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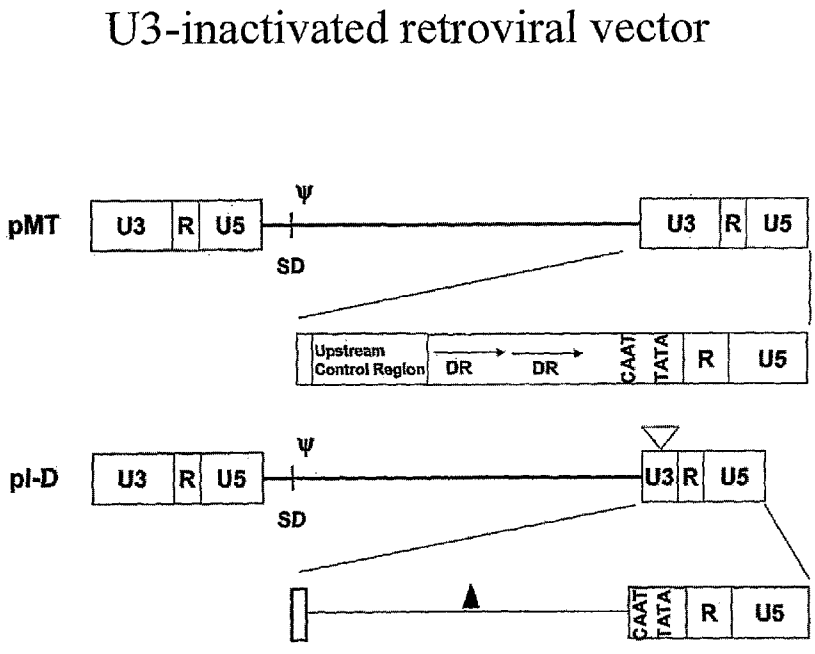

(57) Abstract: The present invention relates to the use of internal promoters in mammalian expression vectors including plasmid vectors and enhancer-deleted retroviral vectors. The retroviral vectors have improved safety and optimal levels of transgene expression and vector titers.

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EXPRESSION VECTORS WITH IMPROVED SAFETY

FIELD OF THE INVENTION

The present invention provides expression vectors comprising internal promoters that can be used for expressing proteins of interest. In one embodiment, the present invention provides retroviral vectors comprising an enhancer-deleted U3 region.

BACKGROUND OF THE INVENTION

Gene transfer involves the transfer of genetic material to a cell, usually for transcription and expression. The method is ideal for protein expression as well as for therapeutic purposes. Various transfer methods are known, such as DNA transfection and viral transduction. Virally-mediated gene transfer is attractive due to the efficiency of transfer and high levels of transgene expression, as well as the potential for targeting particular receptors and/or cell types if needed through natural affinity or pseudotyping.

In particular, retroviral vectors are useful for longer term expression due to their ability to integrate into the cellular genome. Murine leukemia virus-based (MLV) vectors are the most common retroviral vector, with many backbone plasmids and packaging cell lines available to suit most applications (See *e.g.*, Miller and Buttimore, *Mol. Cell. Biol.* *6*:2895 (1986)). Like all "simple" retroviruses, *e.g.* retroviruses that only encode structural and enzymatic viral proteins and do not utilize viral accessory proteins, MLV vectors can only integrate into dividing cells. Other simple retroviruses potentially suitable for use as vectors include other members of the mammalian C-type viruses (*e.g.*, murine stem cell virus, Harvey murine sarcoma virus and spleen necrosis virus), B type viruses (*e.g.*, mouse mammary tumor virus), and D type viruses (*e.g.*, Mason Pfizer monkey virus). Other retroviruses suitable for use as a retroviral vector of the invention include avian retroviruses (*e.g.*, Rous sarcoma virus), spumaviruses (*e.g.*, foamy viruses), and the HTLV-BLV viruses (*e.g.*, HTLV-1).

Lentiviruses are a subgroup of retroviruses that express viral accessory proteins and are capable of infecting and integrating into non-dividing, as well as dividing, cells. Vectors derived from lentiviruses are ideal tools for delivering exogenous genes to target cells because of their ability to stably integrate into the genome of dividing and non-dividing cells and to mediate long-term gene expression (Gilbert et al., *Somat. Cell Mol. Genet. 26*:83 (2001); Mitrophanous *et al., Gene Ther. 6:*1808 (1999); Naldini *et al., Science 272*:263 (1996); Sauter *et al., Somat. Cell Mol. Genet. 26*:99 (2001)).

Lentiviruses have been isolated from many vertebrate species including primates, *e.g.*, human and simian immunodeficiency viruses (HIV-1, HIV-2, SIV), as well as non-primates, *e.g.*, feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), equine infectious virus (EIAV), caprine arthritis encephalitis virus (CAEV) and the visna virus. Of these, HIV and SIV are presently best understood. Among non-primate lentiviral vectors, vectors derived from FIV (Curran et al., *Curr. Top. Microbiol. Immunol. 261*:75 (2002)) and EIAV (U.S. Patent Application No. 2001/0044149) are best characterized.

There are two major safety aspects that have received considerable attention in the context of retroviral gene therapy, regardless of whether the vector is based on murine leukemia virus (MLV) or lentivirus. Specifically, they are the presence of replication competent retrovirus (RCR) and the incidence of insertional mutagenesis. The former problem has greatly been improved by the development of a minimum sized retroviral vector that contains no overlapping viral sequences between the vectors and the packaging genome. However, the latter possibility has recently raised serious concerns, mainly because of the three leukemia cases found in the X-SCID human trial (Hacein-Bey-Abina *et al., Science 302*:415 (2003)). The retrospective analysis of the first two leukemia cases revealed that the leukemia probably resulted from the retroviral integration into the chromosome and the subsequent activation of the LMO2 gene, located in close proximity to the integration site, by the long terminal repeat (LTR). Although it was argued that this vector-mediated tumorigenesis might be restricted to the X-SCID gene therapy case due to the particular nature of this disease and its gene, it is now

clear that the safety of retroviral vectors needs further improvement to become a viable form of therapeutics in the real world.

There have been several approaches for reducing the probability of vector-mediated tumorigenesis. One approach is to remove the U3 region of the LTR (Yu *et al., Proc. Natl. Acad. Sci. USA 83*:3194 (1986); Hawley *et al., Proc. Natl. Acad. Sci. USA 84*:2406 (1987); Yee *et al., Proc. Natl. Acad. Sci. USA 84*:5197 (1987)). The retroviral LTR consists of U3, R, and U5 regions, and the U3 region contains the enhancer and promoter sequence that control gene expression (Sun *et al., J. Virol. 69*:4941 (1995); Wahlers *et al., Mol. Ther. 6*:313 (2002)). Therefore, the insertional activation by a vector can be reduced by removing the U3 region. In that case, an additional promoter should be supplied to the vector to drive the expression of the target gene because the U3-deleted vector no longer contains the promoter sequence in the LTR.

As discussed previously, the U3-inactivated retroviral vector needs an internal promoter for the expression of target gene. One of the most frequently used internal promoters in retroviral vectors is the human cytomegalovirus (HCMV) immediate-early (IE) promoter (Jaalouk *et al., Virol. J. 3*:27 (2006); pQCXIN available from BD Biosciences) or related ones such as CA (HCMV IE enhancer/chicken β-actin promoter) (Ramezani *et al., Mol. Ther. 14*:245 (2006)). However, the HCMV IE promoter is known to be rapidly inactivated in primary human cells, while it does not work for certain genes (Herweijer *et al., J. Gene Med. 3*:280 (2001)). Thus, commonly used promoters have been shown to decrease expression of heterologous genes, be inactive in certain cell types, and potentially activate LTR-driven transcription, all of which decrease the safety and efficacy of the retroviral vector.

Finally, U3-inactivated retroviral vectors have been associated with very low titers due to promoter suppression by commonly-used promoters, such as CMV and SV40, which reduce transcription of genomic RNA for packaging (Jaalouk *et al., Virol. J. 3*:27 (2006)) Indeed, MLV based U3-deleted vectors have been associated with titers up to four orders of magnitude less than the comparable MLV vector with intact U3 regions (Olson *et al., J. Virol. 68*:7060 (1994)). Thus, it is surprising to find promoters that are capable of both

driving high levels of heterologous gene transcription as well as enabling high viral titers to be produced. Therefore, new promoters are needed to be developed for use as an internal promoter in the retroviral vector.

SUMMARY OF THE INVENTION

The present invention provides expression vectors comprising a heterologous internal promoter. In one embodiment, the vector comprises a nucleotide sequence comprising a 5'LTR and a 3'LTR. In further embodiments, the enhancer element of the U3 region of the 3'LTR or both the 3'LTR and 5'LTR is deleted. In one embodiment, the vectors are plasmid vectors. In a further embodiment, the vectors are retroviral vectors comprising one or more enhancer-deleted U3 regions, and which further comprise an internal promoter operably linked to a heterologous gene such that the retroviral vector is capable of producing high viral titers and high levels of transcription of the heterologous gene. Such vectors would also comprise the *cis*-acting elements required for reverse transcription, packaging, *etc.*, as is well known in the art for retroviral vectors. In another embodiment, the vectors encode a retroviral vector comprising one or more enhancer-deleted U3 regions.

In one embodiment of the invention, the internal promoter is a eukaryotic, prokaryotic or viral promoter. In a further embodiment, the internal promoter is a mammalian cellular gene promoter. In a further embodiment, the internal promoter is selected from RPL10 promoter (SEQ ID NO:8), LENG8 promoter (SEQ ID NO:9), SNX3 promoter (SEQ ID NO:10), UQCRQ promoter (SEQ ID NO:17), or ITGB4BP promoter (SEQ ID NO:16). In a further embodiment, the internal promoter is a fragment or variant of the full length promoter and is capable of driving high levels of transcription of the heterologous gene while the vector comprising the promoter is capable of producing high viral titers. In one embodiment, a vector comprising the fragment or variant of the promoter retains substantially the same ability to produce high viral titers and high levels of transcription as a vector comprising the wild-type promoter. In a further embodiment, the internal promoter consists essentially of the TATA box.

In some embodiments, the internal promoter further comprises splicing sites for high levels of gene expression. In another embodiment, the vector is a retroviral vector that further comprises additional sequences, including polyadenylation sites, insulator sequences, splicing sites, an internal ribosomal entry site (IRES) and other transcriptional and translational effector sequences as is well known in the art.

In another embodiment, the vector is a plasmid comprising DNA encoding the retroviral vector comprising a 3' LTR with an enhancer-deleted U3 region. In another embodiment, the plasmid encodes a vector with enhancer-deleted U3 regions in both the 5' and 3' LTRs. In a further embodiment, an infectious retroviral particle encapsulating the vector RNA comprising enhancer-deleted U3 regions in both LTRs is provided. In another embodiment, the vector is in either RNA or DNA form, with one or both U3 regions being enhancer-deleted.

In another embodiment, the heterologous gene encodes a transcript of interest. In a further embodiment, the transcript of interest is a biologically active transcript, such as, but not limited to, a small interfering RNA, a ribozyme, an antisense RNA, or a decoy RNA. In a further embodiment, the heterologous gene encodes a polypeptide. The polypeptide may be any desired protein, *e.g.*, a therapeutic protein or a marker protein. In one embodiment, the heterologous gene encodes eGFP or gp91.

A composition comprising the vector and suitable carriers is also provided. The composition may be suitable for *in vivo* administration.

A cell comprising the vector of the invention is provided, including target cells transformed with the vector or producer cells comprising the vector and additional sequences encoding factors required for the generation of infectious particles, such as retroviral *env*, and *gag-pol*, and other factors as needed. The target cells and producer cells may be any suitable eukaryotic cell type, such as mammalian cells. In a further embodiment, the cells may be of human, primate or murine origin. The cells may be primary cells or cell lines.

The present invention also provides for a method of producing infectious retroviral particles comprising cultivating a producer cell line

comprising the retroviral vector as described above or a plasmid encoding the retroviral vector, collecting the supernatant, and filtering the medium to obtain a cell-free viral supernatant. The packaging cell line used for construction of a producer cell line may be any currently known in the art or one generated by transferring genes encoding the necessary viral proteins into a cell line such that once the retroviral vector, which comprises the packaging signal, is transcribed in the cell, the retroviral vector is packaged in infectious particles.

The present invention also provides for a method of transducing target cells comprising contacting said cells with the viral supernatant prepared as described above and comprising infectious retroviral particles according to the invention. The target cell, as described above, may be, but is not limited to, mammalian cells, human cells, primate cells, or murine cells. The target cells may be primary cells or cell lines.

The present invention further provides for a method of treating a subject comprising administering a composition comprising the vector of the invention and a suitable carrier, wherein the heterologous gene encodes a therapeutically useful polypeptide or transcript. In a further embodiment, the method is for treating a genetic disorder, a proliferation disorder, or an infectious disease.

The present invention also provides kits comprising the polynucleotides and vectors of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic of the enhancer-deleted U3 retroviral vectors pMT and pI-D.

Fig. 2 is a schematic of the enhancer-deleted U3 retroviral vector pI-ND.

Fig. 3 is a schematic of the enhancer-deleted U3 retroviral vector pI-LND-n.

Fig. 4 shows the levels of heterologous gene expression (gp91) of enhancer-deleted U3 retroviral vectors comprising the indicated promoters. The control is MT-gp91-n vector.

Fig. 5 shows the promoter sequence for GAPDH (SEQ ID NO:7).

Fig. 6 shows the promoter sequence for RPL10 (SEQ ID NO:8).

Fig. 7 shows the promoter sequence for LENG8 (SEQ ID NO:9).

Fig. 8 shows the promoter sequence for SNX3 (SEQ ID NO:10).

Fig. 9 shows the promoter sequence for ITGB4BP (SEQ ID NO:16).

Fig. 10 shows the promoter sequence for UQCRQ (SEQ ID NO:17).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to expression vectors comprising a heterologous internal promoter. In one embodiment, the vector is a retroviral vector comprising an enhancer-deleted U3 region in one or both LTRs with a heterologous promoter operably linked to a heterologous gene and which is capable of high levels of transcription of the heterologous gene and high viral titers. In another embodiment, the vector encodes a retroviral vector comprising an enhancer-deleted U3 region in one or both LTRs.

The vector

As used herein, the term "retrovirus" is used in reference to RNA viruses that utilize reverse transcriptase during their replication cycle. The retroviral genomic RNA is converted into double-stranded DNA by reverse transcriptase. This double-stranded DNA form of the virus is capable of being integrated into the chromosome of the infected cell; once integrated, it is referred to as a "provirus." The provirus serves as a template for RNA polymerase II and directs the expression of RNA molecules which encode the structural proteins and enzymes needed to produce new viral particles.

As used herein, the term "vector" refers to any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, retrovirus, virion, *etc.*, which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors in RNA or DNA form. A large number of vectors known in the art may be used to manipulate nucleic acids, incorporate response elements and promoters into genes, *etc.* Possible vectors include, for example, plasmids or modified viruses including, for example bacteriophages such as lambda derivatives, or

plasmids such as pBR322 or pUC plasmid derivatives, or the Bluescript vector. Viral vectors, and particularly retroviral vectors, have been used in a wide variety of gene delivery applications in cells, as well as living animal subjects. Viral vectors that can be used include, but are not limited to, retrovirus, adeno-associated virus, pox, baculovirus, vaccinia, herpes simplex, Epstein-Barr, adenovirus, geminivirus, and caulimovirus vectors. Non-viral vectors include plasmids, liposomes, electrically charged lipids (cytofectins), DNA-protein complexes, and biopolymers. Examples of eukaryotic vectors include, but are not limited to, pW-LNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; pSVK3, pBPV, pMSG and pSVL available from Amersham Pharmacia Biotech; and pCMVDsRed2-express, pIRES2-DsRed2, pDsRed2-Mito, and pCMV-EGFP available from Clontech. Many other vectors are well-known and commercially available. The insertion of the DNA fragments corresponding to response elements and promoters into a suitable vector can be accomplished by ligating the appropriate DNA fragments into a chosen vector that has complementary cohesive termini. Alternatively, the ends of the DNA molecules may be enzymatically modified or any site may be produced by ligating nucleotide sequences (linkers) into the DNA termini. Such vectors may be engineered to contain selectable marker genes that provide for the selection of cells that have incorporated the marker into the cellular genome. Such markers allow identification and/or selection of host cells that incorporate and express the proteins encoded by the marker.

As described previously, the retroviral vector of the present invention or the retroviral vector encoded by a vector of the present invention may be based on simple retroviruses, such as MLV, lentiviruses, such as HIV, or any other retrovirus. These vectors retain the *cis* elements required for production of infectious particles. Such elements include a packaging signal located adjacent to the 5' LTR of the retroviral genome which is required for encapsidation of the viral RNA into the viral capsid or particle. Several retroviral vectors use the minimal packaging signal (also referred to as the psi [ψ] sequence) needed for encapsidation of the viral genome. Additional *cis* elements are well known in the art, such as the primer binding site, the polypurine tract and other sequences, and are included in the retroviral vector.

However, sequences encoding viral proteins are removed from the vector such that no full-length viral protein is expressed. Any viral proteins required for the production of infectious particles are provided in *trans* by the packaging constructs.

