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**WO 03/029412 A2**

(54) Title: METHODS AND COMPOSITIONS RELATING TO RESTRICTED EXPRESSION LENTIVIRAL VECTORS AND THEIR APPLICATIONS

(57) Abstract: The present invention provides HIV-derived lentivectors which are safe, highly efficient, and very potent for expressing transgenes for human gene therapy, especially, in human hematopoietic progenitor cells as well as in all other blood cell derivatives. The lentiviral vectors comprise promoters active to promote expression specific to cell types or tissues. Further, promoters are providing that are amenable to control by activators, enhancers, or repressors. These vectors are in a self-inactivating configuration for biosafety. Additional promoters are also described. The vectors can also comprise additional transcription enhancing elements such as the wood chuck hepatitis virus post-transcriptional regulatory element, without any decrease in the specificity or control exerted by the promoters. These vectors therefore provide useful tools for genetic treatments such as inherited and acquired lympho-hematological disorders, gene-therapies for cancers especially the hematological cancers, as well as for the study of hematopoiesis via lentivector-mediated modification of human HSCs.

**DESCRIPTION****METHODS AND COMPOSITIONS RELATING TO RESTRICTED EXPRESSION  
LENTIVIRAL VECTORS AND THEIR APPLICATIONS****BACKGROUND OF THE INVENTION**

5           The present application claims the benefit of U.S. Provisional Application Serial No. 60/326,593 filed on October 2, 2001, the entire text of which is herein incorporated by reference.

**1. Field of the Invention**

10           The present invention relates to improved lentiviral vectors and their use in gene delivery and high level expression of desired transgenes to target cells, particularly to differentiated blood lineages derived from lentiviral vector-modified human hematopoietic stem cells (hHSC).

**2. Description of Related Art**

15           Gene therapy via the transduction of human hematopoietic stem cells (hHSC) represents a very promising approach for the treatment of a number of inherited and acquired lympho-hematological disorders. The stable genetic manipulation of long term repopulating hHSC with existing gene delivery systems, however, has been impossible to achieve at an efficiency compatible with therapeutic realities. Oncoretroviral vectors derived from Moloney murine leukemia virus (MLV), for instance, although highly appealing since they integrate their cargo into the chromosomes of target cells, cannot transduce hHSC that have not been first treated with inducers of proliferation (Kohn *et al.*, 1991; Mazurier *et al.*, 1998). Indeed, the nuclear transport of the MLV preintegration complex requires the breakdown of the nuclear envelope that occurs at mitosis (Roe *et al.*, 1993; Lewis and Emerman, 1994). Unfortunately hHSCs, whether harvested from the bone marrow (BM), the umbilical cord blood (UCB) or mobilized in the peripheral circulation, are mostly non-dividing and lose their pluripotentiality after continuous stimulation and proliferation (Bhatia *et al.*, 1997; Dao *et al.*, 1997; Dorrell *et al.*, 2000). Recent reports, however, have shown that a significant fraction of pluripotent cells as well as cells capable of long-term engraftment in non-obese diabetic/severe combined immunodeficient (NOD/SCID), also called SCID-repopulating cells (SRC), can be maintained, transduced and

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even expanded using specific stimulation conditions (Dorrell *et al.*, 2000; Dao *et al.*, 1998; Piacibello *et al.*, 1999; Ueda *et al.*, 2000).

5 Lentiviruses are a subgroup of retroviruses that can infect non-dividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Correspondingly, lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) can mediate the efficient delivery, integration and long-term expression of transgenes into non-mitotic cells both *in vitro* and *in vivo* (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b; Blomer *et al.*, 1997). In particular, HIV-based vectors can efficiently transduce human CD34<sup>+</sup> hematopoietic cells in the absence of cytokine stimulation (Akkina *et al.*, 1996; Sutton *et al.*, 1998; Uchida *et al.*, 1998; Miyoshi *et al.*, 1999; Case *et al.*, 1999). These cells are capable of long-term engraftment in NOD/SCID mice (Miyoshi *et al.*, 1999). Bone marrow from these primary recipients can repopulate secondary mice with transduced cells, confirming the lentivector-mediated genetic modification of very primitive hematopoietic precursors, most probably *bona fide* stem cells. Since none of the other currently available gene delivery systems has such an ability, lentiviral vectors provide a previously unexplored basis for the study of hematopoiesis and for the gene therapy of inherited and acquired lympho-hematopoietic disorders via the genetic modification of HSCs.

The demonstration of this important point, however, was provided with an early generation of lentiviral vectors unsuitable for therapeutic applications, either because they failed to meet biosafety requirements (Akkina *et al.*, 1996; Sutton *et al.*, 1998; Uchida *et al.*, 1998) or because they induced levels of transgene expression that were dismissingly low (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000). Accordingly, there is a significant need to develop improved lentiviruses for use as transducing vectors that are capable of effectively transducing hematopoietic cells, particularly hematopoietic progenitor cells, and which are capable of expressing desired transgenes at high levels.

An optimal stem cell gene therapy approach should result in the efficient transduction of HSCs, and considering the plasticity of stem cells, in the restricted expression of therapeutic genes into specific mature blood cell lineages. Third generation lentiviral vectors are currently the most optimized tools for gene delivery into non-cycling human HSC. Moreover, a self-inactivating design (SIN) provides for the use of tissue specific promoters without interference from the upstream LTR.

**SUMMARY OF THE INVENTION**

The present invention is directed to the development of improved lentiviral vectors that both meet biosafety requirements, and which may be stimulated as desired to induce high levels of transgene expression in a tissue or stem cell lineage specific manner. Additionally, the present invention provides for control of transgene expression in transduced cells via the transcriptional activation or repression resulting from contacting the promoter or enhancers with transcriptional regulatory factors.

Accordingly, the present invention describes gene transfer vehicles that appear particularly well suited for the transduction of human hematopoietic precursor cells (HPCs) and for the expression of transgenes in specific differentiated blood lineages or under the control of specific transcription factors. These vectors will facilitate the further use of lentiviral vectors for the genetic manipulation of hematopoietic stem cells, and should be particularly useful for both research and therapeutic applications. Some examples of cell types contemplated include immature blood cells, mature blood cells, neutrophils, monocytes/macrophages, and granulocytes.

However, it will be understood by the skilled artisan that the invention is not limited to the transduction of hematopoietic cells and that one may use the lentiviral vectors of the invention for the cell-specific expression of transgenes in other cell types as well. Some examples of other cell types contemplated include terminally differentiated cells such as neurons, lung cells, muscle cells, liver cells, pancreatic cells, endothelial cells, cardiac cells, skin cells, bone marrow stromal cells, and eye cells. Additionally, stem cells and progenitor cells such as pancreatic ductal cells, neural precursors, and mesodermal stem cells are also contemplated.

The present invention thus concerns, in a general and overall sense, improved vectors that are designed to permit the transfection and transduction of human hematopoietic progenitor cells, or stem cells (hHSC), and provide high level expression of desired transgenes in such cells. Additionally, the present invention provides for restricted expression of these desired transgenes in that expression is regulated to achieve expression in specific descendent lineages of HSC or in response to transcriptional activators. The vectors of the present invention also may be self-inactivating lentivectors in that they may contain certain "self-inactivating" design characteristics that render these vectors safe for human applications. These self-inactivating, or SIN design characteristics may include the modification of the LTRs of the vector so that

reconstitution of a replication competent lentiviral genome is prevented. A particularly preferred embodiment of such a SIN design includes the deletion of nucleotides in the 3' LTR U3 region.

The lentivectors of the present invention provide, for the first time, an efficient means of achieving controlled, cell type specific, and high level expression of desired transgenes in differentiated progeny of genetically modified hHSCs. Human HSCs have been difficult to transduce because when in an unstimulated state they are relatively resistant to transduction by previous vector systems. The lentiviral vectors of the present invention have the ability to infect non-dividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Moreover, preferred lentiviral vectors of the present invention can mediate the efficient delivery, integration and appropriate or long-term expression of transgenes into non-mitotic cells both *in vitro* and *in vivo*, even in the absence of cytokine stimulation. Stem cells transduced by the more preferred lentivectors of the present invention are capable of long-term engraftment, for example, in NOD/SCID mice. Most notably, however, the more preferred lentivectors of the present invention have highly desirable features that permit controlled, yet high level expression of transgenes in specific lineages of human progenitor cells and mature, differentiated cell types, while meeting human biosafety requirements.

The viral vectors of the present invention, therefore, may be generally described as recombinant vectors that include at least lentiviral *gag*, *pol* and *rev* genes, or those genes required for virus production, which permit the manufacture of vector in reasonable quantities using available producer cell lines. To meet important human safety needs, the more preferred vectors in accordance with the present invention will not include any other active lentiviral genes, such as *vpr*, *vif*, *vpu*, *nef*, *tat*. These genes may have been removed or otherwise inactivated. It is preferred that the only active lentiviral genes present in the vector will be the aforementioned *gag*, *pol* and *rev* genes.

The most preferred combination of lentiviral genes and backbone (*i.e.*, long terminal repeats or LTRs) used in preparing lentivectors in accordance with the present invention will be one that is human immunodeficiency virus (HIV) derived, and more particularly, HIV-1 derived. Thus, the *gag*, *pol* and *rev* genes will preferably be HIV genes and more preferably HIV-1 genes. However, the *gag*, *pol* and *rev* genes and LTR regions from other lentiviruses may be employed for certain applications in accordance with the present invention, including the genes and LTRs of HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus, bovine

immunodeficiency virus, equine infectious anemia virus, caprine arthritis encephalitis virus and the like. Such constructs could be useful, for example, where one desires to modify certain cells of non-human origin. However, the HIV based vector backbones (*i.e.*, HIV LTR and HIV *gag*, *pol* and *rev* genes) will generally be preferred in connection with most aspects of the present invention in that HIV-based constructs are the most efficient at transduction of human hematopoietic progenitor cells.

The viral vectors of the present invention also include an expression cassette comprising a transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a human cell. In preferred embodiments the promoter is active in promoting transcription of the transgene in human hematopoietic progenitor cells. More preferred embodiments include promoters that are active to promote transcription in specific cell types or descendent lineages of progenitor cells. Still further preferred embodiments include promoters that are subject to control through either activation or suppression by transcriptional control factors or activators and repressors.

Examples of promoters that may be preferably employed in connection with the present invention include a gp91-phox, gp47-phox, CD11b, EF1- $\alpha$ , PGK, beta-globin promoter, MHC classII, clotting Factor IX, insulin promoters, PDX1 promoter, CD11, CD4, and CD2 promoters. Of these the gp91-phox promoter is particularly preferred. The gp91-phox promoter is an example of a promoter that provides for controllable expression restricted to specific desired cell types in that it promotes expression of the transgene primarily in monocytes and granulocytes and in that its activity may be modulated by contacting the promoter with activators, particularly interferon-gamma (INF-gamma). In any event, however, practice of the present invention is not restricted to the foregoing promoters, so long as the promoter is active in the progenitor, hematopoietic or other cell that one desires to target or is responsive to transcriptional control.

To determine whether a particular promoter is useful, a selected promoter is tested in the construct *in vitro* in a selected progenitor cell and, if the promoter is capable of promoting expression of the transgene at a detectable signal-to-noise ratio, it will generally be useful in accordance with the present invention. A desirable signal-to-noise ratio is one between about 10 and about 200, a more desirable signal-to-noise ratio is one 40 and about 200, and an even more desirable signal-to-noise ratio is one between about 150 and about 200. One means of testing such a promoter, described in more detail hereinbelow, is through the use of a signal generating transgene such as the green fluorescent protein (GFP).

The present invention further provides for increased transduction efficiency through the inclusion of a central polypurine tract (cPPT) in the vector. The transduction efficiency may be 20%, 30%, 40%, 50%, 60%, 70%, or up to and including 80% transduction. In a preferred embodiment, the cPPT is positioned upstream of the promoter of sequence. A cPPT is exemplified by the sequence of nucleotides described by SEQ ID NO:1.

Further preferred aspects of the invention include multiple unique cloning sites. Unique cloning sites are sites of restriction enzyme recognition sequences that are unique within the vector sequence. Several such sites clustered together provide for multiple unique cloning sites. These sites are preferably interposed between the cPPT and the promoter, or upstream of the cPPT, although they may be located where ever it may be convenient for the manipulation of polynucleotides into or out of the vector. For example, these multiple unique cloning sites provide for the facile introduction into the vector sequence elements that are additionally useful and beneficial in practicing the invention.

The promoters mentioned above can comprise additional elements required for transcription and thus be a part of a transcription cassette. A transcription cassette is defined as comprising one or more promoter elements coupled to enhancers and/or locus control regions in order to ensure strong or tissue-restricted expression of a transgene. One or more enhancers may be positioned in the vector anywhere they are most active in modulating expression of a transgene. In order to achieve a high level of transgene expression in target differentiated cell lineages, enhancers may also be specific for target differentiated lineages. Lineage-specific enhancers include HS sites. HS sites are known for beta-globin, CD2 and gp91, but additional HS or HS-type sites may be identified. For example, the GATA-1 enhancer for erythroblasts. Availability of the human genome sequence should greatly facilitate identification of such elements that are contemplated as part of the present invention.

A particularly preferred group of enhancer and insulator elements are those located within locus control region (LCR) and can be identified as DNAase hypersensitive sites. The coordinated enhancer activity of these HS sites is believed to be responsible for the chromatin domain opening activity, thus facilitating transcription factor(s) accessibility in chromatin, stimulating protein-protein interactions between enhancer- and promoter bound factors; and are needed for defining domain boundaries. HS sites present in *cis* to promoter-gene cassettes confer high level, integration-site-independent expression. These elements may be positioned singly or multiply either upstream or downstream of the transgene cassette. In a most preferred

embodiment of the invention, the HS elements are positioned adjacent to and both upstream and downstream of the cPPT element and are wholly upstream of the promoter. These HS sites may therefore be introduced at the position of the multiple unique cloning sites described above. By adjacent is meant that the subject element, e.g. the cPPT element, is the first functionally important element encountered when scanning the vector sequence from the boundaries of the reference element, i.e. the promoter element.

For certain applications, for example, in the case of promoters that are only modestly active in cells targeted for transduction, one will desire to employ a posttranscriptional regulatory sequence positioned to promote the expression of the transgene. One type of posttranscriptional regulatory sequence is an intron positioned within the expression cassette, which may serve to stimulate gene expression. However, introns placed in such a manner may expose the lentiviral RNA transcript to the normal cellular splicing and processing mechanisms. Thus, in particular embodiments it may be desirable to locate intron-containing transgenes in an orientation opposite to that of the vector genomic transcript.

A more preferred method of enhancing transgene expression is through the use of a posttranscriptional regulatory element which does not rely on splicing events, such as the posttranscriptional processing element of herpes simplex virus, the posttranscriptional regulatory element of the hepatitis B virus (HPRE) or that of the woodchuck hepatitis virus (WPRE), which contains an additional *cis*-acting element not found in the HPRE. The regulatory element is positioned within the vector so as to be included in the RNA transcript of the transgene, but outside of stop codon of the transgene translational unit. It has been found that the use of such regulatory elements are particularly preferred in the context of modest promoters, but may be contraindicated in the case of very highly efficient promoters.

It is particularly desirable to employ in the lentivectors of the present invention an LTR region that has reduced promoter activity relative to wild-type LTR, in that such constructs provide a "self-inactivating" (SIN) biosafety feature. Self-inactivating vectors are ones in which the production of full-length vector RNA in transduced cells is greatly reduced or abolished altogether. This feature greatly minimizes the risk that replication-competent recombinants (RCRs) will emerge. Furthermore, it reduces the risk that that cellular coding sequences located adjacent to the vector integration site will be aberrantly expressed. Furthermore, a SIN design reduces the possibility of interference between the LTR and the promoter that is driving the



expression of the transgene. It is therefore particularly suitable to reveal the full potential of the internal promoter.

5 Self-inactivation is preferably achieved through in the introduction of a deletion in the U3 region of the 3' LTR of the vector DNA, *i.e.*, the DNA used to produce the vector RNA. Thus, during reverse transcription, this deletion is transferred to the 5' LTR of the proviral DNA. It is desirable to eliminate enough of the U3 sequence to greatly diminish or abolish altogether the transcriptional activity of the LTR, thereby greatly diminishing or abolishing the production of full-length vector RNA in transduced cells. However, it is generally desirable to retain those elements of the LTR that are involved in polyadenylation of the viral RNA, a function spread out  
10 over U3, R and U5. Accordingly, it is desirable to eliminate as many of the transcriptionally important motifs from the LTR as possible while sparing the polyadenylation determinants. In the case of HIV based lentivectors, it has been discovered that such vectors tolerate significant U3 deletions, including the removal of the LTR TATA box (*e.g.*, deletions from -418 to -18), without significant reductions in vector titers. These deletions render the LTR region  
15 substantially transcriptionally inactive in that the transcriptional ability of the LTR is reduced to about 90% or lower. In preferred embodiments the LTR transcription is reduced to about 95% to 99%. Thus, the LTR may be rendered about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, to about 99% transcriptionally inactive.

It is believed that the lentivectors of the present invention may be employed to deliver  
20 any transgene that one desires, depending on the application. In the case of delivery to hematopoietic progenitor cells, one will typically select a transgene that will confer a desirable function on such cells, including, for example, globin genes, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (such as Interleukin-1 (IL-1), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-6 (IL-6), Interleukin-12 (IL-12), *etc.*) and the colony-  
25 stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin  $\alpha$ IIb $\beta$ , multidrug resistance genes, the gp91-phoxor gp 47 genes that are defective in patients with chronic granulomatous disease (CGD), antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation  
30 factors VIII or IX which are mutated in hemophiliacs, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins) as well as combination of T and B cell antigen receptors alone or in combination with single

chain antibodies such as ScFv, tumor necrosis factor (TNF), IL-2, IL-12, gamma interferon, CTLA4, B7 and the like, genes expressed in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100 *etc.*

In a preferred embodiment, the transgene to be introduced in therapy is a gp91-phox gene (Dinauer, *et al.* 1987). In an additional preferred embodiment, the transgene is a gp91-phox gene operably linked to a gp91-phox promoter introduced in therapy for CGD. In a most preferred embodiment, the gp91-phox promoter provides for monocyte and granulocyte expression of the gp91-phox gene and additionally provides for the modulation of gp91-phox expression through the action of the activator INF-gamma. In an additional preferred embodiment, the posttranscriptional regulatory element WPRE is positioned in the vector to enhance the expression of the gp91-phox gene. In a similarly preferred embodiment, the transgene to be introduced in therapy is a gp47-phox gene.

A principal application of the present transgenes will be to deliver desired transgenes to hematopoietic cells for a number of possible reasons. This might include, but of course not be limited to, the treatment of myelosuppression and neutropenias which may be caused as a result of chemotherapy or immunosuppressive therapy or infections such as AIDS, genetic disorders, cancers and the like.

Exemplary genetic disorders of hematopoietic cells that are contemplated include sickle cell anemia, thalassemias (including Beta-thalassemia), hemaglobinopathies, Glanzmann thrombasthenia, lysosomal storage disorders (such as Fabry disease, Gaucher disease, Niemann-Pick disease, and Wiskott-Aldrich syndrome), severe combined immunodeficiency syndromes (SCID), leukocyte adhesion deficiency (LAD), as well as diseases resulting from the lack of systemic production of a secreted protein, for example, coagulation factor VIII and/or IX. In such cases, one would desire to introduce transgenes such as globin genes (including Beta-globins), alpha-galactosidase A, glucocerebrosidase, Sphingomyelin phosphodiesterase-1, cytokine receptor, CD18 integrin subunit, hematopoietic growth factors, which include erythropoietin (EPO), the interleukins (especially Interleukin-1, Interleukin-2, Interleukin-3, Interleukin-6, Interleukin-12, *etc.*) and the colony-stimulating factors (such as granulocyte colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, or stem-cell colony-stimulating factor), the platelet-specific integrin  $\alpha\text{IIb}\beta$ , multidrug resistance genes, the gp91-phox or gp47-phox genes, antiviral genes rendering cells resistant to infections with pathogens such as human immunodeficiency virus, genes coding for blood coagulation factors

VIII or IX which are mutated in hemophiliacs, ligands involved in T cell-mediated immune responses such as T cell antigen receptors, B cell antigen receptors (immunoglobulins), a combination of both T and B cell antigen receptors alone and/or in combination with single chain antibodies (ScFv), IL2, IL12, TNF, gamma interferon, CTLA4, B7 and the like, genes expressed  
5 in tumor cells such as Melana, MAGE genes (such as MAGE-1, MAGE-3), P198, P1A, gp100  
*etc.*

Exemplary cancers are those of hematopoietic origin, for example, arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. Exemplary myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML)  
10 and chronic myelogenous leukemia (CML). Lymphoid malignancies which may be treated utilizing the lentivectors of the present invention include, but are not limited to acute lymphoblastic leukemia (ALL) which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas  
15 contemplated as candidates for treatment utilizing the lentiviral vectors of the present invention include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease.

