NON-IMMUNOGENIC PRODRUGS AND SELECTABLE MARKERS FOR USE IN GENE THERAPY

TECHNICAL FIELD

The present invention relates generally to nucleic acid vectors, and more specifically, to vectors which are capable of delivering a gene of interest to susceptible target cells. These vector constructs are designed to deliver a non-immunogenic gene product which is capable of activating a compound with little or no activity into an active product.

BACKGROUND OF THE INVENTION

Human gene therapy is a clinical strategy wherein the genetic repertoire of cells are altered either to gain an understanding of the cell's function, or for therapeutic benefit. Briefly, gene therapy involves delivering vectors (*e.g.*, a retrovirus, adenovirus, vaccinia virus, or naked DNA alone) to cells so that therapeutically beneficial genetic information that is contained within the vector can be transferred from the vectors to the cells. This strategy has now been widely applied, with clinical trials presently ongoing for a wide range of both hereditary (*e.g.*, ADA deficiency, familial hypercholesterolemia, and cystic fibrosis) and acquired (*e.g.*, tumors) diseases (Crystal *Science 270*:404-410, 1995).

It is now clear, however, that long-term expression of foreign genes introduced by gene therapy may lead to immune responses in patients that destroy the treated cells (*e.g.*, C. Bordignon, *Brit. J. Hematology 93-S2*:306, 1996; S.R. Riddell et al., *Nature Medicine 2*:216, 1996). Although the actual tissue transduced may affect this and although many means of avoiding or minimizing this have been proposed (*see* J.D. Davies et al., *J. Immunol. 157*:529, 1996; D.J. Lenschow et al., *Science* 257. 789, 1992; A. Waisman et al., *Nature Medicine 2*:889, 1996), it is clear that this limitation is not easily overcome. Moreover, this problem extends to genes that are included in gene transfer vectors (*e.g.*, the neomycin resistance gene) that are included

for ease of handling, testing, characterization and manufacturing of gene delivery vehicles. It also extends to the use of genes that are included either to ablate a tumor or tissue or as a "fail-safe" mechanism so that cells that have been treated by genes or in some other way can be destroyed. An archetype of this is the HSV-TK gene.

The present invention provides novel compositions and methods for treating a variety of diseases (*e.g.*, viral diseases, cancer, genetic diseases and others) that overcome previous difficulties associated with the use of vectors in gene therapy, and further provides other, related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides recombinant gene delivery vehicles and methods of using such vehicles for the treatment of a wide variety of pathogenic agents. In particular, utilizing the vectors provided herein one can avoid problems in treating human patients through the use of human genes for selectable markers or activation of prodrugs. The selectable marker can allow biochemical selection (*e.g.*, hypoxanthine phosphoribosyltransferase) color selection (*e.g.*, alkaline phosphatase or beta galactosidase) or selection by antibody binding (*e.g.*, membrane bound alkaline phosphatase, CD 34). The activation of prodrugs can be of various pyrimidine or purine analogues (*e.g.*, deoxycytidine kinase and cytosine arabinoside), other prodrugs from the cancer field. (See for example A.K. Sinhabubu and D.R. Thakker, *Advanced Drug Delivery Reviews 19*:241, 1996 and M.A. Graham et al., *Pharmac. Ther. 51*:275, 1991 (both incorporated by reference) such as alkaline phosphatase acid phosphatase, beta-glucuronidase, carboxypeptidase A, cytosine deaminase, nitroreductase (a.k.a. azoredactase or DT diaphorase) plasmin and γ-glutamyl transpeptidase.

Within one aspect of the present invention, methods are provided delivering a gene delivery vehicle to a warm-blooded animal, comprising administering to a warm-blooded animal (*e.g.*, human) a gene delivery vehicle which directs the expression of a non-immunogenic selectable marker. Within other related aspects methods are provided for delivering a gene delivery vehicle to a warm-blooded animal,

comprising administering to a warm-blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic molecule which is capable of activating an otherwise inactive compound into an active compound. Within one embodiment, the non-immunogenic molecule is selected from the group consisting of alkaline phosphatase, α-Galactosidase, β-glucosidase, β-glucuronidase, Carboxypeptidase A, Cytochrome P450, γ-glutamyl transferase; reductases such as Azoreductase, DT diaphorase and Nitroreductase; and oxidases such as glucose oxidase and xanthine oxidase. Within a preferred embodiment, the gene delivery vehicle is a recombinant retroviral particle which is made in a non-mouse (*e.g.*, human or dog) packaging cell line. Within further embodiments, the retroviral particle is made utilizing a crossless retroviral vector backbone, and/or is substantially free of replication competent retrovirus. Within yet further embodiments, the above retroviral vector particle, upon introduction into a cell, directs the expression of human placental alkaline phosphatase.

Within other aspects of the invention, gene delivery vehicles are provided which direct the expression of a protein that is toxic upon processing or modification by a protein derived from a pathogenic agent. Within one embodiment, the protein which is toxic upon processing or modification is proricin.

Within yet certain embodiments of the invention, gene delivery vehicles are provided carrying a vector construct comprising a cytotoxic gene under the transcriptional control of an event-specific promoter, such that upon activation of the event-specific promoter the cytotoxic gene is expressed. Within various embodiments, the event-specific promoter is a cellular thymidine kinase promoter, or a thymidylate synthase promoter. Within another embodiment, the event-specific promoter is activated by a hormone. Within yet other embodiments, the cytotoxic gene is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

Within another embodiments of the present invention, gene delivery vehicles are provided comprising a cytotoxic gene under the transcriptional control of a tissue-specific promoter (including tissue-specific elements, such as for example, a locus control region), such that upon activation of the tissue-specific promoter the

cytotoxic gene is expressed. Within various embodiments, the tissue-specific promoter is the PEPCK promoter, HER2/neu promoter, casein promoter, IgG promoter, Chorionic Embryonic Antigen promoter, elastase promoter, porphobilinogen deaminase promoter, insulin promoter, growth hormone factor promoter, tyrosine hydroxylase promoter, albumin promoter, alphafetoprotein promoter, acetyl-choline receptor promoter, alcohol dehydrogenase promoter, α or β globin promoter, T-cell receptor promoter (including the CD2 LCR), the osteocalcin promoter the IL-2 promoter, IL-2 receptor promoter, whey (wap) promoter, and the MHC Class II promoter.

Within yet another embodiment of the present invention, gene delivery vehicles are provided comprising a cytotoxic gene under the transcriptional control of both an event-specific promoter and a tissue-specific promoter, such that the cytotoxic gene is maximally expressed only upon activation of both the event-specific promoter and the tissue-specific promoter. Representative event-specific and tissue-specific promoters have been discussed above. Within one preferred embodiment, the event-specific promoter is thymidine kinase, and the tissue-specific promoter is selected from the group consisting of the casein promoter and the HER2/neu promoter.

Within other embodiments of the present invention, the gene delivery vehicles described herein may also direct the expression of additional non-vector derived genes (*i.e.*, a heterologous nucleic acid sequence). Within one embodiment the heterologous nucleic acid sequence encodes a protein, such as an immune accessory molecule. Representative examples of immune accessory molecules include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, B7, B7-2, GM-CSF, CD3, ICAM-1, ICAM-2, β-microglobulin, LFA-3, HLA Class I, and HLA Class II molecules. Within one preferred embodiment, the protein is gamma-interferon.

Within other embodiments, the gene delivery vehicle may also direct the expression of an antisense sequence or ribozyme. Within further embodiments, the gene delivery vehicle may direct the expression of a replacement gene such as Factor VIII, glucocerebrosidase, FIX, ADA, HPRT, CFTCR or the LDL Receptor. Within yet other embodiments, the gene delivery vehicle may direct the expression of a disease

associated antigen, such as an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV and HIV.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of KT-1.

Figure 2 is a schematic illustration of KS2+Eco571-LTR(+).

Figure 3 is a schematic illustration of BA5.

Figure 4 is a schematic illustration of pBa6B-L1.

Figures 5A and 5B depict a sequence of human beta galactosidase (SEQ ID NOS: 20 and 21).

Figure 6 is a schematic illustration of pKT/βGal.

Figures 7A and 7B depict a sequence of human placental alkaline phosphatase (SEQ ID NOS: 22 and 23).

Figure 8 is a schematic illustration of pMGA/PLAP.

Figure 9 is a sequence of human cytochrome P-450 2B (CYP2B) (SEQ ID NOS: 24 and 25).

Figure 10 is a schematic illustration of pBA6B/CYP2A.

Figures 11A and 11B depict a sequence of human furin cDNA (SEQ ID NOS: 26 and 27).

Figure 12 is a schematic illustration of pBA6B/Xfur.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Gene delivery vehicle" refers to a construct which is capable of delivering, and, within preferred embodiments expressing, one or more gene(s) or sequence(s) of interest in a host cell. Representative examples of such vehicles include viral vectors, nucleic acid expression vectors in combination with facilatating agents such as liposomes or polycation condensing agents, naked DNA, and certain eukaryotic cells (*e.g.*, producer cells). Within particularly preferred embodiments of the invention, the gene delivery vehicle includes a member of the high affinity binding pair (discussed below), either expressed on, or included as, an integral part of the exterior of the gene delivery vehicle.

"Retroviral vector construct" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest. Preferably, the retroviral vector construct should include a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (*e.g.*, cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement protein), or which are useful as a molecule itself (*e.g.*, as a ribozyme or antisense sequence). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence, which is of a size sufficient to allow production of viral particles containing the RNA genome. Preferably, the heterologous sequence is at least 1, 2, 3, 4, 5, 6, 7 or 8 kB in length.

The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally, the retroviral vector construct may also include non-immunogenic selectable markers such as described in this application, as well as one or more specific restriction sites and a translation termination sequence.

"Nucleic Acid Expression Vector" refers to an assembly which is capable of directing the expression of a sequence or gene of interest. The nucleic acid

expression vector must include a promoter which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest, as well as a polyadenylation sequence.

Within certain embodiments of the invention, the nucleic acid expression vectors described herein may be contained within a plasmid construct. In addition to the components of the nucleic acid expression vector, the plasmid construct may also include a bacterial origin of replication, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication), a multiple cloning site, and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication).

"Non-immunogenic" refers to a selectable marker or prodrug activating enzyme that does not cause an unwanted immune reaction in the majority of patients when it is administered as part of a gene delivery vehicle. Such genes may be human genes, non-human genes, or, mutated human genes that lack sufficient difference from normal human genes (normally less than 10% amino acid sequence difference), may be genes that although not of human origin do not carry epitopes that allow effective presentation of the protein sequence through MHC class I or class 2 presentation in patients, or may be genes that carry sequences that prevent the effective presentation of otherwise immunogenic epitopes. It is important to note that at least some non-immunogenic selectable markers will be species specific. In general, for clinical use, non-immunogenic markers will preferably be of human origin.

"Selectable marker" refers to genes that are included in a gene delivery vehicle and that have no therapeutic acitvity, but rather is included to allow for simpler preparation, manufacturing, characterization or testing of the gene delivery vehicle.

As noted above, the present invention provides compositions and methods for delivering a gene delivery vehicle to a warm-blooded animal. Within one aspect, such methods comprise the step of administering to a warm-blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic selectable marker. Within other aspects, methods are provided for delivering a gene delivery vehicle to a warm-blooded animal, comprising the step of administering to a warm-

blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic molecule which is capable of activating an otherwise inactive compound into an active compound (*i.e.*, a "prodrug activating enzyme" or "PAE"). As discussed in more detail below, a wide variety of non-immunogenic selectable markers / prodrug activating enzymes may be utilized within the context of the present invention.

A. NON-IMMUNOGENIC MARKERS / PRODRUG ACTIVATING ENZYMES

A wide variety of non-immunogenic markers and/or prodrug activating enzymes may be expressed by the gene delivery vehicles of the present invention. Briefly, the markers and PAE of the present invention may be readily tested for immunogenicity by a variety of assays, including for example, CTL assays for antigens to which the organism has previously generated immunity, and *in vitro* generation of T-cell response utilizing dendritic cells transduced with the antigen for antigens to which the organism does not have a previously existing response (see Henderson et al., *Canc. Res.* *56*:3763-3770,1996; Hsu et al., *Nat. Med.* *2*:52-58,1995; CTL assays can be conducted as described in WO 91/02805). Another method for ensuring that an antigen is non-immunogenic is to administer the antigen in a standard skin test such as one utilized to test allergic reactions. It should be noted however, that while the above tests may be utilized in order to ascertain markers or PAE which are non-immunogenic within the context of the present invention (*i.e.*, do not produce statistically significant results), that some small percentage of patients may nevertheless react against the markers or PAE described herein.

Markers and PAEs of the present invention may be obtained from a variety of sources. For example, the marker or PAE may be, in its native state, a human enzyme, and thus, by its very nature be non-immunogenic. Similarly, markers or PAE closesly related species such as macaques may likewise be non-immunogenic. Within further embodiments of the invention, the marker or PAE may be of non-human origin, and can be made non-imunogenic by mutation (*e.g.*, substition, deletion or insertion). Representative examples of such PAE's and associated prodrug molecules include Alkaline phosphatase and various toxic phosphorylated compounds such as

phenolmustard phosphate, doxorubicin phosphate, mitomycin phosphate and etoposide phosphate; α-Galactosidase and N-[4-(α-D-galactopyranosyl) Benyloxycarbonyl]-daunorubicin; Azoreductase and azobenzene mustards; β-glucosidase and amygdalin; β-glucuronidase and phenolmustard-glucuronide and epirubicin-glucuronide; Carboxypeptidase A and methotrexate-alanine; Cytochrome P450 and cyclophosphamide or ifosfamide; DT diaphorase and 5-(Aziridine-1-yl)-2,4,dinitrobenzamide (CB1954) (Cobb et al., *Biochem. Pharmacol 18*:1519-1527, 1969; Knox et al., *Cancer Metastasis Rev. 12*:195-212, 1993; γ-glutamyl transferase and γ-glutamyl p-phenylenediamine mustard; Nitroreductase and CB1954 or derivatives of 4-Nitrobenzyloxycarbonyl; glucose oxidase and glucose; xanthine oxidase and hypoxanthine; and plasmin and peptidyl-p-phenylenediamine-mustard. Non-immunogenic markers or PAE's may also be made by expressing an enzyme in a compartment of the cell where it is not normally expressed. For example, the enzyme furin, normally expressed in the trans Golgi, can be made to express on the cell surface. There it can activate drugs than normally may not reach the trans-Golgi. In order to further a more complete understanding of such selectable markers and/or prodrugs, certain of these markers or prodrugs are discussed in more detail below.

1. *Use of Human Deoxycytidine Kinase and Human Equilibrative Nucleoside Transporter as Novel Prodrugs for Tumor Therapy*

Deoxycytidine kinase (dCK) is responsible for phosphorylation of several deoxynucleosides and their analogs. dCK has a broad substrate specificity for deoxycytidine, deoxyadenosine and deoxyguanosine and is important in the maintenance of normal dNTP pools. dCK also can phosphorylate a number of anti-tumor and anti-viral nucleoside analogs, including cytosine arabinoside (ara-C) and ddC. T-cells have relatively high levels of dCK activity, although in most other cell types the enzyme is found at low levels and is relatively unstable. The phosphorylation of deoxyadenosine and deoxyguanosine by dCK is the first step in the synthesis of dATP and dGTP which are utilized in DNA synthesis. The human deoxycytidine kinase mRNA contains an open reading frame of 780 nt and encodes a polypeptide with

a predicted size of 30.5 kD. The cDNA was first cloned by Chottiner et al., *PNAS* *88*:1531-1535, 1991.

2. *Cytosine arabinoside (ara-C)*

Ara-C is the prototype nucleoside chemotherapeutic drug and differs from its physiologic counterpart, deoxycytidine, by the presence of an additional -OH group at the 2' position. Ara-C is the most effective agent in the treatment of acute myeloid leukemia. As a single agent, ara-C induces remission in 50% of patients with acute myeloblastic leukemia (AML). Ara-C is also used in blast crisis of chronic granulocytic leukemia (CGL), acute lymphocytic leukemia (ALL) and non-Hodgkins lymphoma. Ara-C incorporates into replicating DNA and terminates DNA strand elongation in dividing cells. Because of its selectivity for rapidly growing tumors and its propensity for deamination by cytosine deaminase, ara-C has not been effective for the treatment of solid tumors.

Ara-C enters cells via the equilbrative nucleoside transporter (hENT). Once in the cell, ara-C can undergo deamination to ara-U or serve as a substrate for salvage pathway enzymes to generate ara-CTP. Ara-CTP competes with dCTP and inhibits DNA polymerase. Intracellular metabolism of ara-C results in three sequential phosphorylation reactions. The first is mediated by dCK to form ara-CMP. dCMP kinase results in the formation of ara-CDP which is phosphorylated by nucleoside diphosphate kinase to generate ara-CTP. There are two limiting steps in the generation of ara-CTP from ara-C: the initial intracellular transport of ara-C by the membrane bound transporter (hENT) and intracellularly, the balance between deamidation by cytidine deaminase versus the initial phosphorylation event by deoxycytidine kinase. The intracellular generation of the toxic ara-CTP metabolite can be enhanced by either expression of the recently cloned hENT1 (Griffiths et al., *Nature Medicine* *3*:89-93, 1997) transporter or increased expression of dCK. It is believed that dCK expression is the rate limiting step in ara-CTP formation intracellularly (Manome et al., *Nature Medicine* *2*:567-573, 1996). The level of cell surface expression of hENT1 imposes a rate limiting transport step on the accumulation of the toxic ara-CTP at drug

concentrations that are used clinically (Wiley et al., *J. Clin. Invest.* 75:632-642, 1985). hENT1 is highly expressed in acute myeloid leukemia whereas normal leukocytes express low levels of hENT1. Co-expression of both of these molecules should have synergistic effects, especially in solid tumors where augmented tumor cell killing mediated by the so-called "bystander" effect will occur. Increased co-expression of hENT1 and dCK in tumor cells will allow therapeutic doses of ara-C to be reduced thereby reducing toxic side effects. Dose limiting toxicities include severe myelosuppression and gastrointestinal epithelial injury.

Because of the high levels of cytidine deaminase in the gastrointestinal epithelium and first pass elimination in the liver, ara-C is not given orally. However, when administered by IV infusion, the drug distributes rapidly in total body water and concentrations in the CSF reach 50% of that in plasma after 2 hours of continuous infusion. This latter feature of penetrating the blood brain barrier as well as relative lack of toxicity against post-mitotic cells makes ara-C an attractive candidate for the treatment of CNS tumors. Currently, ara-C is not widely used against solid tumors, however, potentiation of action of the drug will occur in cells that express augmented levels of dCK and hENT1. Plasma half life of ara-C is less than 20 minutes due to the rapid deamination reaction. Deamination is minimal in the CSF and ara-C is currently used intrathecally for treatment of meningeal leukemia.

3. *Cyclophosphamide*

Cyclophosphamide and its isomer ifosfamide are cell cycle-nonspecific alkylating agents that undergo bioactivation catalyzed by liver cytochrome P-450 enzymes. The therapeutic efficacy of these oxazaphosphorine anticancer drugs is limited by host toxicity resulting from the systemic distribution of activated drug metabolites formed in the liver (see, *e.g.*, Chen and Waxman, *Canc. Res.* 55:581, 1994).

4. *Cytochrome P-450*

Biotransformation involves the metabolism of xenobiotics (pharmaceuticals, plant-derived chemicals, environmental pollutants, pesticides and herbicides) and occurs in the liver, where the xenobiotics are rendered inactive and water soluble prior to elimination. Two series of reactions occur: Phase I reactions result in the addition of a chemical group that can be further modified by the Phase II reaction involving hydrolysis or conjugation. The Phase I reactions are carried out by a group of enzymes called the cytochromes P-450 which are all endoplasmic reticulum integral membrane monooxygenases. The cytochrome P-450 enzymes interact with organic substrates (xenobiotics) resulting in the oxidation of the substrate and generation of water. NADPH is used as the electron donor and catalyzes the reaction. A cytochrome P-450 reductase catalyzes the reduction of the CYC P-450 monooxygenases. The CYC P-450 is a multigene superfamily whereas the reductase is the product of a single gene that interacts with all the CYC P-450s. The Phase II conjugation reactions are important in the detoxification of reactive compounds such as carcinogens. Normally, these reactive compounds are conjugated resulting in: glucuronidation; sulfation; methylation or glutathione conjugation or amino-acid conjugation.

In order to further describe certain preferred embodiments of the invention the cloning of an active human CYC P-450 gene into a retroviral vector is described below within the examples. Briefly, the subcellular localization of the CYC P-450 proteins is re-targeted to allow expression of the protein either extracellularly or bound to the inner surface of the plasma membrane. Xenobiotic compounds, including anti-cancer agents may undergo the Phase I oxidation reactions, however, they are not subjected to the Phase II detoxification conjugation reactions, thereby rendering the anti-cancer agents as active toxic metabolites. Because of the membrane permeability of these reactants, they may diffuse from cell to cell, resulting in a bystander effect.

B. GENE DELIVERY VEHICLES

The non-immunogenic markers / PAE of the present invention may be utilized in a wide variety of gene delivery vehicles. As discussed in more detail below, the gene delivery vehicle may be of either viral or non-viral origin (See generally, Jolly, *Cancer Gene Therapy 1* (1994) 51-64; Kimura, *Human Gene Therapy 5* (1994) 845-852, Connelly, *Human Gene Therapy 6* (1995) 185-193 and Kaplitt, *Nature Genetics 6* (1994) 148-153).

1. *Construction of retroviral gene delivery vehicles*

Within one aspect of the present invention, retroviral vector constructs are provided which are constructed to carry or express a non-immunogenic selectable marker and/or PAE. Numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those described in GB 2200651; EP 0415731; EP 0345242; WO 89/02468; WO 89/05349; WO 89/09271; WO 90/02806; WO 90/07936; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; WO 93/11230; WO 93/10218; WO 91/02805; in U.S. 5,219,740; U.S. 4,405,712; U.S. 4,861,719; U.S. 4,980,289 and U.S. 4,777,127; in U.S. Serial No. 07/800,921 and provisional application 60/053066, filed July 18, 1997; and in Vile, *Cancer Res 53*:3860-3864, 1993; Vile, *Cancer Res 53*:962-967, 1993; Ram, *Cancer Res 53*:83-88, 1993; Takamiya, *J Neurosci Res 33*:493-503, 1992; Baba, *J Neurosurg 79*:729-735, 1993; Mann, *Cell 33*:153, 1983; Cane, *Proc Natl Acad Sci 81*;6349, 1984; and Miller, *Human Gene Therapy 1*, 1990.

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include those

described in RNA Tumor Viruses: Molecular Biology of tumor viruses, Second Edition, Cold Spring Harbor Laboratory, 1985 at pages 2-7, as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB_{9-1} (see O'Neill et al., *J. Vir. 53*:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (see Kelly et al., *J. Vir. 45*(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Gibbon Ape Leukemia Virus, Feline Leukemia Virus, Reticuloendotheliosis virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol. 19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), HIV, HIV-1, HIV-2, SIV, FIV, and Schmidt-Ruppin (*e.g.*, ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retroviral vector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous

Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within one aspect of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks gag/pol or env coding sequences. Representative examples of such vector constructs are described within PCT application Nos. US 95/05789 and US 97/07697.

Packaging cell lines suitable for use with the above described retroviral vector constructs may be readily prepared (see U.S. Application No. 08/240,030 and U.S. Application No. 07/800,921; as well as PCT application Nos. US 95/05789 and US 97/07697), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Within preferred embodiments, transduced packaging cell lines can be selected utilizing a number of titering methods, including PCR titering (*see, e.g.*, Example 5A), or by staining of transduced cells for an appropriate transferred marker (*e.g.*, Fast Red staining as described in Example 5B). Within further embodiments, recombinant vector particles which are made utilizing the above-identified techniques are free of detectable replication competent retrovirus.

2. *Alphavirus gene delivery vehicles*

The present invention also provides a variety of alphavirus-based vectors which can function as gene delivery vehicles. Such vectors can be constructed from a wide variety of alphaviruses, including for example, Sindbis viruses vectors, Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532).

As an example, the Sindbis virus, which is the prototype member of the alphavirus genus of the togavirus family is an unsegmented genomic RNA (49S RNA) of virus of approximately 11,703 nucleotides in length. This virus contains a 5' cap and

a 3' poly-adenylated tail, and displays positive polarity. Infectious enveloped Sindbis virus is produced by assembly of the viral nucleocapsid proteins onto the viral genomic RNA in the cytoplasm and budding through the cell membrane embedded with viral encoded glycoproteins. Entry of virus into cells is by endocytosis through clatharin coated pits, fusion of the viral membrane with the endosome, release of the nucleocapsid, and uncoating of the viral genome. During viral replication the genomic 49S RNA serves as template for synthesis of the complementary negative strand. This negative strand in turn serves as template for genomic RNA and an internally initiated 26S subgenomic RNA. The Sindbis viral nonstructural proteins are translated from the genomic RNA while structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as a polyprotein and processed into individual proteins by post translational proteolytic cleavage. The packaging sequence resides within the nonstructural coding region, therefore only the genomic 49S RNA is packaged into virions.