The vector may further comprise sequences such as polyadenylation sequence, insulator sequences, splicing sites, IRES and other transcriptional and translational effector sequences.

The term "polyadenylation site", "poly A site" or "poly A sequence" as used herein denotes a DNA sequence that directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in the present vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one that is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp BamHI/Bcll restriction fragment and directs both termination and polyadenylation. Suitable polyadenylation sequences of the present invention also include, but are not limited to the bovine growth hormone (bGH) polyadenylation signal, the -globin polyA site, and herpes simplex virus thymidine kinase polyA site (Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, pp. 16.7-16.8 (1989)).

Vectors of the present invention may also contain additional nucleic acid sequences, such as an intron sequence, splicing sequences, a localization sequence, or a signal sequence, sufficient to permit a cell to efficiently and effectively process the protein expressed by the nucleic acid of the vector. Examples of intron sequences include the β-globin intron and the human EF-1α intron (U.S. Pat. No. 7,049,143). Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, pp. 16.7-16.8 (1989)).

As used herein, the term "internal ribosome entry site" or "IRES" refers to a sequence located between polycistronic genes that permits the production of the expression product originating from the second gene by internal initiation of the translation of the dicistronic mRNA. Examples of internal ribosome entry sites include, but are not limited to, those derived from foot and mouth disease virus (FDV), encephalomyocarditis virus, poliovirus and RDV (Scheper *et al., Biochem. 76*:801 (1994); Meyer *et al., J. Virol. 69*:2819 (1995); Jang *et al., J. Virol. 62*:2636 (1998); Haller *et al., J. Virol. 66*:5075 (1995)). Vectors incorporating IRESs may be assembled as is known in the art. For example, a vector containing a polycistronic sequence may contain the following elements in operable association: internal promoter, heterologous gene, an internal ribosome entry site and a second heterologous gene.

Such additional sequences are inserted into the vector such that they are operably linked with the promoter sequence, if transcription is desired, or additionally with the initiation and processing sequence if translation and processing are desired. Alternatively, the inserted sequences may be placed at any position in the vector.

Standard techniques for the construction of the vectors of the present invention are well-known to those of ordinary skill in the art and can be found in such references as Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, (1989). A variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments and which choices can be readily made by the skilled artisan.

Compositions comprising the expression vectors of the present invention and a suitable carrier are also envisioned. Such carriers are well known in the art and refer to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such suitable carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions

can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations and the like. The compositions can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Examples of suitable carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the retroviral vector of the present invention, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

The U3 region

As discussed previously, the retroviral genome comprises long terminal repeats (LTRs) at the 5' and 3' ends of the genome containing sequences important for replication during the retroviral life cycle. The promoter and enhancer elements of the U3 region are important for generating full-length transcripts of the retroviral genome during replication. During reverse transcription, a portion of the 3' LTR serves as a template for both the 3' as well as the 5' LTR, so the sequence of the 3' LTR is copied into the 5' LTR. Therefore, a deletion or mutation in the 3' U3 region is copied into the 5' LTR, rendering both U3 regions substantially inactive. This duplication of the 3' LTR allows a vector sequence to contain the unaltered 5' U3 region during packaging so as to allow generation of full length transcripts from the 5' LTR to be generated and packaged. However, during replication, the 5' U3 is lost due to the duplication of the 3' U3 region comprising the enhancer-deleted

U3 region into the 5' LTR. In this way, full length transcripts are produced for packaging, but the vector is self-inactivating because both LTRs are rendered transcriptionally silent after one round of replication. Alternatively, the U3 regions of both LTRs may be enhancer-deleted, and full length genomic transcripts may be generated using a heterologous promoter.

"Enhancer-deleted" is used herein to refer to U3 regions in which all or a portion of the enhancer has been altered by deletion, addition and/or substitution within and/or around the enhancer such that the enhancer has been substantially inactivated. An enhancer is considered to be substantially inactivated when the alteration of the enhancer is sufficient to substantially eliminate U3-driven transcripts. In one embodiment, less than 1% of the total retroviral transcripts is driven from the U3 region of the LTR as compared to the internal promoter-driven gene expression. In one embodiment of the invention, less than 0.1% of the total transcripts is driven from the U3 region of the LTR. In another embodiment, no detectable U3-driven transcript is found.

In one embodiment of the invention, the enhancer-deleted U3 region is substantially inactivated by deletion of some or all of the enhancer and promoter elements in that region. In another embodiment, the U3 region is substantially inactivated by substitution or insertion of nucleotides in the U3 region. In a further embodiment, the entire U3 region is deleted. The U3 regions of both the known retroviral vectors as well as wild type retroviruses suitable for use in the invention are well known in the art and readily recognized (Coffin, JM, *Fundamental Virology*, pp. 798-800, Fields *et al.* eds., 3rd Ed., Lippincott-Raven Publ. (1996)).

Internal promoters

In the present invention, promoters are provided which can produce high viral titer and high levels of gene expression in the enhancer-deleted U3 retroviral vector environment. In one embodiment, the internal promoter is heterologous to the vector, *i.e.*, is not present in the vector as the vector is found in nature. Various promoters were tested, including HCMV IE promoter (SEQ ID NO:1), MLV U3 region (SEQ ID NO:2), CMV enhancer/ubiquitin promoter (SEQ ID NO:3), cytomegalovirus

enhancer/chicken β-actin (CAG) promoter (SEQ ID NO:4), human elongation factor 1 alpha (EF-1α) promoter (SEQ ID NO:5), human β-actin (ACTB) promoter (SEQ ID NO:6), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (SEQ ID NO:7), human ribosomal protein L10 (RPL10) promoter (SEQ ID NO:8), human leukocyte receptor cluster member 8 (LENG8) promoter (SEQ ID NO:9), human sorting nexin 3 (SNX3) promoter (SEQ ID NO:10), human CCR4-NOT transcription complex, subunit 3 (CNOT3) promoter (SEQ ID NO:11), human copine I (CPNE1) promoter (SEQ ID NO:12), human hypothetical protein (HYPO) promoter (SEQ ID NO:13), human dyskeratosis congenita 1, dyskerin (DKC1) promoter (SEQ ID NO:14), human vacuolar protein sorting 72 (VPS72) promoter (SEQ ID NO:15), integrin beta 4 binding protein (ITGB4BP) promoter (SEQ ID NO:16), and ubiquinol-cytochrome c reductase, complex III subunit VII (UQCRQ) promoter (SEQ ID NO:17). It was found that the UQCRQ, SNX3, ITGB4BP, GAPDH, RPL10 and LENG8 promoters could produce high viral titer and high levels of gene expression. These data show that the cellular promoters of the present invention can drive gene expression in enhancer-deleted U3 retroviral vectors. In one embodiment of the invention, the internal promoter may be a fragment or variant of the above-listed promoters which can produce high viral titer and high levels of gene expression. A fragment of a promoter, as used herein, refers to a polynucleotide comprising a sequence that is identical to but less than the full length of the naturally occurring promoter. In one embodiment, the fragment has at least about 20% (*e.g.*, about 30, 40, 50, 60, 70, 80, 85, 90, or 95%) of the transcriptional activity of the full length promoter. Fragments that may be used include, without limitation, fragments of the RPL10 promoter comprising or consisting of the nucleotide sequences about -50 to about +143 (about nucleotides 951 to 1143 of SEQ ID NO:8), about -100 to about +143 (about nucleotides 901 to 1143 of SEQ ID NO:8), about -200 to about +143 (about nucleotides 801 to 1143 of SEQ ID NO:8), about -350 to about +143 (about nucleotides 651 to 1143 of SEQ ID NO:8), about -500 to about +143 (about nucleotides 501 to 1143 of SEQ ID NO:8), about -1000 to about +143 (about nucleotides 1 to 1143 of SEQ ID NO:8), or about -350 to about +1 (about nucleotides 651 to 1001 of SEQ ID NO:8), and fragments of

the LENG8 promoter comprising the nucleotide sequences about -50 to about +305 (about nucleotides 970 to 1325 of SEQ ID NO:9), about -100 to about +305 (about nucleotides 920 to 1325 of SEQ ID NO:9), about -200 to about +305 (about nucleotides 820 to 1325 of SEQ ID NO:9), about -385 to about +305 (about nucleotides 635 to 1325 of SEQ ID NO:9), about -1020 to about +305 (about nucleotides 1 to 1325 of SEQ ID NO:9), or about -385 to about +1 (about nucleotides 635 to 1020 of SEQ ID NO:9) (with the transcription start site considered to be +1 for each promoter). A variant of a promoter, as used herein, refers to a polynucleotide comprising a sequence that is at least about 70% (*e.g.*, at least about 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99%) identical to the sequence of a naturally occurring promoter and which has at least about 20% (*e.g.*, about 30, 40, 50, 60, 70, 80, 85, 90, or 95%) of the transcriptional activity of the naturally occurring promoter. A variant of a promoter may also be a fragment of that promoter.

"High viral titers" is used herein to mean that the enhancer-deleted U3 retroviral vector produces at least 10% of infectious retroviral particles containing the vector as the same vector that has functional, unaltered U3 regions in both LTRs. In other embodiments, the titer of the enhancer-deleted U3 vector is at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200% or more as compared to the U3-functional vector. Such comparisons of the titers can be made in several ways, such as by the expression of a reporter gene, *e.g.* GFP or luciferase, in transduced cells, selection of transduced cells using a selectable marker expressed by the vectors, or expression of a therapeutic gene by the vectors. Many such methods for measuring viral titers are well known in the art.

"High levels of heterologous gene transcription" or "high levels of gene expression" encompasses the transcription or expression of the heterologous gene that is at least 70% (*e.g.*, at least 80, 90, 95, 96, 97, 98, or 99%) of the total transcripts produced by the retroviral vector and is at least 10% of the transcription or expression as the same gene in the same vector that has functional, unaltered U3 regions in both LTRs. In other embodiments, the transcription or expression of the heterologous gene in the enhancer-deleted U3

vector is at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200% or more as compared to same gene in the U3-functional vector. Such comparisons can be made, *e.g.*, by measuring reporter gene expression or by analyzing the amounts of the heterologous transcript or protein that is made using standard molecular biology techniques, *e.g.* by Northern blot analysis, RT-PCR, Western blot analysis, immunohistochemistry and enzyme-linked immunosorbent assays (ELISA).

A promoter of the present invention may comprise a promoter of eukaryotic, prokaryotic or viral origin, and will be sufficient to direct the transcription of a distally located sequence (a sequence linked to the 3' end of the promoter sequence) in a cell. The internal promoter should drive high levels of transcription of the heterologous gene to which it is operably linked while also allowing high viral titers through generation and packaging of the genomic RNA in the producer cell line. The promoter may also further comprise enhancer elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis *et al., Science 236*:1237 (1987)). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells, and viruses (analogous control elements are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest.

Promoters, enhancers and other regulatory elements may be tissue specific or cell specific. The term "tissue specific" as it applies to a regulatory element refers to a regulatory element that is capable of directing selective expression of a heterologous gene to a specific type of tissue (*e.g.*, liver) to a greater extent than the expression of the same nucleotide sequence of interest in a different type of tissue (*e.g.*, lung). The term "tissue-specific" (*e.g.*, liver-specific) as used herein is a relative term that does not require absolute specificity of expression. In other words, the term "tissue-specific" does not require that one tissue have extremely high levels of expression and another tissue have no expression. It is sufficient that expression is greater in one

tissue than another. By contrast, "strict" or "absolute" tissue-specific expression is meant to indicate expression in a single tissue type (*e.g.*, liver) with no detectable expression in other tissues. Likewise, the term "cell type specific" as applied to a regulatory element refers to a regulatory element which is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell to a greater extent than the expression of the same nucleotide sequence of interest in a different type of cell within the same tissue (*e.g.*, hyperproliferative cells, for example, cancer cells). The term "cell type specific" when applied to a regulatory element also means a regulatory element capable of promoting selective expression of a nucleotide sequence of interest in a region within a single tissue.

While any internal promoter capable of producing high levels of transcription while also allowing high viral titers is contemplated in the present invention, in one embodiment, the internal promoter is a cellular promoter. In a further embodiment, the internal promoter is selected from RPL10 promoter (SEQ ID NO:8), LENG8 promoter (SEQ ID NO:9), SNX3 promoter (SEQ ID NO:10), UQCRQ promoter (SEQ ID NO:17), or ITGB4BP promoter (SEQ ID NO:16). In a further embodiment, the internal promoter is a variant of the full length promoter and is capable of driving high levels of transcription of the heterologous gene while the vector comprising the promoter is capable of producing high viral titers. In a further embodiment, the internal promoter consists essentially of the TATA box. In some embodiments, the internal promoter further comprises one or more splicing sites for high levels of gene expression.

To reduce any possibility of insertional activation from the internal promoter, insulator sequences may be used to block the activation effect of the internal promoter to nearby genes (Ramezani *et al., Mol. Ther. 14*:245 (2006)). Another approach is to modify the vector by inserting additional polyadenylation signals to inhibit the read-through from the internal promoter (Ramezani *et al., Mol. Ther. 14*:245 (2006)).

Heterologous gene

The term "operably linked" is used to describe a linkage between a gene sequence and a promoter or other regulatory or processing sequence such that the transcription of the gene sequence is directed by an operably linked promoter sequence.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or transcript. The term also encompasses the coding region of a structural gene and includes sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' untranslated sequences. The sequences that are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' untranslated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

The term “polynucleotide” or “nucleic acid molecule”, as used interchangeably herein, refers to nucleotide polymers of any length, such as two or more, and includes both DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, nucleotide analogs (including modified phosphate moieties, bases, or sugars), or any substrate that can be incorporated into a polymer by a suitable enzyme, such as a DNA polymerase or an RNA polymerase.

As will be appreciated by one skilled in the art, the nucleotide sequence of the inserted polynucleotide of interest may be of any nucleotide sequence. For example, the polynucleotide sequence may be a reporter gene

sequence or a selectable marker gene sequence. A reporter gene sequence, as used herein, is any gene sequence which, when expressed, results in the production of a protein whose presence or activity can be monitored. Examples of reporter genes include, but are not limited to, luciferase (*See, e.g.,* deWet *et al., Mol. Cell. Biol.* 7:725 (1987) and U.S. Pat Nos. 6,074,859; 5,976,796; 5,674,713; and 5,618,682; all of which are incorporated herein by reference), green fluorescent protein (*e.g.*, GenBank Accession Number U43284; a number of GFP variants are commercially available from CLONTECH Laboratories, Palo Alto, Calif.), chloramphenicol acetyltransferase, β-galactosidase, alkaline phosphatase, and horse radish peroxidase. Alternatively, the reporter gene sequence may be any gene sequence whose expression produces a gene product that affects cell physiology. Polynucleotide sequences of the present invention may comprise one or more gene sequences that already possess one or more promoters, initiation sequences, or processing sequences.

A reporter gene sequence may be a selectable marker, which is any gene sequence capable of expressing a protein whose presence permits one to selectively propagate a cell which contains it. Selectable markers may be "dominant"; a dominant selectable marker encodes an enzymatic activity that can be detected in any eukaryotic cell line. Examples of dominant selectable markers include, but are not limited to, the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as the neo gene) that confers resistance to the drug G418 in mammalian cells, the bacterial hygromycin G phosphotransferase (hyg) gene that confers resistance to the antibiotic hygromycin and the bacterial xanthine-guanine phosphoribosyl transferase gene (also referred to as the gpt gene) that confers the ability to grow in the presence of mycophenolic acid. Other selectable markers are not dominant in that their use must be in conjunction with a cell line that lacks the relevant enzyme activity. Examples of non-dominant selectable markers include the thymidine kinase (tk) gene that is used in conjunction with tk^- cell lines, the CAD gene which is used in conjunction with CAD-deficient cells and the mammalian hypoxanthine-guanine phosphoribosyl transferase (hprt) gene which is used in conjunction with $hprt^-$ cell lines. A review of the use of

selectable markers in mammalian cell lines is provided in Sambrook, J. *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, New York pp. 16.9-16.15 (1989).

Reporter gene sequences are sufficient to permit the recognition or selection of the vector in normal cells. In one embodiment of the invention, the reporter gene sequence may encode an enzyme or other protein which is normally absent from mammalian cells, and whose presence can, therefore, definitively establish the presence of the vector in such a cell.

The retroviral vectors of the present invention provide for the incorporation of heterologous genes into virus particles, thereby providing a means for amplifying the number of infected host cells containing heterologous nucleic acid therein. The incorporation of the heterologous gene facilitates the replication of the heterologous gene within the viral particle, and the subsequent production of a heterologous transcript or protein therein. A gene is said to be heterologous if it is not naturally present in the wild-type of the vector used to deliver the gene into a cell. The term heterologous gene, as used herein, is intended to refer to a nucleic acid molecule.

