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(54) Title: INDUCIBLE ADENO -ASSOCIATED VIRUS VECTOR MEDIATED TRANSGENE ABLATION SYSTEM

(57) Abstract: The present invention relates to gene therapy systems designed for the delivery of a therapeutic product to a subject using replication-defective virus composition(s) engineered with a built-in safety mechanism for ablating the therapeutic gene product, either permanently or temporarily, in response to a pharmacological agent - preferably an oral formulation, e.g., a pill. The invention is based, in part, on the applicants' development of an integrated approach, referred to herein as "PITA" (Pharmacologically Induced Transgene Ablation), for ablating a transgene or negatively regulating transgene expression. In this approach, replication-deficient viruses are used to deliver a transgene encoding a therapeutic product (an RNA or a protein) so that it is expressed in the subject, but can be reversibly or irreversibly turned off by administering the pharmacological agent; e.g., by administration of a small molecule that induces expression of an ablator specific for the transgene or its RNA transcript.

WO 2013/049493 A1

INDUCIBLE ADENO -ASSOCIATED VIRUS VECTOR MEDIATED TRANSGENE ABLATION SYSTEM

BACKGROUND OF THE INVENTION

Gene therapy involves the introduction of genetic material into host cells with the goal of treating or curing disease. Many diseases are caused by "defective" genes that result in a deficiency in an essential protein. One approach for correcting faulty gene expression is to insert a normal gene (transgene) into a nonspecific location within the genome to replace a nonfunctional, or "defective," disease-causing gene. Gene therapy can also be used as a platform for the delivery of a therapeutic protein or RNA to treat various diseases so that the therapeutic product is expressed for a prolonged period of time, eliminating the need for repeat dosing. A carrier molecule called a vector must be used to deliver a transgene to the patient's target cells, the most common vector being a virus that has been genetically altered to carry normal human genes. Viruses have evolved a way of encapsulating and delivering their genes to human cells in a pathogenic manner and thus, virus genomes can be manipulated to insert therapeutic genes.

Stable transgene expression can be achieved following *in vivo* delivery of vectors based on adenoviruses or adeno-associated viruses (AAVs) into non dividing cells, and also by transplantation of stem cells transduced *ex vivo* with integrating and non-integrating vectors, such as those based on retroviruses and lentiviruses. AAV vectors are used for gene therapy because, among other reasons, AAV is nonpathogenic, it does not elicit a deleterious immune response, and AAV transgene expression frequently persists for years or the lifetime of the animal model (see Shyam *et al.,* Clin. Microbiol. Rev. 24(4):583-593). AAV is a small, nonenveloped human parvovirus that packages a linear strand of single stranded DNA genome that is 4.7 kb. Productive infection by AAV occurs only in the presence of a helper virus, either adenovirus or herpes virus. In the absence of a helper virus, AAV integrates into a specific point of the host genome (19q 13-qter) at a high frequency, making AAV the only mammalian DNA virus known to be capable of site-specific integration. *See,* Kotin *et at.,* 1990, PNAS, 87: 2211-2215. However, recombinant AAV, which does not contain any viral genes and only a therapeutic gene, does not

integrate into the genome. Instead the recombinant viral genome fuses at its ends via inverted terminal repeats to form circular, episomal forms which are predicted to be the primary cause of the long term gene expression (see Shyam *et at.,* Clin. Microbiol. Rev. 24(4):583-593).

Virtually all pre-clinical and clinical applications of gene therapy have used vectors that express the transgene from a constitutive promoter, which means it is active at a fixed level for as long as the vector genome persists. However, many diseases that are amenable to gene therapy may need to have expression of the transgene regulated. Several systems have been described which that are based on the general principle of placing a gene of interest under the control of a drug-inducible engineered transcription factor in order to positively induce gene expression (Clackson *et at.,* 1997, Curr Opin Chern BioI, 1 (2): 210-8; Rossi *et at.,* Curr Opin Biotechnol, 1998.9(5): p. 451-6). The various systems can be divided into two classes. In the first, a DNA-binding domain that is allosterically regulated by inducers such as tetracyclines, antiprogestins, or ecdysteroids is coupled to a transactivation domain. The addition (or in some cases removal) of the drug leads to DNA binding and hence transcriptional activation. In the second, allosteric control is replaced with the more general mechanism of induced proximity. DNA binding and activation domains are expressed as separate polypeptides that are reconstituted into an active transcription factor by addition of a bivalent small molecule, referred to as a chemical inducer of dimerization or "dimerizer." While these systems are useful in gene therapy systems that require inducing transgene expression, they have not addressed the need to be able to turn off or permanently ablate transgene expression if it is no longer needed or if toxicity due to long-term drug administration ensues.