A variety of different alphavirus vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those described in U.S.Patent application Nos. 08/198,450, 08/405,627 and 08/679,640, U.S. patent Nos 5,091,309; 5,217,879 and 5,185440, PCT patent application publication numbers WO 92/10578, WO/94/21792, WO 95/27069, WO 95/27044 and WO 95/07994, and PCT application No. US 97/06010.

Particularly preferred alphavirus vectors for use within the present invention include those which are described within U.S. Application No. 08/198,450. Briefly, within one embodiment, alphavirus vector constructs are provided comprising a 5' sequence which is capable of initiating *in vitro* transcription of a alphavirus RNA, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which is active, modified to reduce viral transcription ofthe subgenomic fragment, or inactivated such that viral transcription of the subgenomic fragment is prevented, and a alphavirus RNA polymerase recognition sequence.

In still further embodiments, the vector constructs described above contain no alphavirus structural proteins in the vector constructs. The selected

heterologous sequence may be located downstream from the viral junction region; in the vector constructs having a second viral junction, the selected heterologous sequence may be located downstream from the second viral junction region, where the heterologous sequence is located downstream, the vector construct may comprise a polylinker located between the viral junction region and said heterologous sequence, and preferably the polylinker does not contain a wild-type Sindbis virus restriction endonuclease recognition sequence.

3. *Other viral gene delivery vehicles*

In addition to retroviral vectors and alphavirus-based vectors, numerous other viral vectors systems may also be utilized as a gene delivery vehicle. For example, within one embodiment of the invention adenoviral vectors may be employed as a gene delivery vehicle. Representative examples of such vectors include those described by, for example, Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; WO 93/9191; Kolls et al., *PNAS 91*(1):215-219, 1994; Kass-Eisler et al., *PNAS 90*(24):11498-502, 1993; Guzman et al., *Circulation 88*(6):2838-48, 1993; Guzman et al., *Cir. Res. 73*(6):1202-1207, 1993; Zabner et al., *Cell 75*(2):207-216, 1993; Li et al., *Hum. Gene Ther. 4*(4):403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*(10):1287-1291, 1993; Vincent et al., *Nat. Genet. 5*(2):130-134, 1993; Jaffe et al., *Nat. Genet. 1*(5):372-378, 1992; and Levrero et al., *Gene 101*(2):195-202, 1991; and WO 93/07283; WO 93/06223; and WO 93/07282. Exemplary known adenoviral gene therapy vectors employable in this invention include those described in the above referenced documents and in WO 94/12649, WO 93/03769, WO 93/19191, WO 94/28938, WO 95/11984, WO 95/00655, WO 95/27071, WO 95/29993, WO 95/34671, WO 96/05320, WO 94/08026, WO 94/11506, WO 93/06223, WO 94/24299, WO 95/14102, WO 95/24297, WO 95/02697, WO 94/28152, WO 94/24299, WO 95/09241, WO 95/25807, WO 95/05835, WO 94/18922 and WO 95/09654. Alternatively, administration of DNA linked to killed adenovirus as described in Curiel, *Hum. Gene Ther. 3*:147-154, 1992 may be employed.

Gene delivery vehicles of the present invention also include parvovirus such as adenovirus associated virus (AAV) vectors. Representative examples of such vectors include the AAV vectors disclosed by Srivastava in WO 93/09239, Samulski et al., *J. Vir. 63*:3822-3828, 1989; Mendelson et al., *Virol. 166*:154-165, 1988; Flotte et al., *PNAS 90*(22):10613-10617, 1993. Particularly preferred AAV vectors comprise the two AAV inverted terminal repeats in which the native D-sequences are modified by substitution of nucleotides, such that at least 5 native nucleotides and up to 18 native nucleotides, preferably at least 10 native nucleotides up to 18 native nucleotides, most preferably 10 native nucleotides are retained and the remaining nucleotides of the D-sequence are deleted or replaced with non-native nucleotides. The native D-sequences of the AAV inverted terminal repeats are sequences of 20 consecutive nucleotides in each AAV inverted terminal repeat (*i.e.*, there is one sequence at each end) which are not involved in HP formation. The non-native replacement nucleotide may be any nucleotide other than the nucleotide found in the native D-sequence in the same position. Other employable exemplary AAV vectors are pWP-19, pWN-1, both of which are disclosed in Nahreini, *Gene 124*:257-262, 1993. Another example of such an AAV vector is psub201. See Samulski, *J. Virol. 61*:3096, 1987. Another exemplary AAV vector is the Double-D ITR vector. How to make the Double D ITR vector is disclosed in U.S. Patent No. 5,478,745. Still other vectors are those disclosed in Carter, U.S. Patent No. 4,797,368 and Muzyczka, U.S. Patent No. 5,139,941; Chartejee, U.S. Patent No. 5,474,935; and Kotin, PCT Patent Publication WO 94/288157. Yet a further example of an AAV vector employable in this invention is SSV9AFABTKneo, which contains the AFP enhancer and albumin promoter and directs expression predonimantly in the liver. Its structure and how to make it are disclosed in Su, *Human Gene Therapy 7*:463-470, 1996. Additional AAV gene therapy vectors are described in U.S. 5,354,678; U.S. 5,173,414; U.S. 5,139,941; and US 5,252,479.

Gene delivery vehicles of the present invention also include herpes vectors. Representative examples of such vectors include those disclosed by Kit in *Adv. Exp. Med. Biol. 215*:219-236, 1989; and those disclosed in U.S. Patent No. 5,288,641 and EP 0176170 (Roizman). Additional exemplary herpes simplex virus vectors

include HFEM/ICP6-LacZ disclosed in WO 95/04139 (Wistar Institute), pHSVlac described in Geller, *Science 241*:1667-1669, 1988 and in WO 90/09441 and WO 92/07945; HSV Us3::pgC-lacZ described in Fink, *Human Gene Therapy 3*:11-19, 1992; and HSV 7134, 2 RH 105 and GAL4 described in EP 0453242 (Breakefield), and those deposited with the ATCC as accession numbers ATCC VR-977 and ATCC VR-260.

Gene delivery vehicles may also be generated from a wide variety of other viruses, including for example, poliovirus (Evans et al., *Nature 339*:385-388, 1989; and Sabin, *J. Biol. Standardization 1*:115-118, 1973); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *N. Eng. J. Med. 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); SV40; HIV (Poznansky, *J. Virol. 65*:532-536, 1991); measles (EP 0 440,219); astrovirus (Munroe, S.S. et al., *J. Vir. 67*:3611-3614, 1993); and coronavirus, as well as other viral systems (*e.g.*, EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic(defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910, 1988), and nevertheless induce cellular immune responses, including CTL.

4. *Non-viral gene delivery vehicles*

In addition to the above viral-based vectors, numerous non-viral gene delivery vehicles may likewise be utilized within the context of the present invention. For example, within one embodiment of the invention the gene delivery vehicles is a eukarytic layered expression systems (see WO 95/07994 for a detailed description of eukaryotic layered expression systems).

Other gene delivery vehicles and methods that may be employed such as, for example, nucleic acid expression vectors; polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example see U.S. Serial No. 08/366,787, filed

December 30, 1994 and Curiel, *Hum Gene Ther 3*:147-154, 1992; ligand linked DNA, for example see Wu, *J Biol Chem 264*:16985-16987, 1989; eucaryotic cell delivery vehicles cells, for example see U.S. Serial No.08/240,030, filed May 9, 1994, and U.S. Serial No. 08/404,796; deposition of photopolymerized hydrogel materials; hand-held gene transfer particle gun, as described in US Patent No.5,149,655; ionizing radiation as described in U.S. 5,206,152 and in WO 92/11033; nucleic charge neutralization or fusion with cell membranes. Additional approaches are described in Philip, *Mol Cell Biol 14*:2411-2418, 1994 and in Woffendin, *Proc Natl Acad Sci 91*:1581-1585, 1994.

Particle mediated gene transfer may be employed, for example see U.S. Serial No. 60/023,867. Briefly, the sequence of interest can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu and Wu, *J. Biol. Chem. 262*:4429-4432, 1987, insulin as described in Hucked, *Biochem Pharmacol 40*:253-263, 1990, galactose as described in Plank, *Bioconjugate Chem 3*:533-539, 1992 lactose or transferrin.

Naked DNA may also be employed. Exemplary naked DNA introduction methods are described in WO 90/11092 and U.S. 5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated latex beads are efficiently transported into cells after endocytosis initiation by the beads. The method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

Liposomes that can act as gene delivery vehicles are described in U.S. 5,422,120, WO 95/13796, WO 94/23697, WO 91/144445 and EP 524,968. As described in U.S. Serial No. 60/023,867, nucleic acid sequences can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell

targeting ligands such as asialoorosomucoid, insulin, galactose, lactose, or transferrin. Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters. Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in Woffendin *et al., Proc. Natl. Acad. Sci. USA* *91*(24):11581-11585, 1994. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand-held gene transfer particle gun, as described in U.S. 5,149,655; use of ionizing radiation for activating transferred gene, as described in U.S. 5,206,152 and WO 92/11033

Exemplary liposome and polycationic gene delivery vehicles are those described in U.S. 5,422,120 and 4,762,915, in WO 95/13796, WO 94/23697, and WO 91/14445, in EP 0524968 and in Starrier, Biochemistry, pages 236-240 (1975) W.H. Freeman, San Francisco; Shokai, *Biochem Biophys Acct 600*:1, 1980; Bayer, *Biochem Biophys Acct 550*:464, 1979; Rivet, *Meth Enzyme 149*:119, 1987; Wang, *Proc Natl Acad Sci 84*:7851, 1987; Plant, *Anal Biochem 176*:420, 1989.

D. ADEPT

Within other aspects of the present invention, the prodrugs described herein may be linked to an antibody, and utilized for antibody-directed enzyme prodrug therapy essentially as described in WO 95/13095.

E. HETEROLOGOUS SEQUENCES

Any of the gene delivery vehicles described above may include, contain (and/or express) one or more heterologous sequences, as well as the non-immunogenic selectable marker or PAE. A wide variety of heterologous sequences may be utilized within the context of the present invention, including for example, cytotoxic genes, disease-associated antigens, antisense sequences, sequences which encode gene products corresponding to genetic deficiencies that need to be expressed over a long

period of time (greater than 2-4 weeks), sequences which encode immunogenic portions of disease-associated antigens and sequences which encode immune accessory molecules. Representative examples of cytotoxic genes include the genes which encode proteins such as ricin (Lamb et al., *Eur. J. Biochem. 148*:265-270, 1985), diphtheria toxin (Tweten et al., *J. Biol. Chem. 260*:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature 306*:551-557, 1983; Sanchez & Holmgren, *PNAS 86*:481-485, 1989), and Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem. 262*:8707-8711, 1987).

Within further embodiments of the invention, antisense RNA may be utilized as a cytotoxic gene in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences may be utilized to induce the increased expression of interferons (including gamma-interferon), due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

Within further aspects of the present invention, gene delivery vehicles of the present invention may also direct the expression of one or more sequences which encode immunogenic portions of disease-associated antigens. As utilized within the context of the present invention, antigens are deemed to be "disease-associated" if they are either associated with rendering a cell (or organism) diseased, or are associated with the disease-state in general but are not required or essential for rendering the cell diseased. In addition, antigens are considered to be "immunogenic" if they are capable, under appropriate conditions, of causing an immune response (either cell-mediated or humoral). Immunogenic "portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

A wide variety of "disease-associated" antigens are contemplated within the scope of the present invention, including for example immunogenic, non-tumorigenic forms of altered cellular components which are normally associated with tumor cells (see U.S. Application No. 08/104,424). Representative examples of altered

cellular components which are normally associated with tumor cells include ras* (wherein "*" is understood to refer to antigens which have been altered to be non-tumorigenic), p53*, and Rb*.

"Disease-associated" antigens should also be understood to include all or portions of various eukaryotic (including for example, parasites), prokaryotic (*e.g.*, bacterial) or viral pathogens. Representative examples of viral pathogens include the Hepatitis B Virus ("HBV") and Hepatitis C Virus ("HCV;" see U.S. Application No. 08/102/132), Human Papiloma Virus ("HPV;" see WO 92/05248; WO 90/10459; EPO 133,123), Epstein-Barr Virus ("EBV;" see EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus ("FeLV;" see U.S. Application No. 07/948,358; EPO 377,842; WO 90/08832; WO 93/09238), Feline Immunodeficiency Virus ("FIV;" U.S. Patent No. 5,037,753; WO 92/15684; WO 90/13573; and JP 4,126,085), HTLV I and II, and Human Immunodeficiency Virus ("HIV;" see U.S. Application No. 07/965,084).

Within other aspects of the present invention, the gene delivery vehicles described above may also direct the expression of one or more immune accessory molecules. As utilized herein, the phrase "immune accessory molecules" refers to molecules which can either increase or decrease the recognition, presentation or activation of an immune response (either cell-mediated or humoral). Representative examples of immune accessory molecules include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7 (U.S. Patent No. 4,965,195), IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf et al., *J. Immun. 46*:3074, 1991; Gubler et al., *PNAS 88*:4143, 1991; WO 90/05147; EPO 433,827), IL-13 (WO 94/04680), IL-15 or ETF, GM-CSF, M-CSF-1, G-CSF, CD3 (Krissanen et al., *Immunogenetics 26*:258-266, 1987), CD8, ICAM-1 (Simmons et al., *Nature 331*:624-627, 1988), ICAM-2 (Singer, *Science 255*: 1671, 1992), β-microglobulin (Parnes et al., *PNAS 78*:2253-2 al., *Nature 338*: 521, 1989), LFA3 (Wallner et al., *J. Exp. Med. 166*(4):923-932, 1987), HLA Class I, HLA Class II molecules B7 (Freeman et al., *J. Immun. 143*:2714, 1989), and B7-2. Within a preferred embodiment, the heterologous gene encodes gamma-interferon.

Within preferred aspects of the present invention, the gene delivery vehicles described herein may direct the expression of more than one heterologous sequence. Such multiple sequences may be controlled either by a single promoter, or preferably, by additional secondary promoters (*e.g.*, Internal Ribosome Binding Sites or "IRBS"). Within preferred embodiments of the invention, a gene delivery vehicle directs the expression of heterologous sequences which act synergistically. For example, within one embodiment retroviral vector constructs are provided which direct the expression of a molecule such as IL-12, IL-2, gamma interferon, or other molecule which acts to increase cell-mediated presentation in the T_H1 pathway, along with an immunogenic portion of a disease-associated antigen. In such embodiments, immune presentation and processing of the disease-associated antigen will be increased due to the presence of the immune accessory molecule.

Within other aspects of the invention, gene delivery vehicles are provided which direct the expression of one or more heterologous sequences which encode "replacement" genes. As utilized herein, it should be understood that the term "replacement genes" refers to a nucleic acid molecule which encodes a therapeutic protein that is capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect. Representative examples of such genetic defects include disorders in metabolism, immune regulation, hormonal regulation, and enzymatic or membrane associated structural function. Representative examples of diseases caused by such defects include Cystic Fibrosis (due to a defect in the Cystic Fibrosis Transmembrane Conductance Regulator ("CFTCR"), see Dorin et al., *Nature 326*:614, Parkinson's Disease, Adenosine Deaminase deficiency ("ADA;" Hahma et al., *J. Bact. 173*:3663-3672, 1991), β-globin disorders, Hemophilia A & B (Factor VIII-deficiencies, see Wood et al., *Nature 312*:330, 1984), Factor IX deficiencies, Gaucher disease, diabetes, forms of gouty arthritis and Lesch-Nylan disease (due to "HPRT" deficiencies; see Jolly et al., *PNAS 80*:477-481, 1983) Duchennes Muscular Dystrophy and Familial Hypercholesterolemia (LDL Receptor mutations; see Yamamoto et al., *Cell 39*:27-38, 1984) and Gaucher's Syndrome.

Sequences which encode the above-described heterologous genes may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode immune accessory molecules may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative sources sequences which encode the above-noted immune accessory molecules include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contains a sequence which encodes Interleukin-1), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6). It will be evident to one of skill in the art that one may utilize either the entire sequence of the protein, or an appropriate portion thereof which encodes the biologically active portion of the protein.

Alternatively, known cDNA sequences which encode cytotoxic genes or other heterologous genes may be obtained from cells which express or contain such sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see* U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159. See also PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989 all of which are incorporated by reference herein in their entirety) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq

polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described genes may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, California)).

F. COMPOSITIONS

Within other aspects of the present invention, any of the above gene delivery vehicles are provided in combination with a pharmaceutically acceptable carrier or diluent. Such pharmaceutical compositions may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or nasal, oral, vaginal, sub-lingual, inhalant or rectal administration.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a retroviral vector construct or recombinant viral particle in 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant vector represents approximately 1 mg of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate cell division, and hence, uptake and incorporation of a gene delivery vehicle. Representative examples include Melanocyte Stimulating Hormone (MSH), for melanomas or epidermal growth factor for breast or

other epithelial carcinomas. In addition, pharmaceutical compositions of the present invention may be placed within containers or kits (*e.g.*, one container for the coupled targeting element, and a second container for the coupled gene delivery vehicle), along with packaging material which provides instructions regarding the use of such pharmaceutical compositions. Generally, such instructions will include a tangible expression describing the reagent concentration, as well as within certain embodiments, relative amounts of excipient ingredients or diluents (*e.g.*, water, saline or PBS) which may be necessary to reconstitute the pharmaceutical compositions.

Particularly preferred methods and compositions for preserving certain of the gene delivery vehicles provided herein, such as recombinant viruses, are described in U.S. applications entitled "Methods for Preserving Recombinant Viruses" (U.S. Application No. 08/135,938, filed October 12, 1993, and U.S. Application No. 08/152,342, filed November 15, 1993, which are incorporated herein by reference in their entirety).

G. METHODS OF TREATMENT / ADMINISTRATION

As noted above, the present invention provides compositions and methods for delivering a gene delivery vehicles to a warm-blooded animal. Within one aspect, such methods comprise the step of administering to a warm-blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic selectable marker. Within other aspects, methods are provided for delivering a gene delivery vehicle to a warm-blooded animal, comprising the step of administering to a warm-blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic molecule which is capable of activating an otherwise inactive compound into an active compound. As utilized herein, it should be understood that "administering" refers not only to direct adminstration of a gene delivery vehicle (*e.g.*, by direct injection or intravenous administration), but also to *ex vivo* routes wherein cells are removed from a donor, transduced or transfected with a gene delivery vehicle, and then introduced into the warm-blooded animal.

As discussed in more detail below, such methods may be utilized not only for delivering a desired heterologous sequence to cells, but for ablative therapy, as a fail-safe to lessen the risk of gene therapy, or for the transduction of cells which have been isolated from the body (*e.g.*, T cells, cancer cells, or, stem cells).

1. *Ablative therapy*

Prodrug activating genes can be used to ablate cancerous or hyperproliferative tissue such as in benign prostate hyperplasia, arthritic joints, smooth muscle proliferation in restinosis or immune cell proliferation in autoimmune disease. In any case where expression of the ablative gene is necessary for more than a few days (3-10), or it may be necessary to readminister the ablative gene it will be advantageous to use prodrug activating genes that do not elicit an immune response, in this case human genes or genes closely related to human genes (<10% difference in sequence).

2. *Fail-safe utility of prodrug activating genes*

Gene therapy has been proposed for many disease therapies including cancer, infectious diseases, autoimmune disease including graft versus host disease, cardiovascular disease, connective tissue disease, neurological disease, genetic disease and others. In all cases there is a least some risk involved in adding genes temporarily or permanently to the cells in a patient's body. One way to lessen that risk is to add a gene that is not itself toxic but the product of which can metabolize a prodrug into an active form that kills or inhibits the undesirable function or proliferation of the transduced cells. This approach can also be used to simply control cellular proliferation etc. of cells that have been manipulated (without gene therapy) and have the potential to be abnormal or cause pathology. However, if the period of activity of the transduced cells is larger than a few days or if repeat treatments are needed, the prodrug activating enzyme can cause an unwanted immune response that will destroy the cells that express them. This is not desirable in most cases. Therefore, the use of genes for the prodrug activating enzyme from human sources or from alternative sources that are very similar

(<10% different in amino acid sequence) will allow the timing of cell ablation to be controlled by the physician, not by the immune system.

3. *T cell transduction methodology*

T cell Transduction Allogeneic donor T cells are routinely used in allogeneic bone marrow (hemopoietic stem cells, HSC) transplants, mainly for lymphomas and leukemias. These T cells donate a immediate immune capability and cause increased cytokine production that aids engraftment, but they can also lead to graft versus host disease (GVHD), that currently kills about 1/3 of patients.

Retroviral vectors encoding prodrug activating enzymes are prepared as described in "Production and administration of High titer recombinant retroviruses" U.S. Application No. 08/367,671, or by other means known to those skilled in the art. T cells can be transduced as described in "High efficiency *ex vivo* transduction of cells by high titer recombinant retroviral preparations (U.S. Application No. 08/425,180). Other methods of growing and transducing T cells can be used and are known to those skilled in the art (*e.g.*, A.S. Chuck and B.O. Palsson, *Hum. Gene Ther. 7*:743, 1996; Heslop et al., *Nature Med. 2*:551, 1996; S.R. Riddell et al., *Nature Medicine 2*:216, 1996). T cells can also be transduced by methods used to grow and transduce T cells from HIV patients (*e.g.*, T. Vandenddriessche et al., *J. Virol. 69*:4045, 1995; L.Q. Sun et al., *PNAS 92*:7272, 1995).

Within various embodiments of the invention, the above-described gene delivery vehicles or pharmaceutical compositions may be administered *in vivo*, or *ex vivo*. Representative routes for *in vivo* administration include intradermally ("i.d."), intracranially ("i.c."), intraperitoneally ("i.p."), intrathecally ("i.t."), intravenously ("i.v."), subcutaneously ("s.c."), intramuscularly ("i.m.") or even directly into a tumor or the peri-tumoral area.

The above-described methods for sequential administration may be readily utilized for a variety of therapeutic (and prophylactic) treatments. For example, within one embodiment of the invention, the methods described above may be accomplished in order to inhibit or destroy a pathogenic agent in a warm-blooded

animal. Such pathogenic agents include not only foreign organisms such as parasites, bacteria, and viruses, but cells which are "foreign" to the host, such as cancer or tumor cells, or other cells which have been "altered." Within a preferred embodiment of the invention, the compositions described above may be utilized in order to directly treat pathogenic agents such as a tumor, for example, by direct injection into several different locations within the body of tumor. Alternatively, arteries which serve a tumor may be identified, and the compositions injected into such an artery, in order to deliver the compositions directly into the tumor. Within another embodiment, a tumor which has a necrotic center may be aspirated, and the compositions injected directly into the now empty center of the tumor. Within yet another embodiment, the above-described compositions may be directly administered to the surface of the tumor, for example, by application of a topical pharmaceutical composition containing the retroviral vector construct, or preferably, a recombinant retroviral particle.

Within other aspects of the present invention, methods are provided for generating an immune response against an immunogenic portion of an antigen, in order to prevent or treat a disease (see, *e.g.*, U.S. Application Nos. 08/104,424; 08/102,132, 07/948,358; 07/965,084), for suppressing graft rejection, (see U.S. Application No. 08/116,827), for suppressing an immune response (see U.S. Application No. 08/116,828), and for suppressing an autoimmune response (see U.S. Application No. 08/116,983), utilizing the above-described compositions.

In addition, although warm-blooded animals (*e.g.*, mammals or vertebrates such as humans, macaques, horses, cows, swine, sheep, dogs, cats, chickens, rats and mice) have been exemplified in the methods described above, such methods are also readily applicable to a variety of other animals, including, for example, fish.

4. *Long Term Expression*

Within certain embodiments of the invention, the gene delivery vehicles provided herein are administered in order to generate a sustained, long-term systemic expresssion of therapeutic genes expressed by the gene delivery vehicle. Preferably, long-term *in vivo* systemic expression is obtained by intravenous delivery methods or

other *in vivo* or *ex vivo* methods as is described in detail below. For long term expression from a retroviral vector *in vivo*, the action of human complement on the retroviral vector is suppressed. This can be done by a variety of techniques known to one of skill in the art. Preferably, human packaging cell lines are used in order to inhibit the action of human complement on the retroviral vector particles (see PCT publication number US 91/06852, entitled "Packaging Cells").