The heterologous gene may also comprise the coding sequence of a desired product such as a suitable biologically active protein or polypeptide, immunogenic or antigenic protein or polypeptide, or a therapeutically active protein or polypeptide. The polypeptide may supplement deficient or nonexistent expression of an endogenous protein in a host cell. Such gene sequences may be derived from a variety of sources including DNA, cDNA, synthetic DNA, RNA or combinations thereof. Such gene 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 sequences or polyadenylation sequences. The gene sequences of the present invention are preferably cDNA. Genomic DNA or cDNA may be obtained in any number of ways. Genomic DNA can be extracted and purified from suitable cells by means well-known in the art. Alternatively, mRNA can be isolated from a cell and used to prepare cDNA by reverse transcription, or other means. Alternatively, the polynucleotide sequence may comprise a sequence complementary to an RNA sequence, such as an antisense

RNA sequence, which antisense sequence can be administered to an individual to inhibit expression of a complementary polynucleotide in the cells of the individual.

Expression of the heterologous gene may provide an immunogenic or antigenic protein or polypeptide to achieve an antibody response. The antibodies thus raised may be collected from an animal in a body fluid such as blood, serum or ascites.

The heterologous gene can also be any nucleic acid of interest that can be transcribed. Generally the foreign gene encodes a polypeptide. Preferably the polypeptide has some therapeutic benefit. The polypeptide may supplement deficient or nonexistent expression of an endogenous protein in a host cell. The polypeptide can confer new properties on the host cell, such as a chimeric signaling receptor, see U.S. Pat. No. 5,359,046. One of ordinary skill can determine the appropriateness of a heterologous gene practicing techniques taught herein and known in the art. For example, the artisan would know whether a heterologous gene is of a suitable size for encapsidation and whether the heterologous gene product is expressed properly.

The particular heterologous gene that can be employed in the present invention is not critical thereto. However, in one embodiment, the heterologous gene encodes cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, ribozymes, RNA external guide sequences, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins or variants thereof.

In a further embodiment, the heterologous gene may encode a polypeptide including, but not limited to, immunoglobulins, erythropoietin, alpha-interferon, alpha-1 proteinase inhibitor, angiogenin, antithrombin III, beta-acid decarboxylase, human growth hormone, bovine growth hormone, porcine growth hormone, human serum albumin, beta-interferon, calf intestine alkaline phosphatase, cystic fibrosis transmembrane regulator, Factor VIII, Factor IX, Factor X, insulin, lactoferrin, tissue plasminogen activator, myelin

basic protein, insulin, proinsulin, prolactin, hepatitis B antigen, immunoglobulin fragments (*e.g.*, FABs), monoclonal antibody CTLA4 Ig, Tag 72 monoclonal antibody, Tag 72 single chain antigen binding protein, protein C, cytokines and their receptors, including, for instance tumor necrosis factors alpha and beta, their receptors and their derivatives; renin; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; von Willebrands factor; atrial natriuretic factor; lung surfactant; urokinase; bombesin; thrombin; hemopoietic growth factor; enkephalinase; human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; beta-lactamase; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-beta; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-.β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-12; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; antibodies; chimeric proteins, such as immunoadhesins, and fragments or fusions of any of the above-listed polypeptides. In a further embodiment, the heterologous gene encodes an oxidase, in particular NADPH oxidase, *e.g.* the gp91 subunit of NADPH oxidase. Nucleic acid and protein sequences for these proteins are available in public databases such as GenBank.

Where a particular protein has more than one subunit (such as an immunoglobulin), the genes encoding the sequences may be arranged in a polycistronic sequence in the vector, separated by one or more IRES elements. Alternatively, genes encoding different subunits of a protein may be introduced into the host cell on separate vectors. In accordance with the present invention, the gene encoding the protein of interest preferably comprises one or more introns. The introns may be introns normally associated with the gene or may be synthetic or exogenous introns. In some embodiments, the gene may comprise less than its normal complement of introns. For example, some of the naturally occurring introns may be removed from the gene while others are retained, or one or more of the naturally occurring introns can be replaced by one or more exogenous introns.

By "wild type" or native, it is intended that the nucleotide or amino acid sequence is identical to the sequence found in nature.

By "variant" it is intended to include substantially similar sequences. Thus, for nucleotide sequences or amino acid sequences, variants include sequences that are functionally equivalent, e.g., retain at least 20% (e.g., 30, 40, 50, 60, 70, 80, or 90%) of one or more of the activities of the wild-type sequence. Variant nucleotide sequences also include synthetically derived nucleotide sequences that have been generated, for example, by site directed mutagenesis, but which still retain the function of the native sequence. Generally, nucleotide sequence variants or amino acid sequence variants of the invention will have at least 70%, generally 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to its respective native nucleotide sequence.

One of skill will appreciate that many conservative variations of the nucleic acid constructs disclosed yield a functionally identical construct. Conservative variations of a particular nucleic acid sequence refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For example, due to the degeneracy of the genetic code, "silent

substitutions" (e.g., substitutions of a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of every nucleic acid sequence which encodes an amino acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence of a packaging or packageable construct are substituted with different amino acids with highly similar properties, are also readily identified as being highly similar to a disclosed construct. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in any described sequence. Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) Proteins W. H. Freeman and Company. Finally, the addition of sequences which do not alter the activity of a nucleic acid molecule, such as a non-functional sequence is a conservative modification of the basic nucleic acid. Such conservatively substituted variations of each disclosed sequence are a feature of the present invention.

With respect to the amino acid sequences for the various full-length or mature polypeptides used in the vector system of the present invention, variants include those polypeptides that are derived from the native polypeptides by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native polypeptide; deletion or addition of one or more amino acids at one or more sites in the native polypeptide; or substitution of one or more amino acids at one or more sites in the native polypeptide. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

One of skill will recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation, chemical synthesis of a desired oligonucleotide (e.g., in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques.

A variant of a native nucleotide sequence or native polypeptide has substantial identity to the native sequence or native polypeptide. A variant may differ by as few as 1 to 10 amino acid residues, such as 6-10, as few as 5, as

few as 4, 3, 2, or even 1 amino acid residue. A variant of a nucleotide sequence may differ by as few as 1 to 30 nucleotides, such as 6 to 20, as low as 5, as few as 4, 3, 2, or even 1 nucleotide residue.

It is intended by "sequence identity" that the same nucleotides or amino acid residues are found within the variant sequence and a reference sequence when a specified, contiguous segment of the nucleotide sequence or amino acid sequence of the variant is aligned and compared to the nucleotide sequence or amino acid sequence of the reference sequence. Methods for sequence alignment and for determining identity between sequences are well known in the art. With respect to optimal alignment of two nucleotide sequences, the contiguous segment of the variant nucleotide sequence may have additional nucleotides or deleted nucleotides with respect to the reference nucleotide sequence. Likewise, for purposes of optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference nucleotide sequence or reference amino acid sequence will comprise at least 20 contiguous nucleotides, or amino acid residues, and may be 30, 40, 50, 100, or more nucleotides or amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the variant's nucleotide sequence or amino acid sequence can be made by assigning gap penalties.

Cells

Cells comprising the expression vectors of the present invention are also encompassed. In one embodiment, cells comprising enhancer-deleted U3 retroviral vectors are also encompassed. The target cell, which is transduced by the expression vector of the present invention, can be any eukaryotic cell type capable of being transduced, including mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells, whether located *in vitro* or *in vivo*. In one embodiment, the target cell is a mammalian cell, particularly a human cell. The target cell may be a primary cell or a cell line.

In one embodiment, producer cell lines which further comprise the necessary viral proteins in *trans* to produce infectious retroviral particles comprising the present vector are encompassed by the present invention. Such producer cell lines produce the retroviral capsids and transcribe the retroviral vector, which is then recruited through the packaging signal to the viral capsid. The packaging cell lines required for the construction of producer cell lines are known in the art and typically comprise the retroviral *gag-pol* and *env* genes, which provide the enzymes (e.g., reverse transcriptase) and structural proteins (*e.g.*, Gag and Env) required for the infectious retroviral particles. Many such packaging cells are known, such as PG13, ψCRIP, PA317, GP+envAm12, FLYA13, FLYRD18, Phoenix-Ampho, Phoenix-Eco, Phoenix-GALV, PE501, GP+E86, PT67, BING, BOSC23, ProPak-A, and others, as well as lentiviral packaging cell lines (Logan *et al., J Virol. 78*:8421-8436 (2004)). Further, packaging cell lines can be transiently transfected for short term use or have the viral genes integrated into their genome for long term use.

Many packaging cell lines utilize the native envelope of the retrovirus upon which the retroviral vector is based. It is also possible to alter the host range of cells that the viral vectors of the present invention can infect by utilizing an envelope gene from another closely related virus. In other words, it is possible to expand the host range of the retroviral vectors of the present invention by taking advantage of the capacity of the envelope proteins of certain viruses to participate in the encapsidation of other viruses. Examples of retroviral-derived *env* genes include, but are not limited to: the G-protein of vesicular-stomatitis virus (VSV-G), gibbon ape leukemia virus (GaLV), cat endogenous virus RD114, Rous sarcoma virus (RSV), amphotropic Moloney murine leukemia virus (MoMuLV), ecotropic Moloney murine leukemia virus (MoMuLV), 10A1 murine leukemia virus, Molony mink cell focus-inducing virus (MCFV), Mus dunni endogenous virus (MDEV), mouse mammary tumor virus (MMTV), and human immunodeficiency virus (HIV). All of these viral envelope proteins efficiently form pseudotyped virions with genome and matrix components of other viruses. As used herein, the term "pseudotype" refers to a viral particle that contains nucleic acid of one virus but the envelope

protein of another virus. In general, either VSV-G or GaLV pseudotyped vectors have a very broad host range, and may be pelleted to titers of high concentration by ultracentrifugation while still retaining high levels of infectivity.

Methods of the invention

The present invention further provides methods of producing infectious retroviral particles comprising the retroviral vector of the present invention by cultivating a producer cell line as described above, collecting the supernatant from the cell culture, and filtering the supernatant to obtain a cell-free viral supernatant. One of skill in the art would readily optimize the conditions for obtaining good viral titers, such as using the appropriate culture medium and determining the optimal collection time periods and cell densities in the culture.

It is further provided herein a method for transducing target cells comprising contacting said cells with the viral supernatant prepared as described above and comprising infectious retroviral particles according to the invention. The target cell, as described above, may be, but is not limited to, mammalian cells, human cells, primate cells, or murine cells. The target cells may be primary cells or cell lines. The method may further comprise the addition of substances to increase transduction to the viral supernatant, such as polybrene, retronectin, and/or protamine sulfate. Additionally, the method may further comprise low-speed centrifugation of the cells once the viral supernatant is applied. These and other transduction optimization techniques are well known and routine in the art.

The present invention further provides a method of treating a subject by administering cells transduced with the retroviral vector of the invention, wherein the heterologous gene encodes a therapeutically useful polypeptide or transcript. In one embodiment, the cells are transduced *in vitro* or *ex vivo*.

The present invention further provides a method of treating a subject comprising administering a composition comprising the expression vector of the invention and a suitable carrier, wherein the heterologous gene encodes a therapeutically useful polypeptide or transcript. In one embodiment, the nucleic acid of interest encodes a therapeutic agent. The term "therapeutically

useful" is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents. A therapeutic agent may be considered therapeutic if it improves or prevents at least one symptom of a disease or medical condition. Genetic diseases which may be treated with vectors and/or methods of the present invention include those in which long-term expression of the therapeutic nucleic acid is desired. In a further embodiment, the method is for treating a genetic disorder, a proliferation disorder, or an infectious disease. A further embodiment includes a method for treating one or more diseases, disorders, or conditions, including but not limited to: neural disorders, immune system disorders, muscular disorders, reproductive disorders, gastrointestinal disorders, pulmonary disorders, cardiovascular disorders, renal disorders, proliferative disorders, and/or cancerous diseases and conditions. Further embodiments include the treatment of neurodegenerative diseases or disorders, Alzheimer's, schizophrenia, epilepsy, neoplasms, cancer and AIDS or other diseases requiring replacement or the up or down regulation of a gene of interest.

Methods of administering the expression vector of the present invention include, but are not limited, to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The vector or composition may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Administration can be systemic or localized. In addition, it may be desirable to introduce the vector or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

Kits

It is a further object of this invention to provide a kit or drug delivery system comprising the vectors for use in the methods described herein. All the essential materials and reagents required for administration of the targeted retroviral particle may be assembled in a kit (*e.g.*, packaging cell construct or cell line). The components of the kit may be provided in a variety of formulations. The one or more retroviral vectors of the present invention may be formulated with one or more agents (*e.g.*, a chemotherapeutic agent) into a single pharmaceutically acceptable composition or separate pharmaceutically acceptable compositions.

The components of these kits or drug delivery systems may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent, which may also be provided in another container means. The kits of the invention may also comprise instructions regarding the dosage and or administration information. The kits or drug delivery systems of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits may also comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of a subject. Such an instrument may be an applicator, inhalant, syringe, pipette, forceps, measured spoon, eye-dropper or any such medically approved delivery vehicle.

It must be noted that as used in this specification and the appended claims, the singular forms "a," "an," "the," and the like, include plural references unless the context clearly dictates otherwise. Thus, for example, reference to “a polynucleotide” includes polynucleotides and "a cell" includes a plurality of cells.

As used herein the term, the term "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments can consist of, but are not limited to, test

tubes and cell cultures. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reaction that occur within a natural environment.

The following examples are illustrative, but not limiting, of the methods of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in medical treatment and pharmaceutical science and which are obvious to those skilled in the art are within the spirit and scope of the invention.

EXAMPLE 1

In the present example, the construction process of various promoters is provided.

1) Human cytomegalovirus (HCMV) immediate early (IE) promoter (SEQ ID NO:1)

The HCMV IE promoter was obtained from a pCN plasmid (Lee *et al., Biochem. Biophys. Res. Commun. 272*: 230 (2000)).

2) U3 of MLV (SEQ ID NO:2)

The U3 region of MLV LTR contains strong enhancer and promoter sequences. The U3 region in the MLV 3' LTR was amplified by PCR using an MLV vector, MT (Hong *et al., J. Gene Med. 6*: 724 (2004); U.S. Patent No. 6,451,595) as a template. The following primer pairs were used for PCR.

ME5: ACGCGTGCAAGGCATGGAAAAA (SEQ ID NO:18)
MluI

MP3: ACGCGTAGATCTGAATTCTACCCGGGCGACGCAGT(SEQ ID NO:19)
MluI BglII EcoRI

One hundred microliters of the PCR reaction solution containing 200 ng of the template plasmid DNA and 1 μl each of the primers (10 pmol/μl) were subjected to 35 cycles of PCR amplification reaction by Expend High Fidelity PCR system (Cat# 92351824, Roche). Each cycle was conducted for 30

seconds at 95℃ (denaturation), 30 seconds at 55℃ (annealing) and 30 seconds at 72℃ (polymerization).

The amplified fragment of 455 bps was cloned into the pGEM T easy vector (Cat#A1360, Promega, WI, USA), resulting in pGEM T-MTU3.

3) CMV/Ubiquitin promoter (SEQ ID NO:3)

A. CMV enhancer

The CMV enhancer was amplified by PCR using pCK (PCT/KR99/00855) as a template. The following primer pairs were used for PCR.

CMV5: ACGCGTTGACATTGATTATTG (SEQ ID NO:20)
MluI

KMD1: TCTAGAGCCAAAACAAACTCCCAT (SEQ ID NO:21)
XbaI

Fifty microliters of the PCR reaction solution containing 200 ng of the template plasmid DNA and 2 μl each of the primers (5 pmol/μl) were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 94℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute at 72℃ (polymerization).

The amplified fragment was cloned into the pGEM T easy vector, resulting in pGEM T-Enh. The nucleotide sequence was confirmed by sequencing.

B. Human polyubiquitin C promoter (Gill *et al., Gene Ther.* *8*:1539 (2001))

Human polyubiquitin C promoter (-333 ~ +877) was amplified using genomic DNA isolated from HT1080 cells as a template. The following primer pairs were used for PCR.

KMD4: GCTAGCGGCCTCCGCGCCGGGTTT (SEQ ID NO:22)
NheI

KMD5:ACGCGTAGATCTGAATTCGTCTAACAAAAAAGCCAA(SEQ ID NO:23)
MluI BglII EcoRI

Fifty microliters of the PCR reaction solution containing 200 ng of the template DNA and 2 μl each of the primers (5 pmol/μl) were subjected to 30

cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 94℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment of 1230 bps was cloned into the pGEM T easy vector, resulting in pGEM T-UbC. The nucleotide sequence was confirmed by sequencing.

C. CMV enhancer/UbC promoter

To construct a hybrid promoter consisting of the CMV enhancer and the UbC promoter, the SalI-XbaI fragment from pGEM T Easy-Enh was excised and inserted into the SalI-XbaI site of pGEM T Easy-UbC to generate pGEM T Easy-Enh+UbC.

4) CAG (cytomegalovirus enhancer, chicken β-actin promoter) promoter (SEQ ID NO:4)

To obtain the CAG promoter (cytomegalovirus enhancer, chicken β-actin promoter) (SEQ ID NO:4), the Klenow fragment treated SalI-SwaI fragment from pAxCAwt (Takara Bio, Otsu, Japan) was cloned into pGEM T easy (Promega, WI, USA) to generate pGEM T easy-CAG. The nucleotide sequence was confirmed by sequencing.