SUMMARY OF THE INVENTION

The present invention relates to gene therapy systems designed for the delivery of a therapeutic product to a subject using replication-defective virus composition(s) engineered with a built-in safety mechanism for ablating the therapeutic gene product, either permanently or temporarily, in response to a pharmacological agent - preferably an oral formulation, *e.g.,* a pill.

The invention is based, in part, on the applicants' development of an integrated

approach, referred to herein as "PITA" (Pharmacologically Induced Transgene Ablation), for ablating a transgene or negatively regulating transgene expression. In this approach, replication-deficient viruses are used to deliver a transgene encoding a therapeutic product (an RNA or a protein) so that it is expressed in the subject, but can be reversibly or irreversibly turned off by administering the pharmacological agent.

The invention presents many advantages over systems in which expression of the transgene is positively regulated by a pharmacological agent. In such cases, the recipient must take a pharmaceutic for the duration of the time he/she needs the transgene expressed - a duration that may be very long and may be associated with its own toxicity.

In one aspect, the invention provides a composition for AAV-mediated delivery of a therapeutic product having a controlled transgene expression ablation system, said composition comprising (a) an AAV vector containing a nucleic acid molecule comprising: (i) a nucleic acid sequence encoding a therapeutic product operably linked to a promoter that controls transcription; and (ii) at least one endonuclease ablation site which comprises a sequence of at least 30 nucleic acid base pairs which are specifically and selectively recognized by at least ten (10X) zinc fingers, said at least one endonuclease ablation site being located at least 5' to the sequence encoding the therapeutic product; and (b) at least one ablator which comprises a chimeric endonuclease comprising at least ten copies of a zinc finger domain linked to a functional endonuclease catalytic domain in operative association with a promoter, wherein transcription and/or ablation activity is induced in response to a pharmacological agent, said at least ten (10X) zinc finger domain specifically and selectively recognizing said at least about 30 base pair sequence in said at least one endonuclease ablation site and comprising at least 10 independently selected recognition helices. In one embodiment, the endonuclease catalytic domain is a FokI catalytic domain.

In a further embodiment, the nucleic acid molecule consists of a double-stranded DNA molecule, wherein the at least one endonuclease ablation site is on a first strand of the DNA molecule and at least a second endonuclease ablation is located on the second strand of the DNA molecule, wherein said second endonuclease ablation site is distinct

from said endonuclease ablation said on the first strand and is specifically and selectively recognized by a different zinc finger.

In a further aspect, the ablator (b) is controlled by a cassette that is activated by a transcription factor following being dimerized by a pharmacologic agent, said cassette comprising two transcription units, wherein: (c) one of said two transcription units encoding the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a first promoter; and (d) a second of said two transcription units encoding the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a second promoter.

In one embodiment, the first and second promoters are both constitutive promoters and the pharmacological agent is a dimerizer that dimerizes the domains of the transcription factor.

In another embodiment, the transcription of the promoter is controlled by a rapamycin - regulatable system and the pharmacological agent is rapamycin or a rapalog.

In yet another embodiment, the unique nucleic acid sequence of least about a 30 base pair of (a)(ii) consists of contiguous nucleic acids which are less than 70% identical with any subsequence in the human genome and no more than 8 contiguous identical positions with any sub-sequence in the human genome.

Other aspects and advantages of the invention will be readily apparent from the following Detailed Description of the Invention.

As used herein, the following terms will have the indicated meaning:

"Unit" refers to a transcription unit.

"Transgene unit" refers to a DNA that comprises (1) a DNA sequence that encodes a transgene; (2) an ablation recognition site (ARS) contained within or flanking the transgene; and (3) a promoter sequence that regulates expression of the transgene.