In other embodiments, the present invention is directed to host cells that have been  
20 transduced with one of the foregoing lentivectors. It is believed that the lentivectors of the present invention can be employed to transduce most any cell. Exemplary cells include but are not limited to a CD4<sup>+</sup> T cell, a peripheral blood lymphocyte cell, a peripheral blood mononuclear cell, a hematopoietic stem cell, a fetal cord blood cell, a fibroblast cell, a brain cell, a lung cell, a liver cell, a muscle cell, a pancreatic cell, an endothelial cell, a cardiac cell, a skin cell, a bone  
25 marrow stromal cell, and an eye cells, a pancreatic ductal cell, a neural precursor, a mesodermal stem cell and the like. The cells transduced may further be primate, murine, porcine, or human in origin, or come from another animal species.

For the production of virus particles, one may employ any cell that is compatible with the expression of lentiviral *gag* and *pol* genes, or any cell that can be engineered to support such  
30 expression. For example, producer cells such as 293T cells and HT1080 cells may be used.

Of course, as noted above, the lentivectors of the invention will be particularly useful in the transduction of human hematopoietic progenitor cell or a hematopoietic stem cell, obtained either from the bone marrow, the peripheral blood or the umbilical cord blood, as well as in the transduction of a CD4<sup>+</sup> T cell, a peripheral blood B or T lymphocyte cell, a peripheral blood mononuclear cell, a dendritic cell, and a monocytic cell. Particularly preferred targets are CD34<sup>+</sup> cells, including those isolated from mobilized peripheral blood.

In still other embodiments, the present invention is directed to a method for transducing a human hematopoietic stem cell comprising contacting a population of human cells that include hematopoietic stem cells with one of the foregoing lentivectors under conditions to effect the transduction of a human hematopoietic progenitor cell in said population by the vector. The stem cells may be transduced *in vivo* or *in vitro*, depending on the ultimate application. Even in the context of human gene therapy, such as gene therapy of human stem cells, one may transduce the stem cell *in vivo* or, alternatively, transduce *in vitro* followed by infusion of the transduced stem cell into a human subject. In one aspect of this embodiment, the human stem cell can be removed from a human, *e.g.*, a human patient, using methods well known to those of skill in the art and transduced as noted above. The transduced stem cells are then reintroduced into the same or a different human.

Where a human subject is treated directly by introduction of the vector into the subject, the treatment is typically carried out by intravenous administration of the vector. When cells, for instance CD34<sup>+</sup> cells, dendritic cells, peripheral blood cells or tumor cells are transduced *ex vivo*, the vector particles are incubated with the cells using a dose generally in the order of between 1 to 50 multiplicities of infection (MOI) which also corresponds to 1x10<sup>5</sup> to 50x10<sup>5</sup> transducing units of the viral vector per 10<sup>5</sup> cells. This of course includes amount of vector corresponding to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 MOI. Typically, the amount of vector may be expressed in terms of HeLa transducing units (TU). Other routes for vector administration include intrarterially, endoscopically, intralesionally, percutaneously, subcutaneously, intramuscular, intrathecally, intraorbitally, intradermally, intraperitoneally, transtracheally, subcuticularly, by intrastemal injection, by inhalation or intranasal spraying, by endotracheal route and the like. In embodiments concerning tumor/cancer therapies with the vectors of the invention the expression vector can be delivered by direct injection into the tumor or into the tumor vasculature.

A preferred example of *ex vivo* gene therapy is a patient suffering from chronic granulomatous disease (CGD), whose CD34<sup>+</sup> cells can be isolated from the bone marrow or the peripheral blood and transduced *ex vivo* with a lentivector expressing the gp91-phox gene under the control of the gp91-phox promoter before reimplantation. A similar approach may be taken with the treatment of patients suffering from thalassemias, for example Beta-thalassemia, where cells may be transduced with a lentivector expressing a Beta-globin under the control of a Beta-globin, or other appropriate promoter. Similarly, lentivectors of the present invention expressing the appropriate gene and promoter combination for treatment of Leukocyte adhesion deficiency (LAD) are contemplated.

In the case of patients suffering from severe combined immunodeficiency (SCID), the inventors contemplate a similar approach, using lentivectors of the invention expressing the gene defective in the patient, for example, the gene encoding the common gamma chain of the Interleukin receptor operably linked to an appropriate promoter providing appropriate tissue or cell specificity and operable control. For the genetic treatment of HIV infection, the present inventors contemplate intracellular immunization, wherein cells are rendered resistant to the HIV virus through the introduction of antiviral genes. In embodiments of the intracellular immunization for HIV, targets of the lentivectors of the invention include hematopoietic progenitors, peripheral blood CD4<sup>+</sup> T cells, and monocytes. As will be recognized by the skilled artisan, similar intracellular immunization methods can be used for other viral infections as well. For the immunotherapy of cancers, tumor cells or antigen presenting cells such as dendritic cells will be genetically engineered with the lentivectors of the invention. For cancer therapies some transgenes that may be used in the lentivector constructs of the invention are those that can inhibit, and/or kill, and/or prevent the proliferation, and/or mediate the apoptosis of, the cancer/tumor cell and/or genes such as TNF.

The lentivectors described herein may also be used *in vivo*, by direct injection into the blood or into a specific organ. For example, in one embodiment intracerebral injection of lentivectors expressing the Glial Cell Derived Nerve Growth Factor (GDNF), can be used for the treatment of Parkinson's disease. In another example, intraportal injection of a lentivector expressing coagulation factor VIII for the correction of hemophilia A is envisioned. In yet another example, intravenous or intramuscular injection of a lentivector of the present invention expressing the dystrophin gene for the treatment of Duchenne Muscular Dystrophy is envisioned. In a further, preferred example, a lentivector expressing gp91-phox is injected in treatment of Chronic Granulomatous Disease (CGD). In a particularly preferred embodiment, a lentivector

expressing gp91-phox under the control of the gp91-phox promoter may be injected in treatment of CGD. Thus, one of ordinary skill in the art will appreciate the extensive use of the lentivector constructs of the present invention in terms of gene therapies.

As used herein the specification or claim(s) when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1A** Lentivectors containing gp91-phox promoter. Schematic maps of lentiviral vectors containing the gp91-phox promoter (1540bp) (pHPP91-GFP) and WPRE sequences (pWPP91-GFP).

**FIG. 1B.** Model for the regulation of the gp91-phox promoter. The transcriptional repressor CDP competes with the binding of transcriptional activating factors at four elements. The DNA binding activity of CDP is down-regulated during terminal phagocyte development, thereby permitting the interaction of transcriptional activators with the gp91-phox promoter (*Luo W, Skalnik DG. JBC, 271: 18203, 1996*).

**FIG. 2A.** Interferon- $\gamma$  inducible GFP expression in UCB CD34<sup>+</sup> derived monocytes driven by gp91-phox promoter UCB CD34<sup>+</sup> transduced with pWPT-GFP and pWPP91-GFP lentivectors (MOI 10). Cells were *in vitro* differentiated in a presence of GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) into monocytes (CD14<sup>+</sup> cells) for 3 weeks.

5 Differentiated cells were stimulated with INF- $\gamma$  (1000U/ml) for 6 days and labelled with PE-conjugated monoclonal antibodies. GFP expression in the PE positive population was analysed using FACS. Numbers indicate percentage of cells in the quadrants.

**FIG. 2B.** Interferon- $\gamma$  inducible GFP expression in UCB CD34<sup>+</sup> derived granulocytes driven by gp91-phox promoter UCB CD34<sup>+</sup> transduced with pWPT-GFP and pWPP91-GFP lentivectors (MOI 10). Cells were *in vitro* differentiated in a presence of G-CSF (Granulocyte Colony Stimulating Factor) into granulocytes (CD15<sup>+</sup> cells) for 3 weeks. Differentiated cells were stimulated with INF- $\gamma$  (1000U/ml) for 6 days and labelled with PE-conjugated monoclonal antibodies. GFP expression in the PE positive population was analysed using FACS. Numbers indicate percentage of cells in the quadrants.

15 **FIG. 3A.** GFP expression in bone marrow of NOD/SCID mice transplanted with lentivector transduced UCB CD34<sup>+</sup>. UCB CD34<sup>+</sup> non-transduced were intravenously injected into sublethally irradiated (375 cGy) NOD/SCID mice (8-10 weeks old). After 8 weeks, bone marrow cells obtained from femurs of transplanted mice were, labelled with PerCP-conjugated anti-human CD45 to label engrafted cells, specific human lineages were further identified using  
20 PE-conjugated antibodies against: CD34 (hematopoietic progenitor cells), CD19 (B lymphocytes), CD33 (neutrophils), CD14 (monocytes), CD15 (granulocytes), CD42b (megacaryocytes) and glycophorin A (erythroblasts). GFP expression was analysed in CD45<sup>+</sup> gated and PE-positive cells. Numbers indicate percentage of cells in the quadrants.

**FIG. 3B.** GFP expression in bone marrow of NOD/SCID mice transplanted with  
25 lentivector transduced UCB CD34<sup>+</sup>. UCB CD34<sup>+</sup> transduced with pHPT-GFP lentivectors (MOI 10) were intravenously injected into sublethally irradiated (375 cGy) NOD/SCID mice (8-10 weeks old). After 8 weeks, bone marrow cells obtained from femurs of transplanted mice were, labelled with PerCP-conjugated anti-human CD45 to label engrafted cells, specific human lineages were further identified using PE-conjugated antibodies against: CD34 (hematopoietic  
30 progenitor cells), CD19 (B lymphocytes), CD33 (neutrophils), CD14 (monocytes), CD15 (granulocytes), CD42b (megacaryocytes) and glycophorin A (erythroblasts). GFP expression

was analysed in CD45+ gated and PE-positive cells. Numbers indicate percentage of cells in the quadrants.

**FIG. 3C.** GFP expression in bone marrow of NOD/SCID mice transplanted with lentivector transduced UCB CD34+. UCB CD34+ transduced with pHPP91-GFP lentivectors (MOI 10) were intravenously injected into sublethally irradiated (375 cGy) NOD/SCID mice (8-10 weeks old). After 8 weeks, bone marrow cells obtained from femurs of transplanted mice were, labelled with PerCP-conjugated anti-human CD45 to label engrafted cells, specific human lineages were further identified using PE-conjugated antibodies against: CD34 (hematopoietic progenitor cells), CD19 (B lymphocytes), CD33 (neutrophils), CD14 (monocytes), CD15 (granulocytes), CD42b (megacaryocytes) and glycophorin A (erythroblasts). GFP expression was analysed in CD45+ gated (in red) and PE-positive cells. Numbers indicate percentage of cells in the quadrants.

**FIG. 3D.** GFP expression in bone marrow of NOD/SCID mice transplanted with lentivector transduced UCB CD34+. UCB CD34+ transduced with pWPT-GFP lentivectors (MOI 10) were intravenously injected into sublethally irradiated (375 cGy) NOD/SCID mice (8-10 weeks old). After 8 weeks, bone marrow cells obtained from femurs of transplanted mice were, labelled with PerCP-conjugated anti-human CD45 to label engrafted cells, specific human lineages were further identified using PE-conjugated antibodies against: CD34 (hematopoietic progenitor cells), CD19 (B lymphocytes), CD33 (neutrophils), CD14 (monocytes), CD15 (granulocytes), CD42b (megacaryocytes) and glycophorin A (erythroblasts). GFP expression was analysed in CD45+ gated and PE-positive cells. Numbers indicate percentage of cells in the quadrants.

**FIG. 3E.** GFP expression in bone marrow of NOD/SCID mice transplanted with lentivector transduced UCB CD34+. UCB CD34+ transduced with pWPP91-GFP lentivectors (MOI 10) were intravenously injected into sublethally irradiated (375 cGy) NOD/SCID mice (8-10 weeks old). After 8 weeks, bone marrow cells obtained from femurs of transplanted mice were, labelled with PerCP-conjugated anti-human CD45 to label engrafted cells, specific human lineages were further identified using PE-conjugated antibodies against: CD34 (hematopoietic progenitor cells), CD19 (B lymphocytes), CD33 (neutrophils), CD14 (monocytes), CD15 (granulocytes), CD42b (megacaryocytes) and glycophorin A (erythroblasts). GFP expression was analysed in CD45+ gated and PE-positive cells. Numbers indicate percentage of cells in the quadrants.



**FIG. 4.** WPRE rescues GFP expression driven gp91-phox promoter in myeloid cells in vivo. Data from FIGS. 3B, 3C, and 3D; background fluorescence of control cells was subtracted. Neu – neutrophils, Mo – monocytes, Gr – granulocytes.

**FIGS. 5A, 5B, 5C, and 5D.** Lentiviral vectors carrying GFP marker gene under control of constitutive EF-1 $\alpha$  promoter (5A, 5B) or under control of gp91-phox myeloid-specific promoter (5C, 5D). Vectors 5B and 5D contain WPRE sequences.

**FIGS. 6A, 6B, and 6C.** Construction of lentiviral vector carrying gp91-phox promoter fragment (500bp) and multicloning sites (MCS) and insertion of gp91-phox – specific enhancers beginning with pHPT-GFP. In a first step, a 500bp fragment of the human gp91-phox promoter was generated by PCR using 1540bp fragment as a template (MCS-I sequence was included into 5' forward primer) and inserted into XhoI-BamHI of pHPT-GFP in place of EF-1 $\alpha$ -short promoter generating the pHP500-GFP intermediate vector. Next, a PCR fragment containing MCSII, cPPT, MCS-I and gp91-phox500bp fragment was created using the pHP500-GFP vector as a template (MCS-II sequence was added into 5' forward primer) was inserted into ClaI-BamHI site of pHOX-GFP vector generating the pHPX-GFP vector (FIG. 6B). gp91-phox – specific enhancers were inserted sequentially into both MCSs either upstream or downstream of cPPT sequence generating the pHPHS-GFP vector (FIG. 6C). Finally, the pWPHS-GFP vector was created by insertion of WPRE sequence into the pHPHS-GFP vector. Positions of PCR-amplified HS elements (+1 gp91-phox transcription start) in human genomic DNA sequence are: HS-12 (-11503, -13244), HS-14 (-13244, -14715), HS-26 (-25345, -26529), HS-27 (-26529, -27656), HS-28 (-27657, -28893) (FIG. 6C). The fragments as depicted are slightly larger than calculated due to incorporation of restriction sites at the end to facilitate cloning (HS-12 and HS-14; gta to restore 5' SnaBI after cloning into this site in pHPX-GFP; HS-26, HS27, HS-28 Sall; gcgtcgac and XhoI; ctcgagcggc).

**FIG. 7.** Insertion of the cPPT element into the lentiviral vectors. The central polypurine tract (cPPT) was taken from the pRRLsinb.hPGK.EGFP vector (see Follenzi, A, Ailles, L.E., Bakovic, S., Geuna, M., Naldini, L. (2000) Gene Transfer by Lentiviral Vectors is Limited by Nuclear Translocation and Rescued by HIV-1 pol Sequences. Nat. Genet. 25:217-22.) A NotI-EcoRV fragment containing the cPPT element was cloned into NotI-ClaI sites of pHOX-GFP vector generating pHPT-GFP. NotI and ClaI sites were filled in both ligation fragments, so NotI is not restored in pHPT-GFP. The ClaI site is restored but became dam methylated. Hence, the plasmid vector must be grown in dam(-) bacteria in order to use this ClaI site.

**DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

While lentiviral vectors offer a great potential for gene-therapy and especially the transduction of human hematopoietic stem cells (hHSC), vectors developed so far have failed to meet biosafety standards and are still inefficient in expression of transgenes. For example, while CMV promoter-containing HIV-derived vectors can induce high levels of transgene expression in the central nervous system (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b; Blomer *et al.*, 1997), and allowed the initial demonstration that pluripotent hematopoietic precursors can be efficiently transduced by this gene delivery tool, they are largely useless for transferring therapeutic genes into most lympho-hematopoietic cells, because in these targets their transcriptional activity is prohibitively low (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000). Current lentiviral vectors have multiply attenuated HIV virulence genes which removes the potential for reconstitution of wild-type virus by recombination (Zufferey *et al.*, 1997; Dull *et al.*, 1998). A self-inactivating design rendered the vectors further biologically safe by eliminating the transcriptional elements of HIV (Zufferey *et al.*, 1998). However, this can negatively affect transgene expression, apparently by decreasing the efficiency of polyadenylation (DeZazzo *et al.*, 1991; Valsamakis *et al.*, 1991; Brown *et al.*, 1991; Cherrington and Ganem, 1992; Valsamakis *et al.*, 1992; Gilmartin *et al.*, 1992).

The present invention overcomes such and other deficiencies in the art and describes the development of improved HIV-derived vectors that are optimized in terms of both biosafety and increased gene expression. Thus, in the practice of the present invention, human cells may be transduced with HIV-derived lentivectors that comprise elements preventing the formation of replication competent recombinants (RCR) and further comprise an internal promoter element which induces high levels of transgene expression in both hematopoietic precursors and *in vitro* differentiated blood lineages, as well as in primary T cells. For example, human CD34<sup>+</sup> cells as well as other human hematopoietic lineages can be transduced using the vectors of this invention.

The promoter elements of the vectors described include the gp91-phox promoter, the Beta-thalassemia promoter, the gp47-phox and CD4 promoters, the EF1 $\alpha$  promoter or the CD11b promoter, although, as will be recognized by one of skill in the art, almost any promoter element may be used.

The gp91-phox promoter was active to provide expression in specific cell types, namely differentiated granulocytes and monocytes. Moreover, the gp91-phox promoter may be activated by contacting the promoter with activators, such as INF-gamma. Also contemplated are engraftment and repopulation assays in NOD/SCID mice with these vectors, to confirm the stability of expression from these promoters *in vivo*.

The element that prevents RCR in the lentivectors of the present invention is the self inactivating (SIN) design. This is achieved by the deletion of a major part of U3 in the 3'LTR of the vector plasmid, leading to a self-inactivating (SIN) configuration (Zufferey *et al.*, 1998). This deletion also prevents potential interference between LTR and the internal promoter elements. However, the SIN can induce decreases in transgene expression, especially in promoters that are not very strong such as the PGK promoter. The invention further describes methods that rescue transgene levels in lentivector constructs that do not have strong promoters by inserting other regulatory elements such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) or the hepatitis virus B regulatory element (HPRE) in the vector, immediately upstream of the deleted 3' LTR. Insertion of the WPRE element does not affect the specificity of expression of the promoter elements.

Additional benefits may be obtained by operably incorporating HS elements into the vectors. For example, incorporating the HS series of enhancer elements into pHPX-GFP may result in higher gene expression and less variegation in expression due to the silencer activity of HS. See, for example, May, *et al.* (2000) "Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin," Nature 406:82-86, incorporated herein by reference. May, *et al.* (2000) disclose HS elements included in a lentivector upstream of a beta-globin promoter to drive higher, yet less variegated expression of beta-globin cDNA.

The lentivectors of this invention can efficiently transduce several human blood lineage cells including CD34<sup>+</sup> cells using conditions under which MLV-based vectors are inefficient. Furthermore, it is also demonstrated that human CD34<sup>+</sup> cells can be efficiently transduced at a relatively low MOI, although the efficacy of gene transfer saturates at about 60 to 70% of transduced cells. For example, an MOI of 10 was used to achieve optimal transduction which is significantly lower than that described in previous studies, where it ranged between 60-300 and 1000-3000 (Miyoshi *et al.*, 1999; Case *et al.*, 1999). This may in part be due to enhanced probability of vector-target meeting since the methods of the present invention involve exposure

of CD34<sup>+</sup> cells to the vector particles in a small volume (10<sup>7</sup> cells in 200 μl) and for a duration of about 6 to 24 hrs.