5) Human Elongation Factor 1 alpha (EF1-) promoter (SEQ ID NO:5) (Kim *et al.*, *Gene 91*:217 (1990))

The human Elongation Factor 1 alpha (EF1-) promoter (-341 ~ +1007) was amplified using genomic DNA isolated from HT1080 cells (human fibrosarcoma cell line, ATCC CCL-121) as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

EEF1A1F: ACGCGTGTAAGCCAGCAATGGTAGAGGGAAGATTCTGCACG
MluI (SEQ ID NO:24)

EEF1A1R: GGATCCTTTTGGCTTTTAGGGGTAGTTTTCACGACACC
BamHI (SEQ ID NO:25)

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO (dimethyl sulphoxide) were subjected to 30 cycles of PCR

amplification reaction by Expend High Fidelity PCR system (Cat# 92351824, Roche). Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-EF. The nucleotide sequence was confirmed by sequencing.

6) Human -actin promoter (SEQ ID NO:6) (Nakajima-Iijima *et al.*, *Proc. Natl. Acad. Sci. USA 82*: 6133 (1985); Miyamoto, *Nucleic Acids Res. 15*:9095 (1987))

The human -actin promoter (-387 ~ +944) was amplified using genomic DNA isolated from K562 cells (human myelogenous cell line, ATCC CCL-243) as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

BApF: ACGCGTGAGATGTCCACACCTAGGATGTCC (SEQ ID NO:26)
MluI

BApR: GGATCCGGTGAGCTGCGAGAATAGCCG (SEQ ID NO:27)
BamHI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragments were cloned into pGEM T easy, generating pGEM T easy-BA. The nucleotide sequence was confirmed by sequencing.

7) Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (SEQ ID NO:7) (Ercolani *et al.*, *J. Biol. Chem. 263*:15335 (1988))

The human GAPDH (GlycerAldehyde-3-Phosphate DeHydrogenase) promoter (-350 ~ +315) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

GAPDHF: ACGCGTTTCATCCAAGCGTGTAAGGG (SEQ ID NO:28)
MluI

GAPDHR: GTTTAAACGGTGTCTGAGCGATGTGGCT (SEQ ID NO:29)
PmeI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was cloned into pGEM T easy, producing pGEM T easy-GAPDH. The nucleotide sequence was confirmed by sequencing.

8) Human ribosomal protein L10 (RPL10) promoter (SEQ ID NO:8) (NCBI accession No: NM_006013, NT_011726; Bignon *et al.*, *Biochem. Biophys. Res. Commun. 184*:1165 (1992))

The human RPL10 (ribosomal protein L10) promoter (-350 ~ +143) (nucleotides 651 to 1143 of SEQ ID NO:8) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

RPLF: ACGCGTAGGCCCACCTAGGGTACTTTCCTTT (SEQ ID NO:30)
MluI

RPLR: GGATCCGGCGACACCAGGATCTTCAGTGGCT (SEQ ID NO:31)
BamHI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was cloned into pGEM T easy to generate pGEM T easy-RPL. The nucleotide sequence was confirmed by sequencing.

9) Human leukocyte receptor cluster member 8 (LENG8) promoter (SEQ ID NO:9) (NCBI accession No: AL834532, NT_011109; Cooper *et al.*, *Genome Res. 16*:1 (2006))

The human LENG8 (leukocyte receptor cluster (LRC) member 8) promoter (-385 ~ +305, +1908 ~ +2121) (nucleotides 635 to 1538 of SEQ ID NO:9) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

LENG8F1: ACGCGTAGAATTGTTTGAACCCAGGAGGCGG (SEQ ID NO:32)
MluI

LENG8R1: GTTTAAACAAAGTAGAAGACGACGGCGCACGCG
PmeI (SEQ ID NO:33)

LENG8F2: GTTTAAACCCACACCCAGAACTCTTCAGATCCT
PmeI (SEQ ID NO:34)

LENG8R2: GAATTCCTGGACCTTGGGGTATAAGGGGTGG (SEQ ID NO:35)
EcoRI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragments were initially cloned into pGEM T easy, resulting in pGEM T easy-LENG1 and LENG2, respectively. After the confirmation of their nucleotide sequence, the MluI-PmeI fragment of pGEM T easy-LENG1 was cloned into the MluI-PmeI site of pGEM T easy-LENG2, to make pGEM T easy-LENG8.

10) Human sorting nexin 3 (SNX3) promoter (SEQ ID NO:10) (NCBI accession No: NM_152828, NT_025741; Haft *et al.*, *Mol. Cell. Biol. 18*:7278-87 (1998))

The human SNX3 (sorting nexin 3) promoter (-353 ~ +338) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

SNX3F: GAATTCAATCCAGACGCGTGTCTGGTGCAA (SEQ ID NO:36)
EcoRI

SNX3R: GGATCCTTCGCTGTAGCTGCTG (SEQ ID NO:37)
BamHI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was cloned into pGEM T easy to generate pGEM T easy–SNX. The nucleotide sequence was confirmed by sequencing.

11) Human CNOT3 (SEQ ID NO:11) (CCR4-NOT transcription complex, subunit 3) promoter (NCBI accession No: NM_014516; Albert *et al.*, *Nucleic Acids Res. 28*:809 (2000))

The human CNOT3 (CCR4-NOT transcription complex, subunit 3) promoter (-350 ~ +654, +5076 ~ +5266) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

CNOT3F1: ACGCGTGTAGCTCCTCCCCCAGACCAATTGTTTTAAG
MluI (SEQ ID NO:38)

CNOT3R1: GGATCCTCCATCCTTCCAGCCAGGAGCCAATACCGAC
BamHI (SEQ ID NO:39)

CNOTF2: AGATCTTGGGGCTGGTCTCTTGTCAGATAGC (SEQ ID NO:40)
BglII

CNOTR2: GGATCCCTTCCCTGCCCTACAGACGCACTCT (SEQ ID NO:41)
BamHI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragments were initially cloned into pGEM T easy, resulting in pGEM T easy-CNOT1 and CNOT2, respectively. After the confirmation of their nucleotide sequence, the MluI-BamHI fragment of pGEM T easy-CNOT1 was cloned into the MluI-BglII site of pGEM T easy-CNOT2, to make pGEM T easy-CNOT3.

12) Human CPNE1 (copine I) promoter (SEQ ID NO:12) (NCBI accession No: NM_152926; Creutz *et al.*, *J. Biol. Chem. 273*:1393 (1998))

The human CPNE1 (copine I) promoter (-300 ~ +489, +5612 ~ +5999) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

CPNE1F1: ACGCGTGTCCATTTAATCCTCAAAAAACTTA (SEQ ID NO:42)
MluI

CPNE1R1: GGATCCTTTTTACTGCAGTCCCCGTTATTAGCTC
BamHI (SEQ ID NO:43)

CPNE1F2: AGATCTAGCTGTGAAGCTGAGCTTTATGACT (SEQ ID NO:44)
Bgl II

CPNE1R2: GGATCCCTGATAAAACAAGAGATGAATTTCC (SEQ ID NO:45)
BamHI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragments were initially cloned into pGEM T easy, resulting in pGEM T easy-CPNEF and CPNER, respectively. After the confirmation of their nucleotide sequence, the MluI-BamHI fragment of pGEM T easy-CPNEF was cloned into the MluI-BglII site of pGEM T easy-CPNER, to make pGEM T easy-CPNE1.

13) Human HYPO (hypothetical protein) promoter (SEQ ID NO:13) (NCBI accession No: AF351613)

The human HYPO (hypothetical protein) promoter (-350 ~ +66) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

HYPOF: ACGCGTCTTTTACACGTTTGGTTTTATGGT (SEQ ID NO:46)
MluI

HYPOR: GGATCCGGCTGCAACAGGCCAGGAAACCTTC (SEQ ID NO:47)
BamHI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-HYPO. The nucleotide sequence was confirmed by sequencing.

14) Human DKC1 (dyskeratosis congenita 1, dyskerin) promoter (SEQ ID NO:14) (NCBI accession No: BC 009928; Strausberg *et al.*, *Proc. Natl. Acad. Sci. USA 99*:16899 (2002))

The human DKC1 (dyskeratosis congenita 1, dyskerin) promoter (-473 ~ +91) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

DKC1F: ACGCGTGCACACTACTCCTATTGGC (SEQ ID NO:48)
MluI

DKC1R: GAATTCGTTACCCTGCACCGCGTGC (SEQ ID NO:49)
EcoRI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-DKC1. The nucleotide sequence was confirmed by sequencing.

15) Human VPS72 (vacuolar protein sorting 72) promoter (SEQ ID NO:15) (NCBI accession No: NM_005997; Horikawa *et al.*, *Biochem. Biophys. Res. Commun. 208*:999 (1995))

The human VPS72 (vacuolar protein sorting 72) promoter (-466 ~ +43) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

VPS72F: ACGCGTACAAAAATTAGTTGGGCAT (SEQ ID NO:50)
MluI

VPS72R: GAATTCACCGCCTACCGAGACTGCG (SEQ ID NO:51)
EcoRI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of

DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-VPS72. The nucleotide sequence was confirmed by sequencing.

16) Human ITGB4BP (InTeGrin Beta 4 Binding Protein) promoter (SEQ ID NO:16) (NCBI accession No: BC011845, NT_028392; Strausberg *et al.*, *Proc. Natl. Acad. Sci. USA 99*:16899 (2002))

The human ITGB4BP (InTeGrin Beta 4 Binding Protein) promoter (-350 ~ +304) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

ITGB4BPF: ACGCGTTCTGTCCCTCAAGG CACAGCT (SEQ ID NO:52)
MluI

ITGB4BPR: GTTTAAACGAGGCCTAGGGGCGGCGGAGGCGGGAGTTCAA
PmeI (SEQ ID NO:53)

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-ITGB4BP. The nucleotide sequence was confirmed by sequencing.

17) Human UQCRQ (UbiQuinol-Cytochrome c Reductase, complex III subunit VII) promoter (SEQ ID NO:17) (NCBI accession No: BC090048, NT_034772; Strausberg *et al.*, *Proc. Natl. Acad. Sci. USA 99*:16899 (2002))

The human UQCRQ (UbiQuinol-Cytochrome c Reductase, complex III subunit VII) promoter (-350 ~ +217) was amplified using genomic DNA isolated from HT1080 cells as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

UQCRQF: ACGCGTGTCACCTTTTTGTTCCCTCCC (SEQ ID NO:54)
MluI

UQCRQR: GTTTAAACTGTGGCGGCGGCCCTGCAGG (SEQ ID NO:55)
PmeI

Fifty microliters of the PCR reaction solution containing 500 ng of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of DMSO were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 55℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-UQCRQ. The nucleotide sequence was confirmed by sequencing.

EXAMPLE 2

In the present example, the effects of the promoters prepared in EXAMPLE 1 on enhanced green fluorescence protein (eGFP) gene expression in an expression vector were compared.

1. Construction of eGFP expression vectors

First, rabbit beta-globin polyA sequence was obtained from pAxCAwt (Takara Bio, Japan) by PCR. The nucleotide sequences of the primer used for PCR are as follows:

RGpA F: GGATCCTTTTCCCTCTGCCAAA (SEQ ID NO:56)
BamHI

RGpA R: ACTAGTATAAGAGAAGAGGGACAGC (SEQ ID NO:57)
SpeI

Fifty microliters of the PCR reaction solution containing 100 ng of template pAxCAwt DNA (1 μl), 1 μl each of the primers (10 pmol/μl) and 5 μl of dNTPs (10 mM) were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 50℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-RGpA. The nucleotide sequence was confirmed by sequencing.

Then, the MluI-BglII fragments from pC-LND-GFP-n, pG-LND-GFP-n, pR-LND-GFP-n, pL-LND-GFP-n, pIT-LND-GFP-n, and pU-LND-GFP-n (described in EXAMPLE 7) were cloned into the MluI-BamHI site of pGEM T easy-RGpA, resulting in pC-GFP-RGpA, pG-GFP-RGpA, pR-GFP-RGpA, pL-GFP-RGpA, pIT-GFP-RGpA, and pU-GFP-RGpA. To construct pS-GFP-RGpA, the EcoRI-XhoI fragment of pS-LND-GFP-n was made blunt and then cloned into the blunted BamH I site of pGEM T easy-RGpA.

2. Analysis of eGFP expression

The expression vectors containing the eGFP gene were transfected into 293T cells using FuGene6 according to the manufacturer's instructions. The level of GFP expression was measured by flow cytometry analysis. Flow cytometry was performed as follows: 48 hours after transfection, 293T cells were harvested, and washed once with phosphate-buffered saline (PBS) containing 0.1% sodium azide (FACS buffer). Then the cells were re-suspended in PBS, and analyzed by FACSort (Becton Dickinson, Los Angeles, CA, USA) with the aid of CellQuest (Becton Dickinson) data acquisition and analysis software. The results are given in Table 1.

Table 1. Comparison of GFP expression

Promoter	Relative mean fluorescence intensity
CMV	100
GAPDH	102.5

RPL10	81.9
LENG8	66.6
SNX3	36.4
ITGB4BP	76.4
UQCRQ	47.1

The data show that the HCMV and GAPDH promoters produced comparable levels of GFP expression. Other promoters, such as RPL10, LENG8 and ITGB4BP also induced significant GFP expression, indicating the possibility of their use as a promoter in the eukaryotic gene expression system.

EXAMPLE 3

Among the various promoters prepared in EXAMPLE 1, the RPL10 and LENG8 promoters were selected and further characterized to analyze the promoter sequences required for gene expression.

1. Construction of a series of RPL10 promoters

1) Construction of a series of RPL10 promoters

Various lengths of RPL10 promoter were produced by PCR. RPL promoters were amplified using genomic DNA of HT1080 as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

RPL F50: ACGCGTACGCGCGCAGACAGACCGCCTATATAAGCCAT
MluI (SEQ ID NO:58)

RPL F100: ACGCGTTGACGTCTGACAGAGCGTCCACCCGTCTTCG
MluI (SEQ ID NO:59)

RPL F200: ACGCGTCTGGCCGCCCGCGGCCCTGGTACCCGGTCACC
MluI (SEQ ID NO:60)

RPL F500: ACGCGTGTCTCCCCCTCCGGCCTCCCGGGTTGACAAAGG
MluI (SEQ ID NO:61)

RPL F1000: ACGCGTGTGCGCTCGAGCAGGATTTCCTCCCGTCCTTCC
MluI (SEQ ID NO:62)

RPLR: GGATCCGGCGACACCAGGATCTTCAGTGGCT (SEQ ID NO: 31)
BamHI

RPL R TSS: GGATCCGCGCTCCTCCGCCTGCGCATGGCTTATATA

BamHI (SEQ ID NO:63)

Fifty microliters of the PCR reaction solution containing 1 μg of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of dNTPs (10 mM) were subjected to 30 cycles of PCR amplification reaction by Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 95℃ (denaturation), 1 minute at 60℃ (annealing) and 1 minute 30 seconds at 72℃ (polymerization).

The RPL promoter (-350 ~ +143) (nucleotides 651 to 1143 of SEQ ID NO:8) was described in EXAMPLE 1 (8).

The RPL50 promoter (-50 ~ +143) (nucleotides 951 to 1143 of SEQ ID NO:8) was amplified by using primer pairs RPL F50 and RPLR and cloned into pGEM T easy, resulting in pGem T easy-pRPL50.

The RPL100 promoter (-100 ~ +143) (nucleotides 901 to 1143 of SEQ ID NO:8) was amplified by using primer pairs RPL F100 and RPLR and cloned into pGEM T easy, resulting in pGem T easy-pRPL100.

The RPL200 promoter (-200 ~ +143) (nucleotides 801 to 1143 of SEQ ID NO:8) was amplified by using primer pairs RPL F200 and RPLR and cloned into pGEM T easy, resulting in pGem T easy-pRPL200.

The RPL500 promoter (-500 ~ +143) (nucleotides 501 to 1143 of SEQ ID NO:8) was amplified by using primer pairs RPL F500 and RPLR and cloned into pGEM T easy, resulting in pGem T easy-pRPL500.

The RPL1000 promoter (-1000 ~ +143) (nucleotides 1 to 1143 of SEQ ID NO:8) was amplified by using primer pairs RPL F1000 and RPLR and cloned into pGEM T easy, resulting in pGem T easy-pRPL1000.

The RPL TSS promoter (-350 ~ -1) (nucleotides 651 to 1000 of SEQ ID NO:8) was amplified by using primer pairs RPL F and RPL R TSS and cloned into pGEM T easy, resulting in pGem T easy-pRPL TSS.

The nucleotide sequences were confirmed by sequencing.

2) Construction of a series of eGFP expression vectors containing the RPL promoter

pGem T easy-GFP-RGpA was constructed by inserting the BamHI fragment from pGem T easy-GFP (described in EXAMPLE 4) into the BamHI site of pGem T easyRGpA (described in EXAMPLE 2). Then, the MluI-BamHI fragments of pGem T easy-pRPL promoters were cloned to the MluI-BamHI site of pGem T easy-GFP-RGpA, resulting in pRPL-, pRPL50-, pRPL100-, pRPL200-, pRPL500-, pRPL1000-, and pRPL TSS-GFP-RGpA.

