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(54) Title: GP41 MUTANTS AND THEIR USE AS HIV THERAPEUTICS

(57) Abstract

Methods and therapeutic compositions for the treatment of HIV infection involving the administration of a mutant gp41 polypeptide, particularly a gp41 polypeptide of HIV.

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GP41 MUTANTS AND THEIR USE AS HIV THERAPEUTICS

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This invention was funded at least in part by the United States government and the government has certain rights in the invention.

Background of the Invention

This invention relates to the treatment of infection with the human immunodeficiency virus (HIV), by which we mean to include all of the various viral types and strains denominated by that term, such as HTLV-III, LAV, ARV, HIV-1, HIV-2, and LAV-2.

HIV is an etiological agent of Acquired Immune Deficiency Syndrome (AIDS). An example of HIV (now denominated HIV-1) is generally described in several articles: Barre-Sinoussi et al., Science 220:868, 1983; Gallo et al., Science 224:500, 1984; Popovic et al., Science 224:497, 1984; and Levy et al., Science 225:840, 1984, each of which is hereby incorporated by reference. Various isolates of HIV-1 have been obtained from North America, Western Europe and Central Africa. These isolates differ somewhat in their nucleotide sequence, but the proteins they encode are generally antigenically cross-reactive.

A second virus related to HIV-1 has been isolated and termed HIV-2. This virus is reported by Guyader et al., Nature 326:662, 1987; Brun-Vezinet et al., The Lancet 1:128, 1987; and Clavel et al., Science 233:343, 1986, each of which is hereby incorporated by reference. The genetic organization of HIV-2 is similar to that of HIV-1.

A group of viruses isolated from monkeys, termed simian immunodeficiency virus (SIV or STLV-III), is related to HIV-1 and HIV-2, particularly the latter.

See Daniel et al., Science 228:1201-1204 (1985); Kanki et al., Science 230:951-954 (1985); Chakrabarti et al., Nature 328:543-547 (1987); and Ohta et al., Int'l. J. Cancer 41:115-222 (1988), each of which is hereby incorporated by reference. Members of this viral group exhibit minor variations in their genomic sequences, and have some differences in their restriction enzyme maps.

As with other lentiviruses, HIV encodes an envelope precursor protein that is processed into a transmembrane (TM) protein and an extracellular protein. The TM protein extends further downstream from the transmembrane domain (Gallaher, W. R. et al., AIDS Res. Hum. Retroviruses 5:431-440 (1989); Hunter and Swanstrom, Current Topics in Microbiology and Immunology 157:187-253 (1990)), resulting in a long cytoplasmic domain of more than 100 amino acids. For example, the cytoplasmic domain of HIV-1, HIV-2, and SIV TM proteins consists of approximately 150 amino acids (Gallaher, W. R. et al., AIDS Res. Hum. Retroviruses 5:431-440 (1989); Hunter and Swanstrom, Current Topics in Microbiology and Immunology 157:187-253 (1990)). For SIV and HIV-2, it is reported that a large portion of the cytoplasmic domain of the TM protein is dispensable for viral replication in certain established human cell lines (Chakrabariti et al., Nature 328:543-547 (1987); Fukasawa et al., Nature 333:457-461 (1988); Guyader et al., Nature 236:662-669 (1987); Hirsch et al., Cell 49:307-319 (1987)). Some natural SIV and HIV-2 isolates which have truncated TM proteins propagate better than viruses with full length TM proteins in certain established human cell lines (Chakrabarti et al., J. Virol 63:4395-4403, (1989); Hirsch et al., Nature 341:573-574 (1989); Kodama et al., J. Virol. 63:4709-4714 (1989)). It is also reported that the truncated TM protein has increased fusogenic ability. ((Earl et al., J. Virol. 65:31-41 (1991); Kowalski et al., Science

237:1351-1355 (1987); Mulligan, M.J. et al., J. Virol. 66:3971-3975 (1992); Mulligan et al., J. Virol. 66:3971-3975 (1992)).

In contrast to SIV and HIV-2, infectious HIV-1 clones that have been sequenced to date generally contain a full length TM protein (gp41). Computer analysis indicates that the cytoplasmic domain of gp41 contains two amphipathic α helical structures (Venable et al., AIDS Res. Hum. Retroviruses 5:7-22 (1989)) which can form a secondary association with the membrane bilayer (Haffar et al., Virology 180:439-441 (1991)). It has been reported that deleting large truncations deleting both of the α helices in the cytoplasmic domain of gp41 did not significantly affect viral Env protein biosynthesis, processing, transport or surface expression (Earl et al., J. Virol 65:31-41, (1991)). Syncytium formation (Earl et al., J. Virol. 65:31-41 (1991); Kowalski et al., Science 237:1351-1355 (1987)) and oligomerization (Earl et al., Proc. Natl. Acad. Sci. USA 87:648-652 (1990)) of the mutant Env protein reportedly were also not impaired.

HIV has already entered large segments of the world population, and substantial effort has been directed toward developing treatments for individuals infected with it. In addition to the investigation of synthetic pharmaceuticals, effort has been directed toward utilizing variants of HIV-1 and HIV-2 to design AIDS therapeutics (Trono et al., Cell, 59:113-120, (1989).

Intracellular "immunization" using *gag* p24 core gene mutants or capsid targeted Gag-nuclease fusion molecules have been described as potential anti-retroviral strategies (Trono et al., Cell 59:113-120 (1989); Natsoulis et al., Nature 352:632-635 (1991)).

Summary of the Invention

The invention features a method of treating a patient infected with human immunodeficiency virus (HIV) by administering a mutant of the gp41 polypeptide in an amount effective to reduce infective HIV levels in the patient. The gp41 polypeptides may be delivered by various vehicles as described below.

The mutated gp41 polypeptide may be administered as the polypeptide itself or as an expressible genetic construction including nucleic acid encoding the mutated gp41. The nucleic acid used may be nucleic acid capable of transforming cells of the patient and may be administered as a part of a viral vector. The nucleic acid used may further include a sequence encoding a CD4-binding polypeptide, a sequence encoding a gp120-binding polypeptide, or fragments thereof which enable entry of the therapeutic peptide into the target cell. Such therapeutics are administered in a pharmaceutically acceptable carrier.

Also included as a part of the invention is a method for transforming the cells outside of the patient's body and returning the transformed cells to the patient. Preferably the cells are removed from the patient, before they are transformed with the nucleic acid, and returned to the patient's body.

Alternatively, the nucleic acid may be administered directly to the patient and/or may be delivered in a viral vector which is a derivative of the human immunodeficiency virus-type I.

The invention further includes methods of treating an HIV infected patient by the administration of a mutant gp41 polypeptide which contains a deletion of at least one amino acid in at least one of the following regions of wild type gp41:

-- amino acids 844 to 856;

-- amino acids 814 to 856;
-- amino acids 796 to 856;
-- amino acids 776 to 856;
-- amino acids 753 to 856; or
-- amino acids 710 to 856,

effective to disrupt the viral replication of HIV or assembly of Env proteins in an infected cell. Preferably, such deletions will include between 40 and 60 percent of the stated deletions, and, more preferably, between 60 and 80% of the stated deletions and most preferably, between 80 and 100% of the stated deletions.

Also included as a part of the invention are therapeutic compositions adapted for administration to a patient infected with human immunodeficiency virus-type I (HIV). These compositions include a mutated gp41 polypeptide in a pharmaceutically acceptable carrier, or a nucleic acid encoding a mutated gp41 polypeptide in an expressible genetic construction for transforming cells of a human patient.

The therapeutic composition may include the nucleic acid as part of a viral vector capable of transforming cells of the patient. The nucleic acid may further include a sequence capable of encoding a CD4-binding polypeptide, a sequence encoding a gp120-binding polypeptide, or fragments thereof.

By mutated gp41 is meant a gp41 having a deletion, insertion, substitution, or other modification rendering gp41 effective as an inhibitor of HIV viral assembly and infectivity as demonstrated by one of the assays described below.

The mutated gp41 polypeptides included in the invention may be the equivalent mutated gp41 proteins from other strains of HIV. Where we have designated the mutation site by number, one skilled in the art may determine the equivalent mutations in HIV and proteins

whose sequence differs from Fig. 1; based upon amino acid and nucleic acid homology.

Those mutations which disrupt the incorporation of Env proteins and/or viral replication and are useful therapeutics for patients infected with HIV may be determined using the techniques described in the methods, below. Particularly useful are the transfection, infection, and RT assay and radioimmunoprecipitation, immunoblot, and plus chase analysis procedures. Specifically, useful mutant gp41 polypeptides and nucleic acids encoding polypeptides will be those which disrupt Env protein incorporation and which result in normal levels of the gag and pol proteins, but confer decreased levels of gp120 and gp41 proteins in the virion. Useful mutant gp41 polypeptides which disrupt viral replication are those which decrease viral replication relative to wild-type virus, preferably decreasing replication by 10% and most preferably decreasing replication by 20% or more in a co-transfection assay.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments of the invention and from the claims.

Description of the Preferred Embodiments

The drawings will first be briefly described.

Drawings

Fig. 1 diagrams the sequence of DNA encoding the gp41 polypeptide and the primary amino acid sequence of the gp41 polypeptide.

Fig. 2 is a diagram representing the construction of gp41 mutants. The HIV-1 Env precursor protein contains 856 amino acids in HXB2R3. The darkened areas represent hydrophilic regions. The arrow indicates the cleavage site between gp120 and gp41. The numbers at the end of each diagram indicate the last amino acid that can be synthesized in gp160 by each construct, except for TM812 which terminates after amino acid 813.

Fig. 3 is a series of graphs representing a virus infectivity assay. Cell-free wild type and mutant viruses were prepared from the supernatants of transfected COS-7 cells and tested on SupT1 (Fig. 3A), MT-2 (Fig. 3C), H9(Fig. 3B), and fresh PBMC (Fig. 3D). Reverse Transcriptase (RT) values represent samples form 0.1 ml culture supernatants, measured in counts per minute (cpm).

Fig. 4 depicts analysis of viral proteins by immunoblot. (a) Purified and analyzed. (b) Cell lysates (cell) and sucrose gradient purified virions (virus) blotted with another HIV-1 positive human sera.

Fig. 5 (a) depicts analysis of viral proteins by radioimmunoprecipitation. Cell lysates (cell) and supernatants (sup) from [^{35}S] cysteine metabolically labeled transfected COS-7 cells were reacted with the HIV-1-positive sera. (b) depicts surface expression of HIV-1 Env proteins detected by indirect immunofluorescence using mouse monoclonal antibody against gp120; a and b are mock-transfected cells; *c* and *d* are wt-transfected cells; *e* and *f* are TM752-transfected cells; *g* and *h* are TM709-transfected cells. The corresponding Nomarsky pictures (*a, c, e* and *g*) are adjacent to the florescence pictures (*b, d, f* and *h*).

Fig. 6 depicts a viral construct with the nucleic acid encoding gp41 in an expressible position.

I. gp41 polypeptides for use as anti-HIV therapeutics.

Without wishing to bind ourselves to a specific mechanism of action, it appears that mutant gp41 polypeptides are useful as anti-HIV therapeutics because they interfere with the incorporation of viral env proteins into infectious viral particles in an HIV infected cell. As the examples below demonstrate, mutations which delete or otherwise disrupt the carboxy-terminal domain of gp41 have this property and are anti-HIV therapeutics. While env protein synthesis is not disrupted in such mutants, the incorporation of the viral Env proteins into infectious particles is significantly impaired. This failure to properly incorporate the Env proteins results in noninfectious particles. This dominant quality of the mutants with respect to wild-type virus is also referred to as a "dominant-negative" property.