"Ablation recognition site" or "ARS" refers to a DNA sequence that (1) can be recognized by the ablator that ablates or excises the transgene from the transgene unit; or (2) encodes an ablation recognition RNA sequence (ARRS)

"Ablation recognition RNA sequence" or "ARRS" refers to an RNA sequence that is recognized by the ablator that ablates the transcription product of the transgene or

translation of its mRNA.

"Ablator" refers to any gene product, *e.g.,* translational or transcriptional product, that specifically recognizes/binds to either (a) the ARS of the transgene unit and cleaves or excises the transgene; or (b) the ARRS of the transcribed transgene unit and cleaves or prevents translation of the mRNA transcript.

"Ablation unit" refers to a DNA that comprises (1) a DNA sequence that encodes an Ablator; and (2) a promoter sequence that controls expression of said Ablator.

"Dimerizable transcription factor (TF) domain unit" refers to (1) a DNA sequence that encodes the DNA binding domain of a TF fused to the dimerizer binding domain (DNA binding domain fusion protein) controlled by a promoter; and (2) a DNA sequence that encodes the activation domain of a TF fused to the dimerizer binding domain (activation domain fusion protein) controlled by a promoter. In one embodiment, each unit of the dimerizable domain is controlled by a constitutive promoter and the unit is utilized for control of the promoter for the ablator. Alternatively, one or more of the promoters may be an inducible promoter.

A "Dimerizable fusion protein unit" refers to (1) a first DNA sequence that encodes a unit, subunit or fragment of a protein or enzyme (*e.g.,* an ablator) fused to a dimerizer binding domain and (2) a second DNA sequence that encodes a unit, subunit or fragment of a protein or enzyme, which when expressed and if required, activated, combine to form a fusion protein. This "Dimerizable fusion protein unit" may be utilized for a variety of purposes, including to activate a promoter for the ablator, to provide DNA specificity, to activate a chimeric ablator by bringing together the binding domain and the catalytic domain, or to produce a desired transgene. These units (1) and (2) may be in a single open reading frame separated by a suitable linker (*e.g.,* an IRES or 2A self-cleaving protein) under the control of single promoter , or may be in separate open reading frames under the control of independent promoters. From the following detailed description, it will be apparent that a variety of combinations of constitutive or inducible promoters may be utilized in the two components of this unit, depending upon the use to which this fusion protein unit is put (e.g., for expression of an ablator). In one embodiment, the dimerizable fusion protein unit contains DNA binding domains which include, *e.g.,* zinc finger motifs, homeo domain motifs, HMG-box domains, STAT proteins, B3, helix-loop-helix, winged helix-turn-helix,

leucine zipper, helix-turn-helix, winged helix, POU domains, DNA binding domains of repressors, DNA binding domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs.

"Dimerizer" refers to a compound or other moiety that can bind heterodimerizable binding domains of the TF domain fusion proteins or dimerizable fusion proteins and induce dimerization or oligomerization of the fusion proteins. Typically, the dimerizer is delivered to a subject as a pharmaceutical composition.

"Side effect" refers to an undesirable secondary effect which occurs in a patient in addition to the desired therapeutic effect of a transgene product that was delivered to a patient via administration of a replication-defective virus composition of the invention.

"Replication-defective virus" or "viral vector" refers to a synthetic or artificial genome containing a gene of interest packaged in replication-deficient virus particles; *i.e.,* particles that can infect target cells but cannot generate progeny virions. The artificial genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be "gutless" - containing only the transgene of interest flanked by the signals required for amplification and packaging of the artificial genome). Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

"Virus stocks" or "stocks of replication-defective virus" refers to viral vectors that package the same artificial/synthetic genome (in other words, a homogeneous or clonal population).

A "chimeric engineered ablator" or a "chimeric enzyme" is provided when a sequence encoding a catalytic domain of an endonuclease ablator fused to a binding domain and a sequence encoding a DNA binding domain of the endonuclease fused to a binding domain are co-expressed. The chimeric engineered enzyme is a dimer, the DNA binding domains may be selected from among, for example, zinc finger and other homeodomain motifs, HMG-box domains, STAT proteins, B3, helix-loop-helix, winged helix-turn-helix, leucine zipper, helix-turn-helix, winged helix, POU domains, DNA binding domains of repressors, DNA binding domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs. [US 5,436,150, issued July 25, 1995].