2. Construction of a series of LENG8 promoters

1) Construction of a series of LENG8 promoters

As described in EXAMPLE 1 (9), the LENG8 promoter was produced by joining 2 fragments (LENG1 and LENG2). To make various lengths of LENG8 promoter, a series of LENG1 fragments were obtained by PCR. Then, the LENG1 fragments were joined to the LENG2 fragment which was described in EXAMPLE 1 (9) to generate final LENG promoters. The genomic DNA of HT1080 was used as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

LENG F50: ACGCGTGTGACGTCAGGACGCCGCGGTCAGG (SEQ ID NO:64)
Mlu I

LENG F100: ACGCGTTGGCGTTCATTGGCTGTGCAGGGCC (SEQ ID NO:65)
Mlu I

LENG F200: ACGCGTTTGTCCCCTCGGGGCCACCGTCCCC (SEQ ID NO:66)
Mlu I

LENG F1000: ACGCGTTTGTATCAGAGTCCTGGACGGAAAC (SEQ ID NO:67)
Mlu I

LENG8R1: GTTTAAACAAAGTAGAAGACGACGGCGCACGCG (SEQ ID NO:33)
Pme I

LENG R TSS: GTTTAAACCTCTGGTCTTCTTTGGCTTCGACGT (SEQ ID NO:68)
Pme I

Fifty microliters of the PCR reaction solution containing 1 μg of template genomic DNA, 1 μl each of the primers (10 pmol/μl) and 5 μl of dNTPs (10 mM) were subjected to 30 cycles of PCR amplification reaction by

Expend High Fidelity PCR system. Each cycle was conducted for 1 minute at 94℃ (denaturation), 1 minute at 55℃ (annealing) and 2 minutes at 72℃ (polymerization).

The L50 fragment (-50 ~ +305) (nucleotides 970 to 1325 of SEQ ID NO:9) was amplified by using primer pairs LENG F50 and LENG8R1 and cloned into pGEM T easy, resulting in pGem T easy-pL50.

The L100 fragment (-100 ~ +305) (nucleotides 920 to 1325 of SEQ ID NO:9) was amplified by using primer pairs LENG F100 and LENG8R1 and cloned into pGEM T easy, resulting in pGem T easy-pL100.

The L200 fragment (-200 ~ +305) (nucleotides 820 to 1325 of SEQ ID NO:9) was amplified by using primer pairs LENG F200 and LENG8R1 and cloned into pGEM T easy, resulting in pGem T easy-pL200.

The L1000 fragment (-1020 ~ +305) (nucleotides 1 to 1325 of SEQ ID NO:9) was amplified by using primer pairs LENG F1000 and LENG8R1 and cloned into pGEM T easy, resulting in pGEM T easy-pL1000.

Then, the MluI-PmeI fragment of pGEM T easy-pL50, -pL100, -pL200, and -pL1000 was cloned into the MluI-PmeI site of pGEM T easy-LENG2 which was described in EXAMPLE 1 (9), to generate pGEM T easy-pLENG50P, -pLENG 100P, -pLENG200P, and -pLENG1000P.

The LENG TSS promoter (-385 ~ -1) (nucleotides 635 to 1019 of SEQ ID NO:9) was amplified by using primer pairs LENG F and LENG R TSS and cloned into pGEM T easy, resulting in pGem T easy-pLENG TSS.

The LENG8 promoter (-385 ~ +305, +1908 ~ +2121) (nucleotides 635 to 1538 of SEQ ID NO:9) was described in EXAMPLE 1 (9).

2) Construction of a series of eGFP expression vectors containing the LENG promoter

To construct expression vectors containing the eGFP gene, the MluI-EcoRI fragments (EcoRI site made blunt) of pGEM T easy-pLENG promoters were cloned into the MluI-BamHI site (BamHI site made blunt) of pGem T easy-GFP-RGpA, resulting in pLENG-, pLENG50-, pLENG100-, pLENG200-, and pLENG1000-GFP-RGpA. The MluI-PmeI fragment of pGem T easy-

pLENG TSS was cloned into the MluI-BamHI site (BamHI site made blunt) of pGem T easy-GFP-RGpA, resulting in pLENG TSS-GFP-RGpA.

3. Analysis of eGFP expression

293T cells were transfected with the eGFP expression vectors using FuGene6 (Roche, Germany) according to the manufacturer's instructions, and cultured for 48 hours. The level of GFP expression was measured by flow cytometry analysis. Flow cytometry was performed as follows: 48 hours after transfection, 293T cells were harvested, and washed once with PBS containing 0.1% sodium azide. Then the cells were re-suspended in PBS, and analyzed by FACSort with the aid of CellQuest data acquisition and analysis software. The results are given in Table 2.

Table 2. Comparison of eGFP expression

A. RPL10 promoters

	Relative mean fluorescence intensity
RPL-GFP	100
RPL50-GFP	20
RPL100-GFP	40
RPL200-GFP	70
RPL500-GFP	150
RPL1000-GFP	210
RPL TSS-GFP	20

B. LENG8 promoters

	Relative mean fluorescence intensity
LENG-GFP	100
LENG50-GFP	14

LENG100-GFP	42
LENG200-GFP	87
LENG1000-GFP	170
LENG TSS-GFP	39

The shortest promoter RPL50 or LENG50 could drive GFP expression that was significantly above background, indicating the presence of 50 bp upstream of the transcription start site was sufficient for basal activity. However, the eGFP expression was higher when the longer promoter was used for gene expression for both the RPL10 and LENG8 promoters. The longest RPL10 promoter, RPL1000, could drive higher level of eGFP expression than RPL500 or the others. The longest LENG8 promoter, LENG1000, also showed the highest promoter activity among various LENG promoters. Furthermore, the promoter activity was better when the element between the transcription start site (TSS) and translation start site was included.

EXAMPLE 4

In the present example, the internal promoters were cloned into retroviral vectors, and their effects on viral titer and level of eGFP gene expression were compared.

1. Construction of eGFP expressing retroviral vectors.

1) Construction of retroviral vector

1-1) I-D

A retroviral plasmid with U3 deletion was constructed. First, a normal 3' LTR of MLV was amplified using pMT (Hong *et al., J. Gene Med.* 6:724 (2004); US 6,451,595) as a template. The nucleotide sequences of the primer pairs used for PCR are as follows:

SCV3LB: GGATCCCTCGAGCGATAAAATAAAAGATTTTATTTAGTCTCC

BamHI XhoI (SEQ ID NO:69)

SCV3LRI: GAATTCGTCGACTGAAAGACCCCCGCTGACGG (SEQ ID NO:70)

EcoRI SalI

The amplified fragment was initially cloned into pGEM T easy, resulting in pGEM T easy-3'LTR.

A deleted form of 3' LTR was amplified using pMT as a template. The nucleotide sequences of the primer used for PCR are as follows:

3'LTR-1: GCTAGCCCCTGTGCCTTATTTGAA (SEQ ID NO:71)
NheI

SCV3LRI: GAATTCGTCGACTGAAAGACCCCCGCTGACGG (SEQ ID NO:70)
EcoRI SalI

The amplified fragment was initially cloned into pGEM T easy, resulting in pGEM T easy-3'dLTR-1. The NheI-SalI fragment was cloned into the NheI-SalI site of pGEM T easy-3'LTR, resulting in pPreSIN2. The BamHI-SalI fragment from pPreSIN2 was cloned into the BamHI-SalI site of pMT, to make I-D.

1-2) I-ND

To facilitate the cloning, new multiple cloning sites (MCS) were introduced into the retroviral plasmid I-D. A 52 bp-length fragment of new MCS was made by polymerase reaction without template. The following primer pairs are used:

NEWMCSF: ACGCGTTTAAACCGCGGAATTCGGATCCACATCGTG
MluI SacII BamHI (SEQ ID NO:72)

NEWMCSR: CTCGAGATCTAGGCCTCACGATGTGGATCCGAATTC
XhoI StuI DraIII EcoRI (SEQ ID NO:73)

The amplified fragment containing restriction sites for MluI, PmeI, SacII, EcoRI, BamHI, DraIII, StuI, BglII, and XhoI was initially cloned into pGEM T easy. After the confirmation of the nucleotide sequence, the MluI-XhoI fragment was cloned into the MluI-XhoI site of I-D, resulting in I-ND.

2) Construction of retroviral vectors containing eGFP gene

2-1) eGFP gene

To construct retroviral vectors expressing the eGFP gene, the eGFP gene was amplified from pIRES2-EGFP (CLONTECH Laboratory, Palo Alto, CA, USA, Cat. #6029-1) by using the following primer pairs:

eGFP5: ACGCGTGGATCCATGGTGAGCAAGGGCGAG 3'

MluI BamHI (SEQ ID NO:74)

eGFP3: CTCGAGAGATCTTTACTTGTACAGCTCGTC 3' (SEQ ID NO:75)

XhoI BglII

The amplified eGFP sequence was cloned into pGEM T easy to generate pGEM T easy-eGFP. The BamHI-BglII fragment was cloned into the BamHI site of retroviral vector pI-D, resulting in pI-D-GFP, and inserted into the BamHI site of retroviral vectors I-ND, producing I-ND-GFP.

2-2) Construction of retroviral vectors containing eGFP gene

pMT-GFP (Kim *et al., Biochem. Biophys. Res. Commun. 343*:1017 (2006)) was constructed by inserting the BamHI-BglII fragment of pGEM T easy-eGFP into the BamHI site of retroviral vector pMT which contains wild-type LTR.

For the retroviral vectors with defective LTR, various internal promoters of EXAMPLE 1 were cloned into a retroviral vector containing the GFP sequence, respectively.

The MluI-BamHI fragment, HCMV IE promoter, from pCN plasmid was cloned into the MluI-BamHI site of pI-D-GFP, resulting in pC-D-GFP. The MluI fragment from pGEM T Easy-MTU3 was inserted into the MluI site of pI-D-GFP, producing pM-D-GFP. The MluI fragment from pGEM T Easy-Enh+UbC was inserted into the MluI site of pI-D-GFP, generating pCU-D-GFP.

The MluI-BamHI fragment, HCMV IE promoter, from pCN plasmid was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in C-ND-GFP. The Klenow fragment treated SalI-SwaI fragment from pAxCAwt (Takara Bio, Otsu, Japan) was cloned into the PmeI site of pI-ND-GFP, resulting in pCA-ND-GFP. The MluI-BamHI fragment from pGEM T easy-EF was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in pE-ND-GFP. The MluI-BamHI fragment from pGEM T easy-BA was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in pB-ND-GFP. The MluI-PmeI fragment from pGEM T easy-GAPDH was cloned into the MluI-PmeI site of pI-ND-GFP, resulting in pG-ND-GFP. The MluI-BamHI fragment from pGEM T easy-RPL was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in pR-ND-

GFP. The MluI-EcoRI fragment from pGEM T easy-LENG8 was cloned into the MluI-EcoRI site of pI-ND-GFP, resulting in pLe-ND-GFP. The EcoRI-BamHI fragment from pGEM T easy-SNX was cloned into the EcoRI-BamHI site of pI-ND-GFP, resulting in pS-ND-GFP. The MluI-BamHI fragment from pGEM T easy-CNOT3was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in pCo-ND-GFP. The MluI-BamHI fragment from pGEM T easy-CPNE1 was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in pCP-ND-GFP. The MluI-BamHI fragment from pGEM T easy-HYPO was cloned into the MluI-BamHI site of pI-ND-GFP, resulting in pHY-ND-GFP. The MluI-EcoRI fragment from pGEM T easy-DKC1 was cloned into the MluI-EcoRI site of pI-ND-GFP, resulting in pD-ND-GFP. The MluI-EcoRI fragment from pGEM T easy-VPS72 was cloned into the MluI-EcoRI site of pI-ND-GFP, resulting in pV-ND-GFP. The MluI-PmeI fragment from pGEM T easy-ITGB4BP was cloned into the MluI-PmeI site of pI-ND-GFP, resulting in pIT-ND-GFP. The MluI-PmeI fragment from pGEM T easy-UQCRQ was cloned into the MluI-PmeI site of pI-ND-GFP, resulting in pU-ND-GFP.

2. Analysis of eGFP expression

293T cells were transfected with the respective retroviral vector containing eGFP gene, together with amphotropic packaging constructs, pVM-GP and pVM-AE (Yu *et al., Gene Ther. 10*:706 (2003)), and cultured for 48 hours. Cell-free virus was prepared by filtering the culture supernatant through a 0.45 μm filter paper and used to transduce 2×10^5 HT1080 and K562 cells, respectively. The cells were incubated for 48 hours and harvested for assays. The percentage of GFP positive cells and level of GFP expression (mean fluorescence intensity) were measured by FACS analysis (see Tables 3 and 4).

The FACS analysis was performed as follows: 48 hours after transduction, HT1080 and K562 cells were harvested, and washed once with phosphate-buffered saline (PBS) containing 0.1% sodium azide (FACS buffer). Then the cells were re-suspended in PBS, and analyzed by FACSort (Becton Dickinson, Los Angeles, CA, USA) with the aid of CellQuest (Becton Dickinson) data acquisition and analysis software.

First, the GFP expression from the retroviral vectors with defective LTR was compared with that from retroviral vector MT-GFP containing wild-type LTR. As given in Table 3, C-D-GFP vector performed as well as MT-GFP. The percentage of GFP positive cells from C-D-GFP vector were higher in HT1080 cells, and more than 80% in K562 cells compared with that from MT-GFP vector. The level of GFP expression driven from C-D-GFP vector were higher in HT1080 cells, and around 70% in K562 cells compared with that driven from MT-GFP vector. From these experiments, we confirmed that the HCMV promoter works well for GFP expression. However, we could not find other promoters which would work as well as the HCMV promoter. The CMV/Ubiquitin hybrid promoter also produced high viral titer and high level of GFP expression, although less than HCMV promoter.

Table 3. Comparison of GFP expression

	HT1080		K562	
	Relative % pf GFP^+ cells	Relative mean fluorescence intensity	Relative % pf GFP^+ cells	Relative mean fluorescence intensity
MT-GFP	100	100	100	100
C-D-GFP	110.2	243.3	81.4	67.2
M-D-GFP	29.6	54.4	21.7	19.9
CU-D-GFP	68.2	59.4	46.6	56.1

As a next step, we tested more retroviral vectors containing various internal promoters other than HCMV promoter. The results are given in Table 2.

Table 4. Comparison of GFP expression

	HT1080		K562	
	Relative % pf GFP^+ cells	Relative mean fluorescence intensity	Relative % pf GFP^+ cells	Relative mean fluorescence intensity
C-ND-GFP	100	100	100	100
CA-ND-GFP	7.9	37.1	3.9	94.5
E-ND-GFP	28.4	21.0	19.2	157.1
B-ND-GFP	15.3	1.3	1.5	10.4
G-ND-GFP	84.1	9.8	68.9	51.6
R-ND-GFP	109.7	9.1	107.4	42.6
L-ND-GFP	112.2	6.1	124.4	33.3
S-ND-GFP	83.8	2.1	57.1	12.5
CN-ND-GFP	52.5	1.9	31.4	11.7
CP-ND-GFP	48.2	1.4	40.1	12.2
HY-ND-GFP	19.7	6.5	14.9	44.3
D-ND-GFP	42.2	1.7	44.7	17.0
V-ND-GFP	17.5	1.2	18.0	12.4
IT-ND-GFP	96.0	3.0	79.5	18.1

U-ND-GFP	89.7	2.6	82.9	17.7

As shown in Table 4, the HCMV promoter produced the highest level of gene expression (see the mean fluorescence intensity) and high number of GFP positive cells both in HT1080 and K562 cells. However, the LENG8 and RPL10 promoter gave the highest viral titer (percentage of transduced cells) both in HT1080 and K562 cells. The level of GFP expression driven from these two promoters was relatively low in HT1080 cells, but more than 30% compared with the HCMV promoter in K562 cells. Therefore, the LENG8 and RPL10 promoter can be used for gene expression in a retroviral vector system in some cell types. In addition, the GAPDH, UQCRQ, ITGB4BP, and SNX3 promoters produced relatively high viral titer (more than 80% of CMV promoter).

The CA-ND-GFP and E-ND-GFP vectors produced the highest level of GFP expression in K562 cells, however, the viral titer from these vectors was very low, making the use of these vectors difficult.

EXAMPLE 5

In the present example, the effects of internal promoters on viral titer and level of gp91 gene expression were compared.

1. Construction of gp91 expressing retroviral vectors

1) gp91-phox gene (NCBI accession No: NM_000397)

To construct retroviral vectors expressing human gp91-phox, gp91 cDNA was cloned from the total RNA of human peripheral blood lymphocytes by RT-PCR. The nucleotide sequences of primers used in this step are as follows:

GP91F: GGATCCATGGGGAACTGGGCTGTGAAT (SEQ ID NO:76)
BamHI

GP91R: GGATCCCTCGAGTTAGAAGTTTTCCTTGTTGAAAA
BamHI XhoI (SEQ ID NO:77)

The amplified fragment was initially cloned into pGEM T easy to generate pGEM T easy-gp91 and its nucleotide sequence was confirmed.