Moreover, mutations outside the carboxy terminal domain which disrupt the interaction between gp41 and the Env proteins, particularly mutations in the transmembrane domain, may yield effective therapeutics.

Some gp41 polypeptides useful for this purpose include deletions which remove greater than twelve but no more than 147 amino acids from the carboxy terminus. Most useful for disruption of viral assembly are those mutations which remove between 104 and 147 amino acids of the carboxy terminal cytoplasmic domain.

Those skilled in the art will appreciate that other gp41 mutations (including deletions, substitutions or additions to naturally occurring (wild type) gp41 sequences) can be used according to the invention. Techniques for generating a universe of candidate mutants are well known to those in the art. A variety of useful techniques are described in Current Protocols in

Molecular Biology, (eds. Ausebel et al., 1989, Green Publishing Associates, John Wiley & Sons, NY, pp. 8.1-8.4). The pool of candidate mutants can then be screened from their ability to disrupt incorporation by various techniques including the techniques described in Examples 2, 3, and 4 and using the dominant negative assay provided below.

Also useful as anti-HIV therapeutics are those gp41 mutations which disrupt the viral replication of pathogenic HIV in an infected cell. Disruption of viral replication may be the result of improper viral assembly or total failure to assemble. Whatever the mechanism, the ability to prevent replication is a useful characteristic of gp41 mutants according to the invention. Mutants having this characteristic can be identified by mutagenesis (described above) followed by screening according to Examples 2, 3, 4 and the dominant negative mutant assay provided, below. Mutations useful for therapy by this mechanism include those mutations which disrupt the most carboxy terminal portion of the cytoplasmic domain. For example, a mutation in gp41 which removes the last 12 amino acids of the carboxy terminus has been demonstrated to be useful for this purpose.

Additional polypeptides useful as gp41 derived anti-HIV therapeutics are described under Other Embodiments.

II. Therapeutic Administration of gp41 Polypeptide.

As described in greater detail in the examples below, gp41 mutants according to the invention are effective HIV therapeutics because they may function in a so-called "transdominant" fashion. Specifically, it appears that the gp41 mutants become physically associated with an otherwise pathogenic virion components

being manufactured in an HIV-infected cells. As a result, the load of properly assembled infections virions that cell manufactures is reduced in proportion to the mutant gp41 levels in the cell. When mutant gp41 is delivered to the cell in sufficient excess, the incidence of ineffective viral assembly results from attempts to incorporate mutant gp41 becomes far higher than the incidence of incorporation of wild type gp41.

With the availability of the cloned gene Rekosh et al., Proc. Natl. Acad. Sci. USA 85:334 (1988), Chanda et al., Vaccines :207 (1989), and Hammarskjold et al., Journal of Virology 63:1959 (1989), the substantially pure gp41 polypeptide mutants can be produced in quantity using standard techniques (Scopes, R. Protein Purification: Principles and Practice 1982 Springer-Verlag, NY). Thus, an aspect of the invention is a pharmaceutical comprising the gp41 polypeptide variants together with an acceptable diluent, carrier or excipient and/or in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the polypeptide to patients infected with HIV.

A substantially pure preparation of a polypeptide is a preparation which is substantially free (e.g., to the extent required for formulating the gp41 variant into a therapeutic composition) of the proteins with which it naturally occurs in a cell.

Fragments or analogs of the gp41 protein variant may also be administered to a patient infected with HIV in the manner described herein. Fragments or analogs which are useful for this purpose include fragments and analogs of those mutants in gp41 which are described in the preceding section and in the Examples and which are useful for the treatment of a patient infected with HIV. Fragments and analogs which will be useful for the

therapeutic treatment of patients infected with HIV are determined using the assays provided in the examples, below, among others.

The gp41 polypeptide variants may also be administered to a patient infected with HIV in the form of a fusion protein consisting of the desirable gp41 mutant polypeptide fragment, fused to the gp120 protein, or a fragment thereof which is sufficient to bind the CD4 receptor of T cells. The sequences of both the gp120 and CD4 genes for the sates use may be obtained from Rekosh et al., Proc. Natl. Acad. Sci. USA 85:334 (1988), Chanda et al., Vaccines :207 (1989), and Hammarskjold et al., Journal of Virology 63:1959 (1989). This fusion protein allows delivery of the gp41 polypeptide variant into uninfected T cells, monocytes, macrophages or other cell types infected by HIV and expressing the CD4 receptor.

The gp41 polypeptide may also be administered to a patient infected with HIV in the form of a fusion protein consisting of the gp41 polypeptide variant, or a therapeutically useful fragment or derivative, fused to the CD4 protein, or a fragment thereof, which is sufficient to bind gp120. This fusion protein allows delivery of the gp41 polypeptide into infected T cells expressing gp120 on their surface. The gp41-gp120 fusion polypeptide or the gp41-CD4 fusion polypeptide may be generated using standard techniques of molecular biology to generate fusions encoded from a suitable vector (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989)).

Either the gp120 fragment or the CD4 fragment may enable internalization of the gp41 polypeptide variant through endocytosis. The usefulness of such gene fusion constructs may be determined using the methods described below in the examples, among others. The invention includes administering either fusion polypeptide alone in

a pharmaceutically acceptable carrier, or administering both fusions together in an acceptable carrier.

Thus, the formulations of this invention can be applied for example by parenteral administration, intravenous, subcutaneus, intramuscular, intracranial, intraorbital, opthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration.

Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences". Formulations for parenteral administration may, for example, contain excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycoside copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally.

II B. Construction of HIV Containing gp41

A particularly preferred embodiment features administering to the patient genetic constructions which encode any of the above-described gp41 polypeptides, and (after transformation of patient cells) can express the gp41 polypeptide variant.

In addition to the HIV example below illustrating a preferred viral vehicle, those skilled in the art will readily appreciate that the invention can use other HIV strains of the many that have been fully characterized e.g., MN, HXB2, LAI, NL43, MFA, BRVA and z321.

Moreover, there are numerous other viral vehicles (i.e., nucleic acid vehicles) which can activate or be activated to enter cells of the host organism and, having done so, to be expressed there.

II C. Therapeutic Administration of gp41 variants in a Viral Vector.

Retroviral vectors, or other viral vectors with the appropriate tropisms for cells infected by HIV, may be used as a gene transfer delivery system for the gp41 polypeptide variants. Numerous vectors useful for this purpose are generally known have been described (Miller, Human Gene Therapy 15-14 (1990); Friedman, Science 244:1275-1281 (1989); Eglitis and Anderson, BioTechniques 6:608-614 (1988); Tolstoshev and Anderson, Current Opinion in Biotechnology 1:55-61 (1990); Sharp, The Lancet 337:1277-1278 (1991); Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322 (1987); Anderson, Science 226:401-409 (1984); Moen, Blood Cells 17:407-416 (1991); and Miller and Rosman, Biotechniques 7:980-990 (1989)). Retroviral vectors are particularly well developed and have been used in a clinical setting (Rosenberg, et al N. Engl. J. Med 323:370 (1990)).

The retroviral constructs, packaging cell lines and delivery systems which may be useful for this purpose include, but are not limited to, one, or a combination of, the following: Moloney murine leukemia viral vector types; self inactivating vectors; double copy vectors; selection marker vectors; and suicide mechanism vectors. The Moloney murine leukemia retroviral system of gp41 delivery is particularly useful since it targets delivery of the gp41 protein to the hematopoietic cells which ultimately give rise to the T-cells. The delivery of the gp41 polypeptide can be further restricted to cells which are infected by HIV directly by virtue of utilizing retroviral constructs in which the HIV-LTR is used to drive expression from the gp160 gene. To achieve proper expression from such a construct the 3'LTR of the Moloney murine leukemia vector must be deleted. Vector strategies which include either the entire HXB2R3 construct or the gp160 *env* gene driven by the HIV-LTR (see Figure 6).

Fragments or derivatives of the gp41 polypeptide may also be administered by retroviral gene transfer therapy or another suitable viral vector system. Fragments or derivatives are defined as described above. Useful fragments or derivatives of gp41 may be administered by inserting the nucleic acids encoding these fragments or derivatives in place of the complete gp160 gene in a gene therapy vector, as described above. Such constructs may be tested using the methods for testing the effects of gp41 on viral infectivity described above, among others.

Retroviral delivery of gp41 is particularly appropriate in HIV infected individuals who display the common secondary appearance of B-cell tumors as a result of immunodeficiency. These individuals may undergo bone marrow removal, treatment, and reimplantation as a matter

of course for the treatment of the B-cell tumors. At this time standard techniques for the delivery of gene therapy vectors may be used to transfect stem cells. Such transfection may result in gp41 synthesizing T-cells useful in lowering the infective levels of HIV in the patient.

II D. Non viral methods for the therapeutic delivery of nucleic acid encoding gp41

Nucleic acid encoding gp41, or a fragment thereof, under the regulation of the HIV-LTR and including the appropriate sequences required for insertion into genomic DNA of the patient, or autonomous replication, may be administered to the patient using the following gene transfer techniques: microinjection (Wolff et al., Science 247:1465 (1990)); calcium phosphate transfer (Graham and Van der Eb, Virology 52:456 (1973); Wigler et al., Cell 14:725 (1978); Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413 (1987)); lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413 (1987); Ono et al., Neuroscience Lett 117:259 (1990); Brigham et al., Am. J. Med. Sci. 298:278 (1989); Staubinger and Papahadjopoulos, Meth. Enz. 101:512 (1983)); asialorosonucoid-polylysine conjugation (Wu and Wu, J. Biol. Chem. 263:14621 (1988); Wu et al., J. Biol. Chem. 264:16985 (1989)); and electroporation (Neuman et al., EMBO J. 7:841 (1980)). These references are hereby incorporated by reference.

III. Experimental Methods

1. DNA Constructs.

The 2.75 kb*EcoRI*-*BamHI* fragment (for generating TM709) and the 0.4 Kb *BamHI*-*XhoI* fragment (for generating TM752, TM775, TM795, TM812, and TM844) from HXB2R3 (Yu et al., J. Virol. 66:4966-4971 (1992)) were subcloned into

pGEM7Zf(+) (Promega, Madison, Wis.). Single-stranded uracil-containing DNA was prepared and used for site-directed mutagenesis according to the protocol of the manufacturer (Bio-Rad, Richmond, Calif.). The sequences of primers used for mutagenesis were as follows: TM709, 5'-GAA TAG AGT TAG CTA GCG ATA TTC ACC AT-3'; TM752, 5'GTC CCA GAT AAG TGC CTA GGA TCC-3'; TM775, 5'-GTT ACA ATC TAG AGT AAG TC-3'; TM795, 5'-TAG GAG ATT CCA CTA AAA TTT GAG GGC TTC-3'; TM812, 5'-GTG GCA TTG AGC TAG CTA ACA GCA C-3'; TM844, 5'-GCC CTG TCT TAT TCC TTA AGG TAT GTG GCG AA-3'. Mutants were screened by restriction enzyme digestion and DNA sequencing. The 2.7 kb*Sal*I-*BamH*I fragments and the 0.4 kb *BamH*i-*Xho*I fragments that contained the gp41 mutations were cloned back into the vectors of HXB2R3.

2. Cells and Sera.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing L-glutamine and D-glucose plus 10% fetal calf serum. SupT1 and H9 cells were maintained in RPMI 1640 medium plus 10% fetal calf serum. MT2 cells were maintained in RPMI 1640 medium plus 15% fetal calf serum. PBMC were isolated by density-gradient centrifugation on LSM (Organon Teknika Corp., Durham, NC) and maintained in RPMI 1640 plus 20% fetal calf serum and 20 units/ml of interleukin-2 (Becton Dickinson Labware, Bedford, Mass). PBMC were treated with 5 μg/ml phytohemagglutinin PHA-P (Sigma, St. Louis, MO) for two days before they were used for the infection assay. The HIV positive sera and the sheep anti-gp120 serum have been previously described (Yu et al., J. Virol. 66:4966-4971 (1992)). For the immunoblot using sucrose gradient purified virions, another pooled HIV positive sera was used. Mouse monoclonal anti-gp120 antibody was obtained from Du Pont (Wilmington, DE).