When a heterodimer is formed, the binding domains are specific for a pharmacologic agent that induces dimerization in order to provide the desired enzymatic bioactivity, DNA binding specificity, and/or transcription of the ablator. Typically, an enzyme is selected which has dual domains, *i.e.,* a catalytic domain and a DNA binding domain which are readily separable. In one embodiment, a type II restriction endonuclease is selected. In one embodiment, a chimeric endonuclease is designed based on an endonuclease having two functional domains, which are independent of ATP hydrolysis. Useful nucleases include type II S endonucleases such as FokI, or an endonuclease such as Nae I. Another suitable endonuclease may be selected from among intron endonucleases, such as *e.g.,* I-TevI. Still other suitable nucleases include, *e.g.,* integrases (catalyze integration), serine recombinases (catalyze recombination), tyrosine recombinases, invertases (*e.g.* Gin) (catalyze inversion), resolvases, (*e.g.,* Tn3), and nucleases that catalyze translocation, resolution , insertion, deletion, degradation or exchange. However, other suitable nucleases may be selected.

BRIEF DESCRIPTION OF DRAWINGS

Figs. 1A and 1B. PITA DNA construct containing a dimerizable transcription factor domain unit and an ablation unit. Figure 1A is a map of the following DNA construct, which comprises a dimerizable transcription factor domain unit and an ablation unit: pAAV.CMV.TF.FRB-IRES-1xFKBP.Cre. Fig. 1B is a cartoon of the transcription unit inserted into the plasmid backbone. A description of the various vector domains can be found in Section 8.1 herein.

Figs. 2A and 2B. PITA DNA construct containing a dimerizable transcription factor domain unit and an ablation unit. Fig. 2A is a map of the following DNA construct, which comprises a dimerizable transcription factor domain unit and an ablation unit: pAAV.CMV.TF.FRB-T2A-2xFKBP.Cre. Fig. 2B is a cartoon of the transcription unit inserted into the plasmid backbone. A description of the various vector domains can be found in Section 8.1 herein.

Figs. 3A and 3B. PITA DNA construct containing a dimerizable transcription factor domain unit and an ablation unit. Fig. 3A is map of the following DNA construct, which comprises a dimerizable transcription factor domain unit and an ablation unit: pAAV.CMV173.TF.FRB-T2A-3xFKBP.Cre. Fig. 3B is a cartoon of the transcription unit

inserted into the plasmid backbone. A description of the various vector domains can be found in Section 8.1 herein.

Figs. 4A and 4B. PITA DNA construct containing a dimerizable transcription factor domain unit and an ablation unit. Fig. 4A is a map of the following DNA construct, which comprises a dimerizable transcription factor domain unit and an ablation unit: pAAV.CMV.TF.FRB-T2A-2xFKBP.ISce-I. Fig. 4B is a cartoon of the transcription unit inserted into the plasmid backbone. A description of the various vector domains can be found in Section 8.1 herein.

Figs. 5A and 5B. PITA DNA construct containing a transgene unit. Fig. 5A is a map of the following DNA construct, which comprises a transgene unit: pENN.CMV.PLloxP.Luc.SV40. Fig. 5B is a cartoon of the transcription unit inserted into the plasmid backbone. A description of the various vector domains can be found in Section 8.2 herein.

Figs. 6A and 6B. PITA DNA construct containing a transgene unit. Figure 6A is a map of the following DNA construct, which comprises a transgene unit: pENN.CMV.PISceI.UC.SV40. Fig. 6B is a cartoon of the transcription unit inserted into the plasmid backbone. A description of the various vector domains can be found in Section 8.2 herein.

Fig. 7. PITA DNA construct containing a dimerizable transcription factor domain unit and a transgene unit. Figure 7 is a map of a vector that contains a transgene unit and a dimerizable transcription factor domain unit. A description of the various vector domains can be found in Sections 8.1 and 8.2 herein.