2) pPromoter-ND

The MluI-BamHI fragment, HCMV IE promoter, from pCN plasmid was cloned into the MluI-BamHI site of pI-ND, resulting in pC-ND. The MluI-PmeI fragment from pGEM T easy-GAPDH was cloned into the MluI-PmeI site of pI-ND, resulting in pG-ND. The MluI-BamHI fragment from pGEM T easy-RPL was cloned into the MluI-BamHI site of pI-ND, resulting in pR-ND. The MluI-EcoRI fragment from pGEM T easy-LENG8 was cloned into the MluI-EcoRI site of pI-ND, resulting in pL-ND. The EcoRI-BamHI fragment from pGEM T easy-SNX was cloned into the EcoRI-BamHI site of pI-ND, resulting in pS-ND. The MluI-PmeI fragment from pGEM T easy-ITGB4BP was cloned into the MluI-PmeI site of pI-ND, resulting in pIT-ND.

3) Construction of gp91 expressing retroviral vectors

The MT-gp91 vector was constructed by inserting the BamHI fragment of pGEM T easy-gp91 into pMT.

The retroviral vectors where gp91-phox expression is driven by an internal promoter were constructed by inserting the BamHI-XhoI fragment from pGEM T easy-gp91 into the BamHI-XhoI site of pC-ND, pG-ND, pR-ND, pL-ND, pS-ND and pIT-ND, resulting in pC-ND-gp91, pG-ND-gp91, pR-ND-gp91, pL-ND-gp91, pS-ND-gp91 and pIT-ND-gp91, respectively.

The construction process of retroviral vectors pR-LND-gp91-phox-n, pR1000-LND-gp91-n and pR1000-LND-gp91-pA-n-rev was described in EXAMPLE 7 (3).

2. Analysis of gp91 expression

293T cells were transfected with the respective retroviral vector containing the gp91 gene, together with packaging constructs, pVM-GP and pVM-GeR (Kim *et al., Biochem. Biophys. Res. Commun. 343*:1017 (2006)), by the calcium phosphate precipitation method, and cultured for 48 hours. Cell-

free virus was prepared by filtering the culture supernatant through a 0.45 μm filter paper, and used for transduction of K562 cells.

For the transduction of K562 cells, 2.5×10^5 cells were seeded per well in a 6 well plate the day before the transduction. The same volume of viral supernatants was added per well in the presence of 8 μg/ml polybrene, and the plate was centrifuged (Eppendorf centrifuge 5810R) at 2800 rpm for 90 min at 32°C. After transduction, cells were incubated in a 37°C CO_2 incubator for 2 days.

The gp91 protein expression was analyzed by flow cytometry. K562 or PLB-985/gp91$^{-/-}$ cells were harvested 2 days after transduction, and washed with PBS. Then the cells were re-suspended in 100 μl PBS, and stained with 1 μl anti-gp91 antibody (7D5; MBL, Japan) for 30 minutes at 4°C. Cells were then washed twice with PBS, re-suspended in 100 μl PBS, and stained with 1 μl of FITC-conjugated anti-mouse antibody raised from goat (Southern Biotechnology Associates, Inc, Birmingham, AL, USA) for 30 min at 4°C. Then cells were washed three times with PBS, and suspended in 500 μl of PBS. Flow cytometry was performed by FACSort (BD, San Jose, CA) with the aid of the CellQuest (BD) data acquisition and analysis software.

First, the gp91 expression from the retroviral vectors MT-gp91, C-ND-gp91, G-ND-gp91, R-ND-gp91, L-ND-gp91, S-ND-gp91 and IT-ND-gp91 was compared. The results are given in Table 5. From the various U3-deleted retroviral vectors, R-ND-gp91 and S-ND-gp91 produced higher viral titer (more than 70% of MT-gp91 in K562 cells) than others. The level of gp91 expression driven from R-ND-gp91 vector was higher than that driven from S-ND-gp91 vector in K562 cells. The C-ND-gp91 vector did not produce gp91 positive cells, although C-ND-eGFP could produce a high level of eGFP expression (Table 4).

Table 5. Comparison of gp91 expression

	K562	
	Relative % of gp91$^+$ cells	Relative mean fluorescence intensity

MT-gp91	100	100
C-ND-gp91	2.4	1.9
G-ND-gp91	14.0	3.3
R-ND-gp91	75.5	18.9
L-ND-gp91	35.4	5.8
S-ND-gp91	79.4	9.9
IT-ND-gp91	47.4	5.5

It was observed in EXAMPLE 3 that the longer form of RPL10 promoter could drive higher level of GFP gene expression than the short one. We tested whether it would apply to gp91 expression. We constructed the gp91 expression retroviral vector containing the longest RPL10 promoter, RPL1000 (pR1000-LND-gp91-n), and confirmed the effect on gp91 gene expression. We also constructed the retroviral vector pR1000-LND-gp91-n-rev where the RPL1000 promoter driven gp91 gene expression cassette is inserted in reverse direction, and compared the gp91 gene expression. The results are given in Table 6.

Table 6. Comparison of gp91 expression

	K562	
	Relative % of $gp91^+$ cells	Relative mean fluorescence intensity
R-LND-gp91-n	100	100
R1000-LND-gp91-n	95.5	145.6
R1000-LND-gp91-n-rev	58.6	193.9

The R1000-LND-gp91-n vector could produce comparable amount of viral titer (% of $gp91^+$ cells), and higher level of gp91 gene expression (relative mean fluorescence intensity) compared with R-LND-gp91-n vector. The R1000-LND-gp91-n-rev vector could drive the highest level of gp91 gene expression although the viral titer produced from it was half of R-LND-gp91-n.

EXAMPLE 6

The retroviral vectors of this invention can be used for ex vivo gene delivery.

The $CD34^+$ hematopoietic stem cells are collected from a subject. The source of $CD34^+$ cells can be bone marrow aspirates or mobilized peripheral blood. Collected $CD34^+$ cells are cultured in a Vuelife culture bag in serum-free SCGM media (Cell Gro, Germany) containing 300 ng/ml of human stem cell factor (SCF), 300 ng/ml of human FLT-3L, 100 ng/ml of human thrombopoietin (TPO), and 20 ng/ml of human IL-3 for 2 days at 37℃ in 5% CO_2 (pre-stimulation). The transduction is performed using a Vuelife culture bag precoated with the CH296 fragment of human fibronectin (Retronectin, TaKaRa Bio). Pre-stimulated cells are transferred to the retronectin-coated Vuelife culture bag, and the retroviral supernatant is added on 3 occasions for 2 days. Cells are then harvested, washed 3 times with saline, re-suspended in the infusion solution (saline containing 1% human serum albumin), and infused into the subject.

Example 7

In the present example, the effects of internal promoters on the viral titer and level of gene expression in packaging cell line PG13 were examined.

1. Construction of eGFP expressing retroviral vectors

1) Construction of retroviral vector

1-1) pI-LND

U3-deleted retroviral vectors can not be mobilized following the first round of retroviral transduction because both of the 5' and 3' LTRs become defective after transduction. Accordingly, a stable producer cell line was established by transfecting the retroviral packaging cells with pDNA, thus allowing stable integration of the vectors in the genome of the retroviral packaging cells. Linearization of vector DNA is important in having all transfectants contain the proper DNA arrangement in their chromosome.

To construct retroviral vectors convenient for linearization, two restriction enzyme sites for linearization were introduced into the retroviral plasmid pI-ND. One of the restriction enzyme sites was introduced by inserting a fragment, L1, in front of the 5' LTR at a distance of about 200 bp, and the other site, L2, at the back of the 3'LTR. L1 fragment was produced without a template by performing a polymerase reaction. L2 fragment was amplified using pUC18 (Promega, WI, USA) as a template. The nucleotide sequences of the primer are as follows:

L1F: 5' GCTCTTCCGCTCACGTGTGATCAATTTAAATTTCGAA

SapI PmlI BclI SwaI BstBI (SEQ ID NO:78)

L1R: 5' AGCGGAAGAGCTTCGAAATTTAAATTGATCACACGTG

SapI BstBI SwaI BclI PmlI (SEQ ID NO:79)

L2F: 5' AGGCCTGGTCACCGGCCATTATGGCCACGTGATCATTTAAATTTG

StuI BstEII SfiI BclI

AAGCATTTATCAGGGTTA (SEQ ID NO:80)

L2R: 5' TATTCGCGCGTTTCGGTGATGAATATT (SEQ ID NO:81)

SspI

The amplified L1 fragment containing the restriction sites for SapI, PmlI, BclI, SwaI, BstBI and SapI was initially cloned into pGEM T easy (Promega, WI, USA), resulting in pGem T easy-L1. After the confirmation of the nucleotide sequence, the SapI fragment released from pGem T easy-L1 was inserted into the SapI site of pI-ND, resulting in pI-L1ND. The amplified L2 fragment containing restriction sites for StuI, BstEII, SfiI, PmlI, BclI, SwaI and SspI was initially cloned into pGEM T easy (Promega, WI, USA), resulting in pGem T easy-L2. After the confirmation of the nucleotide sequence, the StuI-SspI fragment released from pGem T easy-L2 was cloned into the SspI site of pI-L1ND, resulting in pI-LND.

1-2) pI-LND-n

To construct a producer cell line with high viral titer, it is important to select transfectants containing the retroviral vector DNA. The drug-resistance genes are frequently used for this purpose. However, it is not favorable to have the drug-resistant gene inside the vector genome because, if included, this gene is going to be expressed *in vivo*. Therefore, we prepared the vector

construct harboring the drug-resistant gene cassette outside of the retroviral genome.

First, we used the neomycin-resistance gene for the selection of transfectants. For the expression of the neomycin-resistance gene, the human β-actin promoter and polyadenylation sequence were linked to the bacterial Neo coding sequence.

The human β-actin promoter was amplified using genomic DNA from K562. The nucleotide sequences of the primer used for PCR are as follows:

BApF: 5' GTCGACATTAATGCCGGTGAGTGAGCGGCGCGGGGCCAA
SalI PshBI (SEQ ID NO:82)

BApR: 5' GGATCCGGTGGCGCGTCGCGCCGCTGGGTTTT (SEQ ID NO:83)
BamHI

The amplified fragment was cloned into pGEM T easy, resulting in pGEM T easy-pBA.

The bacterial Neo coding gene was amplified using pcDNA 3.1 (Invitrogen, CA, USA). The nucleotide sequences of the primer used for PCR are as follows:

NeoF: 5' AGATCTATGGGATCGGCCATTGAACAA (SEQ ID NO:84)
BglII

pAR: 5' CATATGTCATAATCAGCCATACCACATTT (SEQ ID NO:85)
NdeI

The amplified fragment was cloned into pGEM T easy, resulting in pGEM T easy-Neo.

The polyadenylation signal sequence was amplified using pTet-On (Clontech, TAKARA bio, Japan). The nucleotide sequences of the primer used for PCR are as follows:

SVpAF: CTCGAGATGGGATCGGCCATTGAACAA (SEQ ID NO:86)
XhoI

SVpAR: CATATGAGTAATCAGCCATACCACATTT (SEQ ID NO:87)
NdeI

The amplified fragment was cloned into pGEM T easy, resulting in pGEM T easy-pA.

After the confirmation of the nucleotide sequence, the XhoI-NdeI fragment released from pGEM T easy-pA was inserted into the XhoI-NdeI site of pGEM T easy-Neo, to make pGEM T easy-NeopA. The BglII-NdeI fragment was cloned into the BamHI-NdeI site of pGEM T easy-pBA, resulting in pGEM T easy-pBA-Neo-pA. The MluI-EcoRI-Klenow-treated fragment was clone into the SspI site of pI-LND, resulting in pI-LND-n.

2) Construction of retroviral vectors containing eGFP gene

2-1) pI-LND-GFP-n

The BamHI-BglII fragment released from pGem T easy-eGFP (described in EXAMPLE 2) was cloned into the BamHI site of pI-LND-n, resulting in pI-LND-GFP-n.

2-2) Construction of retroviral vectors containing eGFP gene

Various internal promoters of EXAMPLE 1 were cloned into the retroviral vectors containing GFP sequence, respectively.

The MluI-BamHI fragment, HCMV IE promoter, from pCN plasmid was cloned into the MluI-BamHI site of pI-LND-GFP-n, resulting in pC-LND-GFP-n. The MluI-PmeI fragment from pGEM T easy-GAPDH was cloned into the MluI-PmeI site of pI-LND-GFP-n, resulting in pG-LND-GFP-n. The MluI-BamHI fragment from pGEM T easy-RPL was cloned into the MluI-BamHI site of pI-LND-GFP-n, resulting in pR-LND-GFP-n. The MluI-EcoRI fragment from pGEM T easy-LENG8 was cloned into the MluI-EcoRI site of pI-LND-GFP-n, resulting in pL-LND-GFP-n. The EcoRI-BamHI fragment from pGEM T easy-SNX was cloned into the EcoRI-BamHI site of pI-LND-GFP-n, resulting in pS-LND-GFP-n. The MluI-PmeI fragment from pGEM T easy-ITGB4BP was cloned into the MluI-PmeI site of pI-LND-GFP-n, resulting in pIT-LND-GFP-n. The MluI-PmeI fragment from pGEM T easy-UQCRQ was cloned into the MluI-PmeI site of pI-LND-GFP-n, resulting in pU-LND-GFP-n.

3) Construction of retroviral vectors containing gp91-phox gene

3-1) pPromoter-LND

Various internal promoters of EXAMPLE 1 were cloned into pI-LND.

The MluI-BamHI fragment, HCMV IE promoter, from pCN plasmid was cloned into the MluI-BamHI site of pI-LND, resulting in pC-LND. The MluI-PmeI fragment from pGEM T easy-GAPDH was cloned into the MluI-PmeI site of pI-LND, resulting in pG-LND. The MluI-BamHI fragment from pGEM T easy-RPL was cloned into the MluI-BamHI site of pI-LND, resulting in pR-LND. The MluI-EcoRI fragment from pGEM T easy-LENG8 was cloned into the MluI-EcoRI site of pI-LND, resulting in pL-LND. The EcoRI-BamHI fragment from pGEM T easy-SNX was cloned into the EcoRI-BamHI site of pI-LND, resulting in pS-LND. The MluI-PmeI fragment from pGEM T easy-ITGB4BP was cloned into the MluI-PmeI site of pI-LND, resulting in pIT-LND. The MluI-PmeI fragment from pGEM T easy-UQCRQ was cloned into the MluI-PmeI site of pI-LND, resulting in pU-LND. The MluI-BamHI fragment from pGEM T easy-pR1000 was cloned into the MluI-BamHI site of pI-LND, resulting in pR1000-LND.

3-2) pPromoter-LND-n

The MluI-EcoRI-Klenow-treated fragment from pGEM T easy-pBA-Neo-pA was cloned into the SspI site of pC-LND, pG-LND, pR-LND, pL-LND, pS-LND, pIT-LND, pU-LND, and pR1000-LND, generating pC-LND-n, pG-LND-n, pR-LND-n, pL-LND-n, pS-LND-n, pIT-LND-n, pU-LND-n, and pR1000-LND-n.

3-3) Construction of retroviral vectors containing gp91-phox gene

The BamHI fragment from pGEM T easy-gp91 was cloned into the BamHI site of pC-LND-n, pG-LND-n, pR-LND-n, pL-LND-n, pS-LND-n, pIT-LND-n, pU-LND-n, and pR1000-LND-n, generating pC-LND-gp91-phox-n, pG-LND-gp91-phox-n, pR-LND-gp91-phox-n, pL-LND-gp91-phox-n, pS-LND-gp91-phox-n, pIT-LND-gp91-phox-n, pU-LND-gp91-phox-n, and pR1000-LND-gp91-phox-n.

The retroviral vectors where the gp91 gene expression cassette is inserted in reverse direction were also constructed. pC-LND-gp91-pA-n-rev was constructed by i) inserting the MluI-BamHI fragment from pC-LND-gp91-n into the BamHI-StuI site of pI-LND-n, generating pC-LND-n-rev, ii) inserting the BamHI fragment of pGemT easy-gp91 cloned to the BamHI site of pC-LND- n-rev, producing pC-LND-gp91-n-rev, then by iii) inserting the

EcoRI fragment of pGEM T easy-RGpA into the Pme I site of pC-LND-gp91-n-rev, resulting in pC-LND-gp91-pA-n-rev. pR1000-LND-gp91-pA-n-rev was constructed by i) inserting the MluI-BamHI fragment from pR1000-LND-gp91-n into the BamHI-StuI site of pI-LND-n, generating p R1000-LND-n-rev, ii) inserting the BamHI fragment of pGemT easy-gp91 cloned to the BamHI site of pR1000-LND- n-rev, producing pR1000-LND-gp91-n-rev, then by iii) inserting the EcoRI fragment of pGEM T easy-RGpA into the Pme I site of pR1000-LND-gp91-n-rev, resulting in pR1000-LND-gp91-pA-n-rev.

As a control, pMT-gp91-n was constructed by inserting the MluI-EcoRI fragment into the SapI site of pMT-gp91.