3. Transfection, Infection, and Reverse Transcriptuse (RT) Assay.

Transfection and RT assay were performed as previously described (Yu et al., J. Virol. 66:4966-4971 (1992)). Briefly, subconfluent COS-7 cells ($5X10^6$) were trypsinized and transfected with 2 μg wild type or mutant plasmid DNA by the DEAE-dextran method as previously described (Yu et al., J. Virol. 66:4966-4971 (1992)). Culture supernatants from transfected or infected cells were used to concentrate virus pellets by the polyethylene glycol method and subjected to reverse transcriptase (RT) assay as previously described (Yu et al., J. Virol. 66:4966-4971 (1992)).

4. Radioimmunoprecipitation analysis of viral proteins.

At 60 h posttransfection, COS-7 cells were incubated for 12 h in cysteine-free RPMI 1640 medium containing 10% fetal calf serum and [^{35}S] cysteine (0.1 mCi/ml; Du Pont, Mass). Cells were then lysed with lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and centrifuged at 40,000 rpm (Beckman Ti70) rotor) for 1 h to remove cell debris. Culture supernatants were precleaned at 1,000 X *g* for 30 min and centrifuged at 40,000 rpm (Beckman Ti70 rotor) for 1 h to remove virus pellets. Cell lysates and culture supernatants were reacted with HIV positive sera that had been preabsorbed with protein A-sepharose CL-4B (Sigma, St. Louis, MO) for 12 h at 4°C. Samples were then washed three times with lysis buffer (without sodium deoxycholate) and once with washing buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2). Sixty microliters of sample buffer (0.08 M Tris-HCl, pH 6.8, 0.1 M DTT, 2% SDS, 10% Glycerol, 0.2% bromophenol blue) were added to each sample tube. Samples were boiled for

2 min before loading and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

5. Purification of virions and analysis of viral proteins by immunoblot.

At 72 h posttransfection, culture media from transfected COS-7 cells were centrifuged at 1000 X *g* for 30 min. Supernatants were filtered through 0.2 μm filtration units (Nalge Company, Rochester, NY) and centrifuged through 3 ml 20% sucrose cushion at 20,000 rpm (Beckman SW28 rotor) for 2 h. Virus-free supernatants were discarded and residual liquid was removed from the centrifuge tubes with dry swabs. Virus pellets were dissolved in sample buffer and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For further purification of the virions by sucrose gradient, virus pellets were dissolved in TNE buffer (0.01 M Tris-HCl, pH 7.2, 0.1 M NaCl, 0.001 M EDTA) and overlaid on the top of the sucrose gradients. Sucrose gradients were prepared by a stepwise overlay of 2 ml of sucrose in TNE buffer that decreased from 60% to 20% by 2.5% increments. Samples were centrifuged at 20,000 rpm (Beckman SW28 rotor) for 20 h and 18 fractions were collected dropwise from the bottom of the centrifuge tubes. The fractions with the highest RT activity were used to isolate the virions and analyzed by immunoblot as described above.

6. Indirect Immunofluorescence Assay.

Cells were washed twice with phosphate-buffered saline 60 hours posttransfection and incubated with 1:10 diluted mouse monoclonal antibody against gp120 (Du Pont, Wilmington, DE) for 60 minutes at room temperature. Cells were then washed three times with phosphate-buffered saline and incubated with a 1:25 dilution of fluorescent isothiocyanate conjugated goat anti-mouse antibody (Becton Dickinson, San Jose, Calif) for 45 min

at room temperature. Cells were washed three times with phosphate-buffered saline and fixed with 1% paraformaldehyde before examination under a fluorescent microscope.

7. Dominant Negative Mutant Assay

To determine the presence or absence of a dominant negative phenotype conferred by a given gp41 mutant wild type or wild type-plus-mutant DNA's are transfected into SupT1 cells. Virus replication is monitored by the RT activity in the supernatant of transfected cells.

SupT1 cells (10^7) are washed once with phosphate-buffered saline and resuspended in 3 ml of TD buffer containing 600 μg of DEAE-dextran and 6 μg of DNA. The DNAs used for each SupT1 transfection are: mock, 6 μg of pUC18; wild type 1 μg of HXB2R3 plus μg of pUC18; wild type + mutant 1 μg of HXB2R3 plus approximately 5 μg of mutant in a suitable vector as indicated above.

Transfections are carried out at room temperature for 20 min. Virus infectivity is tested with SupT1 cells by using cell-free supernatants of transfected COS-7 cells. Samples used in the reverse transcriptase (RT) assay (provided above) are prepared from polyethylene glycol-precipitated viral pellets from the supernatant of transfected or infected cells. This assay is performed as previously described (Yu, et al., J. Virol 64:5688-5693, 1990).

IV. Mode of Therapeutic gp41 Action

The results presented herein indicate that the cytoplasmic domain of gp41 plays a critical role in HIV infectivity. Truncation of 43 (TM812), 61 (TM795), 81 (TM775), 104 (TM752), and 147 (TM709) amino acids from the cytoplasmic domain of gp41 generated noninfectious virions (Fig. 3). Analysis of these mutant virions indicated that the incorporation of viral Env proteins

was significantly impaired, although other viral structural proteins were present at normal ratios when compared to the wild type virions (Fig. 4). Truncation of the last 12 amino acids from gp41 (TM844) did not significantly decrease virus assembly and release or the incorporation of viral Env proteins (Fig. 4). However, the infectivity of mutant TM844 virus was dramatically decreased compared to the wild type virus (Fig. 3). This observation suggests that the cytoplasmic domain of gp41 may also function in viral replication steps other than assembly. Since the defect of TM844 virus was more severe in H9 cells and fresh PBMC than in SupT1 and MT2 cells (Fig. 3), it appears that cellular factors could also influence the function of the gp41 cytoplasmic domain in viral replication. It has been suggested that the cytoplasmic domain of gp41 is cleaved by Vif, a potential cysteine protease (Guy et al., J. Virol. 65:1325-1331 (1987)). The putative cleavage site is very close to the stop codon introduced in the mutant TM844 (Guy et al., J. Virol. 65:1325-1331, (1987)). The observation that the wild type gp41 migrated slower than the TM844 gp41 (Fig. 3) suggests that, at least at the virion purification stage in our study, the cleavage of the cytoplasmic domain of gp41 had not occurred.

Truncation of 147 amino acids from the C-terminus of gp41 dramatically decreased the steady state level of viral Env proteins in COS-7 cells (Fig. 5). Mutant Env protein synthesis was not significantly affected as shown by pulse-chase experiments. Thus, the stability of TM709 Env proteins was apparently reduced compared to the wild type Env proteins. It was reported recently that mutations in the cytoplasmic domain of gp41 could decrease the stability of mutant Env proteins (Gabuzda et al., J. Virol. 66:3306-3315 (1992)). The TM709 gp120 migrated slightly slower than the wild type gp120 (Fig.

5), suggesting the polypeptide post-translation modifications of this gp120 in this mutant might be different from that of the wild type gp120. The decreased stability of the mutant Env proteins and the aberrant modification of mutant gp120 may indicate that the cytoplasmic domain of gp41 is important for mediating Env protein intracellular transport.

V. Examples

The following examples are provided to illustrate the invention not to limit it.

Example 1: Construction of gp41 mutants.

The wild type provirus clone, HXB2R3, has been described before (Yu et al., J. Virol. 66:4966-4971 (1992)). In frame stop codons were generated at different positions in the gp41 coding region (Fig. 1, SEQ ID No. 1 and Figure 2). The stop codon in TM709 is two amino acids downstream from the putative transmembrane region. Two positively charged arginine residues at positions 707 and 709, which are presumably important for the stop transfer signal, were preserved. TM752 and TM775 retained the first 45 and 68 amino acids of the gp41 cytoplasmic domain, respectively. Both of the predicted amphipathic 2-helices (Venable et al., AIDS Res. Hum. Retroviruses 5:7-22 (1989)) were deleted from the cytoplasmic domain of TM752 and TM775 TM proteins. Mutations in TM795 and TM812 preserved the first 88 and 106 amino acids of the gp41 cytoplasmic domain and deleted the predicted distal amphipathic α-helix (Venable et al., AIDS Res. Hum. Retroviruses 5:7-22 (1989)). The stop codon in TM844 is just upstream from the putative Vif cleavage site (Guy et al., J. Virol. 65:1325-1331 (1991)) and truncates the last 12 amino acids of gp41. The amino acids of the overlapping *rev* open reading frame

were not affected by the nucleotide substitutions in any of the mutants.

Example 2: Infectivity of gp41 mutant viruses.

Viruses were generated from COS-7 cells after transfection with wild type and mutant plasmid DNA (data not shown), suggesting that mutations in the cytoplasmic domain of gp41 did not block virus assembly and release. The infectivity of mutant viruses was compared with the wild type virus in several T-lymphoid cell lines and PBMC. Cell-free wild type and mutant viruses were prepared and analyzed for infectivity as previously described (Yu et al., J. Virol. 66:4966-4971 (1992)). Except for mutant TM844, none of the mutants established a productive infection in SupT1, MT2, and H9 cells, or fresh PBMC (Fig. 3). Although virus production was detected in TM844-infected MT-2 and SupT1 cells, the kinetics of TM844 replication in these cells was dramatically slower than that of the wild type virus (Fig. 3). The TM844 virus was at least two logs of magnitude less infectious than the wild type virus in SupT1 cells (data not shown). TM844 virus replication was even more dramatically impaired in H9 cells and PBMC, so that mutant virus production never reached the level of the wild type virus production during the thirty day follow-up period (Fig. 3).

Example 3: Analysis of virion proteins by immunoblot.

To study the defect of the mutant viruses, virions harvested from culture supernatants of wild type and mutant-transfected COS-7 cells were analyzed by immunoblot. As was the case with the wild type virions, the *gag* gene-encoded proteins, p24 and p17, and the *pol* gene-encoded proteins, p66, p51, and p34, were readily

detected in all of the mutant virions using a pooled HIV positive human sera (Fig. 4a). However, in sharp contrast to the wild type virions, the level of *env* gene-encoded protein (gp120) detected was dramatically decreased in mutant virions TM709, TM752, TM775, or TM795 as detected by a sheep anti-gp120 serum (Fig. 4a upper panel) or the pooled HIV positive sera (Fig. 4a lower panel). Mutant gp41 was not detected in these mutant virions by HIV positive sera (Fig. 4a lower panel). For mutant TM812, no gp41 and a lesser amount of gp120 as compared to wild type virions were detected by the HIV positive sera (Fig. 3a lower panel). Comparable amounts of gp120 and gp41 were detected in wild type and mutant TM844 virions (Fig. 3a). The TM844 gp41 migrated slightly faster than the wild type gp41 (Fig. 3a).

Wild type, TM844, and TM752 virions were also purified by sucrose gradients and analyzed by immunoblot using another pooled HIV positive sera. This pooled HIV positive sera reacted to gp120 better than the one used above under the stated assay conditions. The amount of gp120 and gp41 detected in mutant TM844 virions remained comparable to that of the wild type virions after purification by sucrose gradient (Fig. 4b). In contrast, the amount of gp120 detected in mutant TM752 virions was significantly less than that seen in the wild type virions (Fig. 4b). The amount of gp120 detected in TM752 virions was estimated to be less than 20% of that detected in the wild type virions when compared to a fourfold dilution of the wild type virion sample. (Fig. 4b). gp41 was not detected in the mutant TM752 virions (Fig. 4b). When cell lysates were analyzed with the same HIV positive sera, comparable amounts of gp120, and wild type and truncated gp41 were detected in wild type and TM752-transfected COS-7 cells (Fig. 4b). This suggests that the decreased detection of Env proteins in mutant

TM752 virions was less likely due to a decreased immunoreactivity of the HIV positive sera.