Thus, the present invention provides HIV-derived vectors which are safe, highly efficient, and very potent for expressing transgenes in human hematopoietic progenitor cells as well as in all other blood cell derivatives, even in a self-inactivating configuration. Furthermore, these vectors provide for cell type specific expression and controllable expression in differentiated cells. These vectors therefore provide useful tools for genetic treatments such as inherited and acquired lympho-hematological disorders, gene-therapies for cancers especially the hematological cancers, the treatment and prevention of HIV infection, as well as for the study of hematopoiesis *via* lentivector-mediated modification of human HSCs.

### 1. Lentiviral Vectors and Gene Therapy

Lentiviruses are complex retroviruses, which, in addition to the common retroviral genes *gag*, *pol*, and *env*, contain other genes with regulatory or structural function. The higher complexity enables the virus to modulate its life cycle, as in the course of latent infection. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1, HIV-2 and the Simian Immunodeficiency Virus: SIV. Lentiviral vectors have been generated by multiply attenuating the HIV virulence genes, for example, the genes *env*, *vif*, *vpr*, *vpu* and *nef* are deleted making the vector biologically safe.

Lentiviral vectors offer great advantages for gene therapy. They integrate stably into chromosomes of target cells which is required for long-term expression. Further, they do not transfer viral genes therefore avoiding the problem of generating transduced cells that can be destroyed by cytotoxic T-cells. Furthermore, they have a relatively large cloning capacity, sufficient for most envisioned clinical applications. In addition, lentiviruses, in contrast to other retroviruses, are capable of transducing non-dividing cells. This is very important in the context of gene-therapy for tissues such as the hematopoietic system, the brain, liver, lungs and muscle. For example, vectors derived from HIV-1 allow efficient *in vivo* and *ex vivo* delivery, integration and stable expression of transgenes into cells such a neurons, hepatocytes, and myocytes (Blomer *et al.*, 1997; Kafri *et al.*, 1997; Naldini *et al.*, 1996; Naldini *et al.*, 1998).

The lentiviral genome and the proviral DNA have the three genes found in retroviruses: *gag*, *pol* and *env*, which are flanked by two long terminal repeat (LTR) sequences. The *gag* gene

encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the *pol* gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease and an integrase; and the *env* gene encodes viral envelope glycoproteins. The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other *cis*-acting sequences necessary for viral replication. Lentiviruses have additional genes including *vif*, *vpr*,  
5 *tat*, *rev*, *vpu*, *nef* and *vpx*.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the *Psi* site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into  
10 infectious virions) are missing from the viral genome, the *cis* defect prevents encapsidation of genomic RNA. However, the resulting mutant remains capable of directing the synthesis of all virion proteins.

Lentiviral vectors are known in the art, see Naldini *et al.*, (1996 and 1998); Zufferey *et al.*, (1997); Dull *et al.*, (1998), Ramezani *et al.*, (2000), all incorporated herein by reference.  
15 Also see U.S. Pat. Nos. 5,994,136; 6,013,516; 6,165,782; 6,207,455; 6,218,181; 6,218,186; and 6,277,633; all of which are incorporated herein by reference. In general, these vectors are plasmid-based or virus-based, and are configured to carry the essential sequences for incorporating foreign nucleic acid, for selection and for transfer of the nucleic acid into a host cell.

Two components are involved in making a virus-based gene delivery system: first, the packaging elements, encompassing the structural proteins as well as the enzymes necessary to generate an infectious particle, and second, the vector itself, i.e., the genetic material to be transferred. Biosafety safeguards can be introduced in the design of both of these components. Thus, the packaging unit of the first generation HIV-based vectors comprised all HIV-1 proteins  
25 except the envelope proteins (Naldini *et al.*, 1998). Subsequently it was shown that the deletion of four additional viral genes that are responsible for virulence including, *vpr*, *vif*, *vpu* and *nef* did not alter the utility of the vector system (Zufferey *et al.*, 1997). It was also shown that Tat, the main transactivator of HIV is also dispensable for the generation of a fully efficient vector (Dull *et al.*, 1998). Thus, the third-generation packaging unit of the HIV-based lentiviral vectors  
30 comprise only three genes of the parental virus: *gag*, *pol* and *rev*, which eliminates the possibility of reconstitution of a wild-type virus through recombination.

This system was further improved by removing HIV transcriptional units from the vector (Zufferey *et al.*, 1998). It was demonstrated therein that introducing a deletion in the U3 region of the 3' LTR of the DNA used to produce the vector RNA generated self-inactivating (SIN) vectors. During reverse transcription this deletion is transferred to the 5' LTR of the proviral DNA. Enough sequence was eliminated, including the removal of a TATA box, which abolished the transcriptional activity of the LTR, which prevents production of full-length vector RNA in transduced cells. This however did not affect vector titers or the *in vitro* or *in vivo* properties of the vector.

The present invention provides several improvements to the existing lentivectors as described above and in other parts of this specification. Introducing a lentivector providing a heterologous gene, such as genes to treat hematopoietic and lympho-hematopoietic disorders in this invention, into a packaging cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest.

The *env* gene can be derived from any virus, including retroviruses. The *env* preferably is an amphotropic envelope protein which allows transduction of cells of human and other species. Examples of retroviral-derived *env* genes include, but are not limited to: Moloney murine leukemia virus (MoMuLV or MMLV), Harvey murine sarcoma virus (HaMuSV or HSV), murine mammary tumor virus (MuMTV or MMTV), gibbon ape leukemia virus (GaLV or GALV), human immunodeficiency virus (HIV) and Rous sarcoma virus (RSV). Other *env* genes such as Vesicular stomatitis virus (VSV) protein G (VSV G), that of hepatitis viruses and of influenza also can be used.

While VSV G protein is a desirable *env* gene because VSV G confers broad host range on the recombinant virus, VSV G can be deleterious to the host cell. Thus, when a gene such as that for VSV G is used, it is preferred to employ an inducible promoter system so that VSV G expression can be regulated to minimize host toxicity when VSV G is expression is not required. For example, the tetracycline-regulatable gene expression system of Gossen & Bujard, (1992) can be employed to provide for inducible expression of VSV G when tetracycline is withdrawn from the transferred cell. Thus, the tet/VP16 transactivator is present on a first vector and the VSV G coding sequence is cloned downstream from a promoter controlled by tet operator sequences on another vector.

The vector providing the viral *env* nucleic acid sequence is associated operably with regulatory sequences, e.g., a promoter or enhancer. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, EF1 $\alpha$ , PGK, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus enhancer, the vaccinia P7.5 promoter or the like (also see examples listed in Tables 1 and 2 below). In some cases, such as the Moloney murine leukemia virus promoter-enhancer element, the promoter-enhancer elements are located within or adjacent to the LTR sequences. Preferably, the regulatory sequence is one which is not endogenous to the lentivirus from which the vector is being constructed. Thus, if the vector is being made from SIV, the SIV regulatory sequence found in the SIV LTR would be replaced by a regulatory element which does not originate from SIV.

One may further target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell-type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific. Retroviral vectors can be made target-specific by inserting, for example, a glycolipid or a protein. Targeting often is accomplished by using an antigen-binding portion of an antibody or a recombinant antibody-type molecule, such as a single chain antibody, to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a retroviral vector to a specific target.

The heterologous or foreign nucleic acid sequence, such as a polynucleotide sequence encoding a gene such as a therapeutic gene for inherited or acquired hematopoietic disorders herein, is linked operably to a regulatory nucleic acid sequence. Preferably, the heterologous sequence is linked to a promoter, resulting in a chimeric gene.

Marker genes may be utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of a marker gene ensures the selection and growth of only those host cells which express the inserts. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances, e.g., histidinol, puromycin, hygromycin, neomycin, methotrexate, and cell surface markers.

The recombinant virus of the invention is capable of transferring a nucleic acid sequence into a mammalian cell. The term, "nucleic acid sequence", refers to any nucleic acid molecule, preferably DNA, as discussed in detail herein. The nucleic acid molecule may be derived from a

variety of sources, including DNA, cDNA, synthetic DNA, RNA or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, poly A sequences or other associated sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription or other means.

The vectors are introduced via transfection or infection into the packaging cell line. The packaging cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After cotransfection of the packaging vectors and the transfer vector to the packaging cell line, the recombinant virus is recovered from the culture media and tittered by standard methods used by those of skill in the art. Thus, the packaging constructs can be introduced into human cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neomycin, DHFR, Glutamine synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones. The selectable marker gene can be linked physically to the packaging genes in the construct.

Stable cell lines wherein the packaging functions are configured to be expressed by a suitable packaging cell are known. For example, see U.S. Pat. No. 5,686,279; and Ory *et al.*, (1996), which describe packaging cells. The packaging cells with a lentiviral vector incorporated in them form producer cells. Producer cells are thus cells or cell-lines that can produce or release packaged infectious viral particles carrying the therapeutic gene of interest. These cells can further be anchorage dependent which means that these cells will grow, survive, or maintain function optimally when attached to a surface such as glass or plastic. The producer cells may also be neoplastically transformed cells. Some examples of anchorage dependent cell lines used as lentiviral vector packaging cell lines when the vector is replication competent are HeLa or 293 cells and PERC.6 cells.

In some applications, particularly when the virus is to be used for gene therapy applications, it is preferable that the vector be replication deficient (or replication defective) to avoid uncontrolled proliferation of the virus in the individual to be treated. In such instances mammalian cell lines are selected which have been engineered, either by modification of the producer cell's genome to encode essential viral functions or by the co-infection of the producer



cell with a helper virus, to express proteins complementing the effect of the sequences deleted from the viral genome. For example, for HIV-1 derived vectors, the HIV-1 packaging cell line, PSI422, may be used as described in Corbeau, *et al.* (1996). Similarly, where the viral vector to be produced is a retrovirus, the human 293-derived retroviral packaging cell line (293GPG) capable of producing high titers of retroviral particles may be employed as described in Ory, *et al.* (1996). In the production of minimal vector systems, the producer cell is engineered (either by modification of the viral genome or by the use of helper virus or cosmid) to complement the functions of the parent virus enabling replication and packaging into virions in the producer cell line.

10           Lentiviral transfer vectors Naldini *et al.*, (1996), have been used to infect human cells growth-arrested *in vitro* and to transduce neurons after direct injection into the brain of adult rats. The vector was efficient at transferring marker genes *in vivo* into the neurons and long term expression in the absence of detectable pathology was achieved. Animals analyzed ten months after a single injection of the vector showed no decrease in the average level of transgene expression and no sign of tissue pathology or immune reaction (Blomer *et al.*, 1997).

## 2. The SIN Design

The SIN design increases the biosafety of the lentiviral vectors. The majority of the HIV LTR is comprised of the U3 sequences. The U3 region contains the enhancer and promoter elements that modulate basal and induced expression of the HIV genome in infected cells and in response to cell activation. Several of these promoter elements are essential for viral replication. Some of the enhancer elements are highly conserved among viral isolates and have been implicated as critical virulence factors in viral pathogenesis. The enhancer elements may act to influence replication rates in the different cellular target of the virus (Marthas *et al.*, 1993).

As viral transcription starts at the 3' end of the U3 region of the 5' LTR, those sequences are not part of the viral mRNA and a copy thereof from the 3' LTR acts as template for the generation of both LTR's in the integrated provirus. If the 3' copy of the U3 region is altered in a retroviral vector construct, the vector RNA is still produced from the intact 5' LTR in producer cells, but cannot be regenerated in target cells. Transduction of such a vector results in the inactivation of both LTR's in the progeny virus. Thus, the retrovirus is self-inactivating (SIN) and those vectors are known as SIN transfer vectors.

The SIN design is described in further detail in Zufferey *et al.*, 1998 and U.S. Pat. No. 5,994,136 both incorporated herein by reference. As described therein, there are, however, limits to the extent of the deletion at the 3' LTR. First, the 5' end of the U3 region serves another essential function in vector transfer, being required for integration (terminal dinucleotide+att sequence). Thus, the terminal dinucleotide and the att sequence may represent the 5' boundary of the U3 sequences which can be deleted. In addition, some loosely defined regions may influence the activity of the downstream polyadenylation site in the R region. Excessive deletion of U3 sequence from the 3' LTR may decrease polyadenylation of vector transcripts with adverse consequences both on the titer of the vector in producer cells and the transgene expression in target cells. On the other hand, limited deletions may not abrogate the transcriptional activity of the LTR in transduced cells.

The lentiviral vectors described herein may carry deletions of the U3 region of the 3' LTR spanning from nucleotide -418 to -18. This is the most extensive deletion and extends as far as to the TATA box, therefore abrogating any transcriptional activity of the LTR in transduced cells. The titer of vector in producer cells as well as transgene expression in target cells was unaffected in these vectors. This design therefore provides an enormous increase in vector safety.

SIN-type vectors with such extensive deletions of the U3 region cannot be generated for murine leukemia virus (MLV) or spleen necrosis virus (SNV) based retroviral vectors without compromising efficiency of transduction.

Elimination of the -418 to -18 nucleotide sequence abolishes transcriptional activity of the LTR, thereby abolishing the production of full length vector RNA in transduced cells. However, in the HIV-derived lentivectors none of the *in vitro* or *in vivo* properties were compromised by the SIN design.

### 3. Posttranscriptionally Regulating Elements (PRE)

Enhancing transgene expression may be required in certain embodiments, especially those that involve lentiviral constructs of the present invention with modest promoters.

One type of PRE is an intron positioned within the expression cassette, which can stimulate gene expression. However, introns can be spliced out during the life cycle events of a lentivirus. Hence, if introns are used as PRE's they have to be placed in an opposite orientation

to the vector genomic transcript.

Posttranscriptional regulatory elements that do not rely on splicing events offer the advantage of not being removed during the viral life cycle. Some examples are the posttranscriptional processing element of herpes simplex virus, the posttranscriptional regulatory element of the hepatitis B virus (HPRE) and the woodchuck hepatitis virus (WPRE). Of these the WPRE is most preferred as it contains an additional *cis*-acting element not found in the HPRE (Donello *et al.*, 1998). This regulatory element is positioned within the vector so as to be included in the RNA transcript of the transgene, but outside of stop codon of the transgene translational unit. As demonstrated in the present invention and in Zufferey *et al.*, 1999, the WPRE element is a useful tool for stimulating and enhancing gene expression of desired transgenes in the context of the lentiviral vectors.

The WPRE is characterized and described in U.S. Pat. No. 6,136,597, incorporated herein by reference. As described therein, the WPRE is an RNA export element that mediates efficient transport of RNA from the nucleus to the cytoplasm. It enhances the expression of transgenes by insertion of a *cis*-acting nucleic acid sequence, such that the element and the transgene are contained within a single transcript. Presence of the WPRE in the sense orientation was shown to increase transgene expression by up to 7 to 10 fold. Retroviral vectors transfer sequences in the form of cDNAs instead of complete intron-containing genes as introns are generally spliced out during the sequence of events leading to the formation of the retroviral particle. Introns mediate the interaction of primary transcripts with the splicing machinery. Because the processing of RNAs by the splicing machinery facilitates their cytoplasmic export, due to a coupling between the splicing and transport machineries, cDNAs are often inefficiently expressed. Thus, the inclusion of the WPRE in a vector results in enhanced expression of transgenes.

#### 25           4. Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that

a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a *cis*-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or

5 mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR<sup>TM</sup>, in connection with the compositions disclosed herein (see U.S. Patents 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well. Control sequences comprising promoters, enhancers and other locus or transcription controlling/modulating elements are also referred to as "transcriptional cassettes".

10 Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, (see, for example Sambrook *et al.*, 1989, incorporated herein by reference). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous for gene therapy or for applications such as the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

20 Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

25 Tables 1 lists non-limiting examples of elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a RNA. Table 2 provides non-limiting examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

| TABLE 1                              |  |
|--------------------------------------|--|
| Promoter and/or Enhancer             |  |
| Promoter/Enhancer                    | References   |
| Immunoglobulin Heavy Chain           | Banerji <i>et al.</i> , 1983; Gilles <i>et al.</i> , 1983; Grosschedl <i>et al.</i> , 1985; Atchinson <i>et al.</i> , 1986, 1987; Imler <i>et al.</i> , 1987; Weinberger <i>et al.</i> , 1984; Kiledjian <i>et al.</i> , 1988; Porton <i>et al.</i> , 1990 |
| Immunoglobulin Light Chain           | Queen <i>et al.</i> , 1983; Picard <i>et al.</i> , 1984  |
| T-Cell Receptor                      | Luria <i>et al.</i> , 1987; Winoto <i>et al.</i> , 1989; Redondo <i>et al.</i> , 1990  |
| HLA DQ a and/or DQ $\beta$           | Sullivan <i>et al.</i> , 1987  |
| $\beta$ -Interferon                  | Goodbourn <i>et al.</i> , 1986; Fujita <i>et al.</i> , 1987; Goodbourn <i>et al.</i> , 1988  |
| Interleukin-2                        | Greene <i>et al.</i> , 1989  |
| Interleukin-2 Receptor               | Greene <i>et al.</i> , 1989; Lin <i>et al.</i> , 1990  |
| MHC Class II 5                       | Koch <i>et al.</i> , 1989  |
| MHC Class II HLA-Dra                 | Sherman <i>et al.</i> , 1989   |
| $\beta$ -Actin                       | Kawamoto <i>et al.</i> , 1988; Ng <i>et al.</i> , 1989   |
| Muscle Creatine Kinase (MCK)         | Jaynes <i>et al.</i> , 1988; Horlick <i>et al.</i> , 1989; Johnson <i>et al.</i> , 1989  |
| Prealbumin (Transthyretin)           | Costa <i>et al.</i> , 1988   |
| Elastase I                           | Omitz <i>et al.</i> , 1987   |
| Metallothionein (MTII)               | Karin <i>et al.</i> , 1987; Culotta <i>et al.</i> , 1989   |
| Collagenase                          | Pinkert <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987   |
| Albumin                              | Pinkert <i>et al.</i> , 1987; Tronche <i>et al.</i> , 1989, 1990   |
| $\alpha$ -Fetoprotein                | Godbout <i>et al.</i> , 1988; Campere <i>et al.</i> , 1989   |
| $\gamma$ -Globin                     | Bodine <i>et al.</i> , 1987; Perez-Stable <i>et al.</i> , 1990   |
| $\beta$ -Globin                      | Trudel <i>et al.</i> , 1987  |
| c-fos                                | Cohen <i>et al.</i> , 1987   |
| c-HA-ras                             | Triesman, 1986; Deschamps <i>et al.</i> , 1985   |
| Insulin                              | Edlund <i>et al.</i> , 1985  |
| Neural Cell Adhesion Molecule (NCAM) | Hirsh <i>et al.</i> , 1990   |
| $\alpha_1$ -Antitrypsin              | Latimer <i>et al.</i> , 1990   |
| H2B (TH2B) Histone                   | Hwang <i>et al.</i> , 1990   |
| Mouse and/or Type I Collagen         | Ripe <i>et al.</i> , 1989  |