2. Construction of producer cell lines

1) Linearization of retroviral vectors

To linearize retroviral vectors containing eGFP or gp91, 10 μg retroviral plasmid was treated with restriction enzyme (SwaI) for 16 hours. DNA fragment containing retroviral vector was eluted from agarose gel, precipitated and resuspended in 30 μl distilled water. After measuring the DNA concentration, it was used for electroporation.

2) Electroporation

PG13 cell line was used for electroporations. 7.5 x 10^5 cells were added in a 0.5 ml volume of serum-free DMEM media to the 0.4-cm cuvette (Bio-Rad Laboratories, Hercules, CA) and incubated with linearized retroviral plasmid of 10 μl (1 μg/μl) for 5 min preceding electroporation. Electroporations were conducted using Gene Pulser Xcell™ (Bio-Rad) with voltage of 200 V during 20 msec. Following electroporation, the suspensions were immediately plated in DMEM media with 10% premium FBS.

3) Selection

Cells were selected for neomycin resistance using G-418 (final concentration of 1 μg/ml) following electroporation. After 10 days of selection, cells with integrated plasmids were obtained.

3. Analysis of eGFP expression

5 x 10^6 cells of PG13 producer cell line are plated on a 100 mm dish in DMEM media with 10% premium FBS. After 48 h, the supernatants are harvested and filtered through a 0.45 μm filter. These supernatants are used to measure viral titer using real time PCR and to transduce HT1080 and K562 cells, respectively. The cells are incubated for 48 h, and harvested for assays. The percentage of GFP positive cells and the level of GFP expression (mean fluorescence intensity) are measured by FACS analysis.

4. Analysis of gp91 expression

5 x 10^6 cells of PG13 producer cell line were plated on a 100 mm dish in DMEM media with 10% premium FBS. After 48 h, supernatants containing viruses were harvested and filtered through a 0.45 μm filter. These supernatants were used to transduce K562 cells.

For the transduction of K562 cells, 2.5 × 10^5 cells were seeded per well in 6 well plates the day before the transduction. The same amount of viral supernatants was added per well in the presence of 8 μg/ml polybrene, and the plate was centrifuged (Eppendorf centrifuge 5810R) at 2800 rpm for 90 min at 32°C. After transduction, cells were incubated in a 37°C CO_2 incubator for 2 days.

For the quantitative analysis of gp91 protein expression, FACS was performed. K562 cells were harvested, and washed with PBS. Then the cells were re-suspended in 100 μl PBS, and stained with 1 μl anti-gp91 antibody (7D5) for 30 minutes at 4°C. Cells were then washed twice with PBS, re-suspended in 100 μl PBS, and stained with 1 μl of PE-conjugated anti-mouse antibody raised from goat (Southern Biotechnology Associates, Inc, Birmingham, AL, USA) for 30 min at 4°C. Then cells were washed three times with PBS, and suspended in 500 μl of PBS. Flow cytometry was performed by FACSort with the aid of the CellQuest data acquisition and analysis software. The results are given in Fig. 4. The percentage of gp91 positive cells and the mean fluorescence intensity (the value in parenthesis) are indicated in the figure. As presented in Fig. 4, the R1000 promoter could drive a high level of gp91 gene expression. Furthermore, the R1000 promoter

could produce a higher viral titer and a higher level of gene expression when the expression cassette was inserted in the reverse direction. In fact, the viral titer produced from the vector containing the R1000 promoter in reverse direction was higher than that from MT-gp91-n which has wild-type MLV LTR (see the percentage of gp91 positive cells).

The level of NADPH oxidase activity is measured by Dihydrorhodamine-123 (DHR) assay after inducing cell differentiation by treating cells with 0.5% DMF (Dimethylforamide, C_3H_7NO). The cells are incubated for 6 days in the presence of DMF, and harvested for assays. The harvested cells are washed 2 times with phosphate-buffered saline (PBS). The cells are suspended with PBS and mixed with 1.8 μl DHR (Molecular Probes, USA , 29 mM). After incubating for 5 minutes at 37℃, the cells are stimulated with 10 μl of phorbol myristate acetate (PMA, 1 μg/ml). Then the percentage of active phagocytic cells and their oxidase activity are measured by FACS analysis.

Having now fully described the invention, it will be understood by those of ordinary skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents, patent applications and publications cited herein are fully incorporated by reference herein in their entirety.

WHAT IS CLAIMED IS:

1. An expression vector comprising a heterologous internal promoter selected from the group consisting of LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter or a fragment or variant thereof.

2. The expression vector of claim 1, wherein said vector comprises a nucleotide sequence comprising a 5' long terminal repeat (LTR) and a 3' LTR.

3. The expression vector of claim 1, wherein said promoter is LENG8 or a fragment or variant thereof.

4. The expression vector of claim 1, wherein said promoter is UQCRQ or a fragment or variant thereof.

5. The expression vector of claim 1, wherein said promoter is SNX3 or a fragment or variant thereof.

6. The expression vector of claim 1, wherein said promoter is ITGB4BP or a fragment or variant thereof.

7. The expression vector of claim 1, wherein said promoter is RPL10 or a fragment or variant thereof.

8. The expression vector of claim 1, wherein said fragment has at least 20% of the transcriptional activity of the full length LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter.

9. The expression vector of claim 8, wherein said fragment has at least 40% of the transcriptional activity of the full length LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter.

10. The expression vector of claim 9, wherein said fragment has at least 60% of the transcriptional activity of the full length LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter.

11. The expression vector of claim 1, wherein said variant is at least 70% identical to the sequence of said LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter.

12. The expression vector of claim 11, wherein said variant is at least 80% identical to the sequence of said LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter.

13. The expression vector of claim 12, wherein said variant is at least 90% identical to the sequence of said LENG8, UQCRQ, SNX3, ITGB4BP or RPL10 promoter.

14. The expression vector of claim 3, wherein said fragment comprises the sequence from about -385 to about -1 relative to the transcription start site (about nucleotides 635 to 1019 of SEQ ID NO:9) of LENG8.

15. The expression vector of claim 3, wherein said fragment comprises the sequence from about -50 to about +305 and about +1908 to about +2121 relative to the transcription start site (about nucleotides 970 to 1538 of SEQ ID NO:9) of LENG8.

16. The expression vector of claim 15, wherein said fragment comprises the sequence from about -100 to about +305 and about +1908 to about +2121 relative to the transcription start site (about nucleotides 920 to 1538 of SEQ ID NO:9) of LENG8.

17. The expression vector of claim 16, wherein said fragment comprises the sequence from about -200 to about +305 and about +1908 to about +2121

relative to the transcription start site (about nucleotides 820 to 1538 of SEQ ID NO:9) of LENG8.

18. The expression vector of claim 17, wherein said fragment comprises the sequence from about -385 to about +305 and about +1908 to about +2121 relative to the transcription start site (about nucleotides 635 to 1538 of SEQ ID NO:9) of LENG8.

19. The expression vector of claim 18, wherein said fragment comprises the sequence from about -1020 to about +305 and about +1908 to about +2121 relative to the transcription start site (about nucleotides 1 to 1538 of SEQ ID NO:9) of LENG8.

20. The expression vector of claim 7, wherein said fragment comprises the sequence from about -350 to about -1 relative to the transcription start site (about nucleotides 651 to 1000 of SEQ ID NO:8) of RPL10.

21. The expression vector of claim 7, wherein said fragment comprises the sequence from about -50 to about +143 relative to the transcription start site (about nucleotides 951 to 1143 of SEQ ID NO:8) of RPL10.

22. The expression vector of claim 21, wherein said fragment comprises the sequence from about -100 to about +143 relative to the transcription start site (about nucleotides 901 to 1143 of SEQ ID NO:8) of RPL10.

23. The expression vector of claim 22, wherein said fragment comprises the sequence from about -200 to about +143 relative to the transcription start site (about nucleotides 801 to 1143 of SEQ ID NO:8) of RPL10.

24. The expression vector of claim 23, wherein said fragment comprises the sequence from about -350 to about +143 relative to the transcription start site (about nucleotides 651 to 1143 of SEQ ID NO:8) of RPL10.

25. The expression vector of claim 24, wherein said fragment comprises the sequence from about -500 to about +143 relative to the transcription start site (about nucleotides 501 to 1143 of SEQ ID NO:8) of RPL10.

26. The expression vector of claim 25, wherein said fragment comprises the sequence from about -1000 to about +143 relative to the transcription start site (about nucleotides 1 to 1143 of SEQ ID NO:8) of RPL10.

27. The expression vector of any one of claims 1-26, wherein said vector is a plasmid vector.

28. The expression vector of any one of claims 1-27, wherein said vector encodes an enhancer-deleted retroviral vector.

29. The expression vector of any one of claims 1-27, wherein said vector is an enhancer-deleted retroviral vector.

30. The expression vector of claim 28 or claim 29, wherein said vector comprises a nucleotide sequence comprising a 5'LTR and a 3'LTR, wherein the enhancer element of the U3 region of said 3'LTR is deleted.

31. The expression vector of claim 30, wherein the enhancer element of the U3 region of the 5'LTR is deleted.

32. The expression vector of any one of claims 1-31, wherein said internal promoter is a fragment or variant of said promoter, wherein said vector comprising said fragment or variant retains substantially the same ability to produce high viral titers or high levels of transcription as the wild type promoter.

33. The expression vector of any one of claims 1-32, wherein said promoter comprises one or more splicing sites.

34. The expression vector of any one of claims 28-31, wherein said vector is an oncoretroviral-based retroviral vector.

35. The expression vector of claim 34, wherein said oncoretroviral-based retroviral vector is a MLV-based retroviral vector.

36. The expression vector of any one of claims 28-31, wherein said vector is a lentiviral-based retroviral vector.

37. The expression vector of any one of claims 1-36, wherein said vector comprises a polynucleotide of interest operably linked to said heterologous internal promoter, wherein said vector containing said internal promoter is capable of producing high viral titers and high levels of transcription of the polynucleotide of interest.

38. The expression vector of claim 37, wherein said polynucleotide of interest encodes a polypeptide.

39. The expression vector of claim 38, wherein said polypeptide is a therapeutic protein or a reporter protein.

40. The expression vector of claim 39, wherein said polypeptide is eGFP.

41. The expression vector of claim 39, wherein said polypeptide is gp91.

42. The expression vector of claim 37, wherein said polynucleotide of interest encodes a RNA, anti-sense RNA, small interfering RNA or ribozyme.

43. The expression vector of claim 37, wherein said polynucleotide of interest further comprises a polyadenylation sequence, an IRES, an insulator sequence, splicing sequences or some combination thereof.

44. A composition comprising the expression vector of any one of claims 1-43 and a suitable carrier.

45. A cell comprising the expression vector of any one of claims 1-43.

46. The cell of claim 45, wherein said cell is a mammalian cell.

47. The cell of claim 46, wherein said cell is a human cell.

48. The cell of claim 46, wherein said cell is a producer cell line.

49. A method of producing infectious retroviral particles comprising cultivating the producer cell line of claim 48 in a suitable medium, collecting the medium, and filtering the medium to obtain a cell-free viral supernatant.

50. A method of transducing mammalian cells comprising incubating the mammalian cells with the cell-free viral supernatant of claim 49.

51. A method of delivering a polypeptide or transcript of interest encoded by the polynucleotide of interest to a subject comprising administering the composition of claim 44.

52. A method of delivering a polypeptide or transcript of interest encoded by the polynucleotide of interest to a subject comprising administering the cell of claim 45.

53. A method of treating a subject comprising administering the composition of claim 44, wherein said expression vector comprises a polynucleotide of interest encoding a therapeutically useful polypeptide or transcript.

54. A method of treating a subject comprising administering the cell of claim 45, wherein said expression vector comprises a polynucleotide of interest encoding a therapeutically useful polypeptide or transcript.