Example 4: Analysis of viral Env protein synthesis, processing, transport, and surface expression.

To address the question of whether the decreased incorporation of Env proteins in mutant TM709 and TM752 virions was due to decreased expression of mutant Env proteins in transfected cells, the expression of viral proteins in wild type-, and in mutants TM709, TM752, and TM844-transfected COS-7 cells was analyzed by radioimmunoprecipitation. After transfection in the COS-7 cells, gp160 and gp120 were detected in the wild type-transfected cells (Fig. 5a). Similar amounts of gp160 and gp120, when adjusted for similar amounts of the Gag polyprotein (p55), were also detected in TM844- and TM752-transfected cells (Fig. 5a). The migration of TM844 and TM752 gp120 was similar to the wild type gp120 (Fig. 5a), suggesting that mutations in TM844 and TM752 did not affect the expression and processing of mutant Env proteins. The transport of TM844 and TM752 Env proteins was not significantly affected since gp120 could be detected in the supernatants of TM844 and TM852-transfected cells (Fig. 5a). The amount of gp160 and gp120 detected in the TM709-transfected cells was much less than that detected in the wild type-transfected cells (Fig. 5a). In addition, TM709 gp120 migrated slightly slower than the wild type gp120 (Fig. 5a). TM709 gp120 was detected in the culture supernatant of TM709-transfected COS-7 cells (Fig. 4a). This suggests that Env protein transport to the cell surface could still occur in TM709-transfected cells.

Surface expression of the gp120 in wild type, TM752- and TM709- transfected COS-7 cells were also studied by indirect immunofluorescence staining.

Comparable levels of gp120 were detected on the surface of wild type- and TM752-transfected cells by mouse monoclonal anti-gp120 antibody (Fig. 4b). This indicates that the surface anchorage of gp120 was not affected by the truncation of the cytoplasmic domain in TM752. This observation is in agreement with previous reports where truncation of the last 104 amino acids from the C terminus of gp41 (same as TM752) did not affect the surface expression of the viral Env protein (Earl et al., J. Virol. 65:31-41 (1991)). In contrast to TM752, the ability of mutant TM709 to express Env proteins on the cell surface was greatly diminished as indicated by the lack of immunofluorescence staining of gp120 on the surface of the TM709-transfected COS-7 cells (Fig. 5b).

VI. Other Embodiments

gp41 Polypeptides

As described above, the invention includes therapies using a protein (or nucleic acid encoding a protein), which are mutant HIV gp41 proteins described elsewhere in this application, or which are homologous to such mutants. Also included are: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high (e.g., washing at 2xSSC at 40 C with a probe length of at least 40 nucleotides) stringency conditions to naturally occurring gp41 encoding nucleic acid (for other definitions of high and low stringency see Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989, 6.3.1 - 6.3.6, hereby incorporated by reference). The term also includes chimeric polypeptides that include gp41 together with unrelated sequences.

The invention also includes any biologically active fragment or analog of gp41. By "biologically active" is meant possessing therapeutically useful anti-

HIV activity which is characteristic of the gp41 encoding by the constructs shown in Figs. 2 and 7 and described in Section I of the specification. Therapeutically useful activity of a gp41 fragment or gp41 analog, can be determined in any one (or more) of a variety of assays, for example, those assays described in this application such as the dominant negative mutant assay. A gp41 analog possessing, most preferably greater than a five fold decrease over wild type in the dominant negative assay between the 1 and 10 day assay period, preferably a four fold decrease, or at least twofold decrease over the anti-HIV activity of an unchallenged control infection with wild type HIV in any in vivo or in vitro gp41 assay for anti-HIV activity (e.g., those described), is considered biologically active and useful in the invention.

Preferred analogs include mutant gp41 (or biologically active fragments thereof) whose sequences differ from the indicated gp41 mutant sequences only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not destroy the polypeptide's relevant anti-HIV activity as measured using in vivo or in vitro (e.g., using the infectivity and dominant negative assays described above). Preferred analogs also include gp41 (or active fragments thereof) which are modified for the purpose of increasing peptide stability; such analogs may contain, for example, one or more desaturated peptide bonds or D-amino acids in the peptide sequence.

Analogs can differ from naturally occurring gp41 by amino acid sequence differences or by modifications that do not affect sequence, or by both. Analogs of the

invention will generally exhibit at least 65%, more preferably 80%, even more preferably 90%, and most preferably 95% or even 99%, homology with all or part of a naturally occurring gp41 sequence. The length of comparison sequences will generally be at least about 15 amino acid residues, preferably more than 40 amino acid residues. Modifications include *in vivo*, or *in vitro* chemical derivatization of polypeptides, e.g., acetylation, glycosylation, or carboxylation. Also embraced are versions of the same primary amino acid sequence that have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine. Analogs can differ from naturally occurring gp41 by alterations of their primary sequence. These include genetic variants, both natural and induced. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids. Alternatively, increased stability may be conferred by cyclizing the peptide molecule.

In addition to substantially full-length polypeptides, the invention also includes biologically active fragments of the polypeptides. As used herein, the term "fragment", as applied to a polypeptide, will ordinarily be at least about 10 contiguous amino acids, typically at least about 20 contiguous amino acids, more typically at least about 30 contiguous amino acids, usually at least about 40 contiguous amino acids, preferably at least about 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Fragments of gp41 can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of gp41 can be assessed by methods

described below. Also included are gp41 polypeptides containing amino acids that are normally removed during protein processing, including additional amino acids that are not required for the therapeutic activity of the polypeptide, or including additional amino acids that result from alternative mRNA splicing or alternative protein processing events.

The invention also includes polypeptides (or nucleic acid either encoding polypeptides) which are homologous to the gp41 protein or homologous to the gp160 gene and are useful for the treatment of individuals infected with HIV. Sequences which are considered to be homologous are those which are 70 % homologous. Homologous refers to the sequence similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in both of the two compared sequences is occupied by the same base or amino acid monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences. For example, 6 of 10, of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology.

What is claimed is:

CLAIMS:

1. A method of treating a patient infected with human immunodeficiency virus (HIV) by administering to said patient a mutated gp41 polypeptide in an amount effective to reduce HIV levels in said patient.

2. The method of claim 1 comprising administering to said patient a therapeutic composition comprising said mutated gp41 polypeptide in a pharmaceutically acceptable carrier.

3. The method of claim 1 comprising administering to said patient a therapeutic composition comprising nucleic acid encoding said mutated gp41 polypeptide in an expressible genetic construction.

4. The method of claim 3, wherein said nucleic acid is capable of transforming cells of said patient.

5. The method of claim 4 in which said nucleic acid is part of a viral vector capable of transforming cells of said patient.

6. The method of claim 3 in which said nucleic acid further comprises a sequence encoding a CD4-binding polypeptide.

7. The method in claim 3 in which the said nucleic acid further comprises a sequence encoding a gp120-binding polypeptide.

8. The method of claim 4, claim 5, claim 6, or claim 7 comprising removing cells from said patient, transforming said cells with said nucleic acid, and returning transformed cells to said patient's body.

9. The method of claim 4, claim 5, claim 6 or claim 7 comprising administering said nucleic acid directly to said patient.

10. The method of claim 5, wherein said viral vector is human immunodeficiency virus-type I.

11. A method of claim 1, claim 2 or claim 3 wherein said mutant gp41 polypeptide contains a deletion of at least one amino acid in at least one of the following regions of wild type gp41:

-- amino acids 844 to 856;

-- amino acids 814 to 856;

-- amino acids 796 to 856;

-- amino acids 776 to 856;

-- amino acids 753 to 856; or

-- amino acids 710 to 856, effective to either disrupt viral replication of HIV or disrupt the assembly of viral Env proteins in an HIV infected cell.

12. The method of claim 11 wherein said gp41 polypeptide contains a deletion a region comprising at least one or more of amino acids 844 to 856 effective to disrupt the viral replication of HIV in an infected cell.

13. The method of claim 11 wherein said mutated gp41 polypeptide contains a deletion a region comprising at least one or more of amino acids 814-856 effective to disrupt the viral replication in an HIV infected cell.

14. The method of claim 11 wherein said mutated gp41 polypeptide contains a deletion a region comprising at least one or more of amino acids 796-856 effective to disrupt the assembly of viral Env proteins in an HIV infected cell.

15. The method of claim 11 wherein said mutated gp41 polypeptide contains a deletion a region comprising at least one or more of amino acids 776-856 effective to disrupt the assembly of viral Env proteins in an HIV infected cell.

16. The method of claim 11 wherein said mutated gp41 polypeptide contains a deletion a region comprising at least one or more of amino acids 753-856 effective to disrupt the assembly of viral Env proteins in an HIV infected cell.

17. The method of claim 1 or claim 2 wherein said mutated gp41 polypeptide contains a deletion of amino acids 710-856 effective to disrupt the assembly of viral Env proteins in an HIV infected cell.

18. A therapeutic composition adapted for administration to a patient infected with human immunodeficiency virus-type I (HIV), said composition comprising a mutated gp41 polypeptide in a pharmaceutically acceptable carrier.

19. A therapeutic composition adapted for administration to a patient infected with human immunodeficiency virus-type I (HIV), said composition comprising nucleic acid encoding a mutated gp41 polypeptide in an expressible genetic construction for transforming cells of a human patient.

20. The therapeutic composition of claim 13 comprising said nucleic acid as part of a viral vector capable of transforming cells of said patient.

21. The therapeutic composition of claim 13 wherein said nucleic acid further comprises a sequence capable of encoding a CD4-binding polypeptide.

22. The therapeutic composition of claim 13 wherein said nucleic acid further comprises a sequence encoding a gp120-binding polypeptide.