| TABLE 1                                      |   |
|--|---|
| Promoter and/or Enhancer                     |   |
| Promoter/Enhancer                            | References  |
| Glucose-Regulated Proteins (GRP94 and GRP78) | Chang <i>et al.</i> , 1989  |
| Rat Growth Hormone                           | Larsen <i>et al.</i> , 1986   |
| Human Serum Amyloid A (SAA)                  | Edbrooke <i>et al.</i> , 1989   |
| Troponin I (TN I)                            | Yutzey <i>et al.</i> , 1989   |
| Platelet-Derived Growth Factor (PDGF)        | Pech <i>et al.</i> , 1989   |
| Duchenne Muscular Dystrophy                  | Klamut <i>et al.</i> , 1990   |
| SV40   | Banerji <i>et al.</i> , 1981; Moreau <i>et al.</i> , 1981; Sleight <i>et al.</i> , 1985; Firak <i>et al.</i> , 1986; Herr <i>et al.</i> , 1986; Imbra <i>et al.</i> , 1986; Kadesch <i>et al.</i> , 1986; Wang <i>et al.</i> , 1986; Ondek <i>et al.</i> , 1987; Kuhl <i>et al.</i> , 1987; Schaffner <i>et al.</i> , 1988        |
| Polyoma                                      | Swartzendruber <i>et al.</i> , 1975; Vasseur <i>et al.</i> , 1980; Katinka <i>et al.</i> , 1980, 1981; Tyndell <i>et al.</i> , 1981; Dandolo <i>et al.</i> , 1983; de Villiers <i>et al.</i> , 1984; Hen <i>et al.</i> , 1986; Satake <i>et al.</i> , 1988; Campbell and/or Villarreal, 1988                                      |
| Retroviruses                                 | Kriegler <i>et al.</i> , 1982, 1983; Levinson <i>et al.</i> , 1982; Kriegler <i>et al.</i> , 1983, 1984a, b, 1988; Bosze <i>et al.</i> , 1986; Miksicek <i>et al.</i> , 1986; Celander <i>et al.</i> , 1987; Thiesen <i>et al.</i> , 1988; Celander <i>et al.</i> , 1988; Chol <i>et al.</i> , 1988; Reisman <i>et al.</i> , 1989 |
| Papilloma Virus                              | Campo <i>et al.</i> , 1983; Lusky <i>et al.</i> , 1983; Spandidos and/or Wilkie, 1983; Spalholz <i>et al.</i> , 1985; Lusky <i>et al.</i> , 1986; Cripe <i>et al.</i> , 1987; Gloss <i>et al.</i> , 1987; Hirochika <i>et al.</i> , 1987; Stephens <i>et al.</i> , 1987   |
| Hepatitis B Virus                            | Bulla <i>et al.</i> , 1986; Jameel <i>et al.</i> , 1986; Shaul <i>et al.</i> , 1987; Spandau <i>et al.</i> , 1988; Vannice <i>et al.</i> , 1988   |
| Human Immunodeficiency Virus                 | Muesing <i>et al.</i> , 1987; Hauber <i>et al.</i> , 1988; Jakobovits <i>et al.</i> , 1988; Feng <i>et al.</i> , 1988; Takebe <i>et al.</i> , 1988; Rosen <i>et al.</i> , 1988; Berkhout <i>et al.</i> , 1989; Laspia <i>et al.</i> , 1989; Sharp <i>et al.</i> , 1989; Braddock <i>et al.</i> , 1989                             |
| CD11b  | Hickstein <i>et al.</i> , 1992  |
| Gibbon Ape Leukemia Virus                    | Holbrook <i>et al.</i> , 1987; Quinn <i>et al.</i> , 1989   |

| Element                                      | Inducer                                | References   |
|--|--|--|
| MT II  | Phorbol Ester (TFA)<br>Heavy metals    | Palmiter <i>et al.</i> , 1982; Haslinger <i>et al.</i> , 1985; Searle <i>et al.</i> , 1985; Stuart <i>et al.</i> , 1985; Imagawa <i>et al.</i> , 1987, Karin <i>et al.</i> , 1987; Angel <i>et al.</i> , 1987b; McNeill <i>et al.</i> , 1989 |
| MMTV (mouse mammary tumor virus)             | Glucocorticoids                        | Huang <i>et al.</i> , 1981; Lee <i>et al.</i> , 1981; Majors <i>et al.</i> , 1983; Chandler <i>et al.</i> , 1983; Lee <i>et al.</i> , 1984; Ponta <i>et al.</i> , 1985; Sakai <i>et al.</i> , 1988   |
| $\beta$ -Interferon                          | Poly(rI)x<br>Poly(rc)                  | Tavernier <i>et al.</i> , 1983   |
| Adenovirus 5 <u>E2</u>                       | EIA                                    | Imperiale <i>et al.</i> , 1984   |
| Collagenase                                  | Phorbol Ester (TPA)                    | Angel <i>et al.</i> , 1987a  |
| Stromelysin                                  | Phorbol Ester (TPA)                    | Angel <i>et al.</i> , 1987b  |
| SV40   | Phorbol Ester (TPA)                    | Angel <i>et al.</i> , 1987b  |
| Murine MX Gene                               | Interferon, Newcastle<br>Disease Virus | Hug <i>et al.</i> , 1988   |
| GRP78 Gene                                   | A23187                                 | Resendez <i>et al.</i> , 1988  |
| $\alpha$ -2-Macroglobulin                    | IL-6                                   | Kunz <i>et al.</i> , 1989  |
| Vimentin                                     | Serum                                  | Rittling <i>et al.</i> , 1989  |
| MHC Class I Gene H-2 $\kappa$ b              | Interferon                             | Blanar <i>et al.</i> , 1989  |
| HSP70  | EIA, SV40 Large T<br>Antigen           | Taylor <i>et al.</i> , 1989, 1990a, 1990b  |
| Proliferin                                   | Phorbol Ester-TPA                      | Mordacq <i>et al.</i> , 1989   |
| Tumor Necrosis Factor                        | PMA                                    | Hensel <i>et al.</i> , 1989  |
| Thyroid Stimulating<br>Hormone $\alpha$ Gene | Thyroid Hormone                        | Chatterjee <i>et al.</i> , 1989  |

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Non-limiting examples of such regions include the human LIMK2 gene (Nomoto *et al.*, 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human



CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al.*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al.*, 1997), and human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996).

5 The lentiviral vectors of the present invention are designed, primarily, to transform cells with a therapeutic gene under the control of regulated eukaryotic promoters. Although the gp91-phox promoter is preferred, other promoter and regulatory signal elements as described in the Tables 1 and 2 above may also be used. Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of structural genes encoding the therapeutic gene of interest that is used in context with the  
10 lentiviral vectors of the present invention. Alternatively, a tissue-specific promoter for cancer gene therapy or the targeting of tumors may be employed with the lentiviral vectors of the present invention for treatment of cancers, especially hematological cancers.

Typically promoters and enhancers that control the transcription of protein encoding genes in eukaryotic cells are composed of multiple genetic elements. The cellular machinery is  
15 able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation. Activation or repression of the promoter and enhancer elements may be had through contacting those elements with the appropriate transcriptional activators or repressors, such as those described in FIG. 1B for the gp91-phox promoter and disclosed in Luo and Skalnik (1996) J.  
20 Biol. Chem. 271:18203-210, and Luo and Skalnik (1996) J. Biol. Chem. 271:23445-23451, incorporated herein by reference. With respect to the gp91-phox promoter, the activity of Interferon-gamma in modulating the transcription and expression of the expression cassette is an example of how such promoter or enhancer elements and the factors that interact with them may be employed in the practice of the present invention.

25 Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one  
30 or more transcriptional proteins. See, for example, the model for the regulation of the gp91-phox promoter presented in FIG. 1B. Exemplary enhancers contemplated in the present invention are the DNAase HyperSensitive elements and their homologs described by Lien LL, Lee Y, Orkin

SH, (1997) "Regulation of the myeloid-cell-expressed human gp91-phox gene as studied by transfer of yeast artificial chromosome clones into embryonic stem cells: suppression of a variegated cellular pattern of expression requires a full complement of distant cis elements," *Mol Cell Biol.* 17(4):2279-90, expressly incorporated herein by reference in its entirety. Under the influence of these enhancer elements, gene expression may be higher (due to enhancer activity) and less variegated (due to silencer activity of HS).

Analogs of the HS elements of gp91-phox are active in other promoter-enhancer systems. See, for example, May C, Rivella S, Callegari J, Heller G, Gaensler KM, Luzzatto L, Sadelain M, (2000) Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* 406(6791):82-6, incorporated herein by reference, where analogous beta-globin HS elements were included into lentivector upstream of beta-globin promoter to drive expression of beta-globin cDNA.

Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities. Constructs of elements that control transcription and expression may therefore be comprised of various elements arranged so as to provide means of control of enhanced utility and operation.

A signal that may prove useful is a polyadenylation signal (hGH, BGH, SV40). The use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5'-methylated cap-dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well as an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open

reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

5 In any event, it will be understood that promoters are DNA elements that when positioned functionally upstream of a gene leads to the expression of that gene. Most transgenes that will be transformed using the lentiviral vectors of the present invention are functionally positioned downstream of a promoter element.

10 A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous  
15 translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

### 5. Brief Description of the Sequence Listings

20 SEQ ID NO: 1 provides the nucleotide sequence of the HIV central polypurine tract element, taken from HIV as reported in Follenzi, A, Ailles, L.E., Bakovic, S., Geuna, M., Naldini, L. (2000) "Gene Transfer by Lentiviral Vectors is Limited by Nuclear Translocation and Rescued by HIV-1 pol Sequences," Nat. Genet. 25:217-22. SEQ ID NOS: 2-6 provide the nucleotide sequences comprising DNAase HyperSensitive sites and used as enhancers in certain  
25 embodiments of the invention. See Lien LL, Lee Y, and Orkin SH, "Regulation of the myeloid-cell-expressed human gp91-phox gene as studied by transfer of yeast artificial chromosome clones into embryonic stem cells: suppression of a variegated cellular pattern of expression requires a full complement of distant cis elements," Mol. Cell. Biol. 17(4):2279-90 (1997).

30 SEQ ID NOS:7-16 provide the nucleotide sequences of the PCR primers used to generate the HS elements of SEQ ID NOS:2-6. Sequences of the PCR primers and HS element sequences are based on human genome sequence published by Human Genome Project (contig

NT\_011844; <http://www.ncbi.nlm.nih.gov/genome/guide/human/>). Positions of PCR-amplified HS elements (+1 gp91-phox transcription start) in human genomic DNA sequence are: HS-12 (-11503, -13244), HS-14 (-13244, -14715), HS-26 (-25345, -26529), HS-27 (-26529, -27656), HS-28 (-27657, -28893). Fragments used are slightly larger than calculated due to incorporation of restriction sites at the end to facilitate cloning (HS-12 and HS-14; gta to restore 5' SnaBI after cloning into this site in pHPX-GFP; HS-26, HS27, HS-28 SalI; gcgtcgac and XhoI; ctcgagcggc).

Other sequences incorporated to particular embodiments of the present invention include those encoding gp91-phox and its homologs, including nucleotide sequences disclosed in Genbank Accession number NM000397 for gp91-phox (Dinauer, *et al.*, 1987), SEQ ID NO: 18 and polypeptides of the sequence of SEQ ID NO: 19. Also incorporated in particular embodiments are gp91-phox promoters encoded by the nucleotide sequence of Genbank accession number M66390, SEQ ID NO: 17 and CD11b promoters encoded by the nucleotide sequence of Genbank accession number M82856, SEQ ID NO: 20.

## 6. Nucleic Acids

One embodiment of the present invention is to transfer nucleic acids encoding a therapeutic gene, especially a gene that provides therapy for hematopoietic and lympho-hematopoietic disorders, such as the inherited or acquired disorders described above. In one embodiment the nucleic acids encode a full-length, substantially full-length, or functional equivalent form of such a gene.

Thus, in some embodiments of the present invention, the treatment of a hematopoietic and lympho-hematopoietic disorder involves the administration of a lentiviral vector of the invention comprising a therapeutic nucleic acid expression construct to a cell of hematopoietic origin. It is contemplated that the hematopoietic cells take up the construct and express the therapeutic polypeptide encoded by nucleic acid, thereby restoring the cells normal phenotype.

A nucleic acid may be made by any technique known to one of ordinary skill in the art. Non-limiting examples of synthetic nucleic acid, particularly a synthetic oligonucleotide, include a nucleic acid made by *in vitro* chemical synthesis using phosphotriester, phosphite or phosphoramidite chemistry and solid phase techniques such as described in EP 266,032, incorporated herein by reference, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, 1986, and U.S. Patent Serial No. 5,705,629, each incorporated

herein by reference. A non-limiting example of enzymatically produced nucleic acid include one produced by enzymes in amplification reactions such as PCR<sup>TM</sup> (see for example, U.S. Patent 4,683,202 and U.S. Patent 4,682,195, each incorporated herein by reference), or the synthesis of oligonucleotides described in U.S. Patent No. 5,645,897, incorporated herein by reference. A non-limiting example of a biologically produced nucleic acid includes recombinant nucleic acid production in living cells (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

The term "nucleic acid" will generally refer to at least one molecule or strand of DNA, RNA or a derivative or mimic thereof, comprising at least one nucleobase, such as, for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.*, adenine "A," guanine "G," thymine "T," and cytosine "C") or RNA (*e.g.* A, G, uracil "U," and C). The term "nucleic acid" encompasses the terms "oligonucleotide" and "polynucleotide." The term "oligonucleotide" refers to at least one molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length. These definitions generally refer to at least one single-stranded molecule, but in specific embodiments will also encompass at least one additional strand that is partially, substantially or fully complementary to the at least one single-stranded molecule. Thus, a nucleic acid may encompass at least one double-stranded molecule or at least one triple-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a strand of the molecule.

In certain embodiments, a "gene" refers to a nucleic acid that is transcribed. As used herein, a "gene segment" is a nucleic acid segment of a gene. In certain aspects, the gene includes regulatory sequences involved in transcription, or message production or composition. In particular embodiments, the gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. In other particular aspects, the gene comprises a nucleic acid, and/or encodes a polypeptide or peptide-coding sequences of a gene that is defective or mutated in a hematopoietic and lympho-hematopoietic disorder. In keeping with the terminology described herein, an "isolated gene" may comprise transcribed nucleic acid(s), regulatory sequences, coding sequences, or the like, isolated substantially away from other such sequences, such as

other naturally occurring genes, regulatory sequences, polypeptide or peptide encoding sequences, etc. In this respect, the term "gene" is used for simplicity to refer to a nucleic acid comprising a nucleotide sequence that is transcribed, and the complement thereof. In particular aspects, the transcribed nucleotide sequence comprises at least one functional protein, polypeptide and/or peptide encoding unit. As will be understood by those in the art, this functional term "gene" includes both genomic sequences, RNA or cDNA sequences, or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or enhancer regions of a gene. Smaller engineered gene nucleic acid segments may express, or may be adapted to express using nucleic acid manipulation technology, proteins, polypeptides, domains, peptides, fusion proteins, mutants and/or such like. Thus, a "truncated gene" refers to a nucleic acid sequence that is missing a stretch of contiguous nucleic acid residues.

Various nucleic acid segments may be designed based on a particular nucleic acid sequence, and may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all nucleic acid segments can be created:

$$n \text{ to } n + y$$

where  $n$  is an integer from 1 to the last number of the sequence and  $y$  is the length of the nucleic acid segment minus one, where  $n + y$  does not exceed the last number of the sequence. Thus, for a 10-mer, the nucleic acid segments correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and/or so on. For a 15-mer, the nucleic acid segments correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and/or so on. For a 20-mer, the nucleic segments correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and/or so on.

The nucleic acid(s) of the present invention, regardless of the length of the sequence itself, may be combined with other nucleic acid sequences, including but not limited to, promoters, enhancers, polyadenylation signals, restriction enzyme sites, multiple cloning sites, coding segments, and the like, to create one or more nucleic acid construct(s). The overall length may vary considerably between nucleic acid constructs. Thus, a nucleic acid segment of almost any length may be employed, with the total length preferably being limited by the ease of preparation or use in the intended recombinant nucleic acid protocol.

The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. Vectors of

the present invention are lentivirus based as described above and in other parts of the specification. The nucleic acid molecules carried by the vectors of the invention encode therapeutic genes and will be used for carrying out gene-therapies. One of skill in the art would be well equipped to construct such a therapeutic vector through standard recombinant techniques (see, for example, Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both incorporated herein by reference).

The term "expression vector" refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described below.

#### A. Multiple Cloning Sites

Vectors of the present invention can include a multiple cloning site (MCS), which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, Carbonelli *et al.*, 1999, Levenson *et al.*, 1998, and Cocea, 1997, incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

## B. Splicing Sites

Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, Chandler *et al.*, 1997, herein incorporated by reference.)

## C. Termination Signals

The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues (polyA) to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to be more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that the terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.



#### D. Polyadenylation Signals

In eukaryotic gene expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Some examples include the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

#### E. Origins of Replication

In order to propagate a vector of the invention in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

#### F. Selectable and Screenable Markers

In certain embodiments of the invention, cells transduced with the lentivectors of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the transduced cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genetic constructs that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes

such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

## 7. Host Cells

As used herein, the terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which is any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, "host cell" refers to a prokaryotic or eukaryotic cell, and it includes any transformable organisms that is capable of replicating a vector and/or expressing a heterologous nucleic acid encoded by the vectors of this invention. A host cell can, and has been, used as a recipient for vectors. A host cell may be "transfected" or "transformed," which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. As used herein, the terms "engineered" and "recombinant" cells or host cells are intended to refer to a cell into which an exogenous nucleic acid sequence, such as, for example, a lentivector of the invention bearing a therapeutic gene construct, has been introduced. Therefore, recombinant cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced nucleic acid.

In certain embodiments, it is contemplated that RNAs or proteinaceous sequences may be co-expressed with other selected RNAs or proteinaceous sequences in the same host cell. Co-expression may be achieved by co-transfecting the host cell with two or more distinct recombinant vectors. Alternatively, a single recombinant vector may be constructed to include multiple distinct coding regions for RNAs, which could then be expressed in host cells transfected with the single vector.

Host cells may be derived from prokaryotes or eukaryotes, depending upon whether the desired result is replication of the vector or expression of part or all of the vector-encoded nucleic acid sequences. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an

organization that serves as an archive for living cultures and genetic materials (www.atcc.org). Some examples of host cells used in this invention include but are not limited to virus packaging cells, virus producer cells, 293T cells, human hematopoietic progenitor cells, human hematopoietic stem cells, CD34<sup>+</sup> cells CD4<sup>+</sup> cells, and the like.

5                   **A. Tissues and Cells**

A tissue may comprise a host cell or cells to be transformed or contacted with a nucleic acid delivery composition and/or an additional agent. The tissue may be part or separated from an organism. In certain embodiments, a tissue and its constituent cells may comprise, but is not limited to, blood (*e.g.*, hematopoietic cells (such as human hematopoietic progenitor cells, 10 human hematopoietic stem cells, CD34<sup>+</sup> cells CD4<sup>+</sup> cells), lymphocytes and other blood lineage cells), bone marrow, brain, stem cells, blood vessel, liver, lung, bone, breast, cartilage, cervix, colon, cornea, embryonic, endometrium, endothelial, epithelial, esophagus, facia, fibroblast, follicular, ganglion cells, glial cells, goblet cells, kidney, lymph node, muscle, neuron, ovaries, pancreas, peripheral blood, prostate, skin, skin, small intestine, spleen, stomach, testes.

15                   **B. Organisms**

In certain embodiments, the host cell or tissue may be comprised in at least one organism. In certain embodiments, the organism may be human, primate or murine. In other embodiments the organism may be any eukaryote or even a prokaryote (*e.g.*, a eubacteria, an archaea), as would be understood by one of ordinary skill in the art (see, for example, webpage 20 <http://phylogeny.arizona.edu/tree/phylogeny.html>). Some lentivectors of the invention may employ control sequences that allow them to be replicated and/or expressed in both prokaryotic and eukaryotic cells. One of skill in the art would further understand the conditions under which to incubate all of the above described host cells to maintain them and to permit replication of a vector. Also understood and known are techniques and conditions that would allow large-scale 25 production of the lentivectors of the invention, as well as production of the nucleic acids encoded by the lentivectors and their cognate polypeptides, proteins, or peptides some of which are therapeutic genes or proteins which will be used for gene therapies.

### C. Injectable Compositions and Pharmaceutical Formulations

To achieve gene-therapy using the lentiviral vector compositions of the present invention, one would generally contact a cell in need thereof with a lentiviral vector comprising a therapeutic gene. The cell will further be in an organism such as a human in need of the gene therapy. The routes of administration will vary, naturally, with the location and nature of the disease, and include, *e.g.*, intravenous, intrarterial, intradermal, transdermal, intramuscular, intranasal, subcutaneous, percutaneous, intratracheal, intraperitoneal, intratumoral, perfusion and lavage. The cells will also sometimes be isolated from the organisms, exposed to the lentivector *ex vivo*, and reimplanted afterwards.

Injection of lentiviral nucleic acid constructs of the invention may be delivered by syringe or any other method used for injection of a solution, as long as the expression construct can pass through the particular gauge of needle required for injection. A novel needleless injection system has recently been described (U.S. Patent 5,846,233) having a nozzle defining an ampule chamber for holding the solution and an energy device for pushing the solution out of the nozzle to the site of delivery. A syringe system has also been described for use in gene therapy that permits multiple injections of predetermined quantities of a solution precisely at any depth (U.S. Patent 5,846,225).

Solutions of the nucleic acids as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the

5 maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

10 For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intraarterial, intramuscular, subcutaneous, intratumoral and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

25 Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

30 The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as

acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" or "pharmacologically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic lentiviral vector is delivered to a target cell.

For gene-therapy to discrete, solid, accessible tumors, intratumoral injection, or injection into the tumor vasculature is specifically contemplated. Local, regional or systemic administration also may be appropriate. For tumors of >4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of <4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals. Systemic administration is preferred for conditions such as hematological malignancies.