The terms "long term systemic expression" or "sustained systemic expression" as used herein in reference to *in vivo* expression of protein encoded by a gene delivery vehicle mean measurable or biologically active expression for 30 days, more preferably for 60 days, yet more preferably for 90 days, and still more preferably for six months after administration of the retroviral vector particle to a host. The term "measurable expression" as used herein in reference to *in vivo* expression of a protein encoded by a retroviral vector means that the protein is produced in sufficient amounts so as to be detectable in biological fluids such as serum or in cells such as stem cells, T-cells, and the like, by an assay specific for the expressed protein. The term "systemic expression" as used herein means that the proteins are expressed into the circulation and are thus useful for treatment of certain diseases. A variety of diseases discussed in detail below are amenable to treatment by this type of gene therapy.

For example, measurement of human growth hormone can be determined by an ELISA assay specific for human growth hormone protein. The term "biologically active expression" or "protein expression in biologically or therapeutically active amounts" when used herein in reference to *in vivo* expression of a protein encoded by a gene delivery vehicle vector means that protein is produced in sufficient amounts so as to be detectable in a functional assay. For example, expression of factor VIII can be measured in a clotting assay. Similarly other expressed proteins can be measured by specific assays for each particular protein that are known to those of skill in the art.

Long-term *in vivo* expression of a variety of proteins can be effected by the methods of the invention, preferably by *in vivo* administration of high titer retroviral vectors as described herein. A large variety of different proteins can be expressed for therapeutic applications in a number of different disease states. Preferred proteins

include, cytokines and immune system modulators, hormones, growth factors, vaccine antigens, and proteins for treatment of inherited diseases.

Genes encoding any of the cytokine and immunomodulatory proteins described herein can be expressed in a gene delivery vehicle to achieve long term *in vivo* expression. Other forms of these cytokines which are known to those of skill in the art can also be used. For instance, nucleic acid sequences encoding native IL-2 and gamma-interferon can be obtained as described in U.S. Patent Nos. 4,738,927 and 5,326,859, respectively, while useful muteins of these proteins can be obtained as described in U.S. Patent No. 4,853,332. As an additional example, nucleic acid sequences encoding the short and long forms of mCSF can be obtained as described in U.S. Patent Nos. 4, 847,201 and 4,879,227, respectively. Retroviral vectors expressing cytokine or immunomodulatory genes can be produced as described herein and in PCT publication number US 94/02951 entitled "Compositions and Methods for Cancer Immunotherapy".

Gene delivery vehicles producing a variety of known polypeptide hormones and growth factors can be used in the methods of the invention to produce therapeutic long-term expression of these proteins. A large variety of hormones, growth factors and other proteins which are useful for long term expression by the retroviral vectors of the invention are described, for instance, in EP publication number 0437478B1, entitled "Cyclodextrin-Peptide Complexes." Nucleic acid sequences encoding a variety of hormones can be used, including human growth hormone, insulin, calcitonin, prolactin, follicle stimulating hormone, leutinizing hormone, human chorionic gonadotropin and thyroid stimulating hormone. Gene delivery vehicles expressing polypeptide hormones and growth factors can be prepared by methods known to those of skill in the art and as described herein. For instance, a retroviral vector expressing human growth hormone can be prepared as described in the Examples. As an additional example, nucleic acid sequences encoding different forms of human insulin can be isolated as described in European Patent Publications EP 026598 or 070632, and incorporated into gene delivery vehicles as described herein.

Any of the polypeptide growth factors described herein can also be administered therapeutically by long-term expression *in vivo* with a gene delivery vehicle. For instance, a variety of different forms of IGF-1 and IGF-2 growth factor polypeptides are also well known the art and can be incorporated into gene delivery vehicles for long term expression *in vivo*. See *e. g.*, European Patent No. 0123228B1, grant published on Sept. 19, 1993, entitled "Hybrid DNA Synthesis of Mature Insulin-like Growth Factors." As an additional example, the long term *in vivo* expression of different forms of fibroblast growth factor can also be effected by the methods of invention. See, *eg.* U.S. Patent No. 5,464,774, issued Nov. 7, 1995; U.S. Patent No. 5,155,214, and U. S. Patent No. 4,994,559, for a description of different fibroblast growth factors.

There are a number of proteins useful for treatment of hereditary disorders that can be expressed *in vivo* by the methods of invention. Many genetic diseases caused by inheritance of defective genes result in the failure to produce normal gene products, for example, thalassemia, phenylketonuria, Lesch-Nyhan syndrome, severe combined immunodeficiency (SCID), hemophilia, A and B, cystic fibrosis, Duchenne's Muscular Dystrophy, inherited emphysema and familial hypercholesterolemia (Mulligan et al., *Science 260*:926, 1993; Anderson et al., *Science 256*:808, 1992; Friedman et al., *Science 244*:1275, 1989). Although genetic diseases may result in the absence of a gene product, endocrine disorders, such as diabetes and hypopituitarism, are caused by the inability of the gene to produce adequate levels of the appropriate hormone insulin and human growth hormone respectively.

Gene therapy by the methods of the invention is a powerful approach for treating these types of disorders. This therapy involves the introduction of normal recombinant genes into somatic cells so that new or missing proteins are produced inside the cells of a patient. A number of genetic diseases have been selected for treatment with gene therapy, including adenine deaminase deficiency, cystic fibrosis, α_1-antitrypsin deficiency, Gaucher's syndrome, as well as non-genetic diseases. As an example of the present invention, a gene delivery vehicle can be used to treat Gaucher disease. Gaucher disease is a genetic disorder that is characterized by the deficiency of

the enzyme glucocerebrosidase. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. For a review see *Science 256*:794, 1992 and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver, et al., vol. 2, p. 1677.

As additional examples, long term expression of Factor VIII or Factor IX is useful for treatment of blood clotting disorders, such as hemophilia. Different forms of Factor VIII, such as the B domain deleted Factor VIII construct described in Example 2 herein can be used to produce gene delivery vehicles expressing Factor VIII for use in the methods of the invention. In addition to clotting factors, there are a number of proteins which can be expressed in the gene delivery vehicles of the invention and which are useful for treatment of hereditary diseases. These include lactase for treatment of hereditary lactose intolerance, AD for treatment of ADA deficiency, and alpha-1 antitypsin for treatment of alpha-1 antitrypsin deficiency. See F.D. Ledley, *J. Pediatics, 110*:157-174, 1987; I. Verma, *Scientific American*:68-84, Nov., 1987; and PCT Publication WO 9527512 entitled "Gene Therapy Treatment for a Variety of Diseases and Disorders" for a description of gene therapy treatment of genetic diseases.

There are a variety of other proteins of therapeutic interest that can be expressed *in vivo* by gene delivery vehicles using the methods of the invention. For instance sustained *in vivo* expression of tissue factor inhibitory protein (TFPI) is useful for treatment of conditions including sepsis and DIC and in preventing reperfusion injury. (See PCT Patent Publications Nos. WO 93/24143 ,WO 93/25230 and WO 96/06637.) Nucleic acid sequences encoding various forms of TFPI can be obtained, for example, as described in U.S. Patent Nos. 4,966,852; 5,106,833; and 5,466,783, and can be incorporated in gene delivery vehicles as is described herein.

Other proteins of therapeutic interest such as erythropoietin (EPO) and leptin can also be expressed *in vivo* by gene delivery vehicles according to the methods of the invention. For instance EPO is useful in gene therapy treatment of a variety of disorders including anemia (see PCT publication number WO 9513376 entitled "Gene Therapy for Treatment of Anemia.") Sustained gene therapy delivery of leptin by the methods of the invention is useful in treatment of obesity. (See WO 9605309 entitled

"Obesity Polypeptides able to modulate body weight" for a description of the leptin gene and its use in the treatment of obesity.) Gene delivery vehicles expressing EPO or leptin can readily be produced using the methods described herein and the constructs described in these two patent publications.

A variety of other disorders can also be treated by the methods of the invention. For example, sustained *in vivo* systemic production of apolipoprotein E or apolipoprotein A by the gene delivery vehicles of this invention can be used for treatment of hyperlipidemia. (See J. Breslow et al., *Biotechnology 12*:365, 1994.) In addition, sustained production of angiotensin receptor inhibitor (T.L. Goodfriend et al., *N. Engl. J. Med. 334*:1469, 1996) can effected by the gene therapy methods described herein. As yet an additional example, the long term *in vivo* systemic production of angiostatin by the gene delivery vehicles of the invention is useful in the treatment of a variety of tumors. (See M.S. O'Reilly et al., *Nature Med. 2*:689, 1996).

5. *Routes and Methods of Administration*

A wide variety of routes and methods may be utilized in order to administer the gene delivery vehicles of the present invention. For example, intravenous (IV) administration can occur under a variety of protocols known to those of skill in the art. For instance, gene delivery vectors can be formulated for IV administration and administered as a single injection. Alternatively, the gene delivery vehicles can be delivered in a multiple injection protocol. An example of a multiple injection protocol is administration for three times a day for several consecutive days or on alternate days. The multiple injection schedule can be carried out over a number of days for example a week or 10 days or two weeks. The injection schedule can also be repeated. The total number of vector particles delivered can dispersed in varying amounts of formulation buffer. Depending on the volume delivered, the retroviral vectors can be delivered as an injection or as an IV formulation such as an IV drip which can be delivered over a longer period of time. Similarly, the rate of administration can vary. Details of the administration protocol such as the single or multiple injection schedule and volume and time of delivery can be determined

experimentally by those of skill in the art, and will also vary depending on the particular gene of interest to be delivered. IV administration is a preferred route of administration for retroviral vectors expressing secretory proteins such as Factor VIII and human growth hormone.

Oral administration is easy and convenient, economical (no sterility required), safe (over dosage can be treated in most cases), and permits controlled release of the active ingredient of the composition (the recombinant retrovirus). Conversely, there may be local irritation such as nausea, vomiting or diarrhea, erratic absorption for poorly soluble drugs, and the recombinant retrovirus will be subject to "first pass effect" by hepatic metabolism and gastric acid and enzymatic degradation. Further, there can be slow onset of action, efficient plasma levels may not be reached, a patient's cooperation is required, and food can affect absorption. Preferred embodiments of the present invention include the oral administration of recombinant retroviruses that express genes encoding erythropoietin, insulin, GM-CSF cytokines, various polypeptides or peptide hormones, their agonists or antagonists, where these hormones can be derived from tissues such as the pituitary, hypothalamus, kidney, endothelial cells, liver, pancreas, bone, hemopoetic marrow, and adrenal. Such polypeptides can be used for induction of growth, regression of tissue, suppression of immune responses, apoptosis, gene expression, blocking receptor-ligand interaction, immune responses and can be treatment for certain anemias, diabetes, infections, high blood pressure, abnormal blood chemistry or chemistries (*e.g.*, elevated blood cholesterol, deficiency of blood clotting factors, elevated LDL with lowered HDL), levels of Alzheimer associated amaloid protein, bone erosion/calcium deposition, and controlling levels of various metabolites such as steroid hormones, purines, and pyrimidines. Preferably, the recombinant retroviruses are first lyophilized, then filled into capsules and administered.

Buccal/sublingual administration is a convenient method of administration that provides rapid onset of action of the active component(s) of the composition, and avoids first pass metabolism. Thus, there is no gastric acid or enzymatic degradation, and the absorption of recombinant retroviruses is feasible.

There is high bioavailability, and virtually immediate cessation of treatment is possible. Conversely, such administration is limited to relatively low dosages (typically about 10-15 mg), and there can be no simultaneous eating, drinking or swallowing. Preferred embodiments of the present invention include the buccal/sublingual administration of recombinant retroviruses that contain genes encoding self and/or foreign MHC, or immune modulators, for the treatment of oral cancer; the treatment of Sjogren's syndrome via the buccal/sublingual administration of such recombinant retroviruses that contain IgA or IgE antisense genes; and, the treatment of gingivitis and periodontitis via the buccal/sublingual administration of IgG or cytokine antisense genes.

Rectal administration provides a negligible first pass metabolism effect (there is a good blood/lymph vessel supply, and absorbed materials drain directly into the inferior vena cava), and the method is suitable of children, patients with emesis, and the unconscious. The method avoids gastric acid and enzymatic degradation, and the ionization of a composition will not change because the rectal fluid has no buffer capacity (pH 6.8; charged compositions absorb best). Conversely, there may be slow, poor or erratic absorption, irritation, degradation by bacterial flora, and there is a small absorption surface (about $0.05m^2$). Further, lipidophilic and water soluble compounds are preferred for absorption by the rectal mucosa, and absorption enhancers (*e.g.*, salts, EDTA, NSAID) may be necessary. Preferred embodiments of the present invention include the rectal administration of recombinant retroviruses that contain genes encoding colon cancer antigens, self and/or foreign MHC, or immune modulators.

Nasal administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. In a preferred embodiment, nasal administration is useful for recombinant retrovirus administration wherein the recombinant retrovirus is capable of expressing a polypeptide with properties as described herein. Conversely, such administration can cause local irritation, and absorption can be dependent upon the state of the nasal mucosa.

Pulmonary administration also avoids first pass metabolism, and gastric acid and enzymatic degradation, and is convenient. Further, pulmonary administration permits localized actions that minimize systemic side effects and the dosage required

for effectiveness, and there can be rapid onset of action and self-medication. Conversely, at times only a small portion of the administered composition reaches the brochioli/alveoli, there can be local irritation, and overdosing is possible. Further, patient cooperation and understanding is preferred, and the propellant for dosing may have toxic effects. Preferred embodiments of the present invention include the pulmonary administration of recombinant retroviruses that express genes encoding IgA or IgE for the treatment of conditions such as asthma, hay fever, allergic alveolitis or fibrosing alveolitis, the CFTR gene for the treatment of cystic fibrosis, and protease and collagenase inhibitors such as α-1-antitrypsin for the treatment of emphysema. Alternatively, many of the same types of polypeptides or peptides listed above for oral administration may be used..

Ophthalmic administration provides local action, and permit prolonged action where the administration is via inserts. Further, avoids first pass metabolism, and gastric acid and enzymatic degradation, and permits self-administration via the use of eye-drops or contact lens-like inserts. Conversely, the administration is not always efficient, because the administration induces tearing. Preferred embodiments of the present invention include the ophthalmic administration of recombinant retroviruses that express genes encoding IgA or IgE for the treatment of hay fever conjunctivitis or vernal and atomic conjunctivitis; and ophthalmic administration of recombinant retroviruses that contain genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators.

Transdermal administration permits rapid cessation of treatment and prolonged action leading to good compliance. Further, local treatment is possible, and avoids first pass metabolism, and gastric acid and enzymatic degradation. Conversely, such administration may cause local irritation, is particularly susceptible to tolerance development, and is typically not preferred for highly potent compositions. Preferred embodiments of the present invention include the transdermal administration of recombinant retroviruses that express genes encoding IgA or IgE for the treatment of conditions such as atopic dermatitis and other skin allergies; and transdermal

administration of recombinant retroviruses encoding genes encoding melanoma specific antigens (such as high molecular weight-melanoma associated antigen), self and/or foreign MHC, or immune modulators.

Vaginal administration provides local treatment and one preferred route for hormonal administration. Further, such administration avoids first pass metabolism, and gastric acid and enzymatic degradation, and is preferred for administration of compositions wherein the recombinant retroviruses express peptides. Preferred embodiments of the present invention include the vaginal administration of recombinant retroviruses that express genes encoding self and/or foreign MHC, or immune modulators. Other preferred embodiments include the vaginal administration of genes encoding the components of sperm such as histone, flagellin, etc., to promote the production of sperm-specific antibodies and thereby prevent pregnancy. This effect may be reversed, and/or pregnancy in some women may be enhanced, by delivering recombinant retroviruses vectors encoding immunoglobulin antisense genes, which genes interfere with the production of sperm-specific antibodies.

Intravesical administration permits local treatment for urogenital problems, avoiding systemic side effects and avoiding first pass metabolism, and gastric acid and enzymatic degradation. Conversely, the method requires urethral catheterization and requires a highly skilled staff. Preferred embodiments of the present invention include intravesical administration of recombinant retrovirus encoding antitumor genes such as a prodrug activation gene such thymidine kinase or various immunomodulatory molecules such as cytokines.

Endoscopic retrograde cystopancreatography (ERCP) (goes through the mouth; does not require piercing of the skin) takes advantage of extended gastroscopy, and permits selective access to the biliary tract and the pancreatic duct. Conversely, the method requires a highly skilled staff, and is unpleasant for the patient.

Many of the routes of administration described herein (*e.g.*, into the CSF, into bone marrow, into joints, intravenous, intra-arterial, intracranial intramuscular, subcutaneous, into various organs, intra-tumor, into the interstitial spaces, intra-

peritoneal, intralymphatic, or into a capillary bed) may be accomplished simply by direct administration using a needle, catheter or related device.

Gene delivery vehicles can also be delivered to a target from outside of the body (as an outpatient procedure) or as a surgical procedure, where the vector is administered as part of a procedure with other purposes, or as a procedure designed expressly to administer the vector. Other routes and methods for administration include the non-pareneral routes as well as administration via multiple sites.

The gene delivery vehicles of the invention can also be delivered in *ex vivo* protocols. *Ex vivo* gene therapy protocols include those in which cells are removed from a patient. transduced *in vitro* and returned to the patient. *Ex vivo* gene therapy also encompasses protocols involving administration of producer cell lines capable of delivering a viral vector to a patient. See U.S. 5,399,346, U.S. 5,529,774, EP 476,953 and WO 96/33282 for a description of *ex vivo* gene therapy administration methods.

H. FORMULATION AND ADMINISTRATION OF GROWTH FACTORS

As is desribed herein, gene delivery vehicles of the present invention can be administered after induction of cell proliferation by a growth factor, or may be co-adminstered with a growth factor. The growth factors of the invention are administered by parenteral, topical, oral or by local administration. For example, the growth factors are adminstered parenterally, *e.g.*, intravenously, subcutaneously, intradermally, or intramuscularly. Preferably, the growth factors are administered intravenously. Administration of the therapeutic agent of the invention can be accomplished by, for example, injection, catheterization, laser-created perfusion channels, cannulization, a particle gun, and a pump.

The growth factors of the invention are typically adminstered with a pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier. The term "liposomes" refers to, for example, the liposome compositions described in U.S. Patent No. 5,422,120, WO

95/13796, WO 94/23697, WO 91/14445 and EP 524,968 B1. Liposomes may be pharmaceutical carriers for the polypeptides of the invention.

The growth factors of the invention are administered in therapeutically effective amounts. The term "therapeutically effective amount" as used herein and applied to polypeptide growth factros refers to an amount of a growth factor that is capable of stimulating cell division in a target tissue *in vivo*. Stimulation of cell proliferation in a target tissue means that the number of dividing cells in the target tissue is greater than in the absence of treatment. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition being treated, recommendations of the treating physician, and particular growth factor that is used. The effective amount for a given situation can be determined by routine experimentation and will vary from growth factor to growth factor. For example, for HGF, a dose of 1 ug/kg to 2 mg/kg body weight, and more preferably from 10 ug/kg to 200 ug/kg body weight is used. In the case of KGF, a dose of 100 ug/kg to 5mg/kg body weight, or more preferably a dose of 1 mg/kg to 50 mg/kg body weight is used. Dose amounts for the other growth factors used in the claimed methods are known to those of skill in the art or can readily be determined experimentally.

Clofibrate, or the other proxisome proliferators, can be administered by IP injection (5-500 mg/kg), or orally (5-500 mg/kg). More preferably the dosages are 10-100 mg/kg. A typical dosing schedule is daily administration for 3-10 days. A tapered dosing can alternatively be employed. Following clofibrate dosing, retroviral vectors can be administered, preferably intravenously, at doses ranging from 1E5 to 1E11 cfu per injection. Injection schedules of one to three times daily, for one to ten days, will be employed. Repeat administrations of retroviral vector with or without repeat clofibrate or growth factor dosing can be performed.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF RETROVIRAL BACKBONES

A. PREPARATION OF RETROVIRAL BACKBONES KT-1

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including *gag* sequences, from the N2 (Armentano et al., *J. Vir. 61*:1647, 1987; Eglitias et al., *Science 230*:1395, 1985) vector was ligated into the plasmid SK^+ (Stratagene, San Diego, CA). The resulting construct was designated N2R5. The N2R5 construct was mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT, preventing *gag* expression. This mutagenized fragment is 200 base pairs (bp) in length and flanked by *Pst* I restriction sites. The *Pst* I-*Pst* I mutated fragment was purified from the SK^+ plasmid and inserted into the *Pst* I site of N2 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is essentially pUC19 (Stratagene, San Diego, CA), except that additional restriction sites *Xho* I, *Bgl* II, *BssH* II and *Nco* I were inserted between the *EcoR* I and *Sac* I sites of the polylinker. This construct was designated pUC31/N2R5gM.

A 1.0 kilobase (Kb) MoMLV 3' LTR EcoR I-EcoR I fragment from N2 was cloned into plasmid SK^+ resulting in a construct designated $N2R3^-$. A 1.0 Kb *Cla* I-Hind III fragment was purified from this construct.

The *Cla* I-*Cla* I dominant selectable marker gene fragment from pAFVXM retroviral vector, comprising a SV40 early promoter driving expression of the neomycin (*neo*) phosphotransferase gene, was cloned into the SK^+ plasmid. This construct was designated SV^+ SV_2-*neo*. A 1.3 Kb *Cla* I-BstB I gene fragment is purified from the SK^+ SV_2-*neo* plasmid.

KT-1 vector was constructed by a three part ligation in which the *Xho* I-*Cla* I fragment containing the gene of interest and the neo gene under the control of the SV40 promoter/enhancer and the 1.0 Kb MoMLV 3' LTR *Cla* I-Hind III fragment are inserted into the *Xho* I-Hind III site of pUC31/N2R5gM plasmid (Figure 1).

B. PREPARATION OF RETROVIRAL BACKBONE pBA5B

1. Preparation of a retroviral vector construct that does not contain an extended packaging sequence (Ψ)

This example describes the construction of a retroviral vector construct using site-specific mutagenesis. Within this example, a MoMLV retroviral vector construct is prepared wherein the packaging signal "Ψ" of the retroviral vector is terminated at basepair 617 of SEQ ID NO: 1, thereby eliminating the ATG start of *gag*.

Briefly, pMLV-K (Miller, *J. Virol 49*:214-222, 1984 - an infectious clone derived from pMLV-1 Shinnick et al., *Nature 293*:543-548, 1981) was digested with *Eco*57I, and a 1.9kb fragment is isolated. (*Eco*571 cuts upstream from the 3' LTR, thereby removing all *env* coding segments from the retroviral vector construct.) The fragment was then blunt ended with T4 polymerase (New England Biolabs), and all four deoxynucleotides, and cloned into the *Eco*RV site of phagemid pBluescript II KS+ (Stratagene, San Diego, Calif.). This procedure yields pKS2+Eco57I-LTR(+) (Figure 2), which was screened by restriction analysis. When the (+) single stranded phagemid was generated, the sense sequence of MoMLV was isolated.

2. Substitution of Nonsense Codons in the Extended Packaging Sequence (Ψ+)

This example describes modification of the extended packaging signal (Ψ+) by site-specific mutagenesis. In particular, the modification substitutes a stop codon, TAA, at the normal ATG start site of *gag* (position 631-633 of SEQ ID NO: 1), and an additional stop codon TAG at position 637-639 of SEQ ID NO: 1.

Briefly, an *Eco*57I - *Eco*RI fragment (MoMLV basepairs 7770 to approx. 1040) from pN2 (Amentano et al., *J. Virol. 61*:1647-1650, 1987) was first cloned into pBluescript II KS+ phagemid at the *Sac*II and *Eco*RI sites (compatible). Single stranded phagemid containing antisense MoMLV sequence, was generated using helper phage M13K07 (Stratagene, San Diego, Calif.). The oligonucleotide 5'-CTG TAT TTG TCT GAG AAT TAA GGC TAG ACT GTT ACC AC (SEQ ID NO: 3) was synthesized, and utilized according to the method of Kunkel (*PNAS 82*:488, 1985), in

order to modify the sequence within the Ψ region to encode stop codons at nucleotides 631-633 and 637-639.

3. Removal of Retroviral Packaging Sequence Downstream from the 3' LTR

Retroviral packaging sequence which is downstream from the 3' LTR was deleted essentially as described below. Briefly, pKS2+Eco57I-LTR(-) was digested with *BalI* and *Hinc*II, and religated excluding the *BalI* to *Hinc*II DNA which contains the packaging region of MoMLV.

4. Construction of Vector Backbones

Constructs prepared in sections B and C above, were combined with a plasmid vector as described below, in order to create a retroviral vector backbone containing all elements required *in cis*, and excluding all sequences of 8 nucleic acids or more contained in the retroviral portion of the *gag-pol* and *env* expression elements.

Briefly, parts B and C are combined as follows: The product of B was digested with *Nhe*I and *Eco*RI and a 1456 basepair fragment containing the LTR and modified Ψ+ region is isolated. The fragment is ligated into the product of C at the unique (compatible) restriction sites *Spe*I and *Eco*RI. The resultant construct was designated pBA5a (Figure 3; see also U.S. Serial No. 08/437,465).