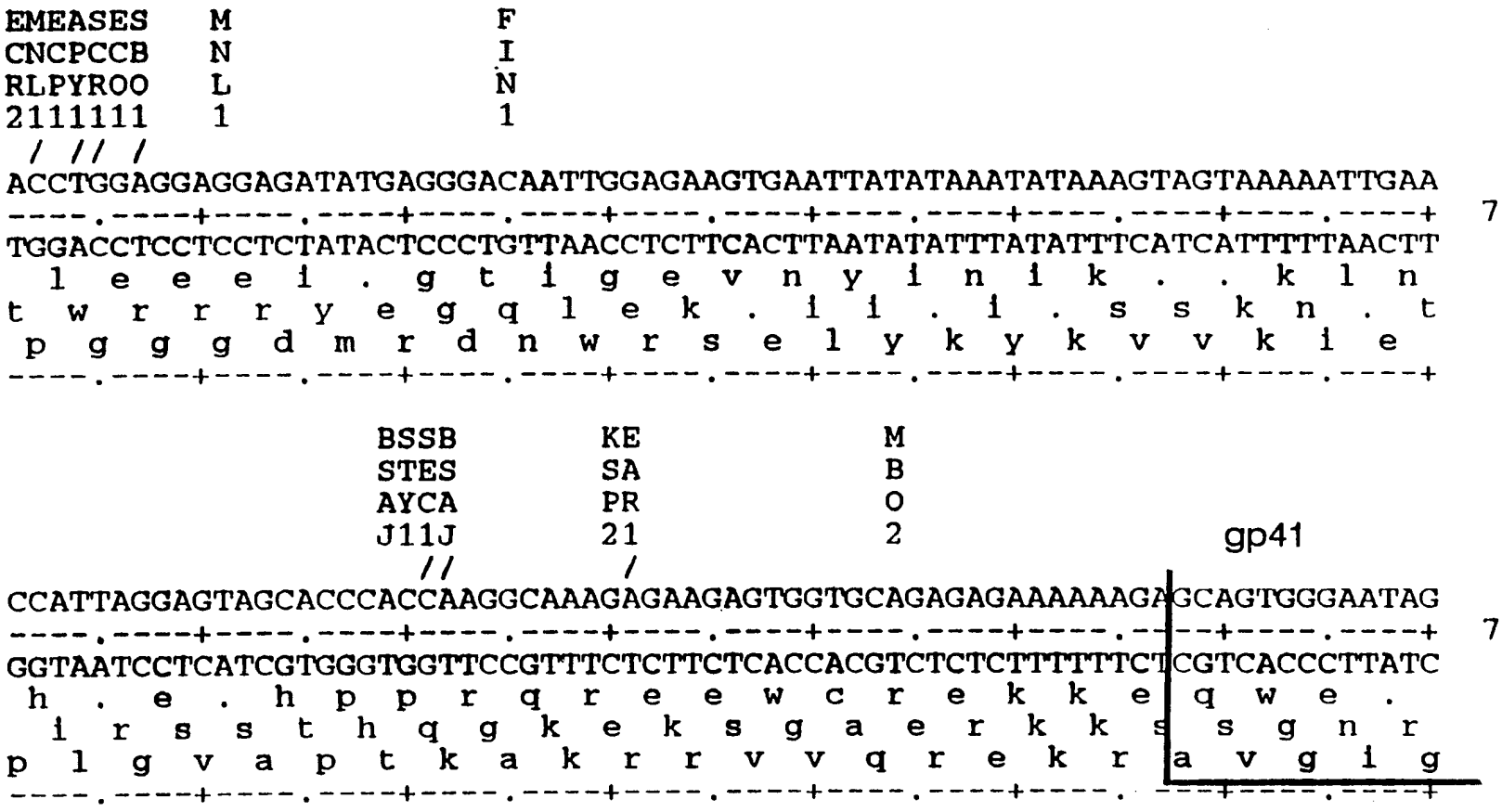


FIG. 1A

```
 A      BSSB          EF      BH      H HF          B       H R
 L      STES          CN      BG      I HN          B       G S
 U      AYCA          1U      VA      N AU          V       A A
 1      J11J          5H      11      P 1H          1       1 1
        //
GAGCTTTGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACGCTGACGGT
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
CTCGAAACAAGGAACCCAAGAACCCTCGTCCTTCGTGATACCCGCGTCGCAGTTACTGCGACTGCCA
e l c s l g s w e q q e a l w a q r q . r . r y
 s f v p w v l g s s s r k h h y g g r s v n d a d g
  a l f l g f l g a a g s t m g a a s m t l t v
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
      HH            EF EF       BM B   D   M         H H
      AA            CN CN       BN B   D   N         I H
      EE            1U 1U       VL V   E   L         N A
      13            5H 5H       11 1   1   1         P 1
      /
ACAGGCCAGACAATTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAA
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
TGTCCGGTCTGTTAATAACAGACCATATCACGTCGTCGTCTTGTTAAACGACTCCCGATAACTCCGCGTT
  r p d n y c l v . c s s r t t l c . g l l r r n
t g q t i i v w y s a a a e e q f a e g g y . g a t
 q a r q l l s g l v q q q n n l l r a l e a q
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
```

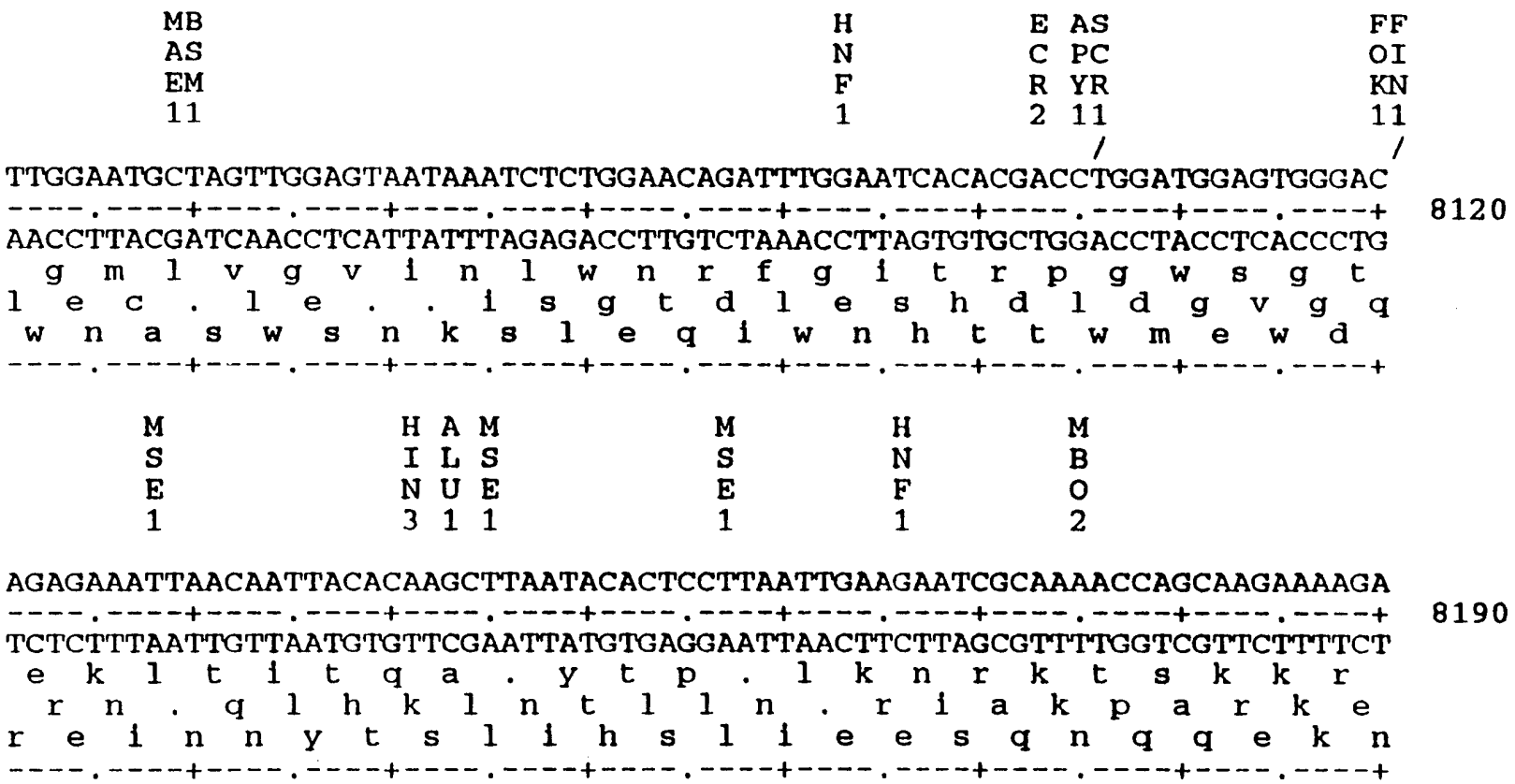
7840

7910

FIG. 1B

```
 A     S                FSA E AS   B H   E AS
 L     F                NFL C PC   B N   C PC
 W     A                UAU R YR   V F   R YR
 N     N                HN1 2 11   1 1   2 11
                                  /
CAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAGAT                7980
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----
GTCGTAGACAACGTTGAGTGTCAGACCCCGTAGTTCGTCGAGGTCCGTTCTTAGGACCGACACCTTTCTA
 s l c c n s q s g a s s s s r q e s w l w k d
  a s v a t h s l g h q a a p g g k n p g c g k l
q h l l q l t v w g l k q l q a r l l a v e r y
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----
                                                          E BSSB
     BM D       A EAASSBB                                 C STES
     IB P       L CLPCESS                                 1 AYCA
     NO N       U RWYRCAA                                 5 J11J
     11 1       1 2N111JJ                                   //
                ////
ACCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACTCATTTGCACCACTGCTGTGCC                8050
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----
TGGATTTCCTAGTTGTCGAGGACCCCTAAACCCCAACGAGACCTTTTGAGTAAACGTGGTGACGACACGG
 t . r l n s s w g f g v a l e n s f a p l l c l
  p k g s t a p g d l g l l w k t h l h h c c a
   l k d q q l l g l w g c s g k l l c t t a v p
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----
```

FIG. 1C


MB AS EM 11
H N F 1
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FF OI KN 11
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8120
AACCTTACGATCAACCTCATTATTTAGAGACCTTGTCTAAACCTTAGTGTGCTGGACCTACCTCACCCTG
g m l v g v i n l w n r f g i t r p g w s g t
l e c . l e . . i s g t d l e s h d l d g v g q
w n a s w s n k s l e q i w n h t t w m e w d
M S E 1
H A M I L S N U E 3 1 1
M S E 1
H N F 1
M B O 2
AGAGAAATTAACAATTACACAAGCTTAATACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGA
8190
TCTCTTTAATTGTTAATGTGTTCGAATTATGTGAGGAATTAACTTCTTAGCGTTTTGGTCGTTCTTTTCT
e k l t i t q a . y t p . l k n r k t s k k r
r n . q l h k l n t l l n . r i a k p a r k e
r e i n n y t s l i h s l i e e s q n q q e k n

FIG. 1D

```
T                                            M
T                                            S
H                                            E
2                                            1

ATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCT
----.----+----.----+----.----+----.----+----.----+----.----+----.----+ 8260
TACTTGTTCTTAATAACCTTAATCTATTTACCCGTTCAAACACCTTAACCAAATTGTATTGTTTAACCGA
m  n  k  n  y  w  n  .  i  n  g  q  v  c  g  i  g  l  t  .  q  i  g  c
 .  t  r  i  i  g  i  r  .  m  g  k  f  v  e  l  v  .  h  n  k  l  a
  e  q  e  l  l  e  l  d  k  w  a  s  l  w  n  w  f  n  i  t  n  w  l
----.----+----.----+----.----+----.----+----.----+----.----+----.----+

                        M                    M                 R
                        N                    S                 S
                        L                    E                 A
                        1                    1                 1

GTGGTATATAAAATTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTT
----.----+----.----+----.----+----.----+----.----+----.----+----.----+ 8330
CACCATATATTTTAATAAGTATTACTATCATCCTCCGAACCATCCAAATTCTTATCAAAAACGACATGAA
  g  i  .  n  y  s  .  .  .  .  e  a  w  .  v  .  e  .  f  l  l  y  f
v  v  y  k  i  i  h  n  d  s  r  r  l  g  r  f  k  n  s  f  c  c  t  f
 w  y  i  k  l  f  i  m  i  v  g  g  l  v  g  l  r  i  v  f  a  v  l
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
```

