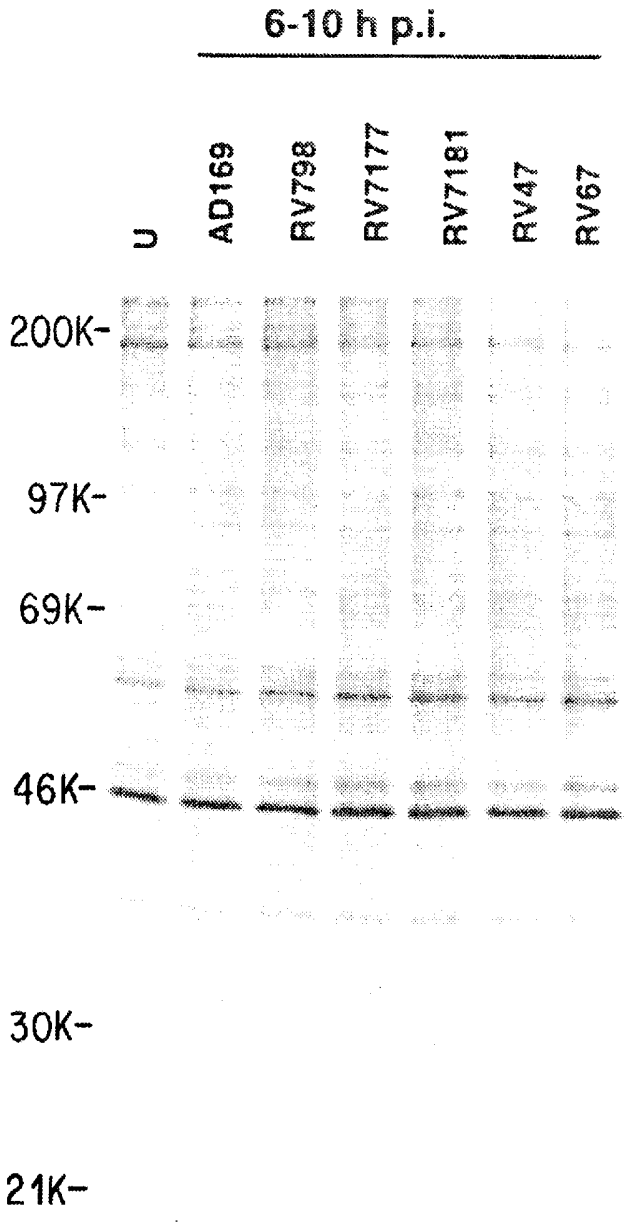

6-10 h p.i.
U
AD169
RV798
RV7177
RV7181
RV47
RV67
200K-
97K-
69K-
46K-
30K-
21K-
1 2 3 4 5 6 7
TOTAL

FIG. 9D

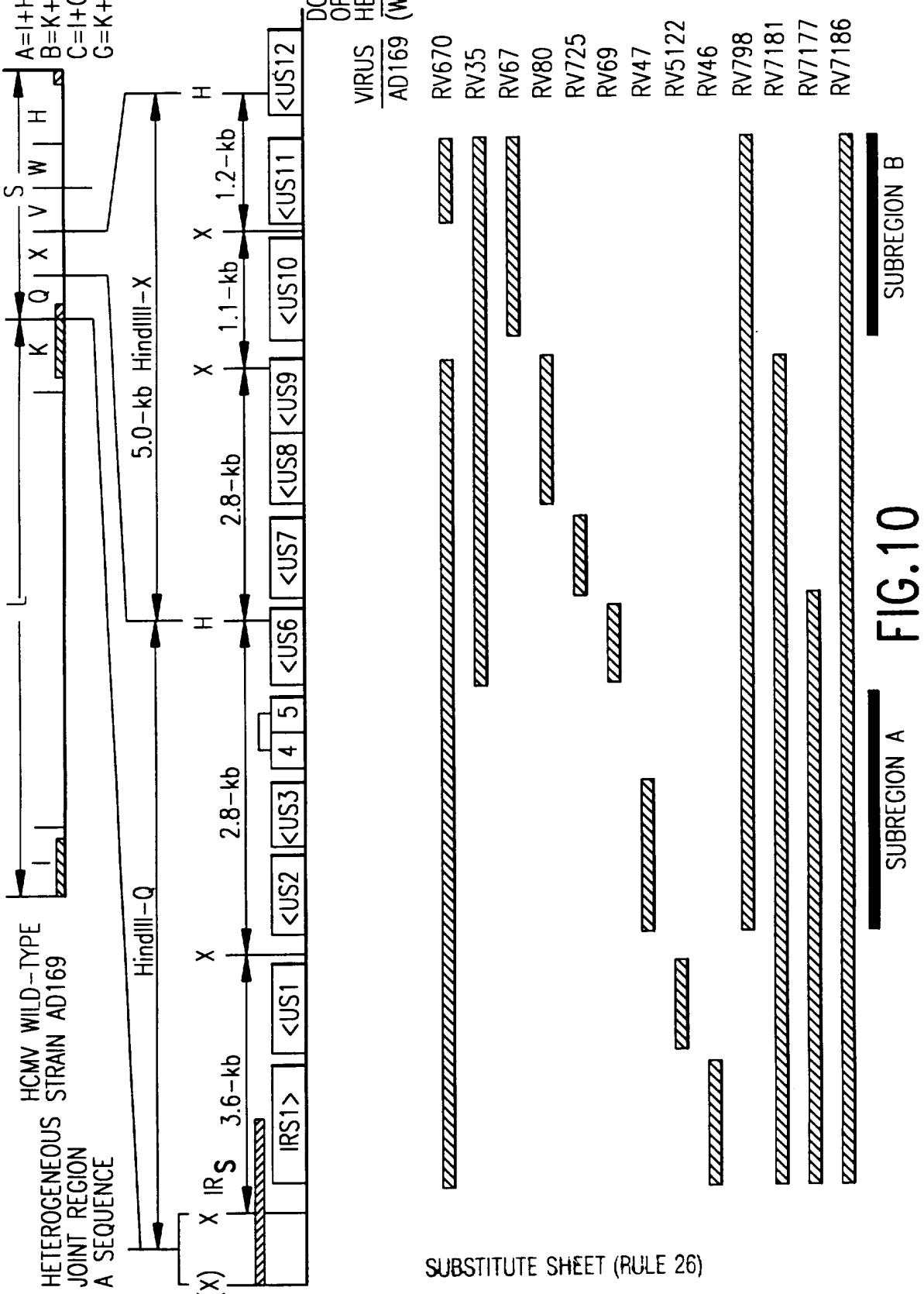

HETEROGENEOUS JOINT REGION A SEQUENCE
HCMV WILD-TYPE STRAIN AD169
A=I+H
B=K+H
C=I+Q
G=K+Q
(X)
X
IRS
3.6-kb
IRS1>
<US1
<US2
<US3
4
5
<US6
<US7
<US8
<US9
<US10
<US11
<US12
HindIII-Q
5.0-kb HindIII-X
2.8-kb
2.8-kb
1.1-kb
1.2-kb
H
X
L
S
I
K
Q
V
W
H
DOWN-REGULATION OF MHC CLASS 1 HEAVY CHAINS
VIRUS
AD169 (WT) YES
RV670 NO
RV35 YES
RV67 YES
RV80 YES
RV725 YES
RV69 YES
RV47 YES
RV5122 YES
RV46 YES
RV798 NO
RV7181 YES
RV7177 YES
RV7186 NO
SUBREGION A
SUBREGION B