The vector pBA5a was cut with NotI and the end was made blunt by filling in the 5' overhang with Klenow (Sambrook et al., *Mol. Cloning, CSH*, 1989) followed by digestion with *EcoR* I. The resulting insert was ligated to pUC18 cut with *Sma* I and *EcoR* I to make pBA5b. The neo resistance marker gene was added by inserting the *Xho* I to *BstB* I fragment from KT-1 into pBA5b, digested with *Xho* I and *Cla* I, to make pBA6b. A polylinker was added by annealing two oligonucleotides: (1) - 5' TCGAGGATCG CGCCGGGCGG CCGCATCGAT GTCGACG (Sequence ID No. 4) and (2) 5' - CGCGTCGACA TCGATGCGGC CGCCCGGGCG GATCC (Sequence ID No. 5) and ligating the product to pBA6b cut with *Xho* I and *Cla* I to make pBA6bL1 (see Figure 4).

EXAMPLE 2

USE OF HUMAN BETA GALACTOSIDASE AS A GENETIC MARKER IN RETROVIRAL VECTOR

Human beta galactosidase mRNA is obtained from human liver tissue prepared with a MicroFastTrak™ kit (Invitrogen, San Diego, CA). The sequence of the cDNA for human beta galactosidase is listed in Figure 5. This is used as a template for RT PCR reaction using the GeneAmp® RNA PCR kit (Perkin Elmer) and primers: 5' GGG GGG CTC GAG ATG ACG CGC GGC TTG CGC AAT GC (Sequence ID No. 6) and 3' GGG GGG ATC GAT TTC ATC ATC ATA CA (Sequence ID No. 7). The resulting 2.0 Kb human ß-galactosidase cDNA, has no signal peptide, *Xho* I at the 5' end, and *Cla* I at the 3' end. It is inserted into the Moloney retroviral vector KT-1 at the *Xho* I and *Cla* I sites to make KT1/hßGal (Figure 6). This removal of the signal peptide from human beta galactosidase converts it from microsomal to cytoplasmic in distribution which allows conversion of the prodrug conjugate to occur in the cytoplasm rather than in microsomes.

KT1/hßGal is used to make a vector producing cell line by pseudotyping with VSV G protein (Burns, J.C. et al., *PNAS 90:*8033-8037, 1993). This method consists of cotransfection of 293 2-3 (Burns, J.C. et al., *PNAS 90:*8033-8037, 1993) with 10 µg of each of retroviral vector KT/hßGAl with 10 µg of the VSV G protein vector, MLPG by $CaPO_4$ transfection with the ProFection kit according to the manufacturer's instructions (Promega, Madison, WI). The $CaPO_4$-containing media is replaced with fresh DMEM/10% FBS after 16 hours then incubated overnight. The resulting culture supernatant containing VSV-G pseudotyped vector is filtered through 0.45 µm filter. This is used for transduction of the retroviral packaging cell line, DA (see PCT Publication No. WO 92/05266). These cells are subjected to cloning by limiting dilution, and the best clones selected by, *e.g.*, PCR titering as described in Example 5. The supernatants of these cell lines are harvested, passed through 0.45 µm filters and stored at -80°C in aliquots until use.

Supernatant from the selected vector producing DA/hßGal is used to transduce HT1080 target cells, which are then fixed and stained with Xgal (Irwin et al., *J. Virol. 68*:50361994).

EXAMPLE 3

USE OF HUMAN BETA GALACTOSIDASE FOR CONVERSION OF PRODRUG TO ACTIVE FORM FOR ABLATION THERAPY

The prodrug conjugate, N-[4-(ß-D-galactopyranosyl) Benzyloxycarbonyl]-daunorubicin, is synthesized in a manner similar to that described in S. Andrianomenjanahary et al., *Bioorganic & Medicinal Chemistry Letters* 2:1093-1096, 1992, using the method of Danishevsky to generate the ß-D-galactopyranoside (S.J. Danishevsky and M.T. Bilodeaux, *Angewante Chemie Int'l Ed. English 35*:1380-1419, 1996).

The relative sensitivity of HT1080 with and without hßGal to daunorubicin and N-[4-(ß-D-galactopyranosyl)Benzyloxycarbonyl]-daunorubicin is measured as follows: HT 1080 cells are transduced with the DA/hßGal supernatant in 8 µg/ml polybrene overnight, then rinsed, fed fresh DMEM/10% FBS, and incubated overnight. The effect of daunorubicin and N-[4-(ß-D-galactopyranosyl) Benzyloxycarbonyl]-daunorubicin is measured by plating 1×10^4 cells per well in 96 well dishes of transduced and untransduced cells (P.D. Senter et al., *PNAS 85*:4842-4846, 1988). These are incubated for six hours in concentrations of daunorubicin, N-[4-(ß-D-galactopyranosyl)Benzyloxycarbonyl]-daunorubicin (0 to 75 µM) or media alone. The wells are washed and incubated in media for 12 hours, then receive a pulse of [^{3}H]thymidine (1µCi/well) for six hours. The cells are transferred to glass fiber filters and counted in a scintillation counter (Beckman).

EXAMPLE 4

USE OF HUMAN PLACENTAL ALKALINE PHOSPHATASE AS A GENETIC MARKER IN RETROVIRAL VECTOR

Human placental alkaline phosphatase cDNA (sequence shown in Figure 7) was cloned from the vector pSVT7/PLAP (C. Hummer and J.L. Millan, *Biochem. J. 274*:91-95, 1991) into pCI (Promega, Madison, WI) at the *EcoR* I and *Kpn* I sites. This insert was then cut out of pCI with *Xho* I and *Cla* I and cloned into the *Xho* I and *Cla* I sites of the retroviral vector pMBA to make pMBA/hPLAP (Figure 8).

MBA/hPLAP was used to make a vector producing cell line by pseudotyping with VSV G protein (Burns, J.C. et al., *PNAS 90:*8033-8037, 1993). This method consists of cotransfection of 293 2-3 with 10 μg of each of retroviral vector MBA/hPLAP with 10 μg of the VSV G protein vector, MLPG by $CaPO_4$ transfection with the ProFection kit according to the manufacturer's instructions (Promega, Madison, WI). Sixteen hours post-transfection the cells were rinsed and fed fresh DMEM/10% FBS. The media was removed after 24 hours of incubation and filtered through 0.45 μm syringe filter. This supernatant was applied to the packaging cell line, DA, with 8 μg/ml of polybrene.

The DA cells were selected by adsorption onto antibody-coated magnetic beads followed by exposure to a magnetized iron column (MACS) using the Miltenyi MiniMACS system (Miltenyi Biotec Inc., Sunnyvale, CA) as follows: the antibody, MIG-P1 (Biosource International, Camarillo, CA), specific for the placental alkaline phosphatase, was bound at a 1:50 dilution to 0.5 to 1 x 10^7 transduced cells in 200 μl PBS/2% FBS on ice for 30 min. The goat polyclonal anti-mouse IgG magnetic beads (Miltenyi cat. #484-01) beads are washed by resuspending 200 μl in cold PBS then loading them on a Miltenyi column (Miltenyi cat. #422-01) held in the magnet. The beads are eluted by removing the column from the magnet and eluting in 200 μl PBS/2% FBS. The beads were then incubated with the antibody-treated transduced DA cells, the cells are collected by centrifugation 10 minutes at 1000 rpm, 4°C, and loaded on a fresh Miltenyi column on the magnet (prepared according to manufacturer's

instructions). Following elution of the non-bound cells, the column was washed with cold PBS/2% FBS, and then removed from the magnet and the bound cells were then washed off the column with cold PBS/2% FBS. The cells were plated in DMEM/10% FBS and allowed to grow out. The percentage of positive cells was measured by FACS analysis using the same monoclonal antibody, MIG-P1, followed by staining with FITC-goat anti-mouse IgG (Fab') fragment, and analysis on a Becton-Dickenson FACS analyzer. Supernatant from the DA/hPLAP cells was collected and filtered through 0.45 μm syringe filter and stored at -80°C. The cells are subjected to cloning by limiting dilution, and the best clones selected by, *e.g.*, PCR titering or Fast Red staining as described in Example 5.

The relative sensitivity of HT1080 with and without hPLAP to etoposide and etoposide phosphate is measured as follows: Etoposide phosphate is generated by phosphorylation of etoposide (Bristol-Myers) using the method described in Senter et al., *PNAS 85*:4842-4846, 1988. HT 1080 cells are transduced with the DA/hPLAP supernatant in 8 μg/ml polybrene overnight, then rinsed, fed fresh DMEM/10% FBS, and incubated overnight. The effect of etoposide and etoposide phosphate is measured by plating 1 x 10^4 cells per well in 96 well dishes of transduced and untransduced cells. These are incubated for six hours in concentrations of etoposide, etoposide phosphate (0 to 75 μM) or media alone. The wells are washed and incubated in media for 12 hours, then receive a pulse of [^{3}H]thymidine (1μCi/well) for six hours. The cells are transferred to glass fiber filters and counted in a scintillation counter (Beckman).

EXAMPLE 5

PRODUCER CELL TITERING METHODS

A. TITERING OF VECTOR VIA PCR AMPLIFICATION USING VECTOR SPECIFIC PRIMERS.

Vector titer can be determined in a PCR assay by correlation of detected provector sequences in transduced cells to a vector standard run in parallel. Briefly, both vector test sample and vector standard are used to transduce target cells (*e.g.*,

HT1080) in parallel (using serial dilutions of both vector test sample and vector standard), and specific DNA sequences of the provectors integrated in the target cells are amplified via PCR. The PCR primers amplify a desired fragment within the vector LTR. The amount of amplified PCR amplicons of the vector test sample is then correlated to the PCR amplicons of the vector standard and expressed as colony forming unit equivalents (cfu-eq). PCR amplicons can be detected via incorporation of radiolabel during the PCR reaction. Radiolabel signals are quantitated using a phosphor imaging system.

For example, HT1080 cells are plated in 6 well plates at 3e5 cells per well. Twenty-four hours later, transductions are performed with the DA Cbßgal vector at 3e5 to 1e4 cfu per well. Vector test sample used for transductions is diluted 1/10 to 1/1000 (depending on the expected titer) to achieve transductions within the range of the assay. DNA is isolated 72 hours after transduction via phenol/chloroform extraction and ethanol precipitation and quantitated via microfluorometry using Hoechst dye 33258.

Genomic DNA (175ng per PCR reaction) is then amplified in a 50µl PCR reaction containing 2mM $MgCl_2$, 0.2mM dATP, dCTP, TTP, and dGTP, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 0.4mM of each primer F2A and R2A, 1.25 units of Amplitaq™ DNA polymerase (previously incubated with Taqstart™ antibody in 1X Taqstart™ buffer (Clonetech, Palo Alto CA), and 0.2µCi of Redivue [α-^{32}P] dCTP. Five microliters from each PCR reaction are blotted onto DE81 Ion Exchange Chromatography Paper (Whatman, Maidstone England) and washed 3 times with a phosphate buffered wash solution. The signals on the membrane are quantitated using a phosphor imager. A standard curve is generated by plotting the PCR signals versus the cfu-eq of the vector standard. The straight line equation is used to extrapolate the cfu-equivalent titer of the test samples.

Primer sequences:

(Sequence ID No. 29)

F2A primer: 5' CTGTAGGTTTGGCAAGCTAGC 3'

(Sequence ID No. 30)
R2A primer: 5' CGCTGACGGGTAGTCAATC 3'

B. FAST RED STAINING OF ADHERENT PLAP CELLS

This assay can be utilized to detect the presence of adherent PLAP cells and hence, can be used to titer PLAP producer lines. Briefly, media is drained from plates containing adherent PLAP cells. One milliliter of fixing solution (PBS + 2% formaldehyde +0.2% glutaraldehyde) is added per well and allowed to incubate for 5 minutes at room temperature. The fixing solution is aspirated and the cells are washed with 2 mls of PBS. The wells are aspirated once more and the plates are incubated at 56° C (with humidity) for 20 min. One milliliter of freshly prepared Fast Red Stain (TR/Naphthol AS_MX Tablet Set, Sigma) is added to each well and the plates are allowed to incubate at room temperature from 2 hours to overnight. The percent transduced/transfected cells is determined by counting red and non- red cells.

EXAMPLE 6

DEMONSTRATION OF FUNCTION OF hPLAP IN ERADICATING TUMOR GROWTH IN NUDE MICE

hPLAP is able to convert the prodrugs, mitomycin phosphate (MOP) and etoposide phosphate (EP), into an active mitomycin C derivative, mitomycin alcohol. and etoposide. 5e5 HT1080 cells or HT/hPLAP cells (HT1080 cells stably expressing hPLAP) are inoculated subcutaneously into nude mice (Balb/c). Tumor development occurs in 7-14 days. Etoposide phosphate is prepared by the method of Senter et al., *Cancer Res. 49*:5789-92, 1988, or obtained from a pharmacy (*e.g.*, manufactured by Bristol-Myers Squibb). Animals are dosed with EP as described (Senter et al., 1988) 2-10 days after inoculation with cells. Control animals inoculated with parental HT1080

cells develop tumors rapidly that are resistant to the effect of EP. However animals inoculated with HT/hPLAP cells demonstrate a dose-dependent reduction in tumor growth after administration of EP. Experiments involving injection of HT1080 cells in one flank and HT/hPLAP cells in the contralateral flank demonstrate that the EP effect is specific for cells expressing hPLAP.

EXAMPLE 7

USE OF HUMAN CYTOCHROME P-450 2B FOR CONVERSION OF PRODRUG TO ACTIVE FORM FOR ABLATION THERAPY

Human cytochrome P-450 2B mRNA is obtained from human liver tissue prepared with a MicroFastTrak™ kit (Invitrogen, San Diego, CA). The sequence of the cDNA for human cytochrome P-450 2B is listed in Figure 9. This is used as a template for RT PCR reaction using the GeneAmp® RNA PCR kit (Perkin Elmer) and primers: 5' GGG GGG CTC GAG GGC ACC ATG GAG CTC AG (Sequence ID No. 8) and 3' GGG GGG ATC GAT CCC TCA GAA GCT GGT GTG (Sequence ID No. 9). The resulting 1.13 Kb human cytochrome P-450 2B cDNA has *Xho* I at the 5' end and *Cla I* at the 3' end, and is inserted into the Moloney retroviral vector pBA6BL1 at the *Xho* I and *Cla I* sites to make BA6/CYP2B (Figure 10).

BA6/CYP2B is used to make a vector producing cell line by pseudotyping with VSV G protein (Burns, J.C. et al., *PNAS 90:*8033-8037, 1993) as described in Example 2. Briefly, 10 μg of each of retroviral vector BA6/CYP2B with 10 μg of the VSV G protein vector, MLPG introduced into 293 2-3 cells by $CaPO_4$ transfection with the ProFection kit according to the manufacturer's instructions (Promega, Madison, WI). The $CaPO_4$-containing media is replaced with fresh DMEM/10% FBS after 16 hours then incubated overnight. The resulting culture supernatant containing VSV-G pseudotyped vector is filtered through 0.45 μm filter. This is used for transduction of the retroviral packaging cell line, DA. The cells are then subjected to cloning by limiting dilution, and the best clones selected by, *e.g.*, PCR

titering or Fast Red staining as described in Example 5. Cells are then grown to confluency, and the supernatants of these cell lines were harvested, passed through 0.45 μm filters and stored at -80°C in aliquots until use.

The relative sensitivity of HT1080 with and without CYP2B to cyclophosphamide is measured as follows: HT 1080 cells are transduced with the DA/ CYP2B supernatant in 8 μg/ml polybrene overnight, then rinsed, fed fresh DMEM/10% FBS, and incubated overnight. The effect of cyclophosphamide is measured by plating 1 x 10^4 cells per well in 96 well dishes of transduced and untransduced cells. These are incubated for six hours in concentrations cyclophosphamide (0 to 1000 μM) or media alone. The wells are washed and incubated in media for 12 hours, then receive a pulse of [^{3}H]thymidine (1 μCi/well) for six hours. The cells are transferred to glass fiber filters and counted in a scintillation counter (Beckman).

EXAMPLE 8

USE OF FURIN AS A CELL-BOUND PRODRUG CONVERTASE FOR ABLATION THERAPY

cDNA encoding furin is made by RT PCR using mRNA prepared by FastTrak™ (Invitrogen, San Diego) from human cell line HT1080 as a template. The primers (5'flanking: 5'CCC CCC CTC GAG ACC TGT CCC CCC CAT GGA G (Sequence ID No. 10), and 3' flanking: 5' CCC CCC ATC GAT GTG GGC TCA CAG AGG GCG C (Sequence ID No. 11)) are used in RT PCR reaction with the GeneAmp kit from Perkin Elmer according to manufacturer's instructions. The resulting PCR product is cloned into the TA vector using the TA cloning kit (Invitrogen) and is verified by DNA sequence analysis. An alteration is made in the cytosolic domain of furin to alter the distribution from *trans*-Golgi to cell surface localization, by deletion of the acidic cluster from residue 766 to 783 (Figure 11) using overlap PCR with the flanking primers above and the deletion primers (5' del: 5'ATA AAG GCG GTC CTT TCA GGG GGC AGC CCC TTC TA (Sequence ID No. 12) and 3' del: 5'GGG GCT GCC CCC TGA AAG GAC CGC CTT TAT CAA AG (Sequence ID No. 13) in the

following PCR reactions: The 5' flanking and 5' del primers in one tube and the 3' flanking and 3' del primers in another, both using the furin construct as template. The resulting left (2.3 Kb) and right (60 bp) bands are purified by agarose gel electrophoresis and the DNA is purified by GeneClean™ kit (Bio101, San Diego, CA). The two fragments are used as templates in a PCR reaction with the 5' and 3' flanking primers. The resulting PCR product with *Xho* I and *Cla I* at the 5' and 3' termini respectively is cloned into the vector pBA6BL1 at the *Xho* I and *Cla I* sites to make pBA6/Xfur (Figure 12). The DNA sequence is verified by automated DNA sequencing methodology (Perkin-Elmer).

BA6/Xfur is used to make a vector producing cell line by pseudotyping with VSV G protein (Burns, J.C. et al., *PNAS 90:*8033-8037, 1993) as described in Example 2. Briefly, 10 μg of each of retroviral vector BA6/Xfur with 10 μg of the VSV G protein vector, MLPG introduced into 293 2-3 cells by $CaPO_4$ transfection with the ProFection kit according to the manufacturer's instructions (Promega, Madison, WI). The $CaPO_4$-containing media is replaced with fresh DMEM/10% FBS after 16 hours then incubated overnight. The resulting culture supernatant containing VSV-G pseudotyped vector is filtered through 0.45 μm filter. This is used for transduction of the retroviral packaging cell line, DA. The cells are subjected to cloning by limiting dilution, and the best clones selected by, *e.g.*, PCR titering or Fast Red staining as described in Example 5. The supernatants of these cell lines were harvested, passed through 0.45 μm filters and stored at -80°C in aliquots until use.

The prodrug substrate for Xfur is synthesized by standard Merrifield peptide synthetic methodology as Arg-Lys-Lys-Arg (Sequence ID No. 28) without deprotection. This is conjugated with phenylenediamine mustard (Everett, J.L. and Ross, W.C.J., *J. Chem. Soc.*:1972, 1949) in a mixed anhydride coupling (Chakravarty, P.K. et al., *J. Med. Chem. 26*:633-638, 1983) followed by deprotection with trifluoroacetic acid to make RKKR-phenylenediamine mustard.

The relative sensitivity of B16 murine melanoma with and without Xfur to RKKR-phenylenediamine mustard *in vitro* is measured as follows: HT 1080 cells are transduced with the DA/Xfur supernatant in 8 μg/ml polybrene overnight, then rinsed,

fed fresh DMEM/10% FBS, and incubated overnight. The effect RKKR-phenylenediamine mustard is measured by plating 1 x 10^4 cells per well in 96 well dishes of transduced and untransduced cells. These are incubated for six hours in concentrations RKKR-phenylenediamine mustard (0 to 500 μM) or media alone. The cells are counted with trypan blue to determine viability and growth.

B16 cells transduced with DA/Xfur and selected as described above. 1 x 10^7 transduced and untransduced B16 cells are implanted subcutaneously in the left and right flanks of BALB/c mice respectively and allowed to establish palpable tumors. RKKR-phenylenediamine mustard from 0 to 8 mg/kg is injected daily into the peritoneum of mice on days 1 to 9 and the survival of the mice is used as a measure.

EXAMPLE 9

VECTORS EXPRESSING DCK

A. GENERATION OF KT1/DCK VECTOR

To generate a retroviral expression vector encoding the human dCK coding sequences, firstly, the dCK cDNA must be obtained. Briefly, cellular mRNA is isolated from human T-cell lines, MOLT-3 (ATCC CRL 1552), MOLT-4 or Jurkat cells using the MicroFastTrak™ kit (Invitrogen, San Diego, CA). The mRNA preparation is used as a template for RT PCR reaction using the GeneAmp® RNA PCR kit (Perkin Elmer) and primers: 5' GGG GGG CTC GAG CCC CGA CAC CGC GGC GGG CCG (Sequence ID No. 14) and 3' GGG GGG ATC GAT GCT GAA GTA TCT GGA ACC (Sequence ID NO. 15). The resulting 1.0 Kb human dCK cDNA has a *Xho* I at the 5' end and *Cla* I at the 3' end. It is inserted into the Moloney retroviral vector KT-1 at the *Xho* I and *Cla* I sites to make KT1/hdCK.

KT1/hdCK is used to make a VCL by pseudotyping with VSV G protein as described above. The relative sensitivity to cytosine arabinoside (ara-C) of 9L glioblastoma cells transduced with the KT/hdCK vector versus control cells transduced with KT1/beta-gal is evaluated as described below. 9L cells are transduced with vector supernatant from VCL specific for KT1/hdCK or KT1/β-gal. Cells are transduced with vector supernatant in the presence of 8 μg/ml polybrene overnight, rinsed and fed with

fresh media and incubated overnight. The effect of dCK is measured by plating 2X103 cells/200 µl in individual wells of 96 well dishes. The cells are incubated for 12 hours and then treated with ara-C for 96 hours. The cells are fixed and stained with 0.05% Methylene blue. The dye is eluted with 0.33 M HCl for 15 minutes with agitation and absorbance measured in a microplate reader at 600 nm. Alternatively, the cells may be stained with Trypan blue and viable/dead cells evaluated.

B. EVALUATION OF THE EFFECT OF HDCK *IN VIVO*

9L cells expressing hdCK or β-gal are injected into Fischer 344 rats and evaluated for their *in vivo* sensitivity to araC. One million stably transduced cells expressing hdCK or β-gal are injected intradermally into opposite flanks of adult rats. Small tumor nodules are evident between days 7-10. At day 9, animals are treated with ara C or PBS. The dose of ara C is 200 mg/kg every 8 hours for 2 days, followed by another dose of ara C 6 days later. Tumor volumes are measured periodically through the course of the experiment.

EXAMPLE 10

GENERATION OF KT1/hENT1 VECTOR

To generate a retroviral expression vector encoding the human hENT1 coding sequences, first the hENT1 cDNA is obtained. Briefly, cellular mRNA is isolated from the acute myelogenous leukemia cell line KG-1 (ATCC CCL 246) using the MicroFastTrak™ kit (Invitrogen, San Diego, CA). The mRNA preparation is used as a template for RT PCR reaction using the GeneAmp® RNA PCR kit (Perkin Elmer) and primers as follows: The upstream primer sequence (from Genbank Accession number (T25352), 5' GGG GGG CTC GAG AAC AAC ATC ACC ATG ACA (Sequence ID No. 16), and the downstream primer sequences taken from Griffiths et al., (*Nature Medicine* 3:89-93, 1997) where the two degenerate primers are combined for a degeneracy of 960 sequences, 5' GGG GGG ATC GAT TCA NAC (G/A/T)AT NGC YCT RAA (Sequence ID No. 17). The abbreviations in the degenerate primers are as

follows R is A or G; Y is C or T; and N is A,T,C,G. The resulting 1.4 Kb human hENT1 cDNA, has a *Xho* I at the 5' end and *Cla I* at the 3' end. It is inserted into the Moloney retroviral vector KT-1 at the *Xho* I and *Cla I* sites to make KT1/hENT1. KT1/hENT1 is used to make a VCL by pseudotyping with VSV G protein.

EXAMPLE 11

INTRAVENOUS ADMINISTRATION OF RECOMBINANT RETROVIRUSES EXPRESSING FACTOR VIII

A. CONSTRUCTION OF FULL-LENGTH AND B DOMAIN DELETED FACTOR VIII cDNA RETROVIRAL VECTORS

The following is a description of the construction of several retroviral vectors encoding a full-length factor VIII cDNA. Further discussion is also provided in U.S. Application No. 08/366,851. Due to the packaging constraints of retroviral vectors and because selection for transduced cells is not a requirement for therapy, a retroviral backbone, *e.g.*, KT-1, lacking a selectable marker gene is employed.