FIG. 1E

```
                   H                                   E         M      AMSBB   DPAA
                   P                                   C         N      VNESS   RPVS
                   H                                   P         L      ALCAA   AUAU
                   1                                   1         1      111JJ   2121
                                                                          ///    ///
TCTATAGTGAATAGAGTTAGGCAGGATATTCACCATTATCGTTTCAGACCCACCTCCCAACCCCGAGGG
----.----+----.----+----.----+----.----+----.----+----.----+----.----+   8400
AGATATCACTTATCTCAATCCGTCCCTATAAGTGGTAATAGCAAAGTCTGGGTGGAGGGTTGGGGCTCCC
 l  .  .  i  e  l  g  r  d  i  h  h  y  r  f  r  p  t  s  q  p  r  g
  y  s  e  .  s  .  a  g  i  f  t  i  i  v  s  d  p  p  p  n  p  e  g
s  i  v  n  r  v  r  q  g  y  s  p  l  s  f  q  t  h  l  p  t  p  r  g
----.----+----.----+----.----+----.----+----.----+----.----+----.----+

NNFP     AH                          M  M B     B     XMD  B  T H
LLIS     SA                          B  B S     S     HBP  I  A N
AANS     UE                          O  O M     M     OON  N  Q F
4411     13                          2  2 2     2     211  1  1 3
  /                                                     /
GACCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGT
----.----+----.----+----.----+----.----+----.----+----.----+----.----+   8470
CTGGGCTGTCCGGGCTTCCTTATCTTCTTCTTCCACCTCTCTCTCTGTCTCTGTCTAGGTAAGCTAATCA
d  p  t  g  p  k  e  .  k  k  k  v  e  r  e  t  e  t  d  p  f  d  .  .
 t  r  q  a  r  r  n  r  r  r  r  w  r  e  r  q  r  q  i  h  s  i  s
  p  d  r  p  e  g  i  e  e  e  g  g  e  r  d  r  d  r  s  i  r  l  v
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
```

FIG. 1F

```
BXBNMDBSSBB          FMD      N M        KEAM       B
IHALBPITESS          IBP      L B        SALN       S
NOMAONNYCAA          NON      A O        PRUL       M
12141111JJ           111      4 2        2111       2
 / // ////            /                   //
GAACGGATCCTTGGCACTTATCTGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGA
----.----+----.----+----.----+----.----+----.----+----.----+----.----+ 8540
CTTGCCTAGGAACCGTGAATAGACCCTGCTAGACGCCTCGGACACGGAGAAGTCGATGGTGGCGAACTCT
 t  d  p  w  h  l  s  g  t  i  c  g  a  c  a  s  s  a  t  t  a  .  e
e  r  i  l  g  t  y  l  g  r  s  a  e  p  v  p  l  q  l  p  p  l  e  r
 n  g  s  l  a  l  i  w  d  d  l  r  s  l  c  l  f  s  y  h  r  l  r
----.----+----.----+----.----+----.----+----.----+----.----+----.----+

          M M                        F      H                  S  M
          N A                        I      G                  S  N
          L E                        N      A                  P  L
          1 3                        1      1                  1  1

GACTTACTCTTGATTGTAACGAGGATTGTGGAACTTCTGGACGCAGGGGGTGGGAAGCCCTCAAATATT
----.----+----.----+----.----+----.----+----.----+----.----+----.----+ 8610
CTGAATGAGAACTAACATTGCTCCTAACACCTTGAAGACCCTGCGTCCCCCACCCTTCGGGAGTTTATAA
 t  y  s  .  l  .  r  g  l  w  n  f  w  d  a  g  g  g  k  p  s  n  i
  l  t  l  d  c  n  e  d  c  g  t  s  g  t  q  g  v  g  s  p  q  i  l
d  l  l  l  i  v  t  r  i  v  e  l  l  g  r  r  g  w  e  a  l  k  y  w
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
```

FIG. 1G

```
    H                   H       P                   A
    N                   N       L                   L
    F                   F       E                   U
    1                   1       1                   1

GGTGGAATCTCCTACAGTATTGGAGTCAGGAACTAAAGAATAGTGCTGTTAGCTTGCTCAATGCCACAGC
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----.----+  8680
CCACCTTAGAGGATGTCATAACCTCAGTCCTTGATTTCTTATCACGACAATCGAACGAGTTACGGTGTCG
g g i s y s i g v r n . r i v l l a c s m p q p
 v e s p t v l e s g t k e . c c . l a q c h s
  w n l l q y w s q e l k n s a v s l l n a t a
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----.----+

     M    AAD       F                   R       A       A       H
     N    LLD       I                   S       L       L       N
     L    WUE       N                   A       U       U       F
     1    N11       1                   1       1       1       3
           //
CATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAGTAGTACAAGGAGCTTGTAGAGCTATTCGCCAC
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----.----+  8750
GTATCGTCATCGACTCCCCTGTCTATCCCAATATCTTCATCATGTTCCTCGAACATCTCGATAAGCGGTG
  . q . l r g q i g l . k . y k e l v e l f a t
h s s s . g d r . g y r s s t r s l . s y s p h
 i a v a e g t d r v i e v v q g a c r a i r h
----.----+----.----+----.----+----.----+----.----+----.----+----.----+----.----+
```

FIG. 1H

```
  M       M
  A       B
  E       O
  1       2                    gp41

ATACCTAGAAGAATAAGACAGGGCTTGGAAAGGATTTTGCTATAAGATGGGTGGCAAGTGGTCAAAAAGT
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
TATGGATCTTCTTATTCTGTCCCGAACCTTTCCTAAAACGATATTCTACCCACCGTTCACCAGTTTTTCA
 y  l  e  e  .  d  r  a  w  k  g  f  c  y  k  m  g  g  g  k  w  s  k  s
 t  .  k  n  k  t  g  l  g  k  d  f  a  i  r  w  v  a  s  g  q  k  v
 i  p  r  r  i  r  q  g  l  e  r  i  l  l  .  d  g  w  q  v  v  k  k  .
----.----+----.----+----.----+----.----+----.----+----.----+----.----+

                F       B          AED   E  EF          BT
                O       S          LSD   C  CN          BT
                K       M          UPE   1  1U          VH
                1       2          111   5  5H          12
                                   /

AGTGTGATTGGATGGCTTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAG
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
TCACACTAACCTACCGAATGACATTCCCTTTCTTACTCTGCTCGACTCGGTCGTCTACCCCACCCTC
s  v  i  g  w  l  t  v  r  e  r  m  r  r  a  e  p  a  a  d  g  v  g  a
v  .  l  d  g  l  l  .  g  k  e  .  d  e  l  s  q  q  q  m  g  w  e
c  d  w  m  a  y  c  k  g  k  n  e  t  s  .  a  s  s  r  w  g  g  s
----.----+----.----+----.----+----.----+----.----+----.----+----.----+
```

8820 8890

FIG. 11

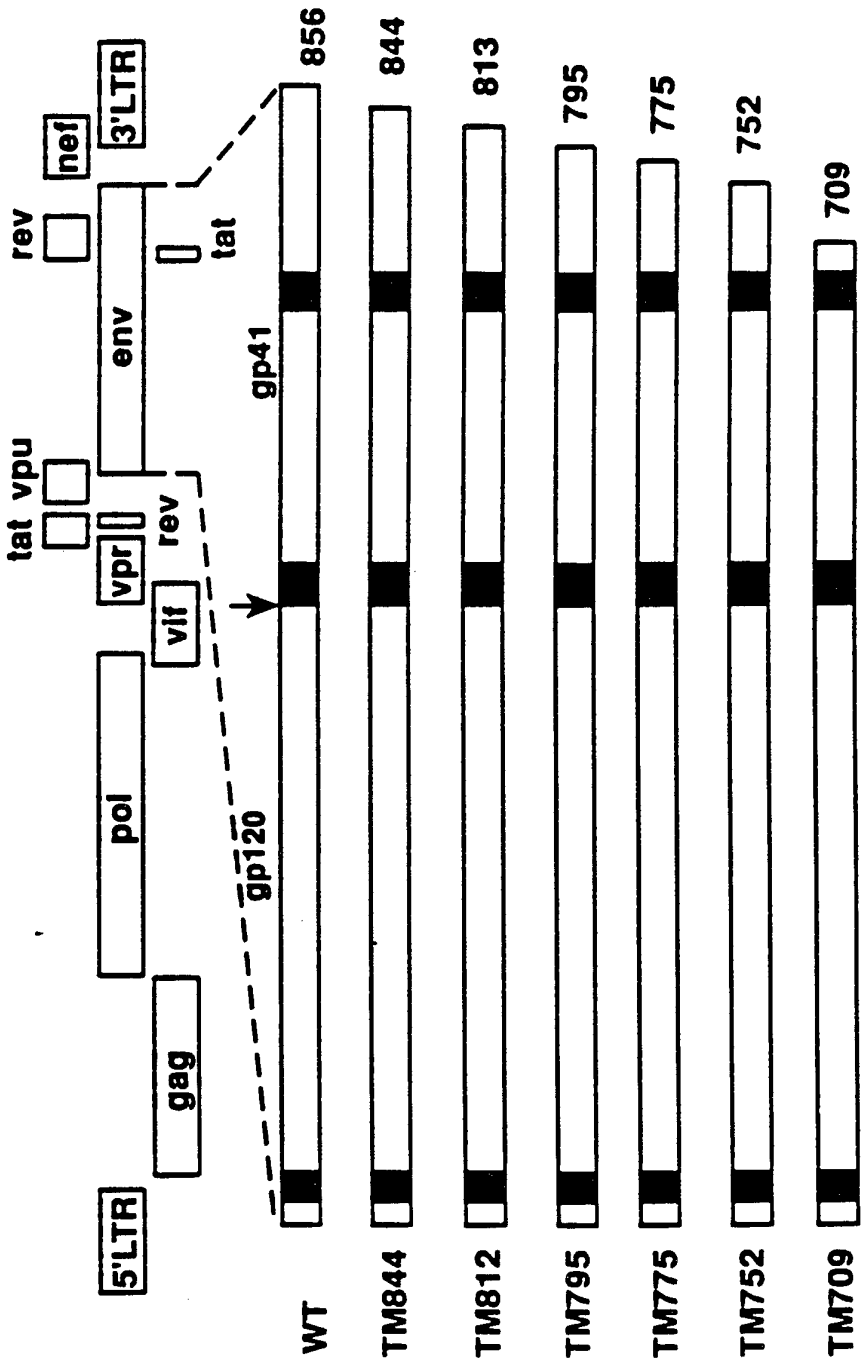
5'LTR
gag
pol
vif
vpr
tat vpu
rev
env
nef
3'LTR
rev
tat
gp120
gp41
856
844
813
795
775
752
709
WT
TM844
TM812
TM795
TM775
TM752
TM709

FIG. 2

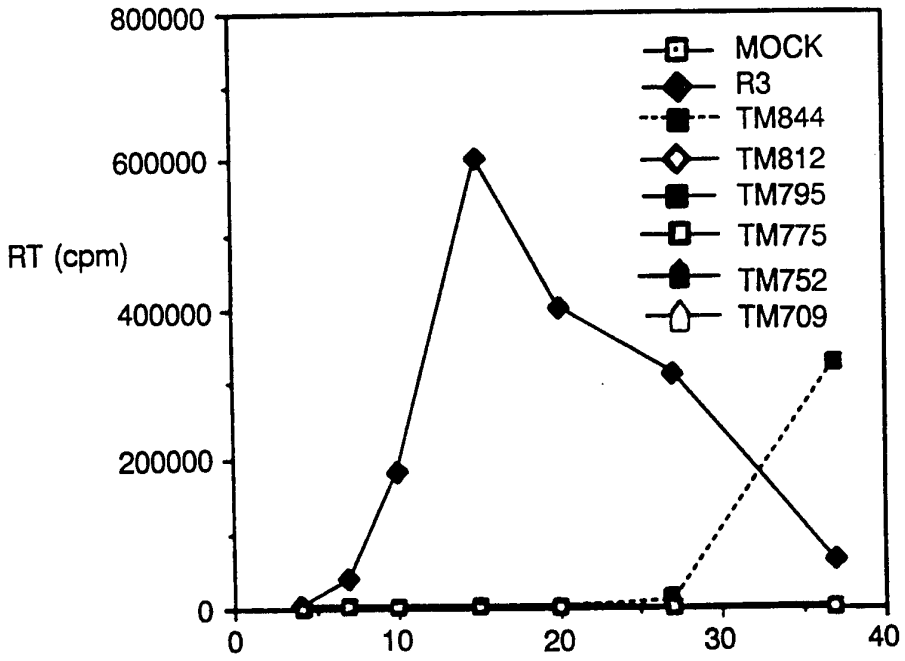

FIG. 3A DAYS AFTER INJECTION (SUP-T1)