55. The method of any one of claims 51-54, wherein the polynucleotide of interest encodes gp91.

56. A kit comprising the expression vector of any one of claims 1-43.

FIG. 1

U3-inactivated retroviral vector

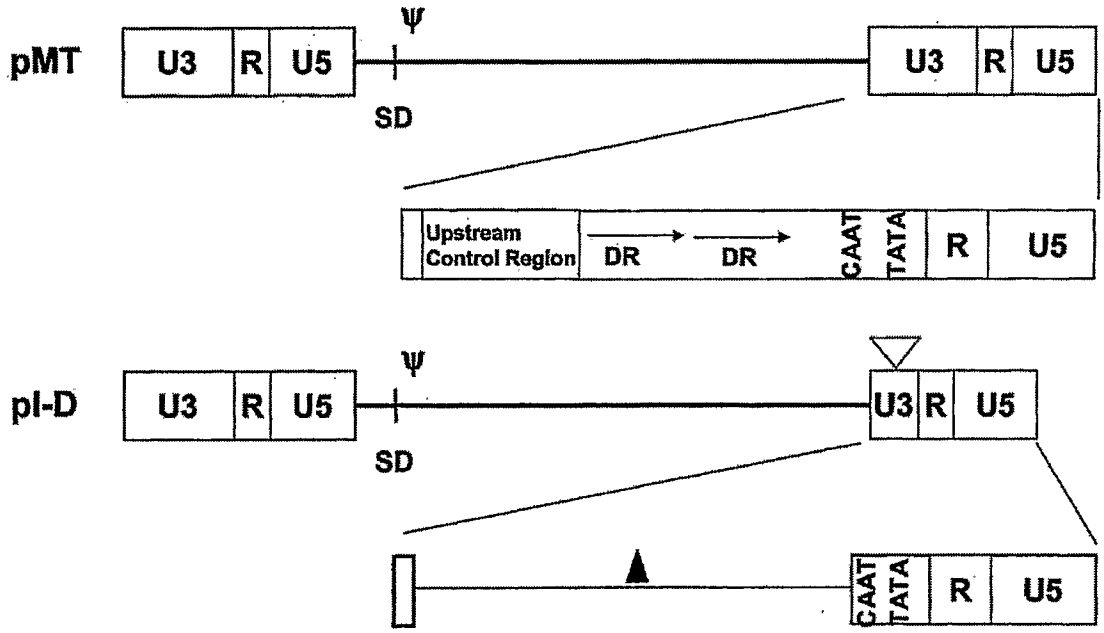

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FIG. 2

Pl-ND

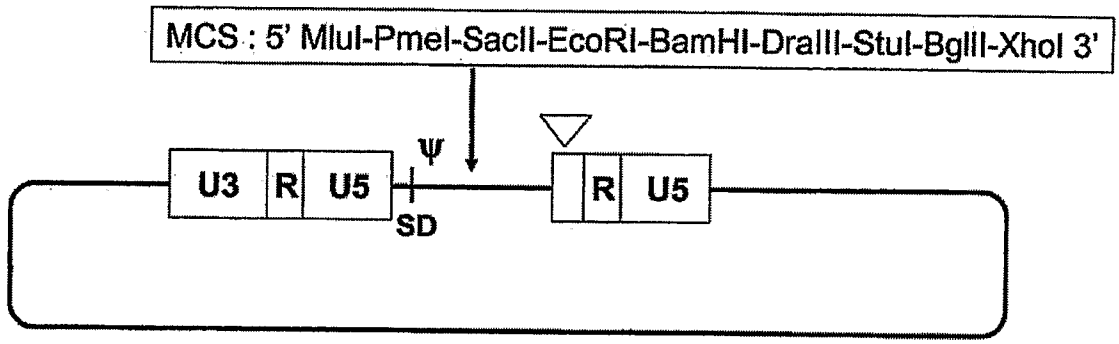

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FIG. 3

pl-LND-n

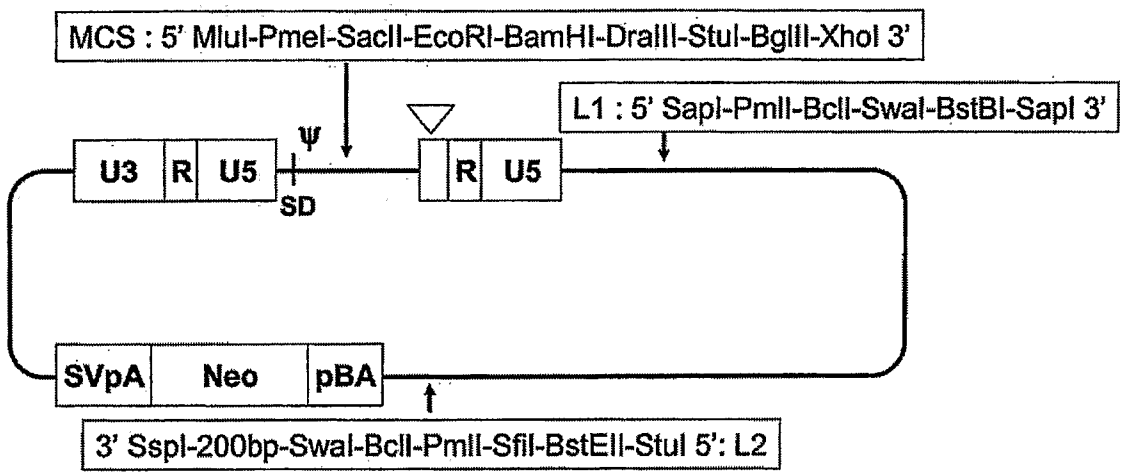

FIG. 4

gp91 expression

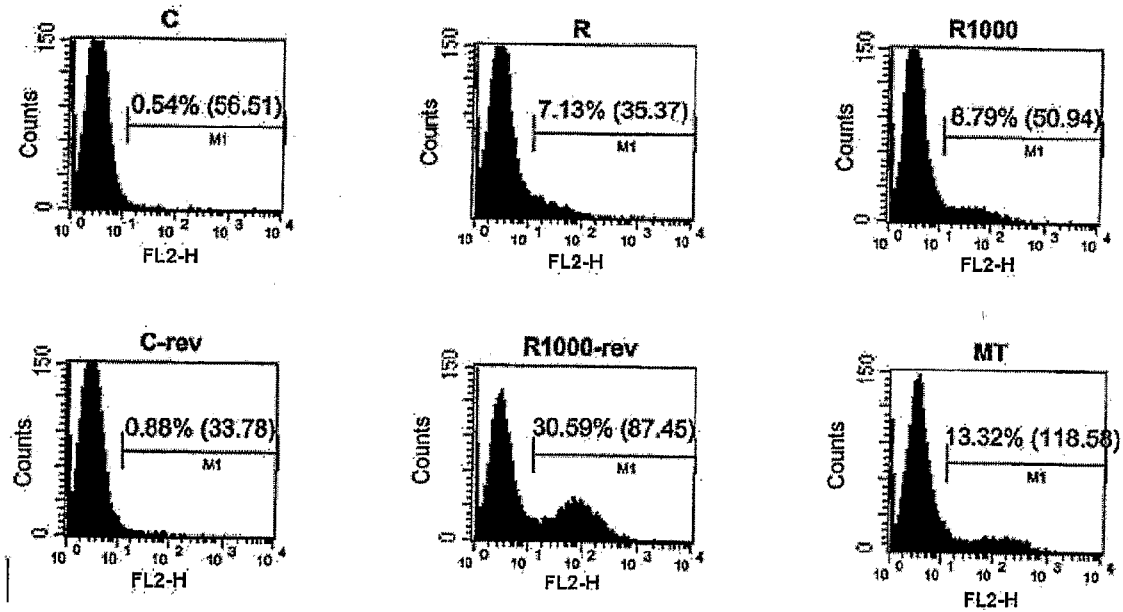

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FIG. 5

GAPDH promoter

TTCATCCAAGCGTGTAAGGGTCCCCGTCCTTGACTCCCTAGTGTCCTGCTGCCCACAGTC
CAGTCCTGGGAACCAGCACCGATCACCTCCCATCGGGCCAATCTCAGTCCCTTCCCCCCT
ACGTCGGGGCCCACACGCTCGGTGCGTGCCCAGTTGAACCAGGCGGCTGCGGAAAAAA
AAAAGCGGGGAGAAAGTAGGGCCCGGCTACTAGCGGTTTTACGGGCGCACGTAGCTCA
GGCCTCAAGACCTTGGGCTGGGACTGGCTGAGCCTGGCGGGAGGCGGGGTCCGAGTCA
CCGCCTGCCGCCGCGCCCCCGGTTTCTATAAATTGAGCCCGCAGCCTCCCGCTTCGCTCT
BRE TATA box +1
CTGCTCCTCCTGTTCGACAGTCAGCCGCATCTTCTTTTGCGTCGCCAGGTGAAGACGGGC
GGAGAGAAACCCGGGAGGCTAGGGACGGCCTGAAGGCGGCAGGGGCGGGCGCAGGC
CGGATGTGTTCGCGCCGCTGCGGGGTGGGCCCGGGCGGCCTCCGCATTGCAGGGGCGG
GCGGAGGACGTGATGCGGCGCGGGCTGGGCATGGAGGCCTGGTGGGGGAGGGGAGGG
GAGGCGTGTGTGTCGGCCGGGGCCACTAGGCGCTCACTGTTCTCTCCCTCCGCGCAGCC
GAGCCACATCGCTCAGACACC

FIG. 6

RPL 10 promoter

GTGCGCTCGAGCAGGATTTCCTCCCGTCCTTCCTGTCAAAGGACGGGAAGACTTTGTTA
CCCCACCGCGCCCCACCTGCAGAATGGTGGACAGATACCTCCAGATGCCACTTCCCCC
AGGACGCCCGCCTGCTCTGCGCACCTCTCCCCGGATGCTGCCCCGTGGGCGGGTGGGGG
CGGCCCTGCTTCCCCACGACCCCCAGACGCACCCGGAGGGACTC
TTGAGCACAGTGGAGTGGGAAGGGCGAGGTGGGGCGGTGCCCAGGCGAGAGCGGCTC
ATGGGAGGCGGCGCCCGAGACGCAGCTGGTCGGGACGGTGCGGGTCAGGGTGGGCGG
AGCGGGGCTAGAGATGCCCCGGGGTTTCCCAGGCCATGAGTCTCCGTGGAGATTTCTCC
TCGACCTCTTCCCCCGCGGCAATGTGCGAACCCTGGGTCTCCAGGAAACGGGGATACGG
GGCATGGCTCCCAGCAAGGCCTGGTCCAGCCTCTCCGGTAGGGGAATGGGTCTCCCCCT
CCGGCCTCCCGGGTTGACAAAGGAACGCGGGCCCAGATCCCCGTATGGCGCTTCACCG
CCGGGGCCTCTAGCCTAGAAGGAGGCACGGAGCGCGTGTCCGAGACCCGTGCAAGCTC
AGGGACACTCTCGCGGTCGCCGGGAGGCCCACCTAGGGTACTTTCCTTTTTTCCACTCTC
AGAAATATACGTCTGTCACAGTTAACGGCAAAGCCTAGGGCAAGAGTTCTACGCCCAAG
ATGGCCAGCCGGAAGCGGGCTTCTCGCGACCATGTGGCGAAGCCCCATTCGTCAGCTGG
CCGCCCGCGGCCCTGGTACCCGGTCACCTCTCTGATCTGCGCATGTGCTGGGCTACGCCC
GGGCGCAAGCGCCAAGAGCGGCTGCGTCTATGGTCATGACGTCTGACAGAGCGTCCAC
CCGTCTTCGACAGGACTCTATGGTTCTTACGCGCGCAGACAGACCGCCTATATAAGCCAT
TATA box
GCGCAGGCCGAGGAGCGCCTCTTTCCCTTCGGTGTGGTGAGTAAGCGCAGTTGTCGTCT
+1
CTTGCGGTGCCGTTGCTGGTTCTCACACCTTTTAGGTCTGTTCTCGTCTTCCGTTCCGACT
CTCTCTTTTTCGTTGCAGCCACTGAAGATCCTGGTGTCGCC

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FIG. 7

LENG8 promoter

TTGTATCAGAGTCCTGGACGGAAACAGATGGCACTCAAAAGGTGGCGCGCAGTTCAGA
GAAATGCCTATGTACGGATTTGGTCCAATGCCTCAGCCTGACCTCAGGGACCTTCGGGG
GTCTGCTCCGCGCCCACCCTTACACATCTGTGACCCCACACACTTCCACCCCAGCGCCA
CTGCCAACAGCTACACCCATCCCCCTCCAACCGCGTCAGCTTCCAGCCTCGGTCCATCT
GAACTCGCCGTGCCCCCTCCCCTGCGCCCTTCCAGATTCATTTGCTAGGGAAGCCCGCT
CTTCCGGGTGGAGCTGTTCCTCATCCCCTTTCTTTATCATTCTCTCCCCAGGGCTTCCACA
TCACCGTGCTGTGGACAATCCCGGAACTCCTGTCACGCCAGTTTACATTTAGGAACAGT
AATGGCTCCCACTGACTCAGTCAAAACAAGGCTGCGGCCGGGCACGGTGGCTCACGCC
CGTAATCCCAGCACTTTGGGAAGCCGAGACGGAGGGATCACGAGGTCAGGAGTTCGAG
AACAGCCTGGCCGACATGGTGAAACCCCGTCTTTACAAAAACACAAAAATTAGCCGGG
CATAGTGGCGCGCGCCTGTAATCCCAGCTACTCCGGAGGCTGAGGCAGAATTGTTTGAA
CCCAGGAGGCGGAGGTTGCAGTGAGCAGAGATCACGCCACTGTACTCTATCGTGGGCG
ACGACAGAGCAAGAGCAAGACTCCGTCTCCGAGAACAACAACAACAGCAACAAGAA
AACAACAATAAAAAAAATAAGGCTGCGTGGGAGGCAGAAAGAGCTAATGCGGCCACG
CTTGTCCCCTCGGGGCCACCGTCCCCACCCAGACTTCCGGTCTGCCTTAAAATGTTCATG
CGTAAGTGCGTGGGCAGGAAGGCGGGCTCAAGCGCAGCTCGTGGCGTTCATTGGCTGTG
CAGGGCCGAGGGAGGCGGTGCAAGGCCGCCGCGTGACGTCAGGACGCCGCGGTCAGG
ACGTCGAAGCCAAAGAAGACCAGAGCCAGCCGGGTGGCACAGCGGTGTCGTGGCCGT

(+1) +1

GTTGCTGATCGCCTGGGTGGTTGTTGGCGTGTCCCTGCAGCGAAGGATCCTGGTTGGTAA
GGGGAGCGGCGGGCGAGCAGGCGGGCGGGGATAGCATCTCCTTTTGGTCTTGCGCCCC
GCGAGCCCCCGAGGCCTTCTCGGCCGTCGCAGCAGCAGACGCCGCGCGCGAGCGTCGAC
AGGGTGTGGCGGCGCAGGGGCAGCCACTGCGCCTGCGCACCGGGCCTGGGGCCGCGCG
TTCGGGCACTAGCGCGCGTGCGCCGTCGTCTTCTACTTTCCACACCCAGAACTCTTCAGA
TCCTTGACCCCAGTGGCTTTTCAGTCAGCCTCCCCTTTTCTGCCCAGCTTCTCTTGAGTCC
ATCTACTTTTCTTCCCCACTTTGTGACGTGTTTTTAGCTCCCCCTTAAGTCTCCCTAACTCA
TTCTTTTTCTCATAGGCAGTGAAAAAGCAGTCTGGCTCCCGAGGTCCACCCCTTATACCC
CAAGGTCCAG

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FIG. 8

SNX3 promoter

AATCCAGACGCGTGTCTGGTGCAACGCTCGGGTTTATGGCAAAATCATCTCAGGCATTT
GCTTAACCTTCTCCAGAAAGGCATTTTCAGGGGTTCACAGTGAGACGGTGCACAGGTTG
GCACAGAGTTAGTAGGGGCAGTTTTGTTTCGATTTGCGGGCAAATCTCTAAGATCTCTCC
GTTTAACTTTCGCCCGCAATTCCCAAAGCCGCTAAAGCCGTTTCCGGCGCTCTACCCCG
CCGCAGGCCGAGGCTGGCGCAGAGAGACAGGAAGCGCCAGCTCTGGGCGTCTGGGTC
CTCGCCTCCTCGGCCGCAGCCCCGCGGCGGCGCGCTCGCGGTGCATTGTGGGCGCTGTA
GTCCGGCCGGAACCTGTTTGCGACCCCGAGTCCCATGACACCGCTTCTCCTCACACCCC
+1
AGTCCGCAGTGCCCCTCCCCAGCCTCGGCCGGGCCTCCCGGGAGCCGGGCGTGGCGTTC
CAGCTAGTGAGCCGTTTCTCCCCTGGGCTCGGAGGCGGAAGCTTGAGGGGCGCGGGGA
GGAGCTTCGCGTGCGGGGTGAACGCCCGCTCTACGTGCTCGTTCTCTTCGCGACCGCTG
CGCGCGAGCCCCGTGTCCCCACGGCGGGCAGCAGCGGCGGCGGCGGCGGCTGAACGC
GGAGGGGGCGGAGGGAGCCCGCGGCGGCGGCAGCAGCTACAGCGAA

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FIG. 9

ITGB4BP promoter

TCTGTCCCTCAAGGCACAGCTGGACTCATCCCTTTCCCCAAACCTGCCCTTCCTCCGGCT
TCATTTCCATCAATACCTCCATCATCAACCCTTCCGCGAGACACTCCTGGCCCCTCCTCTC
CCTCATGCCTCACAACCGACCAGCCGGAGGTCTAGGTCGATGACAGCTCCTAAAAAGCT
CCTGAATGAATAATGAATGAATGAACGCGAGCAGGCTAGGCGTGGGGCCAGGCGGGGT
CGCGCCCAGACCGCTCGCGACCATAGAGTCCGCCGGAGGCCGGAGGTAGAGGGGCTGG
ATGCGTGGCGGGGAGCGCCGGGCTCTCCCGGAAGTCTCCCTGGACGGAAGTGGAAACG
+1
GAAACCTTTTTAGGGAGTCCAAGGTACAGTCGCCGCGTGCGGAGCTTGTTACTGGTTACT
(+1)
TGGTAAGCTGGTGTGAGGGGAACCTGGGAGGGTCAGCTCCGGTCCTGGGTCGGGAGGG
GTGGGGGCCAGAGGATTCAGGGCCGGAGGTTCTGGTGGGGGCCCAGTGGGCGGGACCC
GAGGACGGAGGGGCCGGGAGGCCGAGAGGGGCGGGGTCGCGGCGGGGCCTGAGGGA
CGGAGGCCGGGATACTTGGGAAAGGATCCGCCGGCCTTGAACTCCCGCCTCCGCCGCCC
CTAGGCCTC

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FIG. 10

UQCRQ promoter

GTCACCTTTTTGTTCCCTCCCCCGCCTCCCGCATTCGGCCGCTTCCTGACTGGGATTCCAC
AGAAAAGCCGAGGGCTGAGGAGAAGTGTGAGCGCCTCCGCCTGTCCACTGTCCCCCAA
AGTCAGTTCAATCCCCGACGTCCTCCGCTAGGCTCCACCCCACCGGCCCGGGCAGGGCC
TCCAAGGCACCTCCCACCTACGGGTCACCCAGTCAGCCCACTTCTTTCTGGGACAAAGG
CGTCATCCCTTAGAGACAGTAGGAAAATGGTATCTCCCGGAAGTTACCTCACGACCTCC
AAGAGCGGCTTCCAACCTTGCCGGAAATGACGAACGAGTCAACCGGATCGGTGACTGT
(+1) +1
GGAGGGCGAGCTGAGCCCTGTGCGTGAGTGGGGTCTGGTTGTGCAGTGTTCGTGGACCC
TGGGAGGCTAGGGGCGCCCCGCTGGGCTGGGAAAGGATAAGGAGTGCAGGGGCAGGA
GTCTGGGGTTGGGGATGGACCCCCGCGGGGACTGCGGCGCTTCGCGAAAGCGAGCCAA
GCGCCTGTCCACCCTCGG TCCTGCAGGGCCGCCGCCACA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/004940

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [x] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/004940

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 31, 35, 38-43, 46-55
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 31, 35, 38-43, 46-55 are too unclear to make meaningful search possible because the claims refer to claims which do not comply with PCT Rule 6.4(a)

3. ☒ Claims Nos.: 28-30, 32-34, 36-37, 44-45, 56
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

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

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

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/004940

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/64(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 8 : C12N 15/64

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Pubmed, Esp@snet, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIRVEN, A. et al. 'Enhanced transgene expression in cord blood CD34(+)-derived hematopoietic cells, including developing T cells and NOD/SCID mouse repopulating cells, following transduction with modified trip lentiviral vectors' Molecular Therapy, Vol. 3(4), pp. 438-448 (April 2001) Seethe Abstract.	1-27
A	YEE, J.-K. et al. 'Gene expression from transcriptionally disabled retroviral vectors' Proceedings of National Academy of Science, USA. Vol. 84, pp. 5197-5201 (August 1987) See Fig.2	1-27
A	FARMER, A. A. et al. 'Isolation and characterization of the QM promoter' Nucleic Acids Research, Vol. 24(11), pp. 2158-2165 (1 June 1996) See Fig. 2 and Fig.3	1,2,7-13, 20-27
A	KUNG, S. K. et al. 'A murine leukemia virus (MuLV) long terminal repeat derived from rhesus macaques in the context of a lentivirus vector and MuLV gag sequence results in high-level gene expression in human T lymphocytes' Journal of Virology, Vol. 74(8), pp. 3668-3681 (April 2000) See the Abstract.	1-27

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 JANUARY 2008 (21.01.2008)	**21 JANUARY 2008 (21.01.2008)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/004940

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FLAMANT, F. et al. 'Importance of 3' non-coding sequences for efficient retrovirus-mediated gene transfer in avian cells revealed by self-inactivating vectors' Journal of General Virology, Vol. 74, pp. 39-46 (January 1993) See the whole document.	1-27
A	KIM, S. H. et al. 'Construction of retroviral vectors with improved safety, gene expression, and versatility' Journal of Virology, Vol. 72(2), pp. 994-1004 (February 1998) See the Abstract.	1-27
A	LOTTI, F. et al. 'Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement' Journal of Virology, Vol. 76(8), pp. 3996-4007 (April 2002) See the Abstract.	1-27