1. Production of Plasmid Vectors Encoding Full-Length Factor VIII

A gene encoding full length factor VIII can be obtained from a variety of sources. One such source is the plasmid pCIS-F8 (*see* EP 0 260 148), which contains a full length factor VIII cDNA whose expression is under the control of a CMV major immediate-early (CMV MIE) promoter and enhancer. The factor VIII cDNA contains approximately 80 bp of 5' untranslated sequence from the factor VIII gene and a 3' untranslated region of about 500 bp. In addition, between the CMV promoter and the factor VIII sequence lies a CMV intron sequence, or "cis" element. The cis element, spanning about 280 bp, comprises a splice donor site from the CMV major immediate-early promoter about 140 bp upstream of a splice acceptor from an immunoglobulin gene.

More specifically, a plasmid, designated pJW-2, encoding a retroviral vector for expressing full length factor VIII is constructed using the KT-1 backbone from pKT-1. Briefly, in order to facilitate directional cloning of the factor VIII cDNA

insert into pKT-1, the unique *Xho* I site is converted to a Not I site by site directed mutagenesis. The resultant plasmid vector is then opened with Not I and *Cla* I. pCIS-F8 is digested to completion with *Cla* I and Eag I, for which there are two sites, to release the fragment encoding full length factor VIII. This fragment is then ligated into the Not I/Cla I restricted vector to generate a plasmid designated pJW-2.

2. Construction of a Truncated Factor VIII retroviral vector (ND-5)

A plasmid vector encoding a truncation of about 80% (approximately 370 bp) of the 3' untranslated region of the factor VIII cDNA, designated pND-5, is constructed in a pKT-1 vector as follows: As described for pJW-2, the pKT-1 vector employed has its *Xho* I restriction site replaced by that for Not I. The factor VIII insert is generated by digesting pCIS-F8 with *Cla* I and Xba I, the latter enzyme cutting 5' of the factor VIII stop codon. The approximately 7 kb fragment containing all but the 3' coding region of the factor VIII gene is then purified. pCIS-F8 is also digested with Xba I and *Pst* I to release a 121 bp fragment containing the gene's termination codon. This fragment is also purified and then ligated in a three way ligation with the larger fragment encoding the rest of the factor VIII gene and *Cla* I/Pst I restricted BLUESCRIPT® KS^+ plasmid (Stratagene, *supra*) to produce a plasmid designated pND-2.

The unique Sma I site in pND-2 is then changed to a *Cla* I site by ligating *Cla* I linkers (New England Biolabs, Beverly, MA) under dilute conditions to the blunt ends created by a Sma I digest. After recircularization and ligation, plasmids containing two *Cla* I sites are identified and designated pND-3.

The factor VIII sequence in pND-3, bounded by *Cla* I sites and containing the full length gene with a truncation of much of the 3' untranslated region, is cloned as follows into a plasmid backbone derived from a Not I/Cla I digest of pKT-1 (a pKT-1 derivative by cutting at the *Xho* I site, blunting with Klenow, and inserting a Not I linker (New England Biolabs)), which yields a 5.2 kb Not I/Cla I fragment. pCIS-F8 is cleaved with Eag I and Eco RV and the resulting fragment of about 4.2 kb, encoding the 5' portion of the full length factor VIII gene, is isolated. pND-3 is digested with Eco RV and *Cla* I and a 3.1 kb fragment is isolated. The two fragments

containing portions of the factor VIII gene are then ligated into the Not I/Cla I digested vector backbone to produce a plasmid designated pND-5.

3. Construction of Factor VIII vectors with non-immunogenic markers / PAE genes

The above vectors can all be made in the KT-1 backbone, or a crossless backbone (see PCT Application Nos. PCT/US95/05789 and PCT/US97/07697), that has no selectable marker. They can similarly be constructed in the pBA5 vector backbone (Figure 3). Briefly a selectable marker can be introduced into them by cutting at a single *Cla* I site and introducing an expression cassete for the marker as was done for the neomycin marker in Example 1. In this way a cassette expressing any of placental alkaline phosphatase, deoxycytidine kinase, cytochrome P-450 or other suitable non-immunogenic marker / PAE can be introduced. The cassette will have the cDNA linked to a promoter such as the SV40 promoter in Example 1 and no polyadenylation site. Other suitable internal promoters (*e.g.*, CMV from the pCI-PLAP vector in Example 4) can also be utilized. Such vectors are called pJW-2-PLAP, pJW-2-DCK, pND5-PLAP, pBdel1-PLAP and pBdel1-DCK, etc.

4. Construction of pCF8-PLAP

a. Deletion of the 3' end of human factor VIII cDNA

A Xba I to Not I fragment was amplified from retroviral vector pCF8 (also designated pMBF8; see PCT Application No. US 97/11785) utilizing the following primers and PCR:

1. FVIII 3' Xba; (Sequence ID No. 31)

5' GAATGGCAAAGTAAAGGTTTTTCAGGG (33 bp upstream of the 3' Xba I)

2. FVIII 3' Not; (Sequence ID No. 32)

5' ATAGTTAGCGGCCGCAACCCGGGCCACCCTCAGTAGAGGTCCTG

The amplified DNA was digested with Xba I and Not I and cloned into the BlueScript SK⁻ plasmid (Stratagene) which had been digested with Xba I and Not I. The resulting plasmid was named pKS-121.

pCF8 was also digested with PflM I and Not I, and dephosphorylated using CIAP. A 8.3 kb fragment was isolated and gel purified. A 1.3 kb fragment was also isolated from pCF8 by digesting with PflM I and Xba I. A 121 bp fragment was isolated from KS-121 by digesting with Xba I and Not I. All 3 fragments were ligated together to generate the plasmid, pCF8-D3'. This plasmid is similar to pCF8 except that the 3' non-coding region of the FVIII cDNA has been deleted and a short linker was added.

b. Insertion of the PLAP cDNA

pBAAP (containing PLAP) was digested with Xho I, blunted using T4 DNA Polymerase large fragment (Klenow), and dephosphorylated using CIAP. It was then ligated in the presence of excess Not I linker (Phosphorylated). The resulting plasmid, pBAAP X/N, was digested with Not I and the 1.9 kb fragment (Not I to Not I PLAP cDNA) was ligated into pCF8-D3' linearized with Not I. The resulting plasmids were analyzed using restriction mapping to determine the orientation of the insert. The resulting plasmid, named pCF8-PLAP, is a dicistronic vector including both cDNAs separated by a short spacer.

B. ASSAY FOR FACTOR VIII EXPRESSION

1. Assay of KT-ND5 Vector Expression by Transient Packaging and Transduction of Murine Cells

Cell lines, L33, (Dennert, USC Comprehensive Cancer Center, Los Angeles, CA, Patek, *et. al., Int. J. of Cancer 24*:624-628, 1979), BC10ME (Patek, *et al.*, Cell Immuno 72:113, 1982, ATCC# TIB85), L33env, and BCenv (L33*env* and BC*env* express HIV-1 IIIB*env*, Warner et al, *AIDS Res. and Human Retrovirus 7*:645, 1991), transduced with the KT-ND5-DCK vector, carrying the amphotropic or VSVG envelope protein are examined for the expression of factor VIII. Non-transduced cells are also

analyzed for factor VIII expression and compared with KT-ND5-DCK transduced cells to determine the effect of transduction on protein expression.

Murine cell lines, L33-KT-ND5-DCK, L33*env*-KT-ND5-DCK, L33*env*, L33, BC10ME, BC10ME-KT-ND5-DCK, BC*env*, and BC*env*-KT-ND5-DCK, are tested for expression of the KT-ND5-DCK molecule. Cells are grown to subconfluent density and the supernatant is removed following centrifugation at 200 xg. The samples are diluted and assayed by the COATEST® Factor VIII assay (KabiVitrum Diagnostica, Molndal, Sweden).

The assay is performed as follows: 100 µl of culture media sample is mixed with 200µl of working buffer provided in the kit. The mixture is incubated at 37°C for 4 - 5 min., after which 100 µL of a 0.025 M $CaCl_2$ stock solution is added, followed by a 5 min. 37°C incubation. 200 µL of the chromogenic reagent (20 mg S-2222, 335 µg synthetic thrombin inhibitor, I-2581, in 10 mL) is then mixed in. After a 5 min. incubation at 37°C, 100 µL of 20% acetic acid or 2% citric acid is added to stop the reaction. Absorbance is then measured against a blank comprising 50 mM Tris, pH 7.3, and 0.2% bovine serum albumin (BSA). A standard curve based on dilutions of normal human plasma (1.0 IU factor VIII/mL) is used and the assays should be performed in plastic tubes. Serum levels of factor VIII in non-hemophilic patients are in the range of 200 ng/mL.

When this assay is used for patient samples, 9 volumes of blood are mixed with one volume of 0.1 M sodium citrate, at a neutral pH, and centrifuged at 2,000 x g for 5 - 20 min. at 20 - 25°C to pellet cells. Due to heat lability of factor VIII, plasma samples should be tested within 30 min. of isolation or stored immediately at -70°C, although as much as 20% of factor VIII activity may be lost during freezing and thawing.

2. <u>Assay of KT-ND5-DCK Vector Expression by Transient Packaging and Transduction of Human Cells</u>

Cell lines transduced with KT-ND5-DCK are examined for expression of factor VIII. Non-transduced cells are analyzed to compare with KT-ND5-DCK transduced cells and determine the effect that transduction has on expression.

Two human cell lines, JY and JY-KT-ND5-DCK are tested for expression of KT-ND5-DCK. Suspension cells grown to 10^6 cells/ml are removed from culture flasks by pipet and pelleted by centrifugation at 200 xg. The supernatant is removed, diluted, and assayed by the Coatest[R] Factor VIII assay as described above in Example 2B1.

C. TRANSIENT TRANSFECTION AND TRANSDUCTION OF PACKAGING CELL LINES HX AND DA WITH THE VECTOR CONSTRUCT KT-ND5-DCK

1. Plasmid DNA Transfection

The packaging cell line, HX (WO92/05266), are seeded at 5.0 x 10^5 cells on a 10 cm tissue culture dish on day 1 with Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS). On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA co-precipitation is performed by mixing 40.0 µl 2.5 M $CaCl_2$, 10 µg plasmid DNA, and deionized H_2O to a total volume of 400 µl. Four hundred microliters of the DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl precipitation buffer (50 mM HEPES-NaOH, pH 7.1; 0.25 M NaCl and 1.5 mM Na_2HPO_4-NaH_2PO_4). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of cells. The cells are incubated with the DNA precipitate overnight at 37°C. On day 3, the media is aspirated and fresh media is added. The supernatant is removed on day 4, passed through a 0.45 µl filter, and stored at -80°C.

Alternatively, 29 2 3 cells (WO 92/05266) (these are 293 cells expressing gag and pol) are transfected with the vector DNA and the plasmid pMLP-VSVG (or other VSVG encoding plasmids) to yield VSVG psuedotyped vector particles that are harvested and stored as described above.

2. Packaging Cell Line Transduction

DA (an amphotropic cell line derived from a D17 cell line ATCC No. 183, WO 92/05266) cells are seeded at 5.0 x 10^5 cells/10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 µg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 3.0 ml, 1.0 ml and 0.2 ml of the freshly collected virus-containing HX media is added to

the cells. The cells are incubated with the virus overnight at 37°C, followed by cloning by limiting dilution, and the best clones are selected by, *e.g.*, PCR titering or Fast Red staining as described in Example 5.

Using these procedures, cell lines are derived that produce greater than or equal to 10^6 cfu/ml in culture.

The packaging cell line HX can be transduced with vector generated from the DA vector producing cell line in the same manner as described for transduction of the DA cells from HX supernatant.

3. Generation of Producer Cell Line via One Packaging Cell Line

In some situations it may be desirable to avoid using more than one cell line in the process of generating producer lines. In this case, DA cells are seeded at 5.0 x 10^5 cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% irradiated (2.5 megarads minimum) FBS. On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 60 µl 2.0 M $CaCl_2$, 10 µg MLP-G plasmid, 10 µg KT-ND5-DCK retroviral vector plasmid, and deionized water to a volume of 400 µl. Four hundred microliters of the DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl 2X precipitation buffer (50 mM HEPES-NaOH, pH 7.1, 0.25 M NaCl and 1.5 mM Na_2HPO_4-NaH_2PO_4). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of DA cells plated the previous day. The cells are incubated with the DNA precipitate overnight at 37°C, followed by cloning by limiting dilution. The best clones are selected by, *e.g.*, PCR titering or Fast Red staining as described in Example 5.

D. DETECTION OF REPLICATION COMPETENT RETROVIRUSES (RCR)

1. The Extended S^+L^- Assay

The extended S^+L^- assay determines whether replication competent, infectious virus is present in the supernatant of the cell line of interest. The assay is based on the empirical observation that infectious retroviruses generate foci on the indicator cell line $MiCl_1$ (ATCC No. CCL 64.1). The $MiCl_1$ cell line is derived from

the Mv1Lu mink cell line (ATCC No. CCL 64) by transduction with Murine Sarcoma Virus (MSV). It is a non-producer, non-transformed, revertant clone containing a replication defective murine sarcoma provirus, S^+, but not a replication competent murine leukemia provirus, L^-. Infection of $MiCl_1$ cells with replication competent retrovirus "activates" the MSV genome to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45 μ filter to remove any cells. On day 1, Mv1Lu cells are seeded at 1.0×10^5 cells per well (one well per sample to be tested) of a 6 well plate in 2 ml DMEM, 10% FBS and 8 μg/ml polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37°C, 10% CO_2. On day 2, 1.0 ml of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 ml of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 ml media) of MA virus (referred to as pAM in Miller *et al., Molec. and Cell Biol.* 5:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 ml DMEM and 10% FBS is added to the cells. In addition, the $MiCl_1$ cells are seeded at 1.0×10^5 cells per well in 2.0 ml DMEM, 10% FBS and 8 μg/ml polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the $MiCl_1$ cells and incubated overnight at 37°C, 10% CO_2. On day 15, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the $MiCl_1$ cells. Using these procedures, it can be shown that the HBV core producer cell lines are not contaminated with replication competent retroviruses.

2. Cocultivation of Producer Lines and MdH Marker Rescue Assay

As an alternate method to test for the presence of RCR in a vector-producing cell line, producer cells are cocultivated with an equivalent number of *Mus dunni* (NIH NIAID Bethesda, MD) cells. Small scale cocultivations are performed by mixing of 5.0 x 10^5 *Mus dunni* cells with 5.0 x 10^5 producer cells and seeding the mixture into 10 cm plates (10 ml standard culture media/plate, 4 μg/ml polybrene) at day 0. Every 3-4 days the cultures are split at a 1:10 ratio and 5.0 x 10^5 *Mus dunni* cells are added to each culture plate to effectively dilute out the producer cell line and provide maximum amplifcation of RCR. On day 14, culture supernatants are harvested, passed through a 0.45 μ cellulose-acetate filter, and tested in the MdH marker rescue assay. Large scale cocultivations are performed by seeding a mixture of 1.0 x 10^8 *Mus dunni* cells and 1.0 x 10^8 producer cells into a total of twenty T-150 flasks (30 ml standard culture media/flask, 4 μg/ml polybrene). Cultures are split at a ratio of 1:10 on days 3, 6, and 13 and at a ratio of 1:20 on day 9. On day 15, the final supernatants are harvested, filtered and a portion of each is tested in the MdH marker rescue assay.

The MdH marker rescue cell line is cloned from a pool of *Mus dunni* cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer *et al., PNAS 84*: 1055-1059, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One ml of test sample is added to a well of a 6-well plate containing 10^5 MdH cells in 2 ml standard culture medium (DMEM with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing 4 μg/ml polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a 0.45 μ cellulose-acetate filter and transferred to a well of a 6-well plate containing 5.0 x 10^4 *Mus dunni* target cells in 2 ml standard culture medium containing polybrene. After 24 hours, supernatants are replaced with standard culture media containing 250 μg/ml of hygromycin B and subsequently replaced on days 2 and 5 with media containing 200 μg/ml of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection, by staining with 0.2% Coomassie blue.

F. TRANSDUCTION OF HUMAN CELLS WITH KT-ND5-DCK VECTOR CONSTRUCT

On day one, HT1080 cells are set up at 2 x 10^4 cells per well in six well tissue culture plates containing 2 mls standard growth media (DME + 10% FBS). On day two, ND-5 FVIII retroviral vector particles from a confluent vector producing cell line are harvested as a HX-ND-5 clone. They are filtered through .45 μm syringe filters prior to testing the supernatants. (Alternatively the filtered media supernatants may be frozen at 80 in aliquots for later use.) Polybrene is added to each well such that the final concentration is 8 μg per ml. Thirty minutes later, either diluted or undiluted retroviral vector supernatant is added to duplicate wells. Typical volumes and dilutions are 0.5 ml per well and four or more 1:3 serial dilutions in growth media. As a control, two wells are transduced with the same volume of growth media only. On day three, the wells are refeed with 2mls of fresh media and the cells allowed to reach confluence, which may typically be about day four or five. On this day, the cells are again refeed with one ml per well fresh growth media. Twenty four hours later the media is harvested and filtered as above.

G. EXPRESSION OF TRANSDUCED VECTOR FOR FVIII

The expression of vector transduced human cells for FVIII is detected by the Coatest^R assay as described above in Example 2B1. Activity is assayed relative to supernatant from the control wells by counting the cells per well from the two control wells and normalizing FVIII expression data per 1x 10^6 cells per 24 hours.

H. ADMINISTRATION OF VECTOR CONSTRUCT

1. Animal Administration Protocol

The intestinal epithelium is an attractive site for gene delivery due to its rapidly proliferating tissue mass and the known location of stem cells in the crypts of Lieberkuhn. The deep location of the stem cells in the crypts and the protective role of the mucus gel layer, makes the retrovirus relatively inaccessible to the tissue cells. However, the accessibility of the retroviral vector to these stem cells can be improved in

animal models by the *in vivo* mucus removal method of Sandberg, J., *et al.*,(*Human Gene Therapy* 5:3232-329, 1994).

Male Sprague-Dawley rats obtained from Charles River Breeding Laboratories (Portage, MD.) are anesthetized and the cecum is identified upon opening the peritoneal cavity. A 3 cm ileal segment is isolated from the last Peyer's patch in the terminal ileum and ligated at each end. A plastic catheter attached to a syringe is inserted into the segment and two milliliters of the mucolytic agents dithiothreitol and N-acetyl-cysteine is instilled under mild pressure for two minutes, then removed. This procedure is repeated once again before filling the segment with 0.2 to 2.0 ml of retroviral vector particles at 10^6 to 10^{10} cfu/ml. The ligatures are removed 1 to 4 hours later and the abdominal cavity is sutured. Control animals are instilled with formulation buffer only.

Blood is collected from the tail vein and assayed for factor VIII production by a sandwich ELISA specific for human factor VIII (according to the modified procedure of Zatloukal, K., *et al., PNAS 91*:5148-5152, 1994). The ELISA is based on two Diagnostica). ESH 4 (25 µg/ml in 1.0 M $NaHCO_3$/0.5 M NaCl, pH 9.0) is coupled to the ELISA plates overnight at 4°C, washed with 0.1% Tween 20 in PBS, and blocked with 1% BSA in PBS. The samples are applied in 0.05 M Tris-HCl/1 M NaCl/2% BSA, pH 7.5, over 4 hr at room temperature, the plates are washed, and ESH 8 (2.5 µg/ml in 0.05 M Tris-HCl/1 M NaCl/2% BSA, pH 7.5,) which has been biotinylated with *N*- hydroxysuccinimidobiotin (Pierce, Rockford, IL.) is added for 2 hr at room temperature. The color reaction is performed with peroxidase-conjugated streptavidin (Boehringer Mannheim, Indianapolis, IN.) and o- phenylenediamine dihydrochloride as substrate. The human factor VIII:c standard (from the National Institute for Biological Standards and Control, Hertfordshire, U.K.) and normal rat plasma are used as references.

2. Human Administration Protocol

Lyophilized recombinant retrovirus containing the gene for Factor VIII expression is formulated into an enteric coated tablet or gel capsule according to known

methods in the art. These are described in the following patents: US 4,853,230, EP 225,189, AU 9,224,296, AU 9,230,801, and WO 92144,52.

The capsule is administered orally to be targeted to the jejunum. At 1 to 4 days following oral administration of the recombinant retrovirus, expression of Factor VIII is measured in the plasma and blood by the Coatest[R] Factor VIII assay.

EXAMPLE 12

PREPARATION OF RECOMBINANT RETROVIRUS FOR DELIVERY OF HUMAN GROWTH HORMONE

A. PREPARATION OF HGH CONTAINING VECTORS

Vector pDHF828 containing the full-length human growth hormone gene is constructed essentially as follows. Briefly, plasmid pDHF811, was constructed by removing the XhoI- ClaI fragment of the KT-1 retroviral vector described above, and inserting the following oligonucleotide linkers by ligation of the cohesive ends:

Linker sequences:

(SEQUENCE ID# 18) 5' TCGAGGATCC GCCCGGGCGG CCGCATCGAT GTCGACG 3'

(SEQUENCE ID# 19) 5' CGCGTCGA CATCGATGCG GCCGCCCGGG CGGATCC 3'

In particular, the linkers were annealed at 65°C for 20 minutes, 42°C for 20 minutes, 37°C for 20 minutes, and room temperature for 2 hours. The concentrations of both oligonucleotides was 18mM and the salt concentration was 100 mM NaCl. After annealing, 50ml of 1.8 mM annealed linker was digested with ClaI overnight to generate ClaI ends. For ligation, 3nM of KT-1 XhoI - ClaI fragment was mixed with 90nM of linker, and the resultant mixture incubated at 15°C for 3 hours. The ligated

DNA sample was transformed into DH-5α competent cells, followed by screening of transformants.

Plasmid chGH 800 containing the full length cDNA of the hGH gene (Martial, R.A. et al., *Science 205*:602, 1979) was digested with Hind III, blunt-ended with the Klenow fragment enzyme, and cloned into the SrfI site of pDHF811. The resultant plasmid was designated pDHF828.

The above vector is made in the KT-1 backbone that has no selectable marker. It can similarly be constructed in the pBA5 vector backbone (Figure 3). Briefly a selectable marker is introduced into it by cutting at a single *Cla* I site and introducing an expression cassete for the marker as was done for the neomycin marker in Example 1. In this way a cassette expressing any of placental alkaline phosphatase, deoxycytidine kinase, cytochrome P-450 or other suitable non-immunogenic marker / PAE can be introduced. The cassette has the cDNA linked to a promoter such as the SV40 promoter in Example 1 and no polyadenylation site. Other suitable internal promoters (*e.g.*, CMV from the pCI-PLAP vector in Example 4) can also be utilized. Such vectors are called pDHF828-PLAP and pDHF828-DCK.

B. PREPARATION OF HGH EXPRESSING RECOMBINANT RETROVIRUS

The pDHF828-DCK plasmid was then introduced into the HX packaging cell, using standard procedures and assayed using the HGH Chemiluminescence Kit (HGH 100T) (Nichols Institute, San Juan Capistrano, CA.), according to a preferred modification of the kit protocol. On day 1, the kit components were warmed to room temperature and gently mixed by inversion before opening any vials. Test samples were centrifuged for 5' at top speed in a microfuge before using them in order to remove fibrin and other debris. All samples were measured in quadruplicate, including the standards. The incubations are performed in 12 x17 polypropylene tubes that have been stored in the dark. One hundred fifty ul of sample or standard were aliquoted into each tube and ul of antibody is added and the samples were mixed gently. One bead was added to each well using the forceps provided in the kit. The tubes were capped, covered with foil, and shaken on an orbital shaker for 24 hr at room temperature.

Standards contain 530 pg/ml (STD D), and serial dilutions were made in zero standard of Std D of 250, 100, 50, 25, 10, 5, and 2.5 pg/ml.

After 24 hours, the tubes were uncapped and 0.5 ml of wash buffer were added. These wash solution was added with enough force to make the bead bounce up off the bottom of the tube. The samples were washed three times with 2.0 ml nanopure water, and aspirated completely each time. The luminometer determinations were done in 12x75 polycarbonate (clear plastic) tubes stored in the dark. The luminometer was pretested with performance control standards.