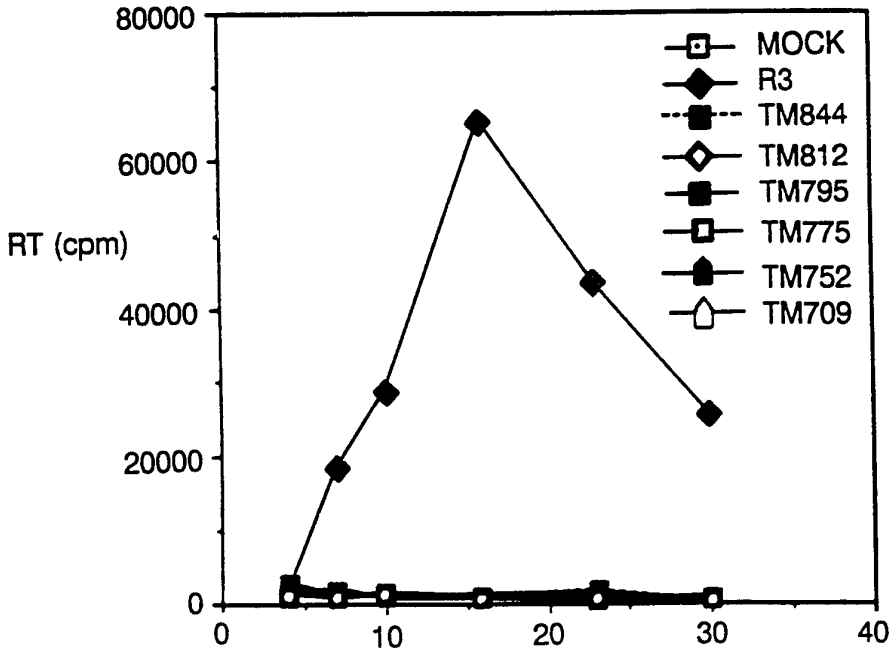

FIG. 3B DAYS AFTER INJECTION (H-9)

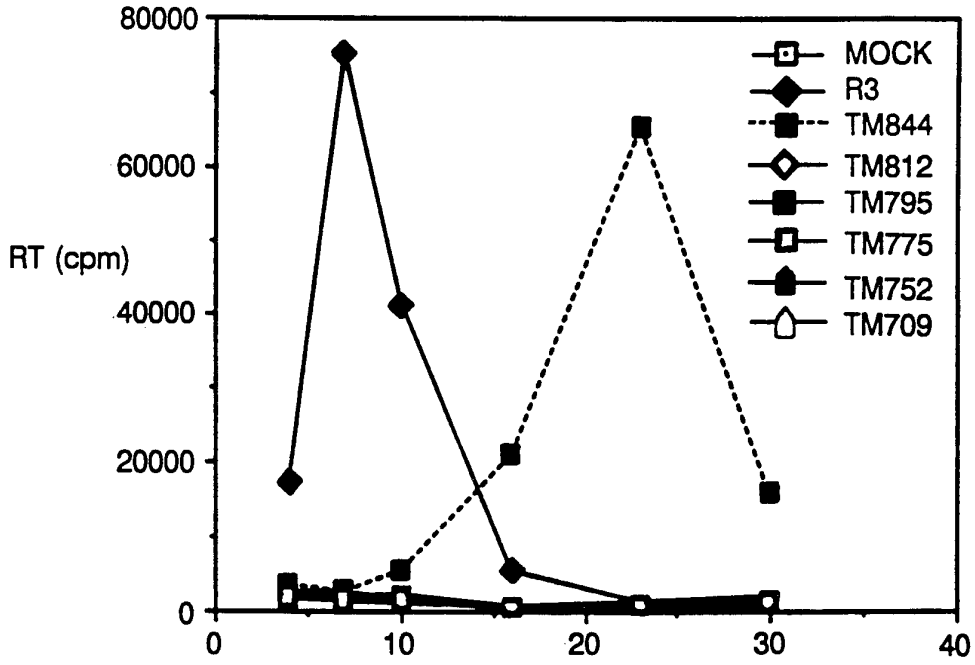
80000
60000
40000
20000
0
RT (cpm)
0
10
20
30
40
DAYS AFTER INJECTION (MT-2)
MOCK
R3
TM844
TM812
TM795
TM775
TM752
TM709

FIG. 3C

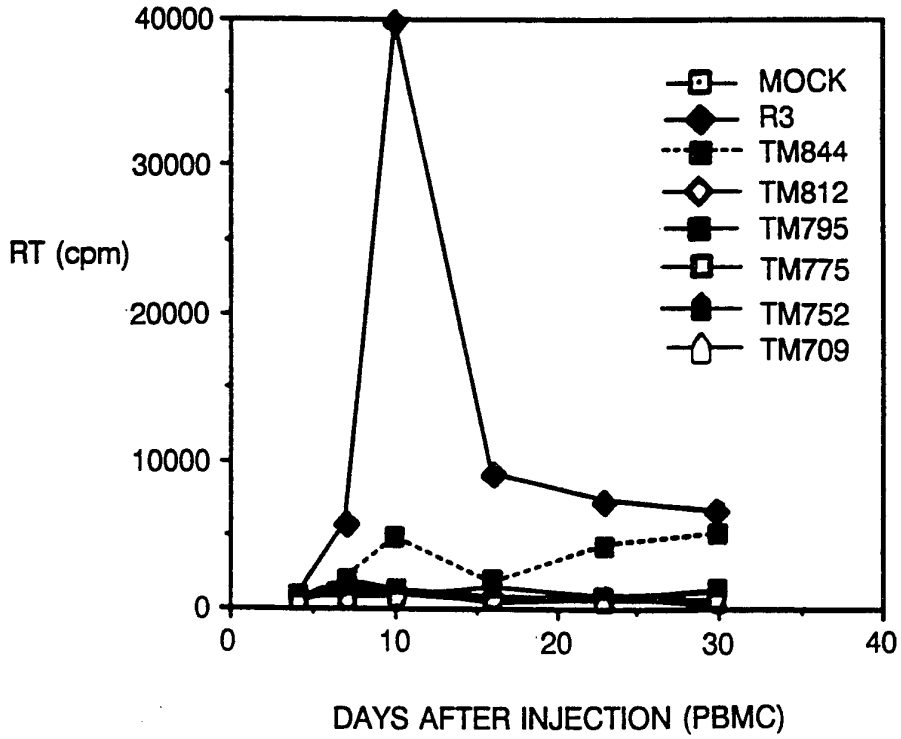
40000
30000
20000
10000
0
RT (cpm)
0
10
20
30
40
DAYS AFTER INJECTION (PBMC)
MOCK
R3
TM844
TM812
TM795
TM775
TM752
TM709

FIG. 3D

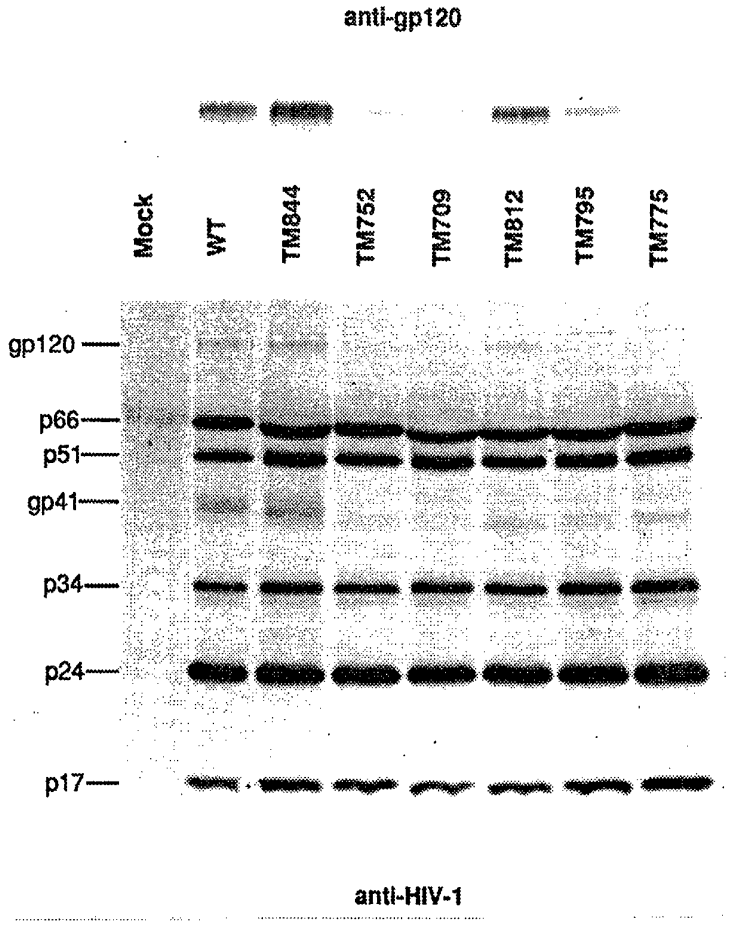
anti-gp120
Mock
WT
TM844
TM752
TM709
TM812
TM795
TM775
gp120
p66
p51
gp41
p34
p24
p17
anti-HIV-1

FIG. 4A

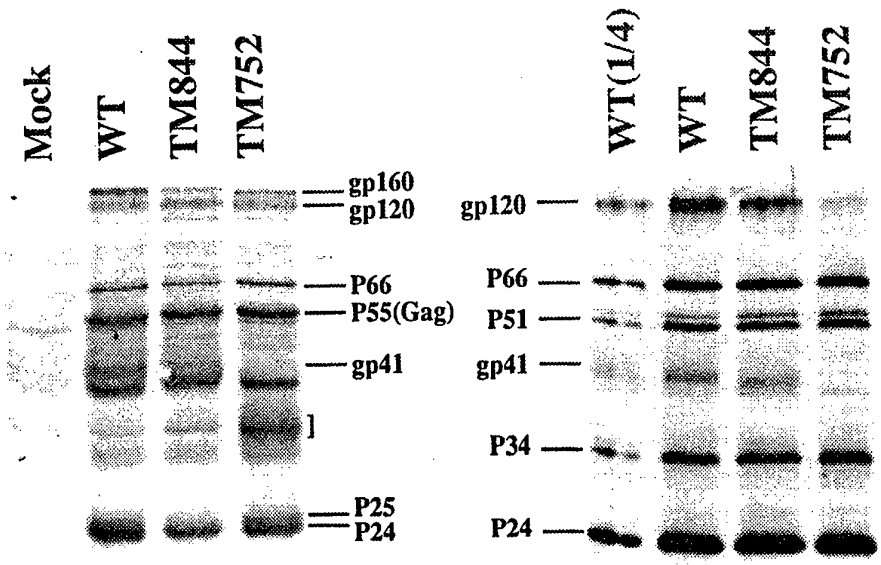
Mock
WT
TM844
TM752
gp160
gp120
P66
P55(Gag)
gp41
P25
P24
WT(1/4)
WT
TM844
TM752
gp120
P66
P51
gp41
P34
P24
cell
virus