Continuous administration also may be applied where appropriate. Delivery *via* syringe or catheterization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs.

Treatment regimens may vary as well, and often depend on type of disease and location of diseased tissue, and factors such as the health and the age of the patient. The clinician will be best suited to make such decisions based on the known efficacy and toxicity (if any) of the therapeutic formulations based on lentiviral vectors of the present invention.

The treatments may include various "unit doses." A unit dose is defined as containing a predetermined-quantity of the therapeutic composition comprising a lentiviral vector of the present invention. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time. Unit dose of the present invention may conveniently be described in terms of transducing units (T.U.) of lentivector, as defined by titering the vector on a cell line such as HeLa or 293. Unit doses range from  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$ ,  $10^7$ ,  $10^8$ ,  $10^9$ ,  $10^{10}$ ,  $10^{11}$ ,  $10^{12}$ ,  $10^{13}$  T.U. and higher.

## 8. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

## A. Materials and Methodology Employed in Examples 1 - 3

### 1. Vector preparation

Production of HIV-derived vectors pseudotyped with the vesicular stomatitis virus (VSV) G envelope protein was achieved by transient co-transfection of three plasmids into 293T epithelial cell line as described in Naldini *et al.* 1996a. The HIV-derived packaging construct used was pCMVΔR8.91, which encodes the HIV-1 Gag and Pol precursors, as well as the regulatory proteins Tat and Rev (Zufferey *et al.*, 1997). VSV G was expressed from pMD.G.

The HIV vector plasmids were derivatives of the original pHR' backbone (Naldini *et al.*, 1996a), with the following modifications. Self-inactivating vectors were produced from the previously described SIN-18 vector, which contains a deletion in the U3 region of the 3'LTR from nt. -418 to nt. -18, removing all the transcriptionally active sequences (Zufferey *et al.*, 1998). Briefly, the pHR'SIN plasmids were generated as follows; a *KpnI-XbaI* fragment containing the polypurine tract and the 3'LTR was excised from a pHR' plasmid and subcloned into the corresponding sites of pUC18. This plasmid was digested completely with *EcoRV* and partially with *PvuII* and self-ligated. A plasmid carrying a 400-nucleotide deletion of U3 was recovered. An *XhoI* linker was inserted into the *EcoRI* site of the deletion plasmid, and an *XhoI-XbaI* fragment was cloned back into the pHR' CMVlacZ plasmid digested with the corresponding enzymes. All other SIN-18 plasmids were obtained by substituting reporter genes (encoding luciferase, GFP and Neo) for lacZ. The pHR' vector plasmids used differed from the plasmids originally described (Naldini *et al.*, 1996) by a *XhoI-KpnI* deletion removing 118 nucleotides from the *Nef* coding sequence upstream of the polypurine tract and a deletion of 1,456 nucleotides of human sequence downstream of the 3' LTR. This human sequence remained from the original cloning of a HXB2 proviral genome. The two deletions did not affect vector titers or transgene expression in dividing 293T cells.

Insertion of the EF1alpha was done by inserting a *Clal-BamHI* cassette containing a EF1-GFP insert, into *Clal-BamHI* site of pHR'-GFP-W-SIN (Zufferey *et al.* 1999) to produce the pHR-EF1-GFP-W-SIN vector. The derivatives of this vector possessed several functional differences. The functional differences are EF-1alpha-short promoter, loxP site at 3'LTR and cPPT. loxP site in 3'LTR is duplicated to 5'LTR in transduced cells and, in a presence of additionally expressed Cre recombinase, allows removal of integrated provirus. However, loxP site and Cre-mediated excision are not required for gp91-phoxpromoter activity, neither, will be



necessary or used in any potential application of the vectors in hematopoietic stem cells-based clinical trials. A difference between EF-1 $\alpha$  present in pHR-EF1-GFP-(+/-)W-SIN and EF-1 $\alpha$ -short promoter in pHPT-GFP and pWPT-GFP is that the shorter version does not contain an intron, which makes the vector smaller.

- 5 The maps shown in FIGS. 5A through 5D depict the plasmid constructs pHPT-GFP, pWPT-GFP, pHPP91-GFP and pWPP91-GFP.

After transient transfection of the plasmids by calcium phosphate in 293T cells, the supernatant was harvested, concentrated by ultracentrifugation using sucrose gradient, resuspended in serum-free Cellgro® SCGM medium (Cellgenix, Germany) and filtered through  
10 0.45 $\mu$ m SpinX filter. Viral stocks were stored at -70° C and titers determined by transduction and flow cytometry analysis of GFP expression in HeLa cells as previously described (Zufferey *et al.*, 1997). Titters were comprised between  $5 \times 10^7$  and  $10^8$  HeLa-transducing units (TU) per ml.

## 15 2. Purification and transduction of CD34<sup>+</sup> cells

Cord blood (CB) samples were obtained according to institutional guidelines and CD34<sup>+</sup> cells were purified as described (Arrighi *et al.*, 1999). In brief, CB mononuclear cells recovered after Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation were incubated on ice with anti-CD34 M450 Dynabeads (Dyna, Norway) as described by the manufacturer. After  
20 several washes to eliminate unbound cells, CD34<sup>+</sup> cells were recovered from the beads by incubation for 15 minutes at 37°C with the "Detach-a-bead" included in the kit. Cells were immediately washed, and analyzed by flow cytometry. The percentage of purified CD34<sup>+</sup> cells was  $89 \pm 7.0$ . For transduction,  $10^5$  cells were seeded in 96-well plates in 100  $\mu$ l of Cellgro® SCGM medium supplemented with antibiotics (Gibco BRL, Life Technologies LTD, Paisley,  
25 Scotland, U.K.), with  $10^{-4}$  M dithiothreitol (Fluka Biochemika, Buchs, Switzerland) and TPO (10ng/ml), SCF (50ng/ml), Flt3L (50ng/ml). After overnight incubation,  $10^6$  (typically), or  $10^5$  to  $5 \times 10^6$  (for dose-response analysis) HeLa-transducing units (TU) of vector were added per well, and the volume was adjusted to 200  $\mu$ l with Cellgro® SCGM medium containing TPO. After 24 hours, cells were washed, diluted to 400  $\mu$ l in Cellgro® SCGM medium supplemented  
30 with antibiotics,  $10^{-4}$  M dithiothreitol and TPO (10ng/ml), SCF (50ng/ml), Flt3L (50ng/ml) for 3

days. Cells were either directly analyzed for GFP and CD34 expression, or further cultured with the 3 growth factors.

### 3. Cytokines

All cytokines were recombinant human material and were obtained from Peprotech  
5 (London, U.K.)

### 4. Antibodies and immunoreactants

All antibodies were obtained from Becton Dickinson Pharmingen (USA). Anti-CD34  
mIgG coated M450 Dynabeads were from Dynal A/S (Oslo, Norway)..

10

### 5. *In vitro* differentiation and INF-gamma stimulation

Differentiation was performed *in vitro* in the presence of GM-CSF and SCF for  
monocytic differentiation, and G-CSF or G-CSF for 3 weeks and SCF for granulocytic  
differentiation. Differentiated cells were stimulated with INF- $\gamma$  (1000U/ml) for 5 days, labelled  
15 with PE-conjugated monoclonal antibodies and GFP expression in PE positive population was  
analysed using FACS. Numbers indicate percentage of cells in the quadrants.

### 6. Flow cytometry analysis

Cells were analyzed as described (Arrighi *et al.*, 1999), on a FACScalibur (Becton-  
Dickinson) with slight modifications. FL-1 was used for GFP, FL-2 for PE-labeled MAbs, FL-3  
20 for PercP-labeled MAbs. Cell suspensions were adjusted to 0.5% paraformaldehyde prior to  
analysis. Data were analyzed using WINMDI software written by J. Trotter at Scripps Institute  
(La Jolla, CA) and CellQuest software (Becton-Dickinson).

## B. Examples 1 – 4

25 **Example 1: Transduction of Human Hematopoietic Progenitors with HIV-Based  
Lentivectors Containing Expression Cassettes Under the Control of the gp91-  
phox Promoter Results in Restricted Expression in Monocytes and  
Granulocytes.**

pWPP91-GFP (with WPRE) and pHPP91-GFP (without WPRE) lentiviral vectors were created by replacing the EF-1 $\alpha$  promoter with a 1540bp fragment of the gp91-phox subunit of phagocyte NADPH oxidase promoter(1) in the pWPT-GFP and pHPT-GFP lentiviral vectors, respectively. Both pWPT-GFP and pHPT-GFP are SIN (self-inactivating) lentiviral vectors and contain central polypurine tract sequences (cPPT) to enhance transduction efficiency of target cells (FIGS. 1 and 5). Recombinant lentivectors were produced and concentrated 100x by previously described standard methods and used for transduction of human hematopoietic CD34+ progenitor/stem cells (HSCs). See Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH, Trono D., "High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors," *Blood* 96(10):3392-8 (2000), incorporated herein by reference.

A high percentage of UCB HSC transduction, up to 80%, was achieved by a lentivector in which a central poly-purine tract was inserted upstream of an EF1-alpha-GFP expression cassette. High numbers of GFP positive cells were retained after in vitro (cytokine cocktail) or in vivo (NOD/SCID mice transplantation) differentiation of transduced HSCs.

Transduction of HSCs with lentivector carrying the gp91-phox-GFP cassette followed by their in vitro (in cytokine cocktail) or in vivo (transplantation into sublethally irradiated NOD/SCID mice) differentiation resulted in GFP expression restricted exclusively to mature monocytes and granulocytes. GFP expression from the gp91-phoxpromoter in mature neutrophils was enhanced by insertion of WPRE without loss of specificity.

The gp91-phoxpromoter delivered into HP/SCs by a lentivector, with or without WPRE, exhibited its physiological responsiveness to INF- $\gamma$  in mature neutrophils in vitro (FIG. 2).

**Example 2: HS enhancer elements produce higher and less variegated expression.**

An important issue for the genetic treatment of a variety of lympho-hematological disorders is the levels of expression of the putatively therapeutic transgene in the appropriate subset of differentiated cells. In order to achieve higher and non-variegated gene expression, gp91-specific enhancer sequences located 30kb upstream of gp91-phoxgene within four DNAase hypersensitivity sites (HS) were cloned into lentiviral vectors.

First, in order to decrease the size of viral genome, the 1540bp gp91-promotor fragment was replaced with a functionally equal 0.5kb fragment (Skalnik, *et al.* 1991), generating a

pHP500-GFP vector (FIG. 6A). To facilitate cloning multiple unique restriction sites were introduced allowing introduction of enhancer on both sites of the cPPT (that would retain its central position and functionality after cloning of the enhancers), generating the pHPX-GFP vector (FIG. 6B).

5           The fragments covering 4 individual HS sites were generated by PCR using genomic DNA as a template. Sequence of PCR primers was based on human genome sequence published by Human Genome Project (contig NT\_011844; <http://www.ncbi.nlm.nih.gov/genome/guide/human/>). See SEQ ID NOS:2-16. Location of the gp91-phoxHS sites was based on published data. See Lien LL, Lee Y, and Orkin SH (1997).  
10       Approximately 1-1.5 kb sequences flanking specified site were amplified and cloned into the lentivector generating finally pPHS-GFP. See SEQ ID NOS: 2 through 16. A version with WPRE sequences was also generated (pWPHS-GFP, FIG. 6C). Upon transduction of HSCs and differentiation, the inclusion of the HS elements in this; and other configurations, will result in the higher overall expression and/or less variegated expression of the transgene.

### 15   **Example 3: Development of Therapy for chronic granulomatous disease (CGD)**

          Chronic Granulomatous Disease is highly correlated with deficiencies in the gp91-phox gene, which encodes a subunit of NADPH (Dinauer *et al.*, 1987). The X-linked gp91-phox gene is defective in about 60% of patients with CGD. For a review, see Malech HL, "Progress in gene  
20       therapy for chronic granulomatous disease," *J. Infect. Dis.* 179(Suppl.2):S 318-25, 1999. One approach to therapy will involve the transduction of appropriate cells so that at least one functional copy of gp91-phox is introduced into the patient. As discussed above, this type of therapy may involve the removal of cells from the patient, *ex vivo* transduction, then replacement of those cells back into the patient.

          CD34 cells will be isolated from CGD patients and transduced with vectors of the present  
25       invention that carry a functional copy of the gp91-phox gene, for example, the gp91-phox gene encoded by the sequence of nucleotides comprised by those of SEQ ID NO: 18, or any nucleotide sequence that encodes the functional polypeptides of the amino acid sequences of SEQ ID NO: 19. Appropriate expression of gp91-phox polypeptide will be obtained through the control exerted by the gp91-phox promoter incorporated in the vector. Enhanced expression  
30       may be obtained by including the WPRE element and HS enhancers as described above.

Cells will be transplanted into test systems, such as SCID-NOD mice or into patients if indicated. Further evaluation of the efficiency of this approach will be obtained through transplantation of transduced cells into appropriate knock-out strains of mice.

**Example 4: Therapeutic approaches for Leukocyte adhesion deficiency (LAD)**

5 One approach to therapy will involve the transduction of appropriate cells so that at least one functional copy of myelomonocytic leukocyte integrin is introduced into the patient. As discussed above, this type of therapy may involve the removal of cells from the patient, *ex vivo* transduction, then replacement of those cells back into the patient.

10 CD34 cells will be isolated from LAD patients and transduced with vectors of the present invention that carry a functional copy of the integrin gene. Appropriate expression of the integrin polypeptide will be obtained through the control exerted by a CD11b promoter incorporated in the vector. The CD11b promoter will be selected from the promoters encoded by the polynucleotide sequence of SEQ ID NO: 20. See Hickstein, *et al.* 1992. Enhanced expression may be obtained by including the WPRE element and HS enhancers as described  
15 above.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred  
20 embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be  
25 achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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U.S. Patent No. 5,645,897  
U.S. Patent No. 5,686,279  
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U.S. Patent No. 5,846,225  
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U.S. Patent No. 5,925,565  
U.S. Patent No. 5,928,906  
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**WHAT IS CLAIMED IS:**

1. A recombinant lentiviral vector comprising:
  - (a) lentiviral *gag*, *pol* and *rev* genes;
  - (b) an expression cassette comprising a transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in a human cell; and
  - (c) a central polypurine tract (cPPT) positioned upstream of the expression cassette.
2. The vector of claim 1, wherein the cPPT comprises the nucleotide sequence of SEQ ID NO: 1.
3. The vector of claim 1, wherein the vector is capable of transducing about 20% to about 80% of cells.
4. The vector of claim 1, wherein the vector is capable of transducing about 40% to about 80% of cells.
5. The vector of claim 1, wherein the vector is capable of transducing about 60% to about 80% of cells.
6. The vector of claim 1, further comprising multiple unique cloning sites positioned adjacent to the cPPT.
7. The vector of claim 2, wherein the multiple unique cloning sites are positioned upstream of the cPPT.
8. The vector of claim 2, wherein the multiple unique cloning sites are positioned downstream of the cPPT.
9. The vector of claim 2, wherein multiple unique cloning sites are positioned both upstream and downstream of the cPPT.

10. The vector of claim 1, wherein the *gag*, *pol* and *rev* genes are HIV *gag*, *pol* and *rev* genes.
11. The vector of claim 10, wherein the *gag*, *pol* and *rev* genes are HIV-1 *gag*, *pol* and *rev* genes.
- 5 12. The vector of claim 1, further defined as a self-inactivating vector (SIN).
13. The vector of claim 1, wherein the LTR region has been rendered substantially transcriptionally inactive by virtue of deletions in the U3 region of the 3' LTR.
14. The vector of claim 13, wherein the deletions are of nucleotides at positions -418 through -18 relative to the U3 -R region boundary.
- 10 15. The vector of claim 1, further defined as incapable of reconstituting a wild-type lentivirus through recombination.
16. The vector of claim 15, wherein the vector does not express a functional lentiviral gene other than the *gag*, *pol* and *rev* genes
17. The vector of claim 1, wherein the promoter is a gp91-phox promoter, a gp47-phox promoter, a CD11b promoter, an EF1- $\alpha$  promoter, a PGK promoter, a beta-globin promoter, an MHC classII promoter, a clotting Factor IX promoter, an insulin promoter, a PDX1 promoter, a CD4 promoter, and a CD2 promoter.
- 15 18. The vector of claim 17, wherein the promoter is a gp91-phox promoter.
19. The vector of claim 17, wherein the promoter is a gp47-phox promoter.
- 20 20. The vector of claim 17, wherein the promoter is a CD11b promoter.
21. The vector of claim 17, wherein the promoter is the EF1- $\alpha$  promoter.



22. The vector of claim 17, further comprising at least one enhancer sequence.
23. The vector of claim 22, wherein the at least one enhancer sequence is selected from the group of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.
- 5 24. The vector of claim 22, wherein the at least one enhancer sequence is positioned adjacent to the cPPT.
25. The vector of claim 19.5, wherein the at least one enhancer sequence is positioned upstream of the cPPT.
26. The vector of claim 24, wherein the at least one enhancer sequence is positioned  
10 downstream of the cPPT.
27. The vector of claim 24, wherein enhancer sequences are positioned both upstream and downstream of the cPPT.
28. The vector of claim 27, wherein the enhancer sequences are selected from the group of  
15 SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO:  
7..
29. The vector of claim 1, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200.
30. The vector of claim 29, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 40 and about 200.
- 20 31. The vector of claim 30, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 150 and about 200.

32. The vector of claim 1, wherein the promoter is capable of promoting expression of the transgene in response to a transcriptional activator.
33. The vector of claim 32, wherein the transcriptional activator is INF-gamma.
34. The vector of claim 32, wherein the promoter is capable of promoting expression of the transgene in specific cell types.
35. The vector of claim 1, wherein the promoter is capable of promoting expression of the transgene in specific cell types.
36. The vector of claim 35, wherein the specific cell types are selected from the group of mature blood cells, neutrophils, monocytes, granulocytes, neurons, lung cells, muscle cells, liver cells, pancreatic cells, endothelial cells, cardiac cells, skin cells, bone marrow stromal cells, and eye cells.
37. The vector of claim 35, wherein the cell types are mature blood cells.
38. The vector of claim 35, wherein the cell types are neutrophils.
39. The vector of claim 35, wherein the cell types are monocytes or granulocytes.
40. The vector of claim 35, wherein the cell types are neurons.
41. The vector of claim 1, wherein the transgene is gp91-phox, gp47-phox, erythropoietin, an interleukin, a colony-stimulating factor, integrin  $\alpha\text{IIb}\beta$ , a multidrug resistance gene, , an antiviral gene, a gene coding for blood coagulation factor VIII, a gene coding for blood coagulation factor IX, a T cell antigen receptor, a B cell antigen receptor, a single chain antibody (ScFv), TNF, gamma interferon, CTLA4, B7, Melana, MAGE, a marker gene, luciferase, or GFP.
42. The vector of claim 41, wherein the transgene is gp91-phox.

43. The vector of claim 41, wherein the transgene is gp47-phox.
44. The vector of claim 41, wherein the transgene is a gene coding for a marker gene.
45. The vector of claim 41, wherein the transgene is a gene coding for a GFP.
- 5 46. The vector of claim 1, further comprising a posttranscriptional regulatory sequence positioned to promote the expression of the transgene.
47. The vector of claim 46, wherein the posttranscriptional regulatory sequence is an intron positioned within the expression cassette.
48. The vector of claim 47, wherein the intron is positioned in an orientation opposite the vector genomic transcript.
- 10 49. The vector of claim 46, wherein the posttranscriptional regulatory sequence is a posttranscriptional regulatory element.
50. The vector of claim 49, wherein the posttranscriptional regulatory element is woodchuck hepatitis virus posttranscriptional regulatory element (WPRE).
- 15 51. The vector of claim 50, wherein the posttranscriptional regulatory element is hepatitis B virus posttranscriptional regulatory element (HPRE).
52. A host cell transduced with a vector in accordance with claim 1.
53. The transduced host cell of claim 52, wherein the cell is a virus producer cell.
54. The transduced host cell of claim 53, wherein the producer cell is a 293T cell.
55. The host cell of claim 54, wherein the cell is a human hematopoietic progenitor cell.