Using this assay, HX/HGH-DCK retroviral vector producing cell lines were generated with titers of 4.8×10^6 cfu/ml. Introduction of the plasmid into DX packaging cells resulted in production of clonal producer cells with a titer of 1.6×10^7 cfu/ml.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Chada, Sunil

(ii) TITLE OF INVENTION: NON-IMMUNOGENIC PRODRUGS AND SELECTABLE MARKERS FOR USE IN GENE THERAPY

(iii) NUMBER OF SEQUENCES: 32

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(A) APPLICATION NUMBER: US
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(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 930049.459

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8332 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCCAGTCC TCCGATTGAC TGAGTCGCCC GGGTACCCGT GTATCCAATA AACCCTCTTG 60

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TACCCGTCAG CGGGGGTCTT TCATTTGGGG GCTCGTCCGG GATCGGGAGA CCCCTGCCCA 180

GGGACCACCG ACCCACCACC GGGAGGTAAG CTGGCCAGCA ACTTATCTGT GTCTGTCCGA 240

TTGTCTAGTG TCTATGACTG ATTTTATGCG CCTGCGTCGG TACTAGTTAG CTAACTAGCT 300

CTGTATCTGG CGGACCCGTG GTGGAACTGA CGAGTTCGGA ACACCCGGCC GCAACCCTGG 360

GAGACGTCCC AGGGACTTCG GGGGCCGTTT TTGTGGCCCG ACCTGAGTCC AAAAATCCCG 420

ATCGTTTTGG ACTCTTTGGT GCACCCCCCT TAGAGGAGGG ATATGTGGTT CTGGTAGGAG 480

ACGAGAACCT AAAACAGTTC CCGCCTCCGT CTGAATTTTT GCTTTCGGTT TGGGACCGAA 540

GCCGCGCCGC GCGTCTTGTC TGCTGCAGCA TCGTTCTGTG TTGTCTCTGT CTGACTGTGT 600

TTCTGTATTT GTCTGAGAAT ATGGGCCAGA CTGTTACCAC TCCCTTAAGT TTGACCTTAG 660

GTCACTGGAA AGATGTCGAG CGGATCGCTC ACAACCAGTC GGTAGATGTC AAGAAGAGAC 720

GTTGGGTTAC CTTCTGCTCT GCAGAATGGC CAACCTTTAA CGTCGGATGG CCGCGAGACG 780

GCACCTTTAA CCGAGACCTC ATCACCCAGG TTAAGATCAA GGTCTTTTCA CCTGGCCCGC 840

ATGGACACCC AGACCAGGTC CCCTACATCG TGACCTGGGA AGCCTTGGCT TTTGACCCCC 900

CTCCCTGGGT CAAGCCCTTT GTACACCCTA AGCCTCCGCC TCCTCTTCCT CCATCCGCCC 960

CGTCTCTCCC CCTTGAACCT CCTCGTTCGA CCCCGCCTCG ATCCTCCCTT TATCCAGCCC 1020

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CTCTGATCGA GTCTGTTCTC ATCACCCATC AGCCCACCTG GGACGACTGT CAGCAGCTGT 1440

TGGGGACTCT GCTGACCGGA GAAGAAAAAC AACGGGTGCT CTTAGAGGCT AGAAAGGCGG 1500

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TCGAGCGCCC AGACTGGGAT TACACCACCC AGGCAGGTAG GAACCACCTA GTCCACTATC 1620

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TAAAAGGAAT AACACAAGGG CCCAATGAGT CTCCCTCGGC CTTCCTAGAG AGACTTAAGG 1740

AAGCCTATCG CAGGTACACT CCTTATGACC CTGAGGACCC AGGGCAAGAA ACTAATGTGT 1800

CTATGTCTTT CATTTGGCAG TCTGCCCCAG ACATTGGGAG AAAGTTAGAG AGGTTAGAAG 1860

ATTTAAAAAA CAAGACGCTT GGAGATTTGG TTAGAGAGGC AGAAAAGATC TTTAATAAAC 1920

GAGAAACCCC GGAAGAAAGA GAGGAACGTA TCAGGAGAGA AACAGAGGAA AAAGAAGAAC 1980

GCCGTAGGAC AGAGGATGAG CAGAAAGAGA AAGAAAGAGA TCGTAGGAGA CATAGAGAGA 2040

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CCCGCGAGGC CCCCTAATCC CCTTAATTCT TCTGATGCTC AGAGGGGTCA GTACTGCTTC 5880

GCCCGGCTCC AGTCCTCATC AAGTCTATAA TATCACCTGG GAGGTAACCA ATGGAGATCG 5940

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ACTCAAGCTA GACCAGACAA CTCATAAATC AAATGAGGGA TTTTATGTTT GCCCCGGGCC 6240

CCACCGCCCC CGAGAATCCA AGTCATGTGG GGGTCCAGAC TCCTTCTACT GTGCCTATTG 6300

GGGCTGTGAG ACAACCGGTA GAGCTTACTG GAAGCCCTCC TCATCATGGG ATTTCATCAC 6360

AGTAAACAAC AATCTCACCT CTGACCAGGC TGTCCAGGTA TGCAAAGATA ATAAGTGGTG 6420

CAACCCCTTA GTTATTCGGT TTACAGACGC CGGGAGACGG GTTACTTCCT GGACCACAGG 6480

ACATTACTGG GGCTTACGTT TGTATGTCTC CGGACAAGAT CCAGGGCTTA CATTTGGGAT 6540

CCGACTCAGA TACCAAAATC TAGGACCCCG CGTCCCAATA GGGCCAAACC CCGTTCTGGC 6600

AGACCAACAG CCACTCTCCA AGCCCAAACC TGTTAAGTCG CCTTCAGTCA CCAAACCACC 6660

CAGTGGGACT CCTCTCTCCC CTACCCAACT TCCACCGGCG GGAACGGAAA ATAGGCTGCT 6720

AAACTTAGTA GACGGAGCCT ACCAAGCCCT CAACCTCACC AGTCCTGACA AAACCCAAGA 6780

GTGCTGGTTG TGTCTAGTAG CGGGACCCCC CTACTACGAA GGGGTTGCCG TCCTGGGTAC 6840

CTACTCCAAC CATACCTCTG CTCCAGCCAA CTGCTCCGTG GCCTCCCAAC ACAAGTTGAC 6900

CCTGTCCGAA GTGACCGGAC AGGGACTCTG CATAGGAGCA GTTCCCAAAA CACATCAGGC 6960

CCTATGTAAT ACCACCCAGA CAAGCAGTCG AGGGTCCTAT TATCTAGTTG CCCCTACAGG 7020

TACCATGTGG GCTTGTAGTA CCGGGCTTAC TCCATGCATC TCCACCACCA TACTGAACCT 7080

TACCACTGAT TATTGTGTTC TTGTCGAACT CTGGCCAAGA GTCACCTATC ATTCCCCCAG 7140

CTATGTTTAC GGCCTGTTTG AGAGATCCAA CCGACACAAA AGAGAACCGG TGTCGTTAAC 7200

CCTGGCCCTA TTATTGGGTG GACTAACCAT GGGGGGAATT GCCGCTGGAA TAGGAACAGG 7260

GACTACTGCT CTAATGGCCA CTCAGCAATT CCAGCAGCTC CAAGCCGCAG TACAGGATGA 7320

TCTCAGGGAG GTTGAAAAAT CAATCTCTAA CCTAGAAAAG TCTCTCACTT CCCTGTCTGA 7380

AGTTGTCCTA CAGAATCGAA GGGGCCTAGA CTTGTTATTT CTAAAAGAAG GAGGGCTGTG 7440

TGCTGCTCTA AAAGAAGAAT GTTGCTTCTA TGCGGACCAC ACAGGACTAG TGAGAGACAG 7500

CATGGCCAAA TTGAGAGAGA GGCTTAATCA GAGACAGAAA CTGTTTGAGT CAACTCAAGG 7560

ATGGTTTGAG GGACTGTTTA ACAGATCCCC TTGGTTTACC ACCTTGATAT CTACCATTAT 7620

GGGACCCCTC ATTGTACTCC TAATGATTTT GCTCTTCGGA CCCTGCATTC TTAATCGATT 7680

AGTCCAATTT GTTAAAGACA GGATATCAGT GGTCCAGGCT CTAGTTTTGA CTCAACAATA 7740

TCACCAGCTG AAGCCTATAG AGTACGAGCC ATAGATAAAA TAAAAGATTT TATTTAGTCT 7800

CCAGAAAAAG GGGGGAATGA AAGACCCCAC CTGTAGGTTT GGCAAGCTAG CTTAAGTAAC 7860

GCCATTTTGC AAGGCATGGA AAAATACATA ACTGAGAATA GAGAAGTTCA GATCAAGGTC 7920

AGGAACAGAT GGAACAGCTG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG 7980

CCCCGGCTCA GGGCCAAGAA CAGATGGAAC AGCTGAATAT GGGCCAAACA GGATATCTGT 8040

GGTAAGCAGT TCCTGCCCCG GCTCAGGGCC AAGAACAGAT GGTCCCCAGA TGCGGTCCAG 8100

CCCTCAGCAG TTTCTAGAGA ACCATCAGAT GTTTCCAGGG TGCCCCAAGG ACCTGAAATG 8160

ACCCTGTGCC TTATTTGAAC TAACCAATCA GTTCGCTTCT CGCTTCTGTT CGCGCGCTTC 8220

TGCTCCCCGA GCTCAATAAA AGAGCCCACA ACCCCTCACT CGGGGCGCCA GTCCTCCGAT 8280

TGACTGAGTC GCCCGGGTAC CCGTGTATCC AATAAACCCT CTTGCAGTTG CA 8332

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTAACAGTC TGGCCCGAAT TCTCAGACAA ATACAG 36

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGTATTTGT CTGAGAATTA AGGCTAGACT GTTACCAC 38

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCGAGGATCG CGCCGGGCGG CCGCATCGAT GTCGACG 37

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCGTCGACA TCGATGCGGC CGCCCGGGCG GATCC 35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGGGCTCG AGATGACGCG CGGCTTGCGC AATGC 35

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGGGGATCG ATTTCATCAT CATACA 26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGGGGCTCG AGGGCACCAT GGAGCTCAG 29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGGGGATCG ATCCCTCAGA AGCTGGTGTG 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCCCCCTCG AGACCTGTCC CCCCCATGGA G 31

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCCCCATCG ATGTGGGCTC ACAGAGGGCG C 31

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATAAAGGCGG TCCTTTCAGG GGGCAGCCCC TTCTA 35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGGCTGCCC CCTGAAAGGA CCGCCTTTAT CAAAG 35

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGGGGCTCG AGCCCCGACA CCGCGGCGGG CCG 33

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGGGGATCG ATGCTGAAGT ATCTGGAACC 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGGGGGCTCG AGAACAACAT CACCATGACA 30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGGGGATCG ATTCANACDA TNGCYCTRAA 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCGAGGATCC GCCCGGGCGG CCGCATCGAT GTCGACG 37

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGCGTCGACA TCGATGCGGC CGCCCGGGCG GATCC 35

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 61..2091

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAATTCGGGC GCGAAGCGGC CGGCCTGGGC GCCGACTGCA GAGCCGGGAG GCTGGTGGTC 60

ATG CCG GGG TTC CTG GTT CGC ATC CTC CTT CTG CTG CTG GTT CTG CTG 108
Met Pro Gly Phe Leu Val Arg Ile Leu Leu Leu Leu Val Leu Leu
1 5 10 15

CTT CTG GGC CCT ACG CGC GGC TTG CGC AAT GCC ACC CAG AGG ATG TTT 156
Leu Leu Gly Pro Thr Arg Gly Leu Arg Asn Ala Thr Gln Arg Met Phe
20 25 30

GAA ATT GAC TAT AGC CGG GAC TCC TTC CTC AAG GAT GGC CAG CCA TTT 204
Glu Ile Asp Tyr Ser Arg Asp Ser Phe Leu Lys Asp Gly Gln Pro Phe
35 40 45

CGC TAC ATC TCA GGA AGC ATT CAC TAC TCC CGT GTG CCC CGC TTC TAC 252
Arg Tyr Ile Ser Gly Ser Ile His Tyr Ser Arg Val Pro Arg Phe Tyr
50 55 60

TGG AAG GAC CGG CTG CTG AAG ATG AAG ATG GCT GGG CTG AAC GCC ATC 300
Trp Lys Asp Arg Leu Leu Lys Met Lys Met Ala Gly Leu Asn Ala Ile

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65                  70                  75                  80

CAG ACG TAT GTG CCC TGG AAC TTT CAT GAG CCC TGG CCA GGA CAG TAC      348
Gln Thr Tyr Val Pro Trp Asn Phe His Glu Pro Trp Pro Gly Gln Tyr
                 85                  90                  95

CAG TTT TCT GAG GAC CAT GAT GTG GAA TAT TTT CTT CGG CTG GCT CAT      396
Gln Phe Ser Glu Asp His Asp Val Glu Tyr Phe Leu Arg Leu Ala His
            100                 105                 110

GAG CTG GGA CTG CTG GTT ATC CTG AGG CCC GGG CCC TAC ATC TGT GCA      444
Glu Leu Gly Leu Leu Val Ile Leu Arg Pro Gly Pro Tyr Ile Cys Ala
        115                 120                 125

GAG TGG GAA ATG GGA GGA TTA CCT GCT TGG CTG CTA GAG AAA GAG TCT      492
Glu Trp Glu Met Gly Gly Leu Pro Ala Trp Leu Leu Glu Lys Glu Ser
    130                 135                 140

ATT CTT CTC CGC TCC TCC GAC CCA GAT TAC CTG GCA GCT GTG GAC AAG      540
Ile Leu Leu Arg Ser Ser Asp Pro Asp Tyr Leu Ala Ala Val Asp Lys
145                 150                 155                 160

TGG TTG GGA GTC CTT CTG CCC AAG ATG AAG CCT CTC CTC TAT CAG AAT      588
Trp Leu Gly Val Leu Leu Pro Lys Met Lys Pro Leu Leu Tyr Gln Asn
                165                 170                 175

GGA GGG CCA GTT ATA ACA GTG CAG GTT GAA AAT GAA TAT GGC AGC TAC      636
Gly Gly Pro Val Ile Thr Val Gln Val Glu Asn Glu Tyr Gly Ser Tyr
            180                 185                 190

TTT GCC TGT GAT TTT GAC TAC CTG CGC TTC CTG CAG AAG CGC TTT CGC      684
Phe Ala Cys Asp Phe Asp Tyr Leu Arg Phe Leu Gln Lys Arg Phe Arg
        195                 200                 205

CAC CAT CTG GGG GAT GAT GTG GTT CTG TTT ACC ACT GAT GGA GCA CAT      732
His His Leu Gly Asp Asp Val Val Leu Phe Thr Thr Asp Gly Ala His
    210                 215                 220

AAA ACA TTC CTG AAA TGT GGG GCC CTG CAG GGC CTC TAC ACC ACG GTG      780
Lys Thr Phe Leu Lys Cys Gly Ala Leu Gln Gly Leu Tyr Thr Thr Val
225                 230                 235                 240

GAC TTT GGA ACA GGC AGC AAC ATC ACA GAT GCT TTC CTA AGC CAG AGG      828
Asp Phe Gly Thr Gly Ser Asn Ile Thr Asp Ala Phe Leu Ser Gln Arg
                245                 250                 255

AAG TGT GAG CCC AAA GGA CCC TTG ATC AAT TCT GAA TTC TAT ACT GGC      876
Lys Cys Glu Pro Lys Gly Pro Leu Ile Asn Ser Glu Phe Tyr Thr Gly
            260                 265                 270

TGG CTA GAT CAC TGG GGC CAA CCT CAC TCC ACA ATC AAG ACC GAA GCA      924
Trp Leu Asp His Trp Gly Gln Pro His Ser Thr Ile Lys Thr Glu Ala
        275                 280                 285

GTG GCT TCC TCC CTC TAT GAT ATA CTT GCC CGT GGG GCG AGT GTG AAC      972
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Val Ala Ser Ser Leu Tyr Asp Ile Leu Ala Arg Gly Ala Ser Val Asn
    290                 295                 300

TTG TAC ATG TTT ATA GGT GGG ACC AAT TTT GCC TAT TGG AAT GGG GCC      1020
Leu Tyr Met Phe Ile Gly Gly Thr Asn Phe Ala Tyr Trp Asn Gly Ala
305                 310                 315                 320

AAC TCA CCC TAT GCA GCA CAG CCC ACC AGC TAC GAC TAT GAT GCC CCA      1068
Asn Ser Pro Tyr Ala Ala Gln Pro Thr Ser Tyr Asp Tyr Asp Ala Pro
                325                 330                 335

CTG AGT GAG GCT GGG GAC CTC ACT GAG AAG TAT TTT GCT CTG CGA AAC      1116
Leu Ser Glu Ala Gly Asp Leu Thr Glu Lys Tyr Phe Ala Leu Arg Asn
            340                 345                 350

ATC ATC CAG AAG TTT GAA AAA GTA CCA GAA GGT CCT ATC CCT CCA TCT      1164
Ile Ile Gln Lys Phe Glu Lys Val Pro Glu Gly Pro Ile Pro Pro Ser
        355                 360                 365

ACA CCA AAG TTT GCA TAT GGA AAG GTC ACT TTG GAA AAG TTA AAG ACA      1212
Thr Pro Lys Phe Ala Tyr Gly Lys Val Thr Leu Glu Lys Leu Lys Thr
    370                 375                 380

GTG GGA GCA GCT CTG GAC ATT CTG TGT CCC TCT GGG CCC ATC AAA AGC      1260
Val Gly Ala Ala Leu Asp Ile Leu Cys Pro Ser Gly Pro Ile Lys Ser
385                 390                 395                 400

CTT TAT CCC TTG ACA TTT ATC CAG GTG AAA CAG CAT TAT GGG TTT GTG      1308
Leu Tyr Pro Leu Thr Phe Ile Gln Val Lys Gln His Tyr Gly Phe Val
                405                 410                 415

CTG TAC CGG ACA ACA CTT CCT CAA GAT TGC AGC AAC CCA GCA CCT CTC      1356
Leu Tyr Arg Thr Thr Leu Pro Gln Asp Cys Ser Asn Pro Ala Pro Leu
            420                 425                 430

TCT TCA CCC CTC AAT GGA GTC CAC GAT CGA GCA TAT GTT GCT GTG GAT      1404
Ser Ser Pro Leu Asn Gly Val His Asp Arg Ala Tyr Val Ala Val Asp
        435                 440                 445

GGG ATC CCC CAG GGA GTC CTT GAG CGA AAC AAT GTG ATC ACT CTG AAC      1452
Gly Ile Pro Gln Gly Val Leu Glu Arg Asn Asn Val Ile Thr Leu Asn
    450                 455                 460

ATA ACA GGG AAA GCT GGA GCC ACT CTG GAC CTT CTG GTA GAG AAC ATG      1500
Ile Thr Gly Lys Ala Gly Ala Thr Leu Asp Leu Leu Val Glu Asn Met
465                 470                 475                 480

GGA CGT GTG AAC TAT GGT GCA TAT ATC AAC GAT TTT AAG GGT TTG GTT      1548
Gly Arg Val Asn Tyr Gly Ala Tyr Ile Asn Asp Phe Lys Gly Leu Val
                485                 490                 495

TCT AAC CTG ACT CTC AGT TCC AAT ATC CTC ACG GAC TGG ACG ATC TTT      1596
Ser Asn Leu Thr Leu Ser Ser Asn Ile Leu Thr Asp Trp Thr Ile Phe
            500                 505                 510
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CCA CTG GAC ACT GAG GAT GCA GTG CGC AGC CAC CTG GGG GGC TGG GGA      1644
Pro Leu Asp Thr Glu Asp Ala Val Arg Ser His Leu Gly Gly Trp Gly
        515                 520                 525

CAC CGT GAC AGT GGC CAC CAT GAT GAA GCC TGG GCC CAC AAC TCA TCC      1692
His Arg Asp Ser Gly His His Asp Glu Ala Trp Ala His Asn Ser Ser
    530                 535                 540

AAC TAC ACG CTC CCG GCC TTT TAT ATG GGG AAC TTC TCC ATT CCC AGT      1740
Asn Tyr Thr Leu Pro Ala Phe Tyr Met Gly Asn Phe Ser Ile Pro Ser
545                 550                 555                 560

GGG ATC CCA GAC TTG CCC CAG GAC ACC TTT ATC CAG TTT CCT GGA TGG      1788
Gly Ile Pro Asp Leu Pro Gln Asp Thr Phe Ile Gln Phe Pro Gly Trp
                565                 570                 575

ACC AAG GGC CAG GTC TGG ATT AAT GGC TTT AAC CTT GGC CGC TAT TGG      1836
Thr Lys Gly Gln Val Trp Ile Asn Gly Phe Asn Leu Gly Arg Tyr Trp
            580                 585                 590

CCA GCC CGG GGC CCT CAG TTG ACC TTG TTT GTG CCC CAG CAC ATC CTG      1884
Pro Ala Arg Gly Pro Gln Leu Thr Leu Phe Val Pro Gln His Ile Leu
        595                 600                 605

ATG ACC TCG GCC CCA AAC ACC ATC ACC GTG CTG GAA CTG GAG TGG GCA      1932
Met Thr Ser Ala Pro Asn Thr Ile Thr Val Leu Glu Leu Glu Trp Ala
    610                 615                 620

CCC TGC AGC AGT GAT GAT CCA GAA CTA TGT GCT GTG ACG TTC GTG GAC      1980
Pro Cys Ser Ser Asp Asp Pro Glu Leu Cys Ala Val Thr Phe Val Asp
625                 630                 635                 640

AGG CCA GTT ATT GGC TCA TCT GTG ACC TAC GAT CAT CCC TCC AAA CCT      2028
Arg Pro Val Ile Gly Ser Ser Val Thr Tyr Asp His Pro Ser Lys Pro
                645                 650                 655

GTT GAA AAA AGA CTC ATG CCC CCA CCC CCG CAA AAA AAC AAA GAT TCA      2076
Val Glu Lys Arg Leu Met Pro Pro Pro Pro Gln Lys Asn Lys Asp Ser
            660                 665                 670

TGG CTG GAC CAT GTA TGATGATGAA AGCCTGTGTC TTTGAGGGAT TCTACCCTGA      2131
Trp Leu Asp His Val
        675

ACATACCTCA CAGATCCTCC CTGTCATGCC ACATTTCACT GATTGGAATG TGGAAATGGA     2191

AAAGGAATTT AGGATGTGCA TTTTCACCTG AGGTTTCCCT GCATCCCTGC AGTGCCAAAG     2251

CCCCACCTTC AGGGACCACC TGGAATGTGT GAGGGGCTGA CAGCACAGTA ACGTGCATAC     2311

ATATCTGCAG GGCTGGAATG GAAGCTTTAA AGGTGGTAGT GATTTTTATT TTGGAAGAAT     2371

CATGTTACCT TTTTGTTAAA TAAAATTTGC CCGAATTC                             2409
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 677 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Pro Gly Phe Leu Val Arg Ile Leu Leu Leu Leu Leu Val Leu Leu
  1               5                  10                  15

Leu Leu Gly Pro Thr Arg Gly Leu Arg Asn Ala Thr Gln Arg Met Phe
            20                  25                  30

Glu Ile Asp Tyr Ser Arg Asp Ser Phe Leu Lys Asp Gly Gln Pro Phe
        35                  40                  45

Arg Tyr Ile Ser Gly Ser Ile His Tyr Ser Arg Val Pro Arg Phe Tyr
    50                  55                  60

Trp Lys Asp Arg Leu Leu Lys Met Lys Met Ala Gly Leu Asn Ala Ile
65                  70                  75                  80

Gln Thr Tyr Val Pro Trp Asn Phe His Glu Pro Trp Pro Gly Gln Tyr
                85                  90                  95

Gln Phe Ser Glu Asp His Asp Val Glu Tyr Phe Leu Arg Leu Ala His
            100                 105                 110

Glu Leu Gly Leu Leu Val Ile Leu Arg Pro Gly Pro Tyr Ile Cys Ala
        115                 120                 125

Glu Trp Glu Met Gly Gly Leu Pro Ala Trp Leu Leu Glu Lys Glu Ser
    130                 135                 140

Ile Leu Leu Arg Ser Ser Asp Pro Asp Tyr Leu Ala Ala Val Asp Lys
145                 150                 155                 160

Trp Leu Gly Val Leu Leu Pro Lys Met Lys Pro Leu Leu Tyr Gln Asn
                165                 170                 175

Gly Gly Pro Val Ile Thr Val Gln Val Glu Asn Glu Tyr Gly Ser Tyr
            180                 185                 190

Phe Ala Cys Asp Phe Asp Tyr Leu Arg Phe Leu Gln Lys Arg Phe Arg
        195                 200                 205

His His Leu Gly Asp Asp Val Val Leu Phe Thr Thr Asp Gly Ala His
    210                 215                 220

Lys Thr Phe Leu Lys Cys Gly Ala Leu Gln Gly Leu Tyr Thr Thr Val
225                 230                 235                 240
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Asp Phe Gly Thr Gly Ser Asn Ile Thr Asp Ala Phe Leu Ser Gln Arg
                245                 250                 255

Lys Cys Glu Pro Lys Gly Pro Leu Ile Asn Ser Glu Phe Tyr Thr Gly
            260                 265                 270

Trp Leu Asp His Trp Gly Gln Pro His Ser Thr Ile Lys Thr Glu Ala
        275                 280                 285

Val Ala Ser Ser Leu Tyr Asp Ile Leu Ala Arg Gly Ala Ser Val Asn
    290                 295                 300