FIG.10

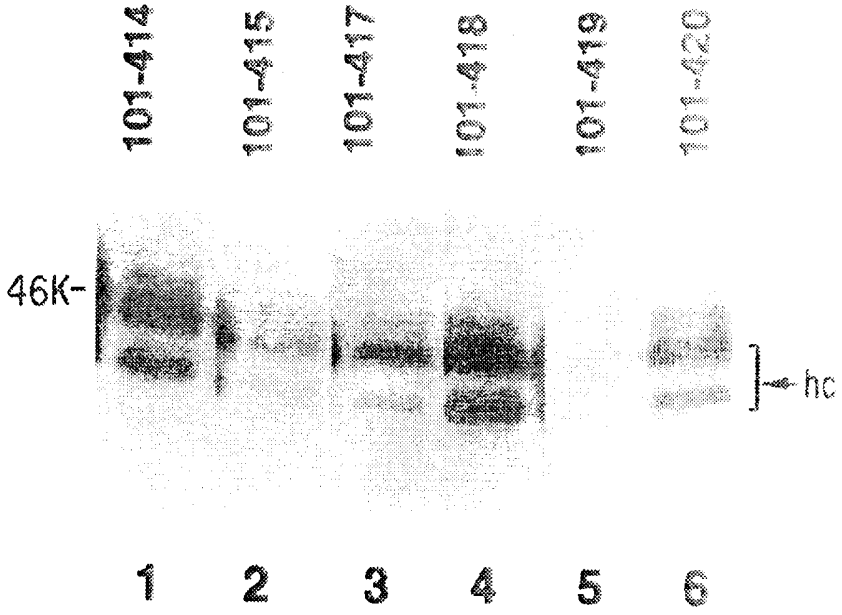


FIG. 11A

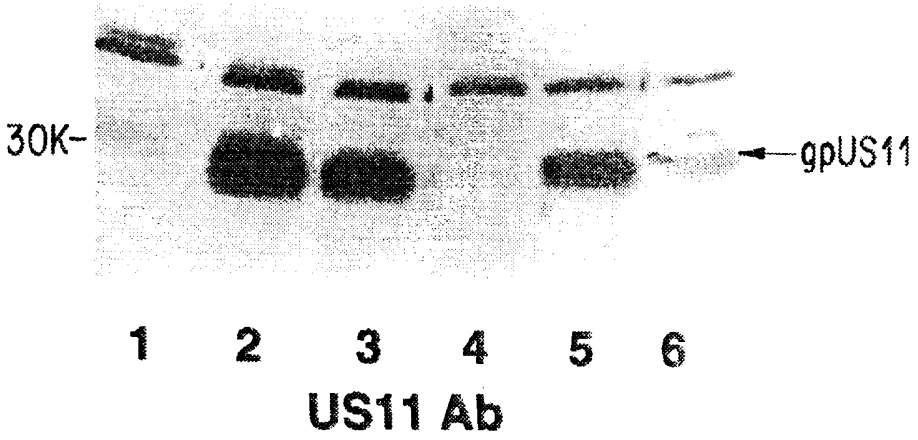


FIG. 11B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/09607

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/38 C12N7/04 C07K14/045 A61K39/245 A61K48/00

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)
IPC 6 C07K C12N A61K

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.VIROLOGY , vol. 65, no. 10, 1991 pages 5184-5189, KOLLERT-JÖNS A. ET AL. 'A 15 kb-pair region of the human cytomegalovirus genome which includes US1 through US13 is dispensable for growth in cell culture'	1
A	see the whole document ---	2-6
X	J.VIROLOGY, vol. 66, no. 4, 1992 pages 2541-2546, JONES T.R. ET AL. 'A cluster of dispensable genes within the human cytomegalovirus genome :IRS1 ,US1 through US5 and the US6 family' cited in the application	1
A	see the whole document ---	2-6

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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 document 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

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Date of the actual completion of the international search	Date of mailing of the international search report
14 November 1995	01. 12. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	EP,A,0 521 427 (AMERICAN CYANAMID CO) 7 January 1993 see the whole document	1,13,19, 20,38-40 2-12
Y	CURRENT TOPICS IN MICROBIOLOGY AND IMMUNOLOGY, vol. 154, 1990 pages 126-169, CHEE M.S. ET AL. 'Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169' cited in the application see page 143, paragraph 2 - page 145, paragraph 1 see page 133 - page 134 see page 158, paragraph 2	1,13,19, 20,38-40
Y	J.VIROLOGY, vol. 67, no. 6, 1993 pages 3461-3469, GILBERT M.J. ET AL. 'Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus' see the whole document	1,13,19, 20
Y A	WO,A,89 10966 (CHILDRENS HOSPITAL INC ;UNIV IOWA RES FOUND (US)) 16 November 1989 see the whole document	19,20 1-18
Y	J.IMMUNOLOGY, vol. 151, no. 9, 1993 pages 4455-4464, BEERSMA M.F.C ET AL. 'Human cytomegalovirus down regulates HLA class I expression by reducing the stability of class I H chains' cited in the application see the whole document	38-40
T	JOURNAL OF VIROLOGY, 1995, vol. 69, no. 8, August 1995 pages 4830-4841, JONES TR ET AL 'MULTIPLE INDEPENDENT LOCI WITHIN THE HUMAN CYTOMEGALOVIRUS UNIQUE SHORT REGION DOWN-REGULATE EXPRESSION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS-I HEAVY-CHAINS' see the whole document	1-12

-/--

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/US 95/09607

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J VIROL, 66 (1). 1992. 95-105., COLBERG-POLEY A M ET AL 'HUMAN CYTOMEGALOVIRUS US3 AND UL36-38 IMMEDIATE-EARLY PROTEINS REGULATE GENE EXPRESSION' see the whole document -----	38-40

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

national application No.

PCT/US95/09607

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. [X] Claims Nos.: 19-37
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 19-37 are directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compositon.

2. [ ] Claims Nos.:
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. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] 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 claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 95/09607

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0521427	07-01-93	AU-B-	659462	18-05-95
		AU-A-	1943992	07-01-93
		JP-A-	6098775	12-04-94
		NZ-A-	243401	28-03-95
WO-A-8910966	16-11-89	AU-B-	3550489	29-11-89
		EP-A-	0413738	27-02-91
		JP-T-	3504197	19-09-91