FIG. 4B

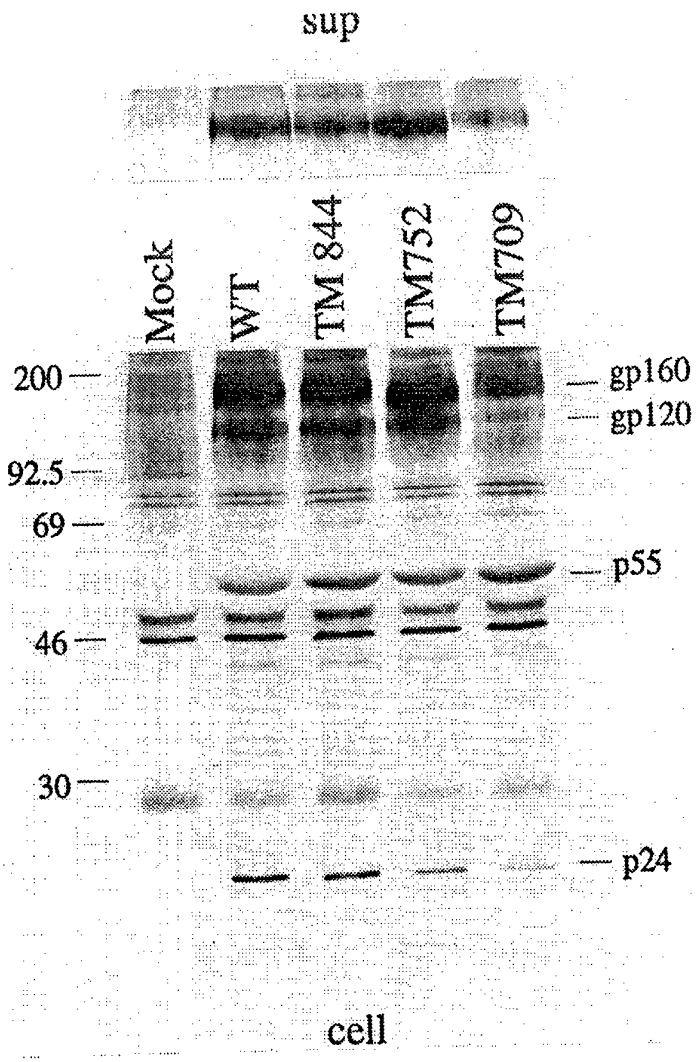
sup
Mock
WT
TM 844
TM752
TM709
200
92.5
69
46
30
gp160
gp120
p55
p24
cell

FIG. 5A

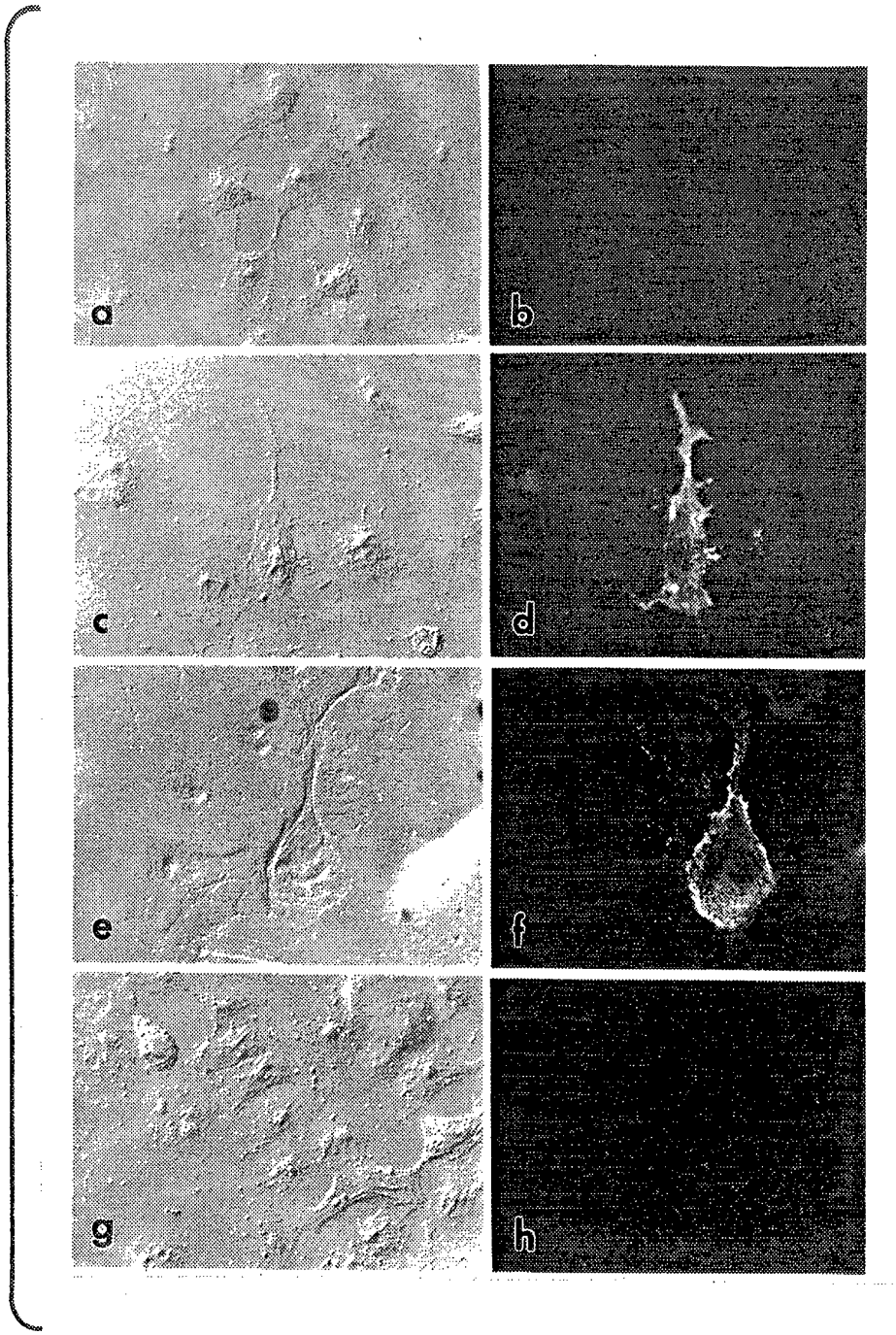

FIG. 5B

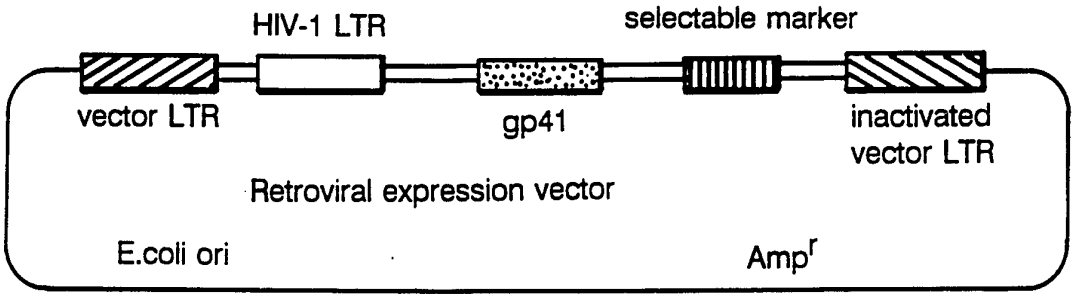
HIV-1 LTR
selectable marker
vector LTR
gp41
inactivated
vector LTR
Retroviral expression vector
E.coli ori
Amp^r

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00212

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00; A61K 39/21; C12P 21/02; C12N 7/01, 15/01, 15/49
US CL :530/350, 825; 424/88, 89; 514/2; 435/69.3, 172.1
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)

U.S. : 530/350, 825; 424/88, 89; 514/2; 435/69.3, 172.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CA, DERWENT,
search terms: HIV, gp41, mutant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Volume 66(6), issued June 1992, D.H. Gabuzda et al, "Effects of deletions in the Cytoplasmic Domain on Biological Functions of Human Immunodeficiency Virus Type I Envelope Glycoproteins", pages 3306-3315, see entire document, especially Figure 4 and Table 1.	1-22
Y	Proceedings of the National Academy of Sciences USA, Volume 87(12), issued June 1990, E.O. Freed et al, "Characterization of the Fusion Domain of the Human Immunodeficiency Virus Type I Envelope Glycoprotein gp41", pages 4650-4654, see entire document.	1-22

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

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 30 April 1993	Date of mailing of the international search report 12 MAY 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MICHAEL S. TUSCAN Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Volume 66(11), issued November 1992, J.W. Dubay et al, "Truncation of the Human Immunodeficiency Virus Type I Transmembrane Glycoprotein Cytoplasmic Domain Blocks Virus Infectivity", pages 6616-6625, see entire document, especially Table 1 and Figure 2.	1-22
Y	AIDS Research and Human Retroviruses, Volume 5, No. 4, issued May 1989, S.J. Lee et al, "Role of the Carboxy-Terminal Portion of the HIV-1 Transmembrane Protein in Viral Transmission and Cytopathogenicity", pages 441-449, see Figure 2, Tables 1 and 2.	1-22
Y	Journal of Virology, Volume 65(5), issued May 1991, M. Ivey-Hoyle et al, "The N-Terminal 31 Amino Acids of the Human Immunodeficiency Virus Type I Envelope Protein gp120 contain a Potential gp41 Contact Site", pages 2682-2685, See Figure 1 and page 2685.	1-22
Y	Journal of Virology, Volume 65(1), issued January 1991, P.L. Earl et al, "Biological and Immunological Properties of Human Immunodeficiency Virus Type I Envelope Glycoprotein: Analysis of Protein with Truncations and Deletions Expressed by Recombinant Vaccinia Viruses", pages 31-41, see entire document.	1-22
Y	Journal of Virology, Volume 65(1), issued January 1991, M. Kowalski et al, "Attenuation of Human Immunodeficiency Virus Type I Cytopathic effect by a Mutation Affecting the Transmembrane Envelope Glycoprotein", pages 281-291, see entire document.	1-22
Y	Virology, Volume 189, No. 2, issued August 1992, H. Shimizu et al, "Analysis of a Human Immunodeficiency Virus Type I Isolate Carrying a Truncated Transmembrane Glycoprotein", pages 534-546, see entire document.	1-22
X Y	Proceedings of the National Academy of Sciences USA, Volume 89(1), issued January 1992, E.O. Freed et al, "A Mutation in the Human Immunodeficiency Virus Type I Transmembrane Glycoprotein gp41 dominantly Interferes with Fusion and Infectivity", pages 70-74, see entire document.	1-5,8-10,18,19 6-7,11-17,20-22
Y	Journal of Virology, Volume 65(3), issued March 1991, B. Guy et al, "A Specific Inhibitor of Cysteine Proteases Impairs a Vif-Dependent Modification of Human Immunodeficiency Virus Type I Env Protein", pages 1325-1331, see entire document.	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/00212

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Volume 64(6), issued June 1990, O.K. Haffar et al, "The Carboxy Terminus of Human Immunodeficiency Virus Type I gp160 Limits its Proteolytic Processing and Transport in Transfected Cell Lines", pages 3100-3103, see entire document, especially Figure 1.	1-22
Y	Science, Volume 244, issued 16 June 1989, T. Friedman, "Progress Toward Human Gene Therapy", pages 1275-1281, see entire document.	1-22
Y	Cell, Volume 50, issued 11 September 1987, L.A. Lasky et al, "Delineation of a Region of the Human Immunodeficiency Virus Type I gp120 Glycoprotein Critical for Interaction with the CD4 Receptor", pages 975-985. see entire document.	6
Y	Cell, Volume 57, issued 05 May 1989, J. Arthos et al, "Identification of the Residues in Human CD4 Critical for the Binding of HIV", pages 469-481, see entire document.	7