56. The transduced host cell of claim 55, wherein the human hematopoietic progenitor cell is a CD34<sup>+</sup> cell.

57. A self-inactivating recombinant lentiviral vector comprising:

- (a) lentiviral *gag*, *pol* and *rev* genes;
- 5 (b) an expression cassette comprising a transgene positioned under the control of a promoter that is active upon contacting the promoter with a transcriptional activator to promote detectable transcription of the transgene in a human cell;
- (c) a central polypurine tract (cPPT) positioned upstream of the expression cassette; and
- 10 (d) a posttranscriptional regulatory element positioned to promote the expression of the transgene.

58. A self-inactivating recombinant lentiviral vector comprising:

- (a) lentiviral *gag*, *pol* and *rev* genes;
- 15 (b) an expression cassette comprising a transgene positioned under the control of a promoter that is active to promote detectable transcription of the transgene in cells of specific human cell types;
- (c) a central polypurine tract (cPPT) positioned upstream of the expression cassette; and
- 20 (d) a posttranscriptional regulatory element positioned to promote the expression of the transgene.

59. A self-inactivating recombinant lentiviral vector comprising:

- (a) lentiviral *gag*, *pol* and *rev* genes;
- (b) an expression cassette comprising a transgene positioned under the control of a promoter wherein,
  - 25 (i) the promoter is active to promote detectable transcription of the transgene in cells of specific human cell types and wherein,
  - (ii) the promoter is active upon contacting the promoter with a transcriptional activator to promote detectable transcription of the transgene in the cells of specific human cell types;

- (c) a central polypurine tract (cPPT) positioned upstream of the expression cassette; and
- (d) a posttranscriptional regulatory element positioned to promote the expression of the transgene.

5 60. A self-inactivating recombinant lentiviral vector comprising:

- (a) lentiviral *gag*, *pol* and *rev* genes;
- (b) an expression cassette comprising a transgene positioned under the control of a promoter wherein,
  - (i) the promoter is active to promote detectable transcription of the transgene in cells of specific human cell types and wherein,
  - 10 (ii) the promoter is active upon contacting the promoter with a transcriptional activator to promote detectable transcription of the transgene in the cells of specific human cell types;
- (c) a central polypurine tract (cPPT) positioned upstream of the expression cassette;
- 15 (d) a posttranscriptional regulatory element positioned to promote the expression of the transgene; and
- (e) at least one enhancer element.

20 61. A method for transducing a human hematopoietic stem cell comprising contacting a population of human cells that include hematopoietic stem cells with a vector in accordance with claim 1 under conditions to effect the transduction of a human hematopoietic progenitor cell in said population by said vector.

25 62. The method of claim 61, wherein the human hematopoietic stem cell population comprises CD34<sup>+</sup> cells.

63. The method of claim 61, wherein the cell population is treated to stimulate cell proliferation without substantial loss of stem cell pluripotency.

64. The method of claim 61, wherein the stem cell is transduced *in vivo*.

65. The method of claim 61, wherein the stem cell is transduced *in vitro*.
66. The method of claim 65, wherein the transduced stem cell is infused into a human subject.
67. A method for expressing a transgene in a restricted set of cell types comprising  
5 transducing a human cell in accordance with claim 61 and providing for the maturation of the desired cell types.
68. The method of claim 67, further comprising the step of stimulating expression of the transgene by contacting the promoter with an activator of transcription.

10



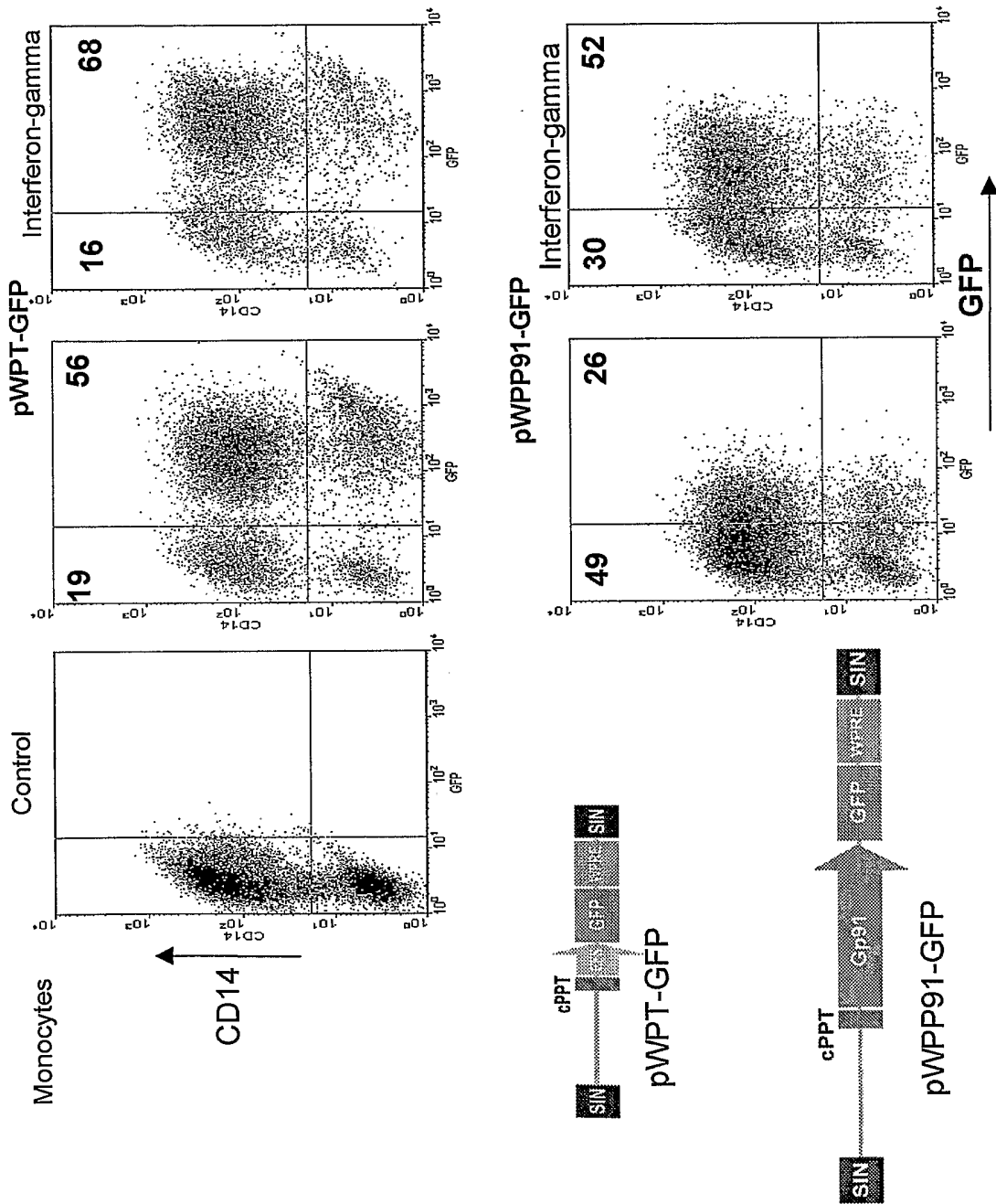


FIG. 2A



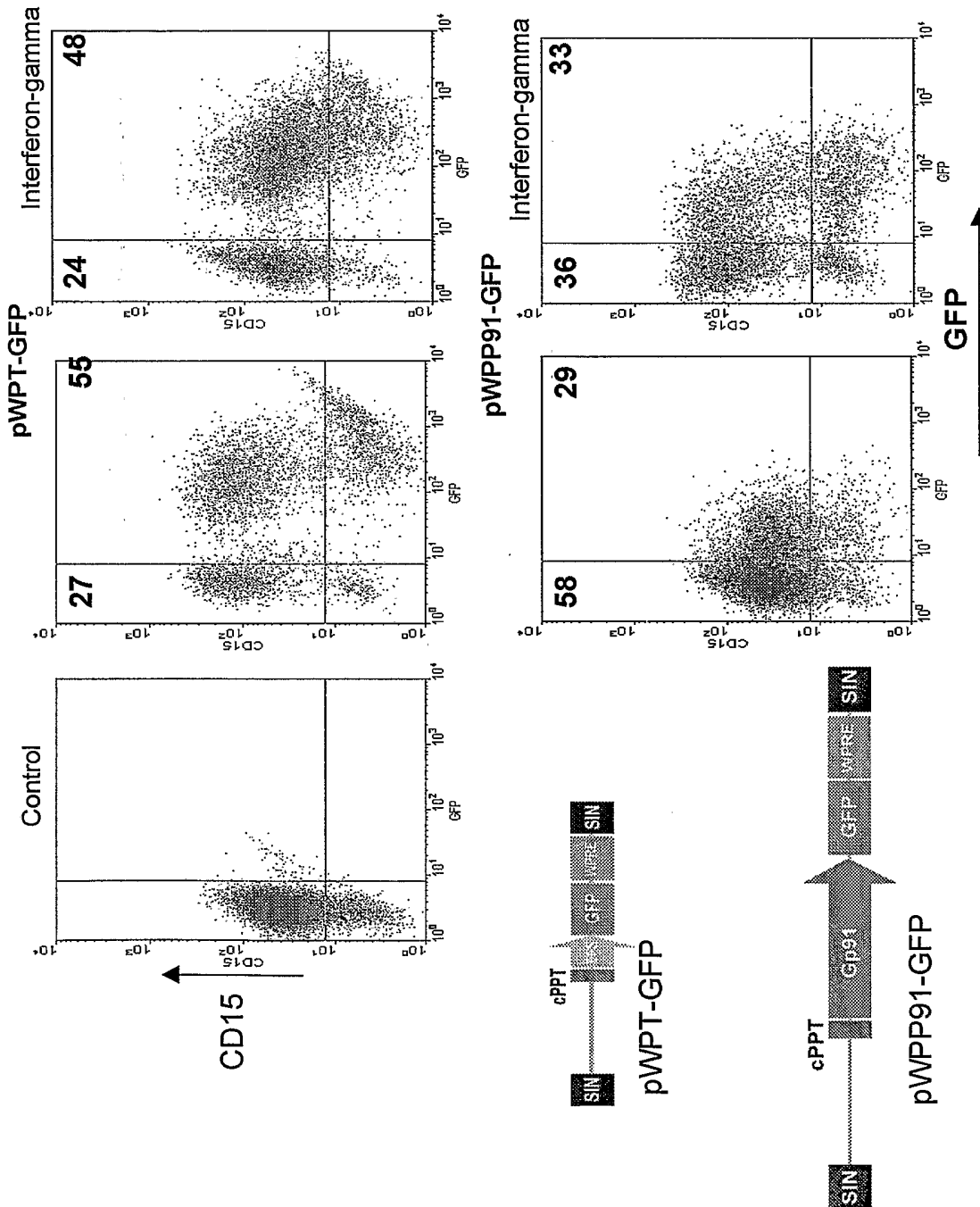
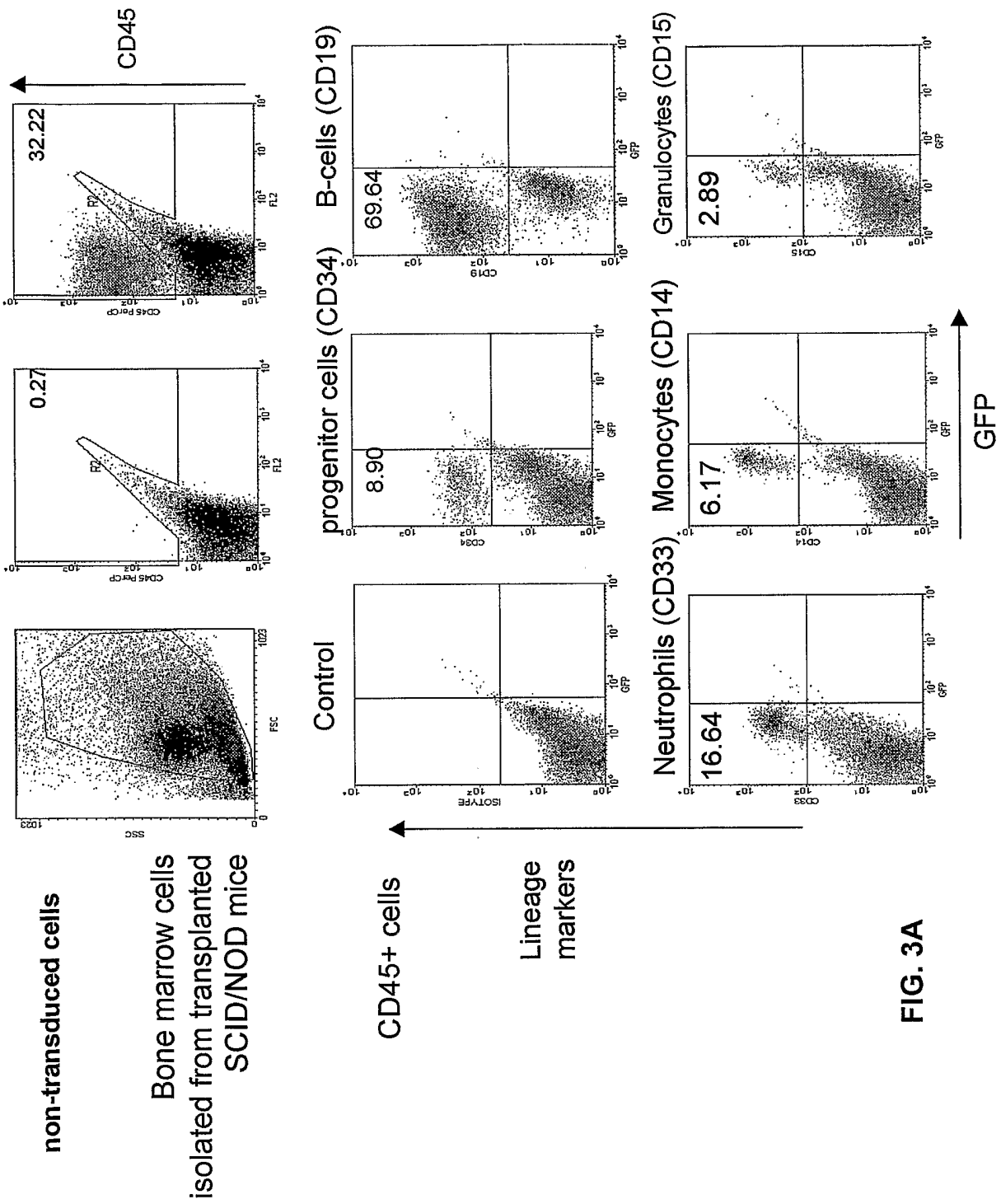