Figs. 8A-B. In vitro induction of luciferase after rapamycin treatment. Fig. 8A is a bar graph showing relative luciferase activity in cells that were transfected with the indicated DNA constructs (DNA constructs 1 to 6) 48 hours after either being treated or not treated with rapamycin. Fig. 8B is a bar graph showing relative luciferase activity in cells that were transfected with the indicated DNA constructs (DNA constructs 1 to 6) 72 hours after either being treated or not treated with rapamycin.

Figs. 9A-D. In the *in vivo* model for a dimerizer-inducible system, four groups of mice received IV injection of AAV vectors containing the following DNA constructs. Fig. 9A is a diagram of a DNA construct encoding GFP-Luciferase under

the control of ubiquitous constitutive CMV promoter, which was delivered to Group 1 mice via AAV vectors. Fig. 9B is a diagram of DNA constructs encoding (1) a dimerizable transcription factor domain unit (FRB fused with p65 activation domain and DNA binding domain ZFHD fused with 3 copies of FKBP) driven by the CMV promoter; and (2) AAV vector expressing GFP-Luciferase driven by a promoter induced by the dimerized TF, which were delivered to Group 2 mice via AAV vectors. Fig. 9C is a diagram of a DNA construct encoding GFP-Luciferase under the control of a liver constitutive promoter, TBG, which was delivered to Group 3 mice via AAV vectors. Fig. 9D is a diagram of DNA constructs encoding (1) AAV vector expressing a dimerizable transcription factor domain unit driven by the TBG promoter; and (2) AAV vector expressing GFP-Luciferase driven by a promoter induced by the dimerized TF, which were delivered to Group 4 mice via AAV vectors.

Figs. 10 A-D. Image of 4 groups of mice that received $3x10^{11}$ particles of AAV virus containing various DNA constructs 30 minutes after injection of luciferin, the substrate for luciferase. Figure 10A shows luciferase expression in various tissues, predominantly in lungs, liver and muscle, in Group 1 mice before ("Pre") and after ("Post") rapamycin administration. Figure 10B shows luciferase expression, predominantly in liver and muscle in Group 2 mice before ("Pre") and after ("Post") rapamycin administration. Figure 10C shows luciferase expression predominantly in liver and muscle after ("Post") rapamycin administration, and shows that there is no luciferase expression before ("Pre") rapamycin administration in Group 3 mice. Figure 10D shows luciferase expression is restricted to the liver ("Post") rapamycin administration and shows that there is no luciferase expression before ("Pre") rapamycin administration.

Figs. 11 A-D. Image of 4 groups of mice that received $1x10^{11}$ particles of AAV virus containing various DNA constructs 30 minutes after injection of luciferin, the substrate for luciferase. Figure 1A shows luciferase expression in various tissues, predominantly in lungs, liver and muscle, in Group 1 mice before ("Pre") and after ("Post") rapamycin administration. Figure 11B shows luciferase expression, predominantly in liver and muscle in Group 2 mice before ("Pre") and after ("Post") rapamycin administration. Figure 11C shows luciferase expression predominantly in liver and muscle after ("Post") rapamycin administration, and shows that there is no luciferase expression before ("Pre") rapamycin

administration in Group 3 mice. Figure 11D shows luciferase expression is restricted to the liver ("Post") rapamycin administration and shows that there is no luciferase expression before ("Pre") rapamycin administration.

Figs. 12 A-C. PITA DNA constructs for treating AMD. Figure 12A shows a DNA construct comprising a transgene unit that encodes a soluble VEGF receptor, sFlt-1. Figure 12B shows a bicistronic DNA construct comprising Avastin IgG heavy chain (AvastinH) and light chain (AvastinL) regulated by IRES. Figure 12C shows a bicistronic DNA construct comprising Avastin IgG heavy chain (AvastinH) and light chain (AvastinL) separated by a T2A sequence.

Figs. 13 A-B. PITA DNA constructs for treating Liver Metabolic Disease. Figure 13A shows a PITA DNA construct for treating hemophilia A and/or B, containing a transgene unit comprising Factor IX. Figure 13B shows a DNA construct for delivery of shRNA targeting the IRES of HCV.