Leu Tyr Met Phe Ile Gly Gly Thr Asn Phe Ala Tyr Trp Asn Gly Ala
305                 310                 315                 320

Asn Ser Pro Tyr Ala Ala Gln Pro Thr Ser Tyr Asp Tyr Asp Ala Pro
                325                 330                 335

Leu Ser Glu Ala Gly Asp Leu Thr Glu Lys Tyr Phe Ala Leu Arg Asn
            340                 345                 350

Ile Ile Gln Lys Phe Glu Lys Val Pro Glu Gly Pro Ile Pro Pro Ser
        355                 360                 365

Thr Pro Lys Phe Ala Tyr Gly Lys Val Thr Leu Glu Lys Leu Lys Thr
    370                 375                 380

Val Gly Ala Ala Leu Asp Ile Leu Cys Pro Ser Gly Pro Ile Lys Ser
385                 390                 395                 400

Leu Tyr Pro Leu Thr Phe Ile Gln Val Lys Gln His Tyr Gly Phe Val
                405                 410                 415

Leu Tyr Arg Thr Thr Leu Pro Gln Asp Cys Ser Asn Pro Ala Pro Leu
            420                 425                 430

Ser Ser Pro Leu Asn Gly Val His Asp Arg Ala Tyr Val Ala Val Asp
        435                 440                 445

Gly Ile Pro Gln Gly Val Leu Glu Arg Asn Asn Val Ile Thr Leu Asn
    450                 455                 460

Ile Thr Gly Lys Ala Gly Ala Thr Leu Asp Leu Leu Val Glu Asn Met
465                 470                 475                 480

Gly Arg Val Asn Tyr Gly Ala Tyr Ile Asn Asp Phe Lys Gly Leu Val
                485                 490                 495

Ser Asn Leu Thr Leu Ser Ser Asn Ile Leu Thr Asp Trp Thr Ile Phe
            500                 505                 510

Pro Leu Asp Thr Glu Asp Ala Val Arg Ser His Leu Gly Gly Trp Gly
        515                 520                 525
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His Arg Asp Ser Gly His His Asp Glu Ala Trp Ala His Asn Ser Ser
    530                 535                 540

Asn Tyr Thr Leu Pro Ala Phe Tyr Met Gly Asn Phe Ser Ile Pro Ser
545                 550                 555                 560

Gly Ile Pro Asp Leu Pro Gln Asp Thr Phe Ile Gln Phe Pro Gly Trp
                565                 570                 575

Thr Lys Gly Gln Val Trp Ile Asn Gly Phe Asn Leu Gly Arg Tyr Trp
            580                 585                 590

Pro Ala Arg Gly Pro Gln Leu Thr Leu Phe Val Pro Gln His Ile Leu
        595                 600                 605

Met Thr Ser Ala Pro Asn Thr Ile Thr Val Leu Glu Leu Glu Trp Ala
    610                 615                 620

Pro Cys Ser Ser Asp Asp Pro Glu Leu Cys Ala Val Thr Phe Val Asp
625                 630                 635                 640

Arg Pro Val Ile Gly Ser Ser Val Thr Tyr Asp His Pro Ser Lys Pro
                645                 650                 655

Val Glu Lys Arg Leu Met Pro Pro Pro Pro Gln Lys Asn Lys Asp Ser
            660                 665                 670

Trp Leu Asp His Val
        675
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1634 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 13..1617

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
TGCCCTCCAG AC ATG CTG GGG CCC TGC ATG CTG CTG CTG CTG CTG CTG          48
              Met Leu Gly Pro Cys Met Leu Leu Leu Leu Leu Leu
                1               5                  10

CTG GGC CTG AGG CTA CAG CTC TCC CTG GGC ATC ATC CCA GTT GAG GAG        96
Leu Gly Leu Arg Leu Gln Leu Ser Leu Gly Ile Ile Pro Val Glu Glu
        15                  20                  25
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GAG AAC CCG GAC TTC TGG AAC CGC GAG GCA GCC GAG GCC CTG GGT GCC        144
Glu Asn Pro Asp Phe Trp Asn Arg Glu Ala Ala Glu Ala Leu Gly Ala
     30                  35                  40

GCC AAG AAG CTG CAG CCT GCA CAG ACA GCC GCC AAG AAC CTC ATC ATC        192
Ala Lys Lys Leu Gln Pro Ala Gln Thr Ala Ala Lys Asn Leu Ile Ile
 45                  50                  55                  60

TTC CTG GGC GAT GGG ATG GGG GTG TCT ACG GTG ACA GCT GCC AGG ATC        240
Phe Leu Gly Asp Gly Met Gly Val Ser Thr Val Thr Ala Ala Arg Ile
                 65                  70                  75

CTA AAA GGG CAG AAG AAG GAC AAA CTG GGG CCT GAG ATA CCC CTG GCC        288
Leu Lys Gly Gln Lys Lys Asp Lys Leu Gly Pro Glu Ile Pro Leu Ala
             80                  85                  90

ATG GAC CGC TTC CCA TAT GTG GCT CTG TCC AAG ACA TAC AAT GTA GAC        336
Met Asp Arg Phe Pro Tyr Val Ala Leu Ser Lys Thr Tyr Asn Val Asp
         95                  100                 105

AAA CAT GTG CCA GAC AGT GGA GCC ACA GCC ACG GCC TAC CTG TGC GGG        384
Lys His Val Pro Asp Ser Gly Ala Thr Ala Thr Ala Tyr Leu Cys Gly
    110                 115                 120

GTC AAG GGC AAC TTC CAG ACC ATT GGC TTG AGT GCA GCC GCC CGC TTT        432
Val Lys Gly Asn Phe Gln Thr Ile Gly Leu Ser Ala Ala Ala Arg Phe
125                 130                 135                 140

AAC CAG TGC AAC ACG ACA CGC GGC AAC GAG GTC ATC TCC GTG ATG AAT        480
Asn Gln Cys Asn Thr Thr Arg Gly Asn Glu Val Ile Ser Val Met Asn
                145                 150                 155

CGG GCC AAG AAA GCA GGG AAG TCA GTG GGA GTG GTA ACC ACC ACA CGA        528
Arg Ala Lys Lys Ala Gly Lys Ser Val Gly Val Val Thr Thr Thr Arg
            160                 165                 170

GTG CAG CAC GCC TCG CCA GCC GGC ACC TAC GCC CAC ACG GTG AAC CGC        576
Val Gln His Ala Ser Pro Ala Gly Thr Tyr Ala His Thr Val Asn Arg
        175                 180                 185

AAC TGG TAC TCG GAC GCC GAC GTG CCT GCC TCG GCC CGC CAG GAG GGG        624
Asn Trp Tyr Ser Asp Ala Asp Val Pro Ala Ser Ala Arg Gln Glu Gly
    190                 195                 200

TGC CAG GAC ATC GCT ACG CAG CTC ATC TCC AAC ATG GAC ATT GAC GTG        672
Cys Gln Asp Ile Ala Thr Gln Leu Ile Ser Asn Met Asp Ile Asp Val
205                 210                 215                 220

ATC CTA GGT GGA GGC CGA AAG TAC ATG TTT CCC ATG GGA ACC CCA GAC        720
Ile Leu Gly Gly Gly Arg Lys Tyr Met Phe Pro Met Gly Thr Pro Asp
                225                 230                 235

CCT GAG TAC CCA GAT GAC TAC AGC CAA GGT GGG ACC AGG CTG GAC GGG        768
Pro Glu Tyr Pro Asp Asp Tyr Ser Gln Gly Gly Thr Arg Leu Asp Gly
            240                 245                 250
```

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AAG AAT CTG GTG CAG GAA TGG CTG GCG AAG CGC CAG GGT GCC CGG TAT        816
Lys Asn Leu Val Gln Glu Trp Leu Ala Lys Arg Gln Gly Ala Arg Tyr
        255                 260                 265

GTG TGG AAC CGC ACT GAG CTC ATG CAG GCT TCC CTG GAC CCG TCT GTG        864
Val Trp Asn Arg Thr Glu Leu Met Gln Ala Ser Leu Asp Pro Ser Val
    270                 275                 280

ACC CAT CTC ATG GGT CTC TTT GAG CCT GGA GAC ATG AAA TAC GAG ATC        912
Thr His Leu Met Gly Leu Phe Glu Pro Gly Asp Met Lys Tyr Glu Ile
285                 290                 295                 300

CAC CGA GAC TCC ACA CTG GAC CCC TCC CTG ATG GAG ATG ACA GAG GCT        960
His Arg Asp Ser Thr Leu Asp Pro Ser Leu Met Glu Met Thr Glu Ala
                305                 310                 315

GCC CTG CGC CTG CTG AGC AGG AAC CCC CGC GGC TTC TTC CTC TTC GTG       1008
Ala Leu Arg Leu Leu Ser Arg Asn Pro Arg Gly Phe Phe Leu Phe Val
            320                 325                 330

GAG GGT GGT CGC ATC GAC CAT GGT CAT CAT GAA AGC AGG GCT TAC CGG       1056
Glu Gly Gly Arg Ile Asp His Gly His His Glu Ser Arg Ala Tyr Arg
        335                 340                 345

GCA CTG ACT GAG ACG ATC ATG TTC GAC GAC GCC ATT GAG AGG GCG GGC       1104
Ala Leu Thr Glu Thr Ile Met Phe Asp Asp Ala Ile Glu Arg Ala Gly
    350                 355                 360

CAG CTC ACC AGC GAG GAG GAC ACG CTG AGC CTC GTC ACT GCC GAC CAC       1152
Gln Leu Thr Ser Glu Glu Asp Thr Leu Ser Leu Val Thr Ala Asp His
365                 370                 375                 380

TCC CAC GTC TTC TCC TTC GGA GGC TAC CCC CTG CGA GGG AGC TCC ATC       1200
Ser His Val Phe Ser Phe Gly Gly Tyr Pro Leu Arg Gly Ser Ser Ile
                385                 390                 395

TTC GGG CTG GCC CCT GGC AAG GCC CGG GAC AGG AAG GCC TAC ACG GTC       1248
Phe Gly Leu Ala Pro Gly Lys Ala Arg Asp Arg Lys Ala Tyr Thr Val
            400                 405                 410

CTC CTA TAC GGA AAC GGT CCA GGC TAT GTG CTC AAG GAC GGC GCC CGG       1296
Leu Leu Tyr Gly Asn Gly Pro Gly Tyr Val Leu Lys Asp Gly Ala Arg
        415                 420                 425

CCG GAT GTT ACC GAG AGC GAG AGC GGG AGC CCC GAG TAT CGG CAG CAG       1344
Pro Asp Val Thr Glu Ser Glu Ser Gly Ser Pro Glu Tyr Arg Gln Gln
    430                 435                 440

TCA GCA GTG CCC CTG GAC GAA GAG ACC CAC GCA GGC GAG GAC GTG GCG       1392
Ser Ala Val Pro Leu Asp Glu Glu Thr His Ala Gly Glu Asp Val Ala
445                 450                 455                 460

GTG TTC GCG CGC GGC CCG CAG GCG CAC CTG GTT CAC GGC GTG CAG GAG       1440
Val Phe Ala Arg Gly Pro Gln Ala His Leu Val His Gly Val Gln Glu
```

```
                465                 470                 475

CAG ACC TTC ATA GCG CAC GTC ATG GCC TTC GCC GCC TGC CTG GAG CCC      1488
Gln Thr Phe Ile Ala His Val Met Ala Phe Ala Ala Cys Leu Glu Pro
            480                 485                 490

TAC ACC GCC TGC GAC CTG GCG CCC CCC GCC GGC ACC ACC GAC GCC GCG      1536
Tyr Thr Ala Cys Asp Leu Ala Pro Pro Ala Gly Thr Thr Asp Ala Ala
        495                 500                 505

CAC CCG GGG CGG TCC GTG GTC CCC GCG TTG CTT CCT CTG CTG GCC GGG      1584
His Pro Gly Arg Ser Val Val Pro Ala Leu Leu Pro Leu Leu Ala Gly
    510                 515                 520

ACC CTG CTG CTG CTG GAG ACG GCC ACT GCT CCC TGAGTGTCCC GTCCCTG       1634
Thr Leu Leu Leu Leu Glu Thr Ala Thr Ala Pro
525                 530                 535
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 535 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Leu Gly Pro Cys Met Leu Leu Leu Leu Leu Leu Leu Gly Leu Arg
  1               5                  10                  15

Leu Gln Leu Ser Leu Gly Ile Ile Pro Val Glu Glu Glu Asn Pro Asp
             20                  25                  30

Phe Trp Asn Arg Glu Ala Ala Glu Ala Leu Gly Ala Ala Lys Lys Leu
         35                  40                  45

Gln Pro Ala Gln Thr Ala Ala Lys Asn Leu Ile Ile Phe Leu Gly Asp
     50                  55                  60

Gly Met Gly Val Ser Thr Val Thr Ala Ala Arg Ile Leu Lys Gly Gln
 65                  70                  75                  80

Lys Lys Asp Lys Leu Gly Pro Glu Ile Pro Leu Ala Met Asp Arg Phe
                 85                  90                  95

Pro Tyr Val Ala Leu Ser Lys Thr Tyr Asn Val Asp Lys His Val Pro
            100                 105                 110

Asp Ser Gly Ala Thr Ala Thr Ala Tyr Leu Cys Gly Val Lys Gly Asn
        115                 120                 125

Phe Gln Thr Ile Gly Leu Ser Ala Ala Ala Arg Phe Asn Gln Cys Asn
```

130 135 140

Thr Thr Arg Gly Asn Glu Val Ile Ser Val Met Asn Arg Ala Lys Lys
145 150 155 160

Ala Gly Lys Ser Val Gly Val Val Thr Thr Thr Arg Val Gln His Ala
165 170 175

Ser Pro Ala Gly Thr Tyr Ala His Thr Val Asn Arg Asn Trp Tyr Ser
180 185 190

Asp Ala Asp Val Pro Ala Ser Ala Arg Gln Glu Gly Cys Gln Asp Ile
195 200 205

Ala Thr Gln Leu Ile Ser Asn Met Asp Ile Asp Val Ile Leu Gly Gly
210 215 220

Gly Arg Lys Tyr Met Phe Pro Met Gly Thr Pro Asp Pro Glu Tyr Pro
225 230 235 240

Asp Asp Tyr Ser Gln Gly Gly Thr Arg Leu Asp Gly Lys Asn Leu Val
245 250 255

Gln Glu Trp Leu Ala Lys Arg Gln Gly Ala Arg Tyr Val Trp Asn Arg
260 265 270

Thr Glu Leu Met Gln Ala Ser Leu Asp Pro Ser Val Thr His Leu Met
275 280 285

Gly Leu Phe Glu Pro Gly Asp Met Lys Tyr Glu Ile His Arg Asp Ser
290 295 300

Thr Leu Asp Pro Ser Leu Met Glu Met Thr Glu Ala Ala Leu Arg Leu
305 310 315 320

Leu Ser Arg Asn Pro Arg Gly Phe Phe Leu Phe Val Glu Gly Gly Arg
325 330 335

Ile Asp His Gly His His Glu Ser Arg Ala Tyr Arg Ala Leu Thr Glu
340 345 350

Thr Ile Met Phe Asp Asp Ala Ile Glu Arg Ala Gly Gln Leu Thr Ser
355 360 365

Glu Glu Asp Thr Leu Ser Leu Val Thr Ala Asp His Ser His Val Phe
370 375 380

Ser Phe Gly Gly Tyr Pro Leu Arg Gly Ser Ser Ile Phe Gly Leu Ala
385 390 395 400

Pro Gly Lys Ala Arg Asp Arg Lys Ala Tyr Thr Val Leu Leu Tyr Gly
405 410 415

Asn Gly Pro Gly Tyr Val Leu Lys Asp Gly Ala Arg Pro Asp Val Thr
420 425 430

```
Glu Ser Glu Ser Gly Ser Pro Glu Tyr Arg Gln Gln Ser Ala Val Pro
        435                 440                 445

Leu Asp Glu Glu Thr His Ala Gly Glu Asp Val Ala Val Phe Ala Arg
    450                 455                 460

Gly Pro Gln Ala His Leu Val His Gly Val Gln Glu Gln Thr Phe Ile
465                 470                 475                 480

Ala His Val Met Ala Phe Ala Ala Cys Leu Glu Pro Tyr Thr Ala Cys
                485                 490                 495

Asp Leu Ala Pro Pro Ala Gly Thr Thr Asp Ala Ala His Pro Gly Arg
            500                 505                 510

Ser Val Val Pro Ala Leu Leu Pro Leu Leu Ala Gly Thr Leu Leu Leu
        515                 520                 525

Leu Glu Thr Ala Thr Ala Pro
    530                 535
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1143 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 7..1137

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
GGAACC ATG GAG CTC AGC GTC CTC CTC TTC CTT GCA CTC CTC ACA GGC          48
       Met Glu Leu Ser Val Leu Leu Phe Leu Ala Leu Leu Thr Gly
         1               5                  10

CTC TTG CTA CTC CTG GTT CAG CGT CAC CCT AAC TCC CAT GGC ACC CTC          96
Leu Leu Leu Leu Leu Val Gln Arg His Pro Asn Ser His Gly Thr Leu
 15                  20                  25                  30

CCA CCA GGG CCC CGC CCT CTG CCC CTT TTG GGG AAC CTT CTG CAG ATG         144
Pro Pro Gly Pro Arg Pro Leu Pro Leu Leu Gly Asn Leu Leu Gln Met
                35                  40                  45

GAC AGA AGA GGC CTA CTC AAA TCC TTT CTG AGG TTC CGA GAG AAA TAT         192
Asp Arg Arg Gly Leu Leu Lys Ser Phe Leu Arg Phe Arg Glu Lys Tyr
            50                  55                  60
```

```
GGG GAC GTC TTC ACG GTA CAC CTG GGA CCG AGG CCC GTG GTC ATG CTG        240
Gly Asp Val Phe Thr Val His Leu Gly Pro Arg Pro Val Val Met Leu
          65                  70                  75

TGT GGA GTA GAG GCC ATA CGG GAG GCC CTG GTG GAC AAC GCT GAG GCC        288
Cys Gly Val Glu Ala Ile Arg Glu Ala Leu Val Asp Asn Ala Glu Ala
      80                  85                  90

TTC TCT GGC CGG GGA AAA ATC GTC ATC ATG GAC CCA GTC TAC CAG GGA        336
Phe Ser Gly Arg Gly Lys Ile Val Ile Met Asp Pro Val Tyr Gln Gly
 95                 100                 105                 110

TAT GGC ATG CTC TTT GCC AAT GGA AAC CGC TGG AAG GTG CTT CGG CGA        384
Tyr Gly Met Leu Phe Ala Asn Gly Asn Arg Trp Lys Val Leu Arg Arg
                115                 120                 125

TTC TCT GTG ACC ACC ATG AGG GAC TTC GGG ATG GGA AAG CGG AGT GTG        432
Phe Ser Val Thr Thr Met Arg Asp Phe Gly Met Gly Lys Arg Ser Val
            130                 135                 140

GAG GAG CGG ATT CAG GAC GAG GCT CAG TGT CTG ATA GAG GAA CTT CGG        480
Glu Glu Arg Ile Gln Asp Glu Ala Gln Cys Leu Ile Glu Glu Leu Arg
        145                 150                 155

AAA TCC AAG GGA GCC CTC GTG GAC CCC ACC TTC CTC TTC CAT TCC ATT        528
Lys Ser Lys Gly Ala Leu Val Asp Pro Thr Phe Leu Phe His Ser Ile
    160                 165                 170

ACC GCC AAC ATC ATC TGC TCC ATC ATC TTT GGA AAA CGC TTC CAC TAC        576
Thr Ala Asn Ile Ile Cys Ser Ile Ile Phe Gly Lys Arg Phe His Tyr
175                 180                 185                 190

CAA GAT CAA GAG TTC CTG AAG ACG CTG AAC TTG TTC TGC CAG AGT TTC        624
Gln Asp Gln Glu Phe Leu Lys Thr Leu Asn Leu Phe Cys Gln Ser Phe
                195                 200                 205

TTA CTC ATC AGC TCT ATA TCC AGC CAG CTG TTT GAG CTC TTC TCT GGC        672
Leu Leu Ile Ser Ser Ile Ser Ser Gln Leu Phe Glu Leu Phe Ser Gly
            210                 215                 220

TTC TTG AAA TAC TTT CCT GGG GCA CAC AGG CAA GTT TAC AAA AAC CTA        720
Phe Leu Lys Tyr Phe Pro Gly Ala His Arg Gln Val Tyr Lys Asn Leu
        225                 230                 235

CAG GAA ATC AAT GCT TAC ATT GGC CAC AGT GTG GAG AAG CAC CGT GAA        768
Gln Glu Ile Asn Ala Tyr Ile Gly His Ser Val Glu Lys His Arg Glu
    240                 245                 250

ACC CTG GAC CCC AGC GCC CCC AGG GAC CTC ATC GAC ACC TAC CTG CTC        816
Thr Leu Asp Pro Ser Ala Pro Arg Asp Leu Ile Asp Thr Tyr Leu Leu
255                 260                 265                 270

CAC ATG GAA AAA GAG AAA TCC AAC CCA CAC AGT GAA TTC AGC CAC CAG        864
His Met Glu Lys Glu Lys Ser Asn Pro His Ser Glu Phe Ser His Gln
                275                 280                 285
```

```
AAC CTC ATC ATC AAC ACG CTC TCG CTC TTC TTT GCT GGC ACT GAG ACC        912
Asn Leu Ile Ile Asn Thr Leu Ser Leu Phe Phe Ala Gly Thr Glu Thr
            290                 295                 300

ACC AGC ACC ACT CTC CGC TAC GGC TTC CTG CTC ATG CTC AAA TAC CCT        960
Thr Ser Thr Thr Leu Arg Tyr Gly Phe Leu Leu Met Leu Lys Tyr Pro
        305                 310                 315

CAT GTC GCA GAG AGA GTC TAC AAG GAG ATT GAA CAG GTG GTT GGC CCA       1008
His Val Ala Glu Arg Val Tyr Lys Glu Ile Glu Gln Val Val Gly Pro
    320                 325                 330

CAT CGC CCT CCA GCG CTT GAT GAC CGA GCC AAA ATG CCA TAC ACA GAG       1056
His Arg Pro Pro Ala Leu Asp Asp Arg Ala Lys Met Pro Tyr Thr Glu
335                 340                 345                 350

GCA GTC ATC CGT GAG ATT CAG AGA TTT GCT GAC CTT CTC CCC ATG GGT       1104
Ala Val Ile Arg Glu Ile Gln Arg Phe Ala Asp Leu Leu Pro Met Gly
                355                 360                 365

GTG CCC CAC ATT GTC ACC CAA CAC ACC AGC TTC TGAGGG                    1143
Val Pro His Ile Val Thr Gln His Thr Ser Phe
            370                 375
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 377 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Met Glu Leu Ser Val Leu Leu Phe Leu Ala Leu Leu Thr Gly Leu Leu
  1               5                  10                  15

Leu Leu Leu Val Gln Arg His Pro Asn Ser His Gly Thr Leu Pro Pro
             20                  25                  30

Gly Pro Arg Pro Leu Pro Leu Leu Gly Asn Leu Leu Gln Met Asp Arg
         35                  40                  45

Arg Gly Leu Leu Lys Ser Phe Leu Arg Phe Arg Glu Lys Tyr Gly Asp
     50                  55                  60

Val Phe Thr Val His Leu Gly Pro Arg Pro Val Val Met Leu Cys Gly
 65                  70                  75                  80

Val Glu Ala Ile Arg Glu Ala Leu Val Asp Asn Ala Glu Ala Phe Ser
                 85                  90                  95
```

Gly Arg Gly Lys Ile Val Ile Met Asp Pro Val Tyr Gln Gly Tyr Gly
100 105 110

Met Leu Phe Ala Asn Gly Asn Arg Trp Lys Val Leu Arg Arg Phe Ser
115 120 125

Val Thr Thr Met Arg Asp Phe Gly Met Gly Lys Arg Ser Val Glu Glu
130 135 140

Arg Ile Gln Asp Glu Ala Gln Cys Leu Ile Glu Glu Leu Arg Lys Ser
145 150 155 160

Lys Gly Ala Leu Val Asp Pro Thr Phe Leu Phe His Ser Ile Thr Ala
165 170 175

Asn Ile Ile Cys Ser Ile Ile Phe Gly Lys Arg Phe His Tyr Gln Asp
180 185 190

Gln Glu Phe Leu Lys Thr Leu Asn Leu Phe Cys Gln Ser Phe Leu Leu
195 200 205

Ile Ser Ser Ile Ser Ser Gln Leu Phe Glu Leu Phe Ser Gly Phe Leu
210 215 220

Lys Tyr Phe Pro Gly Ala His Arg Gln Val Tyr Lys Asn Leu Gln Glu
225 230 235 240

Ile Asn Ala Tyr Ile Gly His Ser Val Glu Lys His Arg Glu Thr Leu
245 250 255

Asp Pro Ser Ala Pro Arg Asp Leu Ile Asp Thr Tyr Leu Leu His Met
260 265 270

Glu Lys Glu Lys Ser Asn Pro His Ser Glu Phe Ser His Gln Asn Leu
275 280 285

Ile Ile Asn Thr Leu Ser Leu Phe Phe Ala Gly Thr Glu Thr Thr Ser
290 295 300

Thr Thr Leu Arg Tyr Gly Phe Leu Leu Met Leu Lys Tyr Pro His Val
305 310 315 320

Ala Glu Arg Val Tyr Lys Glu Ile Glu Gln Val Val Gly Pro His Arg
325 330 335

Pro Pro Ala Leu Asp Asp Arg Ala Lys Met Pro Tyr Thr Glu Ala Val
340 345 350

Ile Arg Glu Ile Gln Arg Phe Ala Asp Leu Leu Pro Met Gly Val Pro
355 360 365

His Ile Val Thr Gln His Thr Ser Phe
370 375

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2410 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 17..2398