FIG. 2B



**FIG. 3A**

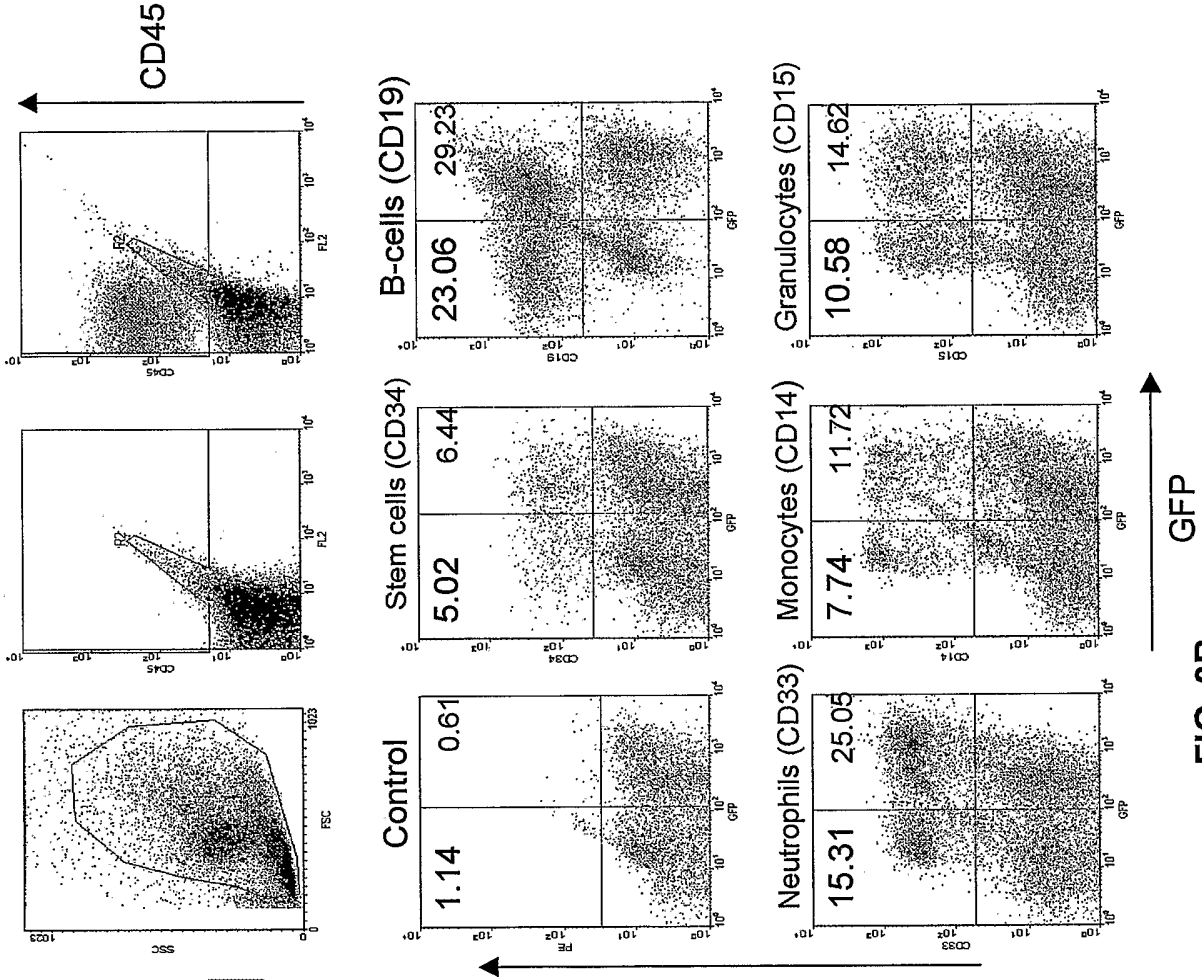


FIG. 3B

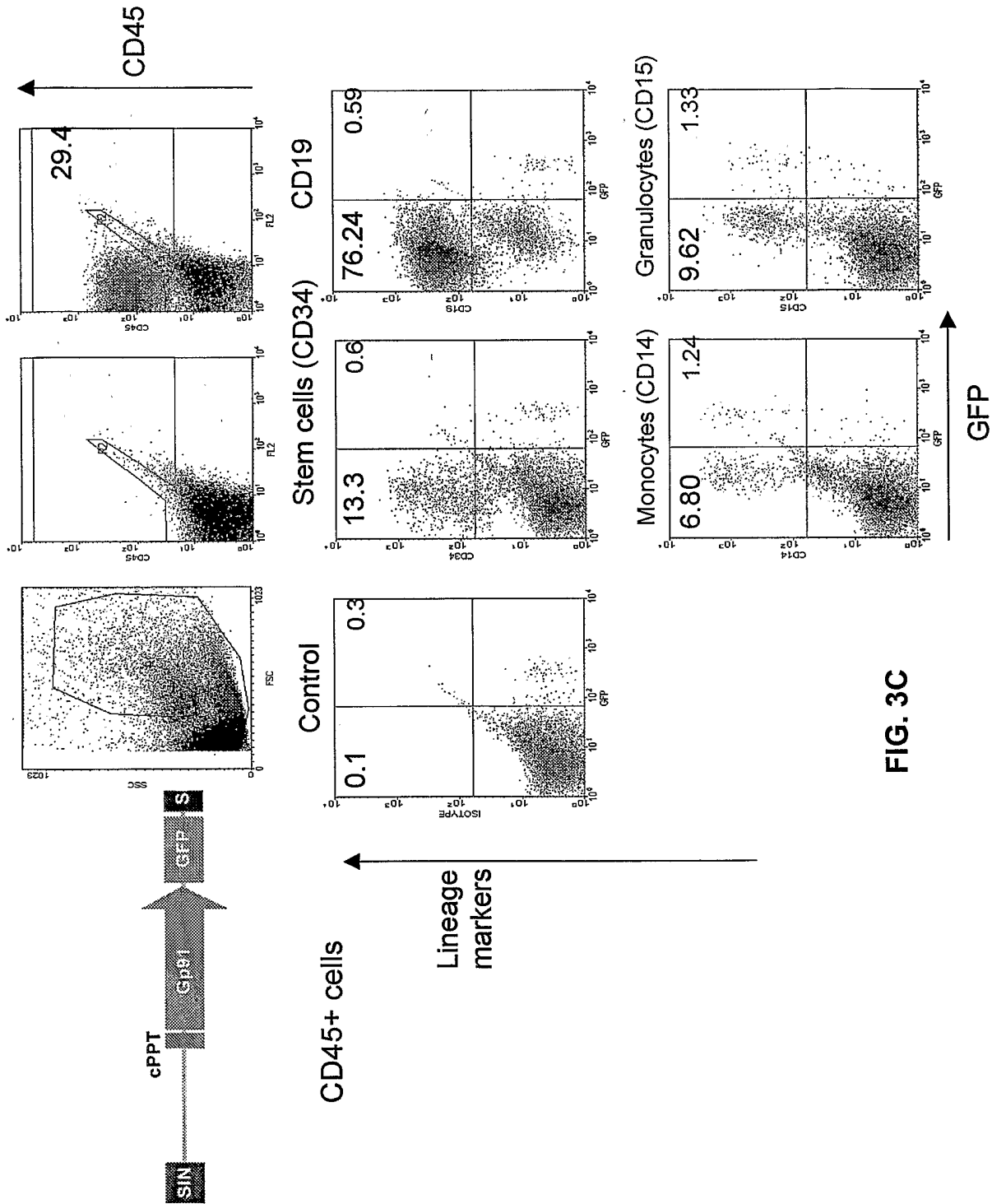


FIG. 3C

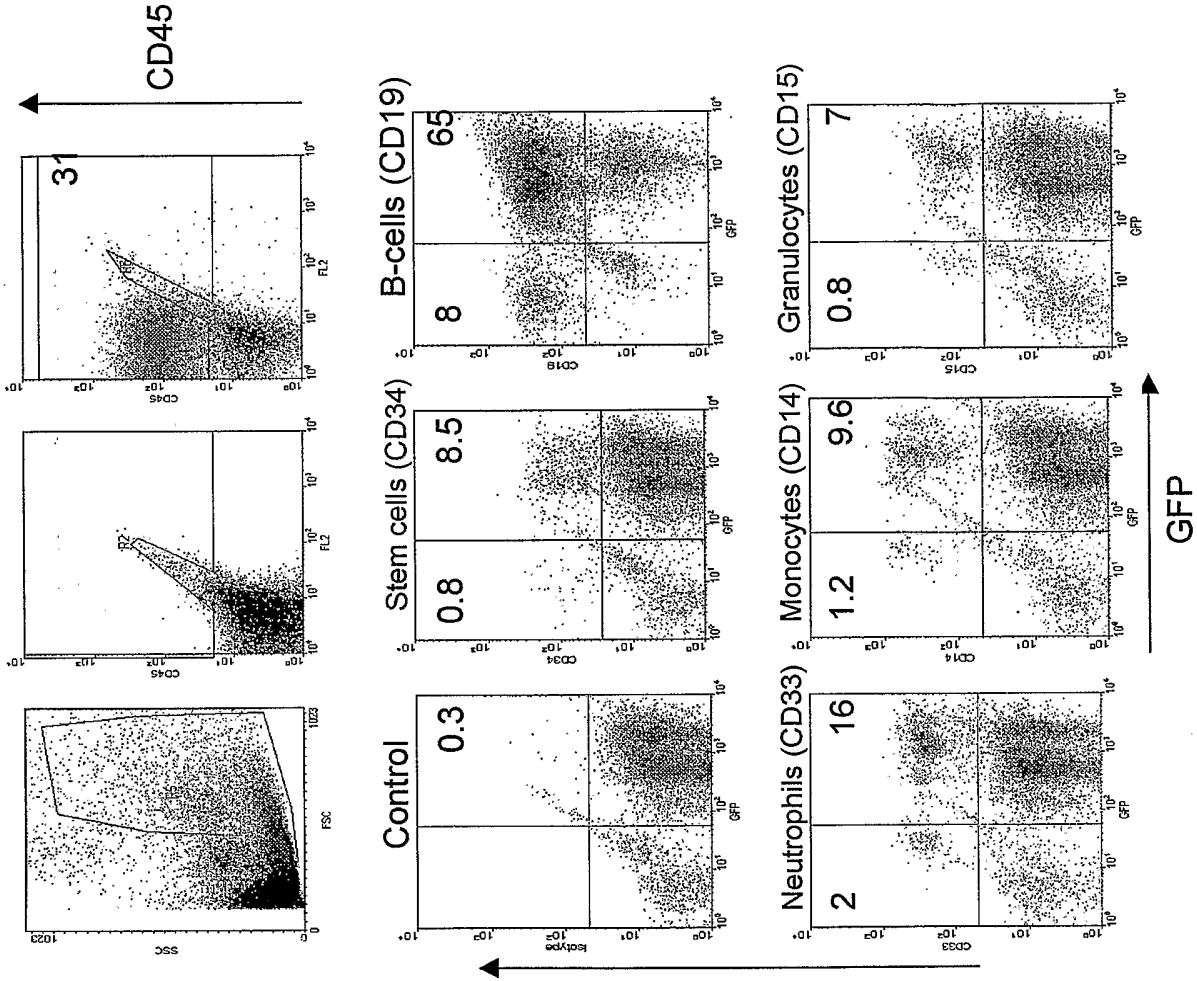


FIG. 3D

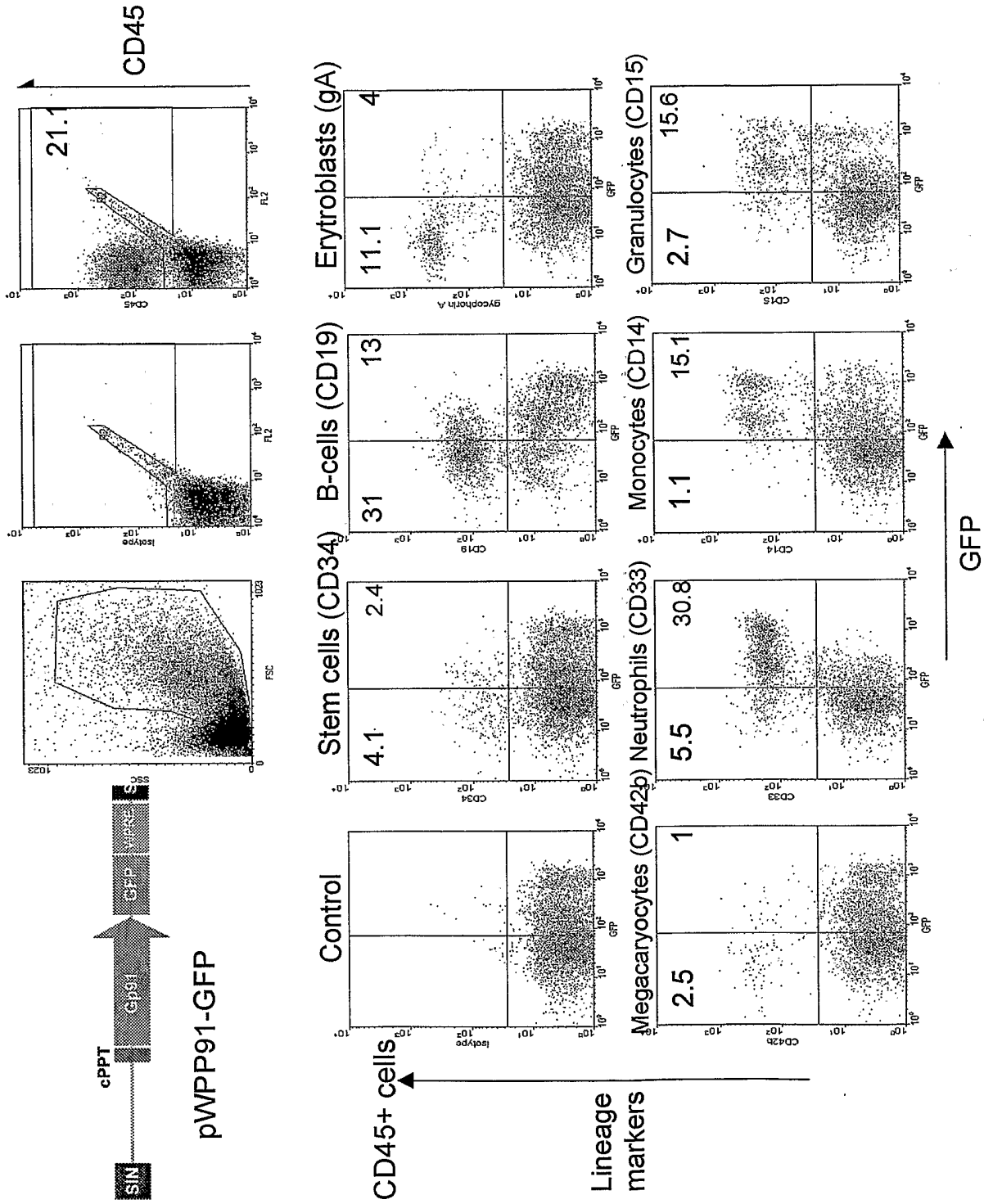


FIG. 3E

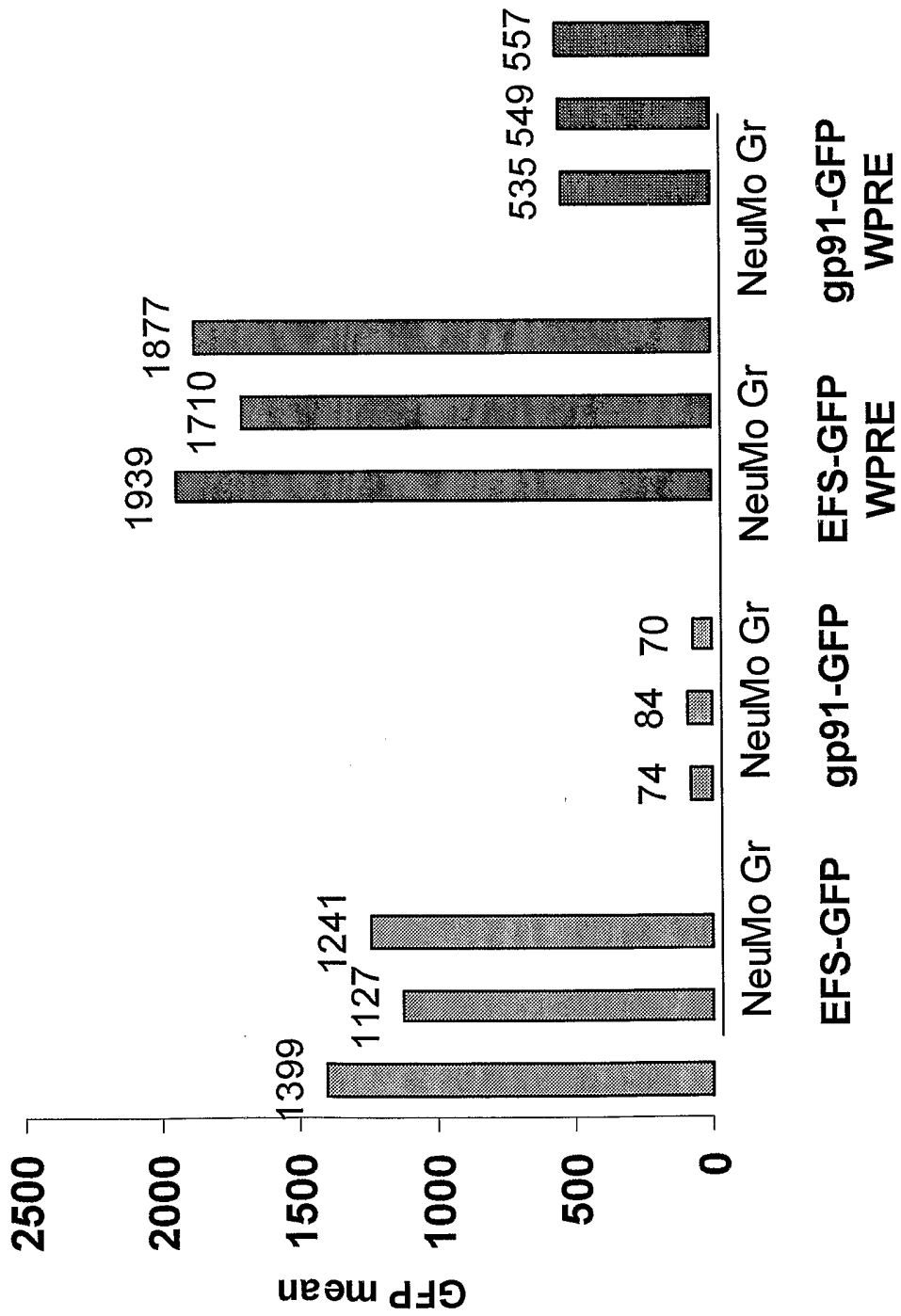


FIG. 4

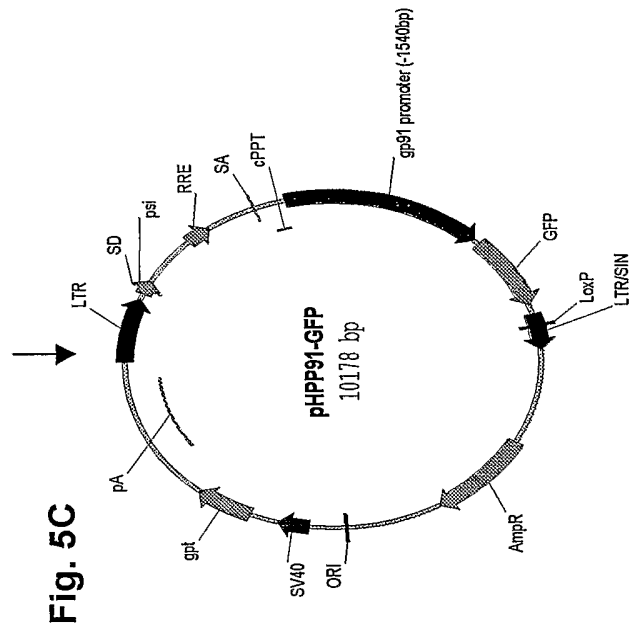
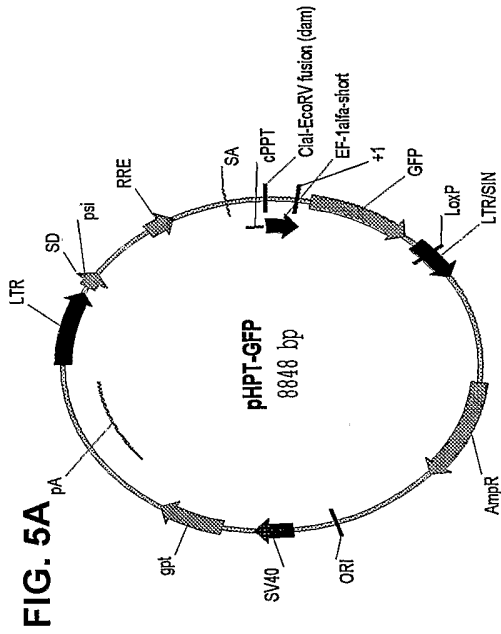
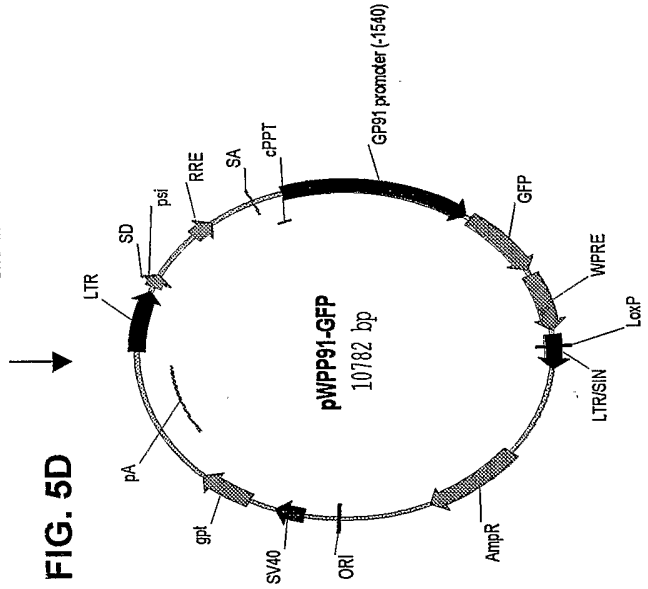
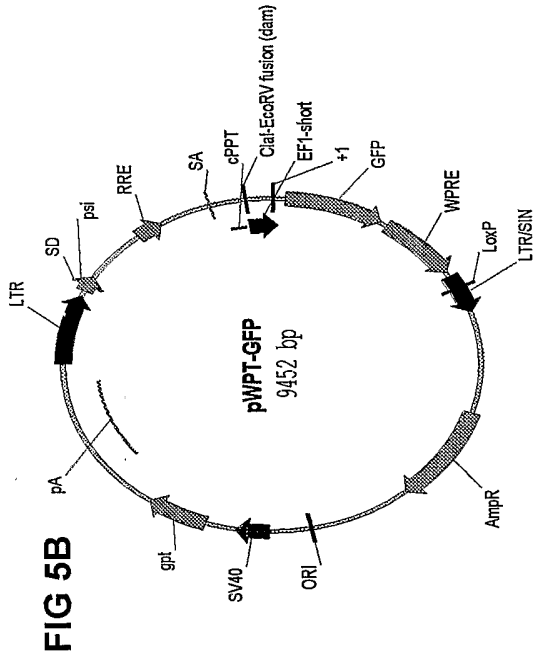




FIG. 6A-1



+

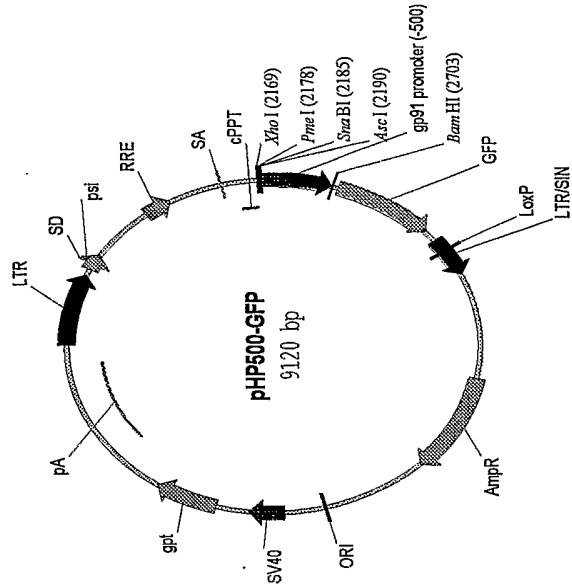
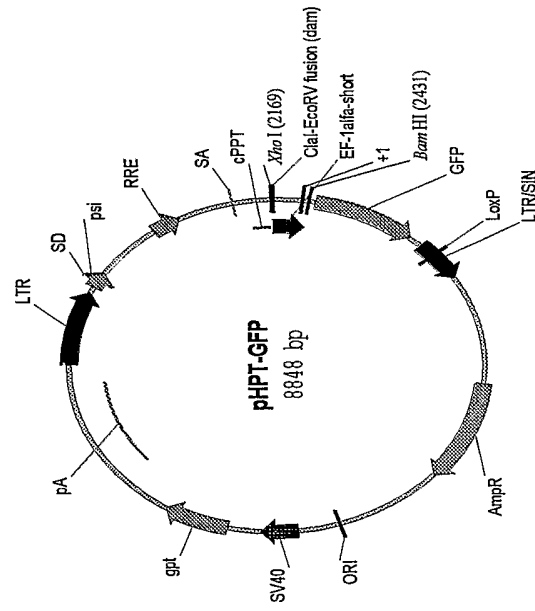
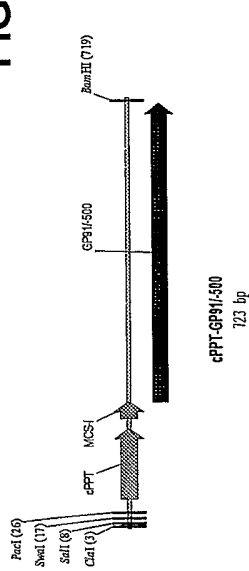