Figs. 14 A-B. PITA DNA constructs for treating Heart Disease. Fig. 14A shows a PITA DNA construct for treating congestive heart failure, containing a transgene unit comprising insulin like growth factor (IGFl). Fig. 14B shows a PITA DNA construct for treating congestive heart failure, containing a transgene unit comprising hepatocyte growth factor (HGF).

Fig. 15. PITA DNA construct for a CNS disease. Fig. 15 shows a PITA DNA construct for treating Alzheimer's disease, containing a transgene unit comprising nerve growth factor (NGF).

Fig. 16. PITA System for HIV treatment. Fig. 16 shows a PITA DNA construct containing a transgene unit comprising the heavy and light chains of an HIV antibody and a PITA DNA construct containing an ablation unit and a dimerizable TF domain unit. Fig. 16 also shows that a rapamycin analog (rapalog) can induce expression of the ablator, cre, to ablate the transgene (heavy and light chains of an HIV antibody) from the PITA DNA construct containing a transgene unit.

Fig. 17. Illustration of one embodiment of the PITA system. Fig. 17 shows a transgene unit encoding a therapeutic antibody that is in operative association with a constitutive promoter, an ablation unit encoding an endonuclease that is in operative association with a transcription factor inducible promoter, and a dimerizable TF domain unit,

with each transcription factor domain fusion sequence in operative association with a constitutive promoter. Prior to administration of rapamycin or a rapalog, there is baseline expression of the therapeutic antibody and of the two transcription factor domain fusion proteins. Upon rapamycin administration, the dimerized transcription factor induces expression of the endonuclease, which cleaves the endonuclease recognition domain in the transgene unit, thereby ablating transgene expression.

Figs. 18A- 18B are bar charts illustrating that wild-type FokI effective ablated expression of a transgene when a DNA plasmid containing a transgene containing ablation sites for FokI was cotransfected into target cells with a plasmid encoding the FokI enzyme. Fig. 18A, bar 1 represents 50 ng pCMV.Luciferase, bar 2 represents 50 ng pCMV.Luciferase + 200 ng pCMV.FokI, bar 3 represents 50 ng pCMV.Luciferase + transfected FokI protein, bar 4 represents transfected FokI protein alone; bar 5 represents untransfected controls. Fig. 18B, bar 1 represents 50 ng pCMV.Luc alone, subsequent bars represent increasing concentrations of a ZFHD-FokI expression plasmid (6.25, 12.5, 25, 50, and 100 ng) cotransfected with pCMV.Luciferase. This study is described in Example 11A.

Figs. 19A-B are bar charts illustrating that a chimeric engineered enzyme tethered to a non-cognate recognition site on the DNA by the zinc finger homeodomain effectively ablates expression of a transgene. Fig. 19A compares increasing concentrations of an expression plasmid encoding un-tethered FokI (6.25 ng, 12.5 ng, 25 ng, 50 ng and 100 ng) co-transfected with pCMV.luciferase. The first bar provides a positive control of 50 ng pCMV.Luc alone. Fig. 19B compares increasing concentrations of an expression plasmid encoding FokI tethered to DNA via fusion with the zinc finger homeodomain (6.25 ng, 12.5 ng, 25 ng, 50 ng and 100 ng) co-transfected with pCMV.luciferase. The first bar provides a control of 50 ng pCMV.Luc alone. This study is described in Example 11B.

Figs. 20A-B are bar charts illustrating that the DNA binding specificity of chimeric FokI can be reproducible changed by fusion with various classes of heterologous DNA binding domains and ablation of target transgene can be further improved by the additional of a heterologous nuclear localization signal (NLS). Fig. 20A illustrates the results of co-transfection of pCMV.Luciferase with increasing concentrations of an expression plasmid encoding FokI tethered to DNA via an HTH fusion (6.25, 12.5, 25, 50, and 100 ng). The first bar is a control showing 50 ng pCMV.Luciferase alone. Fig. 20B illustrates the results

of co-transfection of pCMV.Luciferase with increasing concentrations of an expression plasmid encoding an HTH - FokI fusion, which further has a NLS at its N-terminus (6.25, 12.5, 25, 50, and 100 ng). The first bar is a control showing 50 ng pCMV.Luciferase alone. This study is described in Example 11C.