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
GCCACCTGTC CCCCCC ATG GAG CTG AGG CCC TGG TTG CTA TGG GTG GTA          49
                  Met Glu Leu Arg Pro Trp Leu Leu Trp Val Val
                    1               5                  10

GCA GCA ACA GGA ACC TTG GTC CTG CTA GCA GCT GAT GCT CAG GGC CAG        97
Ala Ala Thr Gly Thr Leu Val Leu Leu Ala Ala Asp Ala Gln Gly Gln
            15                  20                  25

AAG GTC TTC ACC AAC ACG TGG GCT GTG CGC ATC CCT GGA GGC CCA GCG       145
Lys Val Phe Thr Asn Thr Trp Ala Val Arg Ile Pro Gly Gly Pro Ala
        30                  35                  40

GTG GCC AAC AGT GTG GCA CGG AAG CAT GGG TTC CTC AAC CTG GGC CAG       193
Val Ala Asn Ser Val Ala Arg Lys His Gly Phe Leu Asn Leu Gly Gln
    45                  50                  55

ATC TTC GGG GAC TAT TAC CAC TTC TGG CAT CGA GGA GTG ACG AAG CGG       241
Ile Phe Gly Asp Tyr Tyr His Phe Trp His Arg Gly Val Thr Lys Arg
60                  65                  70                  75

TCC CTG TCG CCT CAC CGC CCG CGG CAC AGC CGG CTG CAG AGG GAG CCT       289
Ser Leu Ser Pro His Arg Pro Arg His Ser Arg Leu Gln Arg Glu Pro
                80                  85                  90

CAA GTA CAG TGG CTG GAA CAG CAG GTG GCA AAG CGA CGG ACT AAA CGG       337
Gln Val Gln Trp Leu Glu Gln Gln Val Ala Lys Arg Arg Thr Lys Arg
            95                  100                 105

GAC GTG TAC CAG GAG CCC ACA GAC CCC AAG TTT CCT CAG CAG TGG TAC       385
Asp Val Tyr Gln Glu Pro Thr Asp Pro Lys Phe Pro Gln Gln Trp Tyr
        110                 115                 120

CTG TCT GGT GTC ACT CAG CGG GAC CTG AAT GTG AAG GCG GCC TGG GCG       433
Leu Ser Gly Val Thr Gln Arg Asp Leu Asn Val Lys Ala Ala Trp Ala
    125                 130                 135

CAG GGC TAC ACA GGG CAC GGC ATT GTG GTC TCC ATT CTG GAC GAT GGC       481
Gln Gly Tyr Thr Gly His Gly Ile Val Val Ser Ile Leu Asp Asp Gly
140                 145                 150                 155
```

```
ATC GAG AAG AAC CAC CCG GAC TTG GCA GGC AAT TAT GAT CCT GGG GCC        529
Ile Glu Lys Asn His Pro Asp Leu Ala Gly Asn Tyr Asp Pro Gly Ala
                160                 165                 170

AGT TTT GAT GTC AAT GAC CAG GAC CCT GAC CCC CAG CCT CGG TAC ACA        577
Ser Phe Asp Val Asn Asp Gln Asp Pro Asp Pro Gln Pro Arg Tyr Thr
            175                 180                 185

CAG ATG AAT GAC AAC AGG CAC GGC ACA CGG TGT GCG GGG GAA GTG GCT        625
Gln Met Asn Asp Asn Arg His Gly Thr Arg Cys Ala Gly Glu Val Ala
        190                 195                 200

GCG GTG GCC AAC AAC GGT GTC TGT GGT GTA GGT GTG GCC TAC AAC GCC        673
Ala Val Ala Asn Asn Gly Val Cys Gly Val Gly Val Ala Tyr Asn Ala
    205                 210                 215

CGC ATT GGA GGG GTG CGC ATG CTG GAT GGC GAG GTG ACA GAT GCA GTG        721
Arg Ile Gly Gly Val Arg Met Leu Asp Gly Glu Val Thr Asp Ala Val
220                 225                 230                 235

GAG GCA CGC TCG CTG GGC CTG AAC CCC AAC CAC ATC CAC ATC TAC AGT        769
Glu Ala Arg Ser Leu Gly Leu Asn Pro Asn His Ile His Ile Tyr Ser
                240                 245                 250

GCC AGC TGG GGC CCC GAG GAT GAC GGC AAG ACA GTG GAT GGG CCA GCC        817
Ala Ser Trp Gly Pro Glu Asp Asp Gly Lys Thr Val Asp Gly Pro Ala
            255                 260                 265

CGC CTC GCC GAG GAG GCC TTC TTC CGT GGG GTT AGC CAG GGC CGA GGG        865
Arg Leu Ala Glu Glu Ala Phe Phe Arg Gly Val Ser Gln Gly Arg Gly
        270                 275                 280

GGG CTG GGC TCC ATC TTT GTC TGG GCC TCG GGG AAC GGG GGC CGG GAA        913
Gly Leu Gly Ser Ile Phe Val Trp Ala Ser Gly Asn Gly Gly Arg Glu
    285                 290                 295

CAT GAC AGC TGC AAC TGC GAC GGC TAC ACC AAC AGT ATC TAC ACG CTG        961
His Asp Ser Cys Asn Cys Asp Gly Tyr Thr Asn Ser Ile Tyr Thr Leu
300                 305                 310                 315

TCC ATC AGC AGC GCC ACG CAG TTT GGC AAC GTG CCG TGG TAC AGC GAG       1009
Ser Ile Ser Ser Ala Thr Gln Phe Gly Asn Val Pro Trp Tyr Ser Glu
                320                 325                 330

GCC TGC TCG TCC ACA CTG GCC ACG ACC TAC AGC AGT GGC AAC CAG AAT       1057
Ala Cys Ser Ser Thr Leu Ala Thr Thr Tyr Ser Ser Gly Asn Gln Asn
            335                 340                 345

GAG AAG CAG ATC GTG ACG ACT GAC TTG CGG CAG AAG TGC ACG GAG TCT       1105
Glu Lys Gln Ile Val Thr Thr Asp Leu Arg Gln Lys Cys Thr Glu Ser
        350                 355                 360

CAC ACG GGC ACC TCA GCC TCT GCC CCC TTA GCA GCC GGC ATC ATT GCT       1153
His Thr Gly Thr Ser Ala Ser Ala Pro Leu Ala Ala Gly Ile Ile Ala
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    365                 370                 375

CTC ACC CTG GAG GCC AAT AAG AAC CTC ACA TGG CGG GAC ATG CAA CAC      1201
Leu Thr Leu Glu Ala Asn Lys Asn Leu Thr Trp Arg Asp Met Gln His
380                 385                 390                 395

CTG GTG GTA CAG ACC TCG AAG CCA GCC CAC CTC AAT GCC AAC GAC TGG      1249
Leu Val Val Gln Thr Ser Lys Pro Ala His Leu Asn Ala Asn Asp Trp
                400                 405                 410

GCC ACC AAT GGT GTG GGC CGG AAA GTG AGC CAC TCA TAT GGC TAC GGG      1297
Ala Thr Asn Gly Val Gly Arg Lys Val Ser His Ser Tyr Gly Tyr Gly
            415                 420                 425

CTT TTG GAC GCA GGC GCC ATG GTG GCC CTG GCC CAG AAT TGG ACC ACA      1345
Leu Leu Asp Ala Gly Ala Met Val Ala Leu Ala Gln Asn Trp Thr Thr
        430                 435                 440

GTG GCC CCC CAG CGG AAG TGC ATC ATC GAC ATC CTC ACC GAG CCC AAA      1393
Val Ala Pro Gln Arg Lys Cys Ile Ile Asp Ile Leu Thr Glu Pro Lys
    445                 450                 455

GAC ATC GGG AAA CGG CTC GAG GTG CGG AAG ACC GTG ACC GCG TGC CTG      1441
Asp Ile Gly Lys Arg Leu Glu Val Arg Lys Thr Val Thr Ala Cys Leu
460                 465                 470                 475

GGC GAG CCC AAC CAC ATC ACT CGG CTG GAG CAC GCT CAG GCG CGG CTC      1489
Gly Glu Pro Asn His Ile Thr Arg Leu Glu His Ala Gln Ala Arg Leu
                480                 485                 490

ACC CTG TCC TAT AAT CGC CGT GGC GAC CTG GCC ATC CAC CTG GTC AGC      1537
Thr Leu Ser Tyr Asn Arg Arg Gly Asp Leu Ala Ile His Leu Val Ser
            495                 500                 505

CCC ATG GGC ACC CGC TCC ACC CTG CTG GCA GCC AGG CCA CAT GAC TAC      1585
Pro Met Gly Thr Arg Ser Thr Leu Leu Ala Ala Arg Pro His Asp Tyr
        510                 515                 520

TCC GCA GAT GGG TTT AAT GAC TGG GCC TTC ATG ACA ACT CAT TCC TGG      1633
Ser Ala Asp Gly Phe Asn Asp Trp Ala Phe Met Thr Thr His Ser Trp
    525                 530                 535

GAT GAG GAT CCC TCT GGC GAG TGG GTC CTA GAG ATT GAA AAC ACC AGC      1681
Asp Glu Asp Pro Ser Gly Glu Trp Val Leu Glu Ile Glu Asn Thr Ser
540                 545                 550                 555

GAA GCC AAC AAC TAT GGG ACG CTG ACC AAG TTC ACC CTC GTA CTC TAT      1729
Glu Ala Asn Asn Tyr Gly Thr Leu Thr Lys Phe Thr Leu Val Leu Tyr
                560                 565                 570

GGC ACC GCC CCT GAG GGG CTG CCC GTA CCT CCA GAA AGC AGT GGC TGC      1777
Gly Thr Ala Pro Glu Gly Leu Pro Val Pro Pro Glu Ser Ser Gly Cys
            575                 580                 585

AAG ACC CTC ACG TCC AGT CAG GCC TGT GTG GTG TGC GAG GAA GGC TTC      1825
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Lys Thr Leu Thr Ser Ser Gln Ala Cys Val Val Cys Glu Glu Gly Phe
        590                 595                 600

TCC CTG CAC CAG AAG AGC TGT GTC CAG CAC TGC CCT CCA GGC TTC GCC      1873
Ser Leu His Gln Lys Ser Cys Val Gln His Cys Pro Pro Gly Phe Ala
    605                 610                 615

CCC CAA GTC CTC GAT ACG CAC TAT AGC ACC GAG AAT GAC GTG GAG ACC      1921
Pro Gln Val Leu Asp Thr His Tyr Ser Thr Glu Asn Asp Val Glu Thr
620                 625                 630                 635

ATC CGG GCC AGC GTC TGC GCC CCC TGC CAC GCC TCA TGT GCC ACA TGC      1969
Ile Arg Ala Ser Val Cys Ala Pro Cys His Ala Ser Cys Ala Thr Cys
                640                 645                 650

CAG GGG CCG GCC CTG ACA GAC TGC CTC AGC TGC CCC AGC CAC GCC TCC      2017
Gln Gly Pro Ala Leu Thr Asp Cys Leu Ser Cys Pro Ser His Ala Ser
            655                 660                 665

TTG GAC CCT GTG GAG CAG ACT TGC TCC CGG CAA AGC CAG AGC AGC CGA      2065
Leu Asp Pro Val Glu Gln Thr Cys Ser Arg Gln Ser Gln Ser Ser Arg
        670                 675                 680

GAG TCC CCG CCA CAG CAG CAG CCA CCT CGG CTG CCC CCG GAG GTG GAG      2113
Glu Ser Pro Pro Gln Gln Gln Pro Pro Arg Leu Pro Pro Glu Val Glu
    685                 690                 695

GCG GGG CAA CGG CTG CGG GCA GGG CTG CTG CCC TCA CAC CTG CCT GAG      2161
Ala Gly Gln Arg Leu Arg Ala Gly Leu Leu Pro Ser His Leu Pro Glu
700                 705                 710                 715

GTG GTG GCC GGC CTC AGC TGC GCC TTC ATC GTG CTG GTC TTC GTC ACT      2209
Val Val Ala Gly Leu Ser Cys Ala Phe Ile Val Leu Val Phe Val Thr
                720                 725                 730

GTC TTC CTG GTC CTG CAG CTG CGC TCT GGC TTT AGT TTT CGG GGG GTG      2257
Val Phe Leu Val Leu Gln Leu Arg Ser Gly Phe Ser Phe Arg Gly Val
            735                 740                 745

AAG GTG TAC ACC ATG GAC CGT GGC CTC ATC TCC TAC AAG GGG CTG CCC      2305
Lys Val Tyr Thr Met Asp Arg Gly Leu Ile Ser Tyr Lys Gly Leu Pro
        750                 755                 760

CCT GAA GCC TGG CAG GAG GAG TGC CCG TCT GAC TCA GAA GAG GAC GAG      2353
Pro Glu Ala Trp Gln Glu Glu Cys Pro Ser Asp Ser Glu Glu Asp Glu
    765                 770                 775

GGC CGG GGC GAG AGG ACC GCC TTT ATC AAA GAC CAG AGC GCC CTC          2398
Gly Arg Gly Glu Arg Thr Ala Phe Ile Lys Asp Gln Ser Ala Leu
780                 785                 790

TGATGAGCCC AC                                                       2410
```

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 794 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
Met Glu Leu Arg Pro Trp Leu Leu Trp Val Val Ala Ala Thr Gly Thr
  1               5                  10                  15

Leu Val Leu Leu Ala Ala Asp Ala Gln Gly Gln Lys Val Phe Thr Asn
             20                  25                  30

Thr Trp Ala Val Arg Ile Pro Gly Gly Pro Ala Val Ala Asn Ser Val
         35                  40                  45

Ala Arg Lys His Gly Phe Leu Asn Leu Gly Gln Ile Phe Gly Asp Tyr
     50                  55                  60

Tyr His Phe Trp His Arg Gly Val Thr Lys Arg Ser Leu Ser Pro His
 65                  70                  75                  80

Arg Pro Arg His Ser Arg Leu Gln Arg Glu Pro Gln Val Gln Trp Leu
                 85                  90                  95

Glu Gln Gln Val Ala Lys Arg Arg Thr Lys Arg Asp Val Tyr Gln Glu
            100                 105                 110

Pro Thr Asp Pro Lys Phe Pro Gln Gln Trp Tyr Leu Ser Gly Val Thr
        115                 120                 125

Gln Arg Asp Leu Asn Val Lys Ala Ala Trp Ala Gln Gly Tyr Thr Gly
    130                 135                 140

His Gly Ile Val Val Ser Ile Leu Asp Asp Gly Ile Glu Lys Asn His
145                 150                 155                 160

Pro Asp Leu Ala Gly Asn Tyr Asp Pro Gly Ala Ser Phe Asp Val Asn
                165                 170                 175

Asp Gln Asp Pro Asp Pro Gln Pro Arg Tyr Thr Gln Met Asn Asp Asn
            180                 185                 190

Arg His Gly Thr Arg Cys Ala Gly Glu Val Ala Ala Val Ala Asn Asn
        195                 200                 205

Gly Val Cys Gly Val Gly Val Ala Tyr Asn Ala Arg Ile Gly Gly Val
    210                 215                 220

Arg Met Leu Asp Gly Glu Val Thr Asp Ala Val Glu Ala Arg Ser Leu
225                 230                 235                 240
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Gly Leu Asn Pro Asn His Ile His Ile Tyr Ser Ala Ser Trp Gly Pro
                245                 250                 255

Glu Asp Asp Gly Lys Thr Val Asp Gly Pro Ala Arg Leu Ala Glu Glu
            260                 265                 270

Ala Phe Phe Arg Gly Val Ser Gln Gly Arg Gly Gly Leu Gly Ser Ile
        275                 280                 285

Phe Val Trp Ala Ser Gly Asn Gly Gly Arg Glu His Asp Ser Cys Asn
    290                 295                 300

Cys Asp Gly Tyr Thr Asn Ser Ile Tyr Thr Leu Ser Ile Ser Ser Ala
305                 310                 315                 320

Thr Gln Phe Gly Asn Val Pro Trp Tyr Ser Glu Ala Cys Ser Ser Thr
                325                 330                 335

Leu Ala Thr Thr Tyr Ser Ser Gly Asn Gln Asn Glu Lys Gln Ile Val
            340                 345                 350

Thr Thr Asp Leu Arg Gln Lys Cys Thr Glu Ser His Thr Gly Thr Ser
        355                 360                 365

Ala Ser Ala Pro Leu Ala Ala Gly Ile Ile Ala Leu Thr Leu Glu Ala
    370                 375                 380

Asn Lys Asn Leu Thr Trp Arg Asp Met Gln His Leu Val Val Gln Thr
385                 390                 395                 400

Ser Lys Pro Ala His Leu Asn Ala Asn Asp Trp Ala Thr Asn Gly Val
                405                 410                 415

Gly Arg Lys Val Ser His Ser Tyr Gly Tyr Gly Leu Leu Asp Ala Gly
            420                 425                 430

Ala Met Val Ala Leu Ala Gln Asn Trp Thr Thr Val Ala Pro Gln Arg
        435                 440                 445

Lys Cys Ile Ile Asp Ile Leu Thr Glu Pro Lys Asp Ile Gly Lys Arg
    450                 455                 460

Leu Glu Val Arg Lys Thr Val Thr Ala Cys Leu Gly Glu Pro Asn His
465                 470                 475                 480

Ile Thr Arg Leu Glu His Ala Gln Ala Arg Leu Thr Leu Ser Tyr Asn
                485                 490                 495

Arg Arg Gly Asp Leu Ala Ile His Leu Val Ser Pro Met Gly Thr Arg
            500                 505                 510

Ser Thr Leu Leu Ala Ala Arg Pro His Asp Tyr Ser Ala Asp Gly Phe
        515                 520                 525

Asn Asp Trp Ala Phe Met Thr Thr His Ser Trp Asp Glu Asp Pro Ser
```

530 535 540

Gly Glu Trp Val Leu Glu Ile Glu Asn Thr Ser Glu Ala Asn Asn Tyr
545 550 555 560

Gly Thr Leu Thr Lys Phe Thr Leu Val Leu Tyr Gly Thr Ala Pro Glu
565 570 575

Gly Leu Pro Val Pro Pro Glu Ser Ser Gly Cys Lys Thr Leu Thr Ser
580 585 590

Ser Gln Ala Cys Val Val Cys Glu Glu Gly Phe Ser Leu His Gln Lys
595 600 605

Ser Cys Val Gln His Cys Pro Pro Gly Phe Ala Pro Gln Val Leu Asp
610 615 620

Thr His Tyr Ser Thr Glu Asn Asp Val Glu Thr Ile Arg Ala Ser Val
625 630 635 640

Cys Ala Pro Cys His Ala Ser Cys Ala Thr Cys Gln Gly Pro Ala Leu
645 650 655

Thr Asp Cys Leu Ser Cys Pro Ser His Ala Ser Leu Asp Pro Val Glu
660 665 670

Gln Thr Cys Ser Arg Gln Ser Gln Ser Ser Arg Glu Ser Pro Pro Gln
675 680 685

Gln Gln Pro Pro Arg Leu Pro Pro Glu Val Glu Ala Gly Gln Arg Leu
690 695 700

Arg Ala Gly Leu Leu Pro Ser His Leu Pro Glu Val Val Ala Gly Leu
705 710 715 720

Ser Cys Ala Phe Ile Val Leu Val Phe Val Thr Val Phe Leu Val Leu
725 730 735

Gln Leu Arg Ser Gly Phe Ser Phe Arg Gly Val Lys Val Tyr Thr Met
740 745 750

Asp Arg Gly Leu Ile Ser Tyr Lys Gly Leu Pro Pro Glu Ala Trp Gln
755 760 765

Glu Glu Cys Pro Ser Asp Ser Glu Glu Asp Glu Gly Arg Gly Glu Arg
770 775 780

Thr Ala Phe Ile Lys Asp Gln Ser Ala Leu
785 790

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Arg Lys Lys Arg
1

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGTAGGTTT GGCAAGCTAG C 21

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGCTGACGGG TAGTCAATC 19

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GAATGGCAAA GTAAAGGTTT TTCAGGG 27

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATAGTTAGCG GCCGCAACCC GGGCCACCCT CAGTAGAGGT CCTG 44

CLAIMS



We claim:

1. A method of delivering a gene delivery vehicle to a warm-blooded animal, comprising administering to a warm-blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic selectable marker.

2. A method of delivering a gene delivery vehicle to a warm-blooded animal, comprising administering to a warm-blooded animal a gene delivery vehicle which directs the expression of a non-immunogenic molecule which is capable of activating an otherwise inactive compound into an active compound.

3. The method according to claims 1 or 2 wherein said vector construct also directs the expression of a selected heterologous nucleic acid seqeunce.

4. The method according to claim 1 wherin said selectable marker is selected from the group consisting of alkaline phosphatase, α-Galactosidase, β-glucosidase, β-glucuronidase, Carboxypeptidase A, Cytochrome P450, γ-glutamyl transferase; reductases such as Azoreductase, DT diaphorase and Nitroreductase; and oxidases such as glucose oxidase and xanthine oxidase.

5. The method according to claim 1 wherin said compound capable of activating an otherwise inactive compound into an active compound is selected from the group consisting of alkaline phosphatase, α-Galactosidase, β-glucosidase, β-glucuronidase, Carboxypeptidase A, Cytochrome P450, γ-glutamyl transferase; reductases such as Azoreductase, DT diaphorase and Nitroreductase; and oxidases such as glucose oxidase and xanthine oxidase.

6. The method according to any one of claim 1 or 2 wherein said gene delivery vehicle is a retroviral vector construct.

7. The method according to any one of claim 1 or 2 wherein said gene delivery vehicle is selected from the group consisting of poliovirus vectors, rhinovirus vectors, pox virus vectors, canary pox virus vectors, vaccinia virus vectors, influenza virus vectors, adenovirus vectors, parvovirus vectors, adeno-associated viral vectors, herpesvirus vectors, SV 40 vectors, lenti virus vectors, measles virus vectors, astrovirus vectors, corona virus vectors and Alphavirus vectors.

8. The method according to any one of claim 1 or 2 wherein said gene delivery vehicle is selected from the group consisting of polycation condensed nucleic acids, liposome entrapped nucleic acids, naked DNA or RNA and producer cell lines.

9. The method according to claim 3 wherein said heterologous sequence is a gene encoding a cytotoxic protein.

10. The method according to claim 9 wherein said cytotoxic protein is selected from the group consisting of ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed, antiviral protein, tritin, Shigella toxin and Pseudomonas exotoxin A.

11. The method according to claim 3 wherein said heterologous sequence is an antisense sequence.

12. The method according to claim 3 wherein said heterologous sequence encodes an immune accessory molecule.

13. The method according to claim 12 wherein said immune accessory molecule is selected from the group consisting of α interferon, β interferon, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 and IL-13.

14. The method according to claim 12 wherein said immune accessory molecule is selected from the group consisting of IL-2, IL-12 and gamma-interferon.

15. The method according to claim 12 wherein said immune accessory molecule is selected from the group consisting of ICAM-1, ICAM-2, β-microglobin, LFA3, and HLA class I and HLA class II molecules.

16. The method according to claim 3 wherein said heterologous sequence is a ribozyme.

17. The method according to claim 3 wherein said heterologous sequence is a replacement gene.

18. The method according to claim 17 wherein said replacement gene encodes a protein selected from the group consisting of Factor VIII, ADA, HPRT, CFTCR and the LDL Receptor.

19. The method according to claim 3 wherein said heterologous sequence encodes an immunogenic portion of a virus selected from the group consisting of HBV, HCV, HPV, EBV, FeLV, FIV and HIV.

20. The method according to claims 1 or 2 wherein said gene delivery vehicle is introduced into cells *ex vivo*, followed by administration of said gene delivery vehicle containing cells to said warm-blooded animal.