FIG. 6B-3

FIG. 6B-2

FIG. 6B-1



+

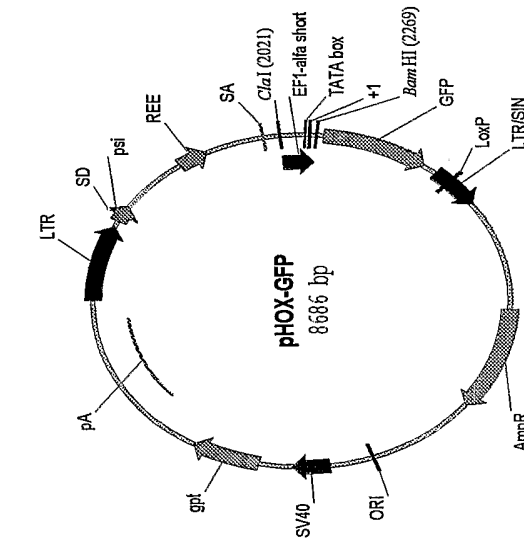


FIG. 6B-2

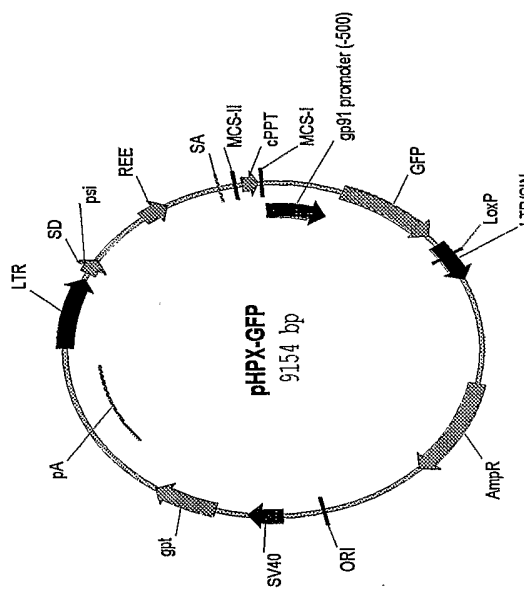


FIG. 6B-3

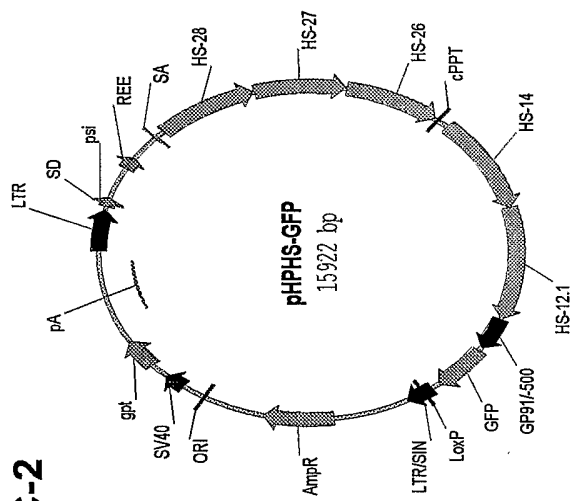


FIG. 6C-2

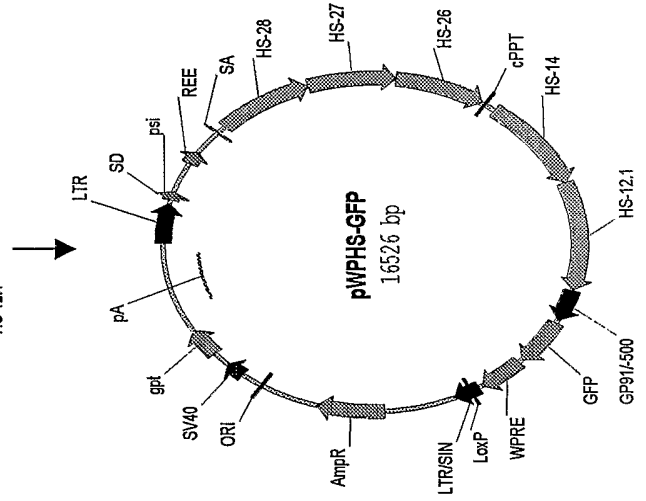


FIG. 6C-3

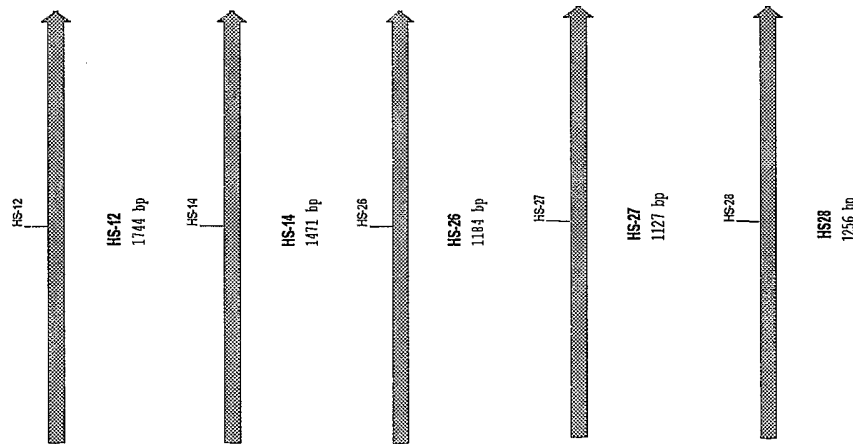


FIG. 6C-1

FIG. 7-2

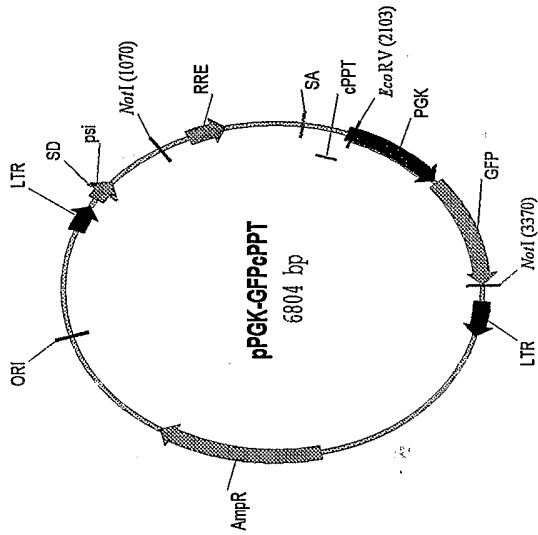


FIG. 7-1

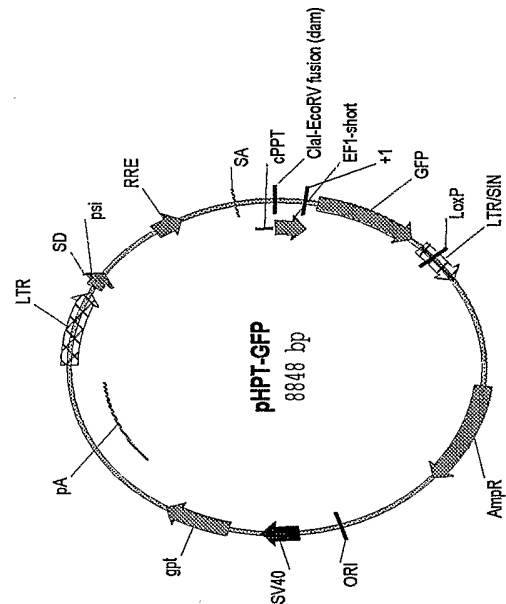
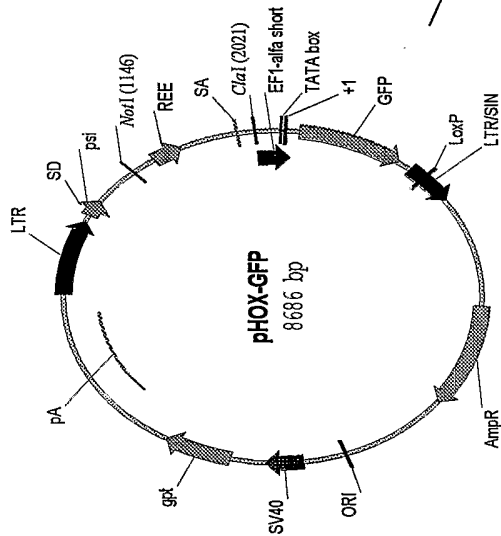


FIG. 7-3

## SEQUENCE LISTING

<110> TRONO, DIDIER  
MACIEJ WIZNEROWICZ

<120> METHODS AND COMPOSITIONS RELATING TO LENTIVIRAL  
RESTRICTED EXPRESSION VECTORS AND THEIR APPLICATIONS

<130> CLFR:014WO

<140> UNKNOWN  
<141> 2002-09-30

<140> 60/326,593  
<141> 2001-10-02

<160> 20

<170> PatentIn Ver. 2.1

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Primer

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<212> DNA  
<213> Homo sapiens

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cagctgttgt taataagacc aagttgtttg taaaaaaga tttttcaagg aaataagcca 240  
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atgtaaactc tcatcgccag acatcttcat tttctattaa gtgaaatgcc agtaaaagcc 420  
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agcaaaactc ttcaactttt attaactctt gagattctgg tgatgaaata aaagtgagtt 600  
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actgttttcc ttaagcagaa agtggcagtg gtgggaattt atggcttctg tagccaggtt 720  
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agcttcagat ctccctcgtgg gattgctgag gccttattgt gaccacatga taactcaact 840  
gctccttctg cccacttctg ctttcttatt tttcattgcc ttttacagct atttacctct 900

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gcaatttcat acatgcacct tgacgtaatt aggaacataa ttttgtgggt gaaatttatt 1560
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ccctggttta ccaataggaa gttgacagca acctcagtaa ggtcttactg gagagaaatg 300
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<211> 1201
<212> DNA
<213> Homo sapiens

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cttagtcctc ccaccaaccc aatgaagatg gtattaatac ccacctccca ttgtacagat 240
gaggagactg gggctaaatg aggtcaaata ggttgctcaa ggtcttatag cttgttagtg 300
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c
1201

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&lt;210&gt; 5

&lt;211&gt; 1143

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 5

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gaaaaagata aacttcccac atgatttttt tttttatfff gcctcgtttt gtccccttaga 180
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gtctttaaaa gggtagcaca atcagctgta ggttacacca tagatatggt taactcgagc 1140
ggc
1143

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&lt;210&gt; 6

&lt;211&gt; 1256

&lt;212&gt; DNA

<213> Homo sapiens

<400> 6

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taattcttat cttggctatt ggctattgtg aatagtgcta cagtaaacad ggtagtgcag 180
ctatctcttc aatactgact tgctttatct tggatatata cccagtgatg ggattcctgg 240
atcatatggt acttctatct tttgtttttt tgaggaatct ccatactggt ctctatagtg 300
gttgtactac tttacattcc caccaacagt gtacaatcat tcccctttct ctacatcctt 360
accattatct actatTTTTT gtctTTTTga ttaaagccat tttatctggg gtgagggtgat 420
agctcattgt ggttttgatt tgcatttttc taatgattag tgatgggtgag catttttcat 480
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tctgggcatg cctcaggaaa tttacaatca tggcagaagg tgaaggggga gcagacacat 720
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aactgggcaa gagaatttgg ataatttagt gcagtctacc caaaaattct taacaggaac 1200
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<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
Primer

<400> 7

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<210> 8

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

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ggctataatc tcaagaaagc aacc 24

<210> 9

<211> 27

<212> DNA



<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic  
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<400> 9

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27

<210> 10

<211> 24

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
Primer

<400> 10

cctgcaaagt ccctttaacc gtat

24

<210> 11

<211> 32

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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32

<210> 12

<211> 27

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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<400> 12

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27

<210> 13

<211> 30

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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<400> 13

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30

<210> 14

<211> 33

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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<400> 14

ggttacacca tagatatggt taactcgagc ggc

33

<210> 15

<211> 32

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic  
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<210> 16

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

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Primer

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<210> 17

<211> 1557

<212> DNA

<213> Homo sapiens

<400> 17

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<210> 18
<211> 4266
<212> DNA
<213> Homo sapiens

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<220>
<221> CDS
<222> (15)..(1727)

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                  1                    5                    10

ttt gtc att ctg gtt tgg ctg ggg ttg aac gtc ttc ctc ttt gtc tgg 98
Phe Val Ile Leu Val Trp Leu Gly Leu Asn Val Phe Leu Phe Val Trp
    15                    20                    25

tat tac cgg gtt tat gat att cca cct aag ttc ttt tac aca aga aaa 146
Tyr Tyr Arg Val Tyr Asp Ile Pro Pro Lys Phe Phe Tyr Thr Arg Lys
    30                    35                    40

ctt ctt ggg tca gca ctg gca ctg gcc agg gcc cct gca gcc tgc ctg 194
Leu Leu Gly Ser Ala Leu Ala Leu Ala Arg Ala Pro Ala Ala Cys Leu
    45                    50                    55                    60

aat ttc aac tgc atg ctg att ctc ttg cca gtc tgt cga aat ctg ctg 242
Asn Phe Asn Cys Met Leu Ile Leu Leu Pro Val Cys Arg Asn Leu Leu
    65                    70                    75

```

|   |     |
|---|-----|
| tcc ttc ctc agg ggt tcc agt gcg tgc tgc tca aca aga gtt cga aga | 290 |
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| 80 85 90  |     |
| caa ctg gac agg aat ctc acc ttt cat aaa atg gtg gca tgg atg att | 338 |
| Gln Leu Asp Arg Asn Leu Thr Phe His Lys Met Val Ala Trp Met Ile |     |
| 95 100 105  |     |
| gca ctt cac tct gcg att cac acc att gca cat cta ttt aat gtg gaa | 386 |
| Ala Leu His Ser Ala Ile His Thr Ile Ala His Leu Phe Asn Val Glu |     |
| 110 115 120   |     |
| tgg tgt gtg aat gcc cga gtc aat aat tct gat cct tat tca gta gca | 434 |
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| Leu Ser Glu Leu Gly Asp Arg Gln Asn Glu Ser Tyr Leu Asn Phe Ala |     |
| 145 150 155   |     |
| cga aag aga ata aag aac cct gaa gga ggc ctg tac ctg gct gtg acc | 530 |
| Arg Lys Arg Ile Lys Asn Pro Glu Gly Gly Leu Tyr Leu Ala Val Thr |     |
| 160 165 170   |     |
| ctg ttg gca ggc atc act gga gtt gtc atc acg ctg tgc ctc ata tta | 578 |
| Leu Leu Ala Gly Ile Thr Gly Val Val Ile Thr Leu Cys Leu Ile Leu |     |
| 175 180 185   |     |
| att atc act tcc tcc acc aaa acc atc cgg agg tct tac ttt gaa gtc | 626 |
| Ile Ile Thr Ser Ser Thr Lys Thr Ile Arg Arg Ser Tyr Phe Glu Val |     |
| 190 195 200   |     |
| ttt tgg tac aca cat cat ctc ttt gtg atc ttc ttc att ggc ctt gcc | 674 |
| Phe Trp Tyr Thr His His Leu Phe Val Ile Phe Phe Ile Gly Leu Ala |     |
| 205 210 215 220   |     |
| atc cat gga gct gaa cga att gta cgt ggg cag acc gca gag agt ttg | 722 |
| Ile His Gly Ala Glu Arg Ile Val Arg Gly Gln Thr Ala Glu Ser Leu |     |
| 225 230 235   |     |
| gct gtg cat aat ata aca gtt tgt gaa caa aaa atc tca gaa tgg gga | 770 |
| Ala Val His Asn Ile Thr Val Cys Glu Gln Lys Ile Ser Glu Trp Gly |     |
| 240 245 250   |     |
| aaa ata aag gaa tgc cca atc cct cag ttt gct gga aac cct cct atg | 818 |
| Lys Ile Lys Glu Cys Pro Ile Pro Gln Phe Ala Gly Asn Pro Pro Met |     |
| 255 260 265   |     |
| act tgg aaa tgg ata gtg ggt ccc atg ttt ctg tat ctc tgt gag agg | 866 |
| Thr Trp Lys Trp Ile Val Gly Pro Met Phe Leu Tyr Leu Cys Glu Arg |     |
| 270 275 280   |     |
| ttg gtg cgg ttt tgg cga tct caa cag aag gtg gtc atc acc aag gtg | 914 |
| Leu Val Arg Phe Trp Arg Ser Gln Gln Lys Val Val Ile Thr Lys Val |     |
| 285 290 295 300   |     |

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| gtc act cac cct ttc aaa acc atc gag cta cag atg aag aag aag ggg | 962  |
| Val Thr His Pro Phe Lys Thr Ile Glu Leu Gln Met Lys Lys Lys Gly |      |
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| ttc aaa atg gaa gtg gga caa tac att ttt gtc aag tgc cca aag gtg | 1010 |
| Phe Lys Met Glu Val Gly Gln Tyr Ile Phe Val Lys Cys Pro Lys Val |      |
| 320 325 330   |      |
| tcc aag ctg gag tgg cac cct ttt aca ctg aca tcc gcc cct gag gaa | 1058 |
| Ser Lys Leu Glu Trp His Pro Phe Thr Leu Thr Ser Ala Pro Glu Glu |      |
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| gac ttc ttt agt atc cat atc cgc atc gtt ggg gac tgg aca gag ggg | 1106 |
| Asp Phe Phe Ser Ile His Ile Arg Ile Val Gly Asp Trp Thr Glu Gly |      |
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| Leu Phe Asn Ala Cys Gly Cys Asp Lys Gln Glu Phe Gln Asp Ala Trp |      |
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| Lys Leu Pro Lys Ile Ala Val Asp Gly Pro Phe Gly Thr Ala Ser Glu |      |
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| gat gtg ttc agc tat gag gtg gtg atg tta gtg gga gca ggg att ggg | 1250 |
| Asp Val Phe Ser Tyr Glu Val Val Met Leu Val Gly Ala Gly Ile Gly |      |
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| gtc aca ccc ttc gca tcc att ctc aag tca gtc tgg tac aaa tat tgc | 1298 |
| Val Thr Pro Phe Ala Ser Ile Leu Lys Ser Val Trp Tyr Lys Tyr Cys |      |
| 415 420 425   |      |
| aat aac gcc acc aat ctg aag ctc aaa aag atc tac ttc tac tgg ctg | 1346 |
| Asn Asn Ala Thr Asn Leu Lys Leu Lys Lys Ile Tyr Phe Tyr Trp Leu |      |
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| tgc cgg gac aca cat gcc ttt gag tgg ttt gca gat ctg ctg caa ctg | 1394 |
| Cys Arg Asp Thr His Ala Phe Glu Trp Phe Ala Asp Leu Leu Gln Leu |      |
| 445 450 455 460   |      |
| ctg gag agc cag atg cag gaa agg aac aat gcc ggc ttc ctc agc tac | 1442 |
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| Asn Ile Tyr Leu Thr Gly Trp Asp Glu Ser Gln Ala Asn His Phe Ala |      |
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| gtg cac cat gat gag gag aaa gat gtg atc aca ggc ctg aaa caa aag | 1538 |
| Val His His Asp Glu Glu Lys Asp Val Ile Thr Gly Leu Lys Gln Lys |      |
| 495 500 505   |      |
| act ttg tat gga cgg ccc aac tgg gat aat gaa ttc aag aca att gca | 1586 |
| Thr Leu Tyr Gly Arg Pro Asn Trp Asp Asn Glu Phe Lys Thr Ile Ala |      |

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| Ser Gln His Pro Asn Thr Arg Ile Gly Val Phe | Leu Cys Gly Pro Glu     |      |     |
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| gcc ttg gct gaa acc ctg agt aaa caa agc atc | tcc aac tct gag tct     | 1682 |     |
| Ala Leu Ala Glu Thr Leu Ser Lys Gln Ser Ile | Ser Asn Ser Glu Ser     |      |     |
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| Gly Pro Arg Gly Val His Phe Ile Phe Asn Lys | Glu Asn Phe             |      |     |
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| attgataata taaatacccc ctgcttaaaa atggacaaaa | agaaactata atgtaatggg   | 1847 |     |
| ttcccttaa aggaatgtca aagattgttt gatagtgata  | agttacatth atgtggagct   | 1907 |     |
| ctatggtttt gagagcactt ttacaaacat tatttcattt | ttttcctctc agtaatgtca   | 1967 |     |
| gtggaagtta gggaaaagat tcttggactc aattttagaa | tcaaaaaggga aaggatcaaaa | 2027 |     |
| aggttcagta acttccctaa gattatgaaa ctgtgaccag | atctagccca tcttactcca   | 2087 |     |
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