Fig. 21 is a flow chart of a method for selecting a unique nucleic acid sequence for use in an ablation recognition site as described herein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to gene therapy systems designed for the delivery of a therapeutic product to a subject using replication-defective virus composition(s) engineered with a built-in safety mechanism for ablating the therapeutic gene product, either permanently or temporarily, in response to a pharmacological agent - preferably an oral formulation, *e.g.,* a pill.

In the PITA system, one or more replication-defective viruses are used in a replication-defective virus composition in which the viral genome(s) have been engineered to contain: (a) a first transcription unit that encodes a therapeutic product in operative association with a promoter that controls transcription, said unit containing at least one ablation recognition site; and (b) a second transcription unit that encodes an ablator (or a fragment thereof as part of a fusion protein unit) specific for the ablation recognition site in operative association with a promoter that induces transcription in response to a pharmacological agent. Any pharmacological agent that specifically dimerizes the domains of the selected binding domain can be used. In one embodiment, rapamycin and its analogs referred to as "rapalogs" can be used.

A viral genome containing a first transcription unit may contain two or more of the same ablation recognition site or two or more different ablation recognition sites (*i.e.,* which are specific sites for a different ablator than that which recognizes the other ablation recognition site(s)). Whether the same or different, such two or more ablation recognition sites may be located in tandem to one another, or may be located in a position non-contiguous to the other. Further, the ablation recognition site(s) may be located at any position relative the coding sequence for the transgene, *i.e.,* within the transgene coding sequence, 5' to the coding sequence (either immediately 5' or separated by one or more

bases, e.g., upstream or downstream of the promoter) or 3' to the coding sequence (*e.g.*, either immediately 3' or separated by one or more bases, *e.g.*, upstream of the poly A sequence).

An ablator is any gene product, *e.g.*, translational or transcriptional product, that specifically recognizes/binds to either (a) the ablation recognition site(s) (ARS) of the transgene unit and cleaves or excises the transgene; or (b) the ablation recognition RNA sequence (ARRS) of the transcribed transgene unit and cleaves or inhibits translation of the mRNA transcript. As described herein, an ablator may be selected from the group consisting of: an endonuclease, a recombinase, a meganuclease, or a zinc finger endonuclease that binds to the ablation recognition site in the first transcription unit and excises or ablates DNA and an interfering RNA, a ribozyme, or an antisense that ablates the RNA transcript of the first transcription unit, or suppresses translation of the RNA transcript of the first transcription unit. In one specific embodiment, the ablator is Cre (which has as its ablation recognition site loxP), or the ablator is FLP (which has as its ablation recognition site FRT). In one embodiment, an endonuclease is selected which functions independently of ATP hydrolysis. Examples of such ablators may include a Type II S endonuclease (e.g., FokI), NaeI, and intron endonucleases (such as *e.g.*, I-TevI), integrases (catalyze integration), serine recombinases (catalyze recombination), tyrosine recombinases, invertases (*e.g.* Gin) (catalyze inversion), resolvases, (*e.g.*, Tn3), and nucleases that catalyze translocation, resolution , insertion, deletion, degradation or exchange. However, other suitable nucleases may be selected.

For permanent shut down of the therapeutic transgene, the ablator can be an endonuclease that binds to the ablation recognition site(s) in the first transcription unit and ablates or excises the transgene. Where temporary shutdown of the transgene is desired, an ablator should be chosen that binds to the ablation recognition site(s) in the RNA transcript of the therapeutic transgene and ablates the transcript, or inhibits its translation. In this case, interfering RNAs, ribozymes, or antisense systems can be used. The system is particularly desirable if the therapeutic transgene is administered to treat cancer, a variety of genetic disease which will be readily apparent to one of skill in the art, or to mediate host immune response.

Expression of the ablator may be controlled by one or more elements, including, e.g., an inducible promoter and/or by use of a chimeric ablator that utilizes a homodimer or heterodimer fusion protein system, such as are described herein. Where use of a homodimer system is selected, expression of the ablator is controlled by an inducible promoter. Where use of heterodimer system is selected, expression of the ablator is controlled by additional of a pharmacologic agent and optionally, a further inducible promoter for one or both of the fusion proteins which form the heterodimer system. In one embodiment, a homo- and hetero-dimizerable ablator is selected to provide an additional layer for safety to constructs with transcription factor regulators. These systems are described in more detail later in this specification.

Any virus suitable for gene therapy may be used, including but not limited to adeno-associated virus ("AAV"); adenovirus; herpes virus; lentivirus; retrovirus; *etc.* In preferred embodiments, the replication-defective virus used is an adeno-associated virus ("AAV"). AAV1, AAV6, AAV7, AAV8, AAV9 or rh10 being particularly attractive for use in human subjects. Due to size constraints of the AAV genome for packaging, the transcription units can be engineered and packaged in two or more AAV stocks. Whether packaged in one viral stock which is used as a virus composition according to the invention, or in two or more viral stocks which form a virus composition of the invention, the viral genome used for treatment must collectively contain the first and second transcription units encoding the therapeutic transgene and the ablator; and may further comprise additional transcription units. For example, the first transcription unit can be packaged in one viral stock, and second, third and fourth transcription units packaged in a second viral stock. Alternatively, the second transcription unit can be packaged in one viral stock, and the first, third and fourth transcription units packaged in a second viral stock. While useful for AAV due to size contains in packaging the AAV genome, other viruses may be used to prepare a virus composition according to the invention. In another embodiment, the viral compositions of the invention, where they contain multiple viruses, may contain different replication-defective viruses (*e.g.,* AAV and adenovirus).

In one embodiment, a virus composition according to the invention contains two or more different AAV (or another viral) stock, in such combinations as are described above. For example, a virus composition may contain a first viral stock comprising the

therapeutic gene with ablator recognition sites and a first ablator and a second viral stock containing an additional ablator(s). Another viral composition may contain a first virus stock comprising a therapeutic gene and a fragment of an ablator and a second virus stock comprising another fragment of an ablator. Various other combinations of two or more viral stocks in a virus composition of the invention will be apparent from the description of the components of the present system.

In one embodiment, a composition contains one or more AAV vectors in a system for delivery of a therapeutic product having a controlled transgene expression ablation system. At least one AAV vector in the composition contains a nucleic acid molecule comprising: (i) a nucleic acid sequence encoding a therapeutic product operably linked to a promoter that controls transcription; and (ii) at least one endonuclease ablation site which comprises a sequence of at least 30 nucleic acid base pairs which are specifically and selectively recognized by at least ten (10X) zinc fingers, said at least one endonuclease ablation site being located at least 5' to the sequence encoding the therapeutic product; and (b) at least one ablator which comprises a chimeric endonuclease comprising at least ten copies of a zinc finger domain linked to a functional endonuclease catalytic domain in operative association with a promoter, wherein transcription and/or ablation activity is induced in response to a pharmacological agent, said at least ten (10X) zinc finger domain specifically and selectively recognizing said at least about 30 base pair sequence in said at least one endonuclease ablation site and comprising at least 10 independently selected recognition helices. In one embodiment, the endonuclease catalytic domain is a FokI catalytic domain. In a further embodiment, the ablator (b) is controlled by a cassette that is activated by a transcription factor following being dimerized by a pharmacologic agent, said cassette comprising two transcription units, wherein one of said two transcription units encoding the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a first promoter; and a second of said two transcription units encoding the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a second promoter. Any pharmacological agent that specifically dimerizes the domains of the selected binding

domain can be used. In one embodiment, rapamycin and its analogs referred to as "rapalogs" can be used.

In order to conserve space within the viral genome(s), bicistronic transcription units can be engineered. For example, transcription units that can be regulated by the same promoter, *e.g.,* the third and fourth transcription units (and where applicable, the first transcription unit encoding the therapeutic transgene) can be engineered as a bicistronic unit containing an IRES (internal ribosome entry site) or a 2A peptide, which self-cleaves in a post-translational event (e.g., furin -2A), and which allows coexpression of heterologous gene products by a message from a single promoter when the transgene (or an ablator coding sequence) is large, consists of multi-subunits, or two transgenes are co-delivered, recombinant AAV (rAAV) carrying the desired transgene(s) or subunits are co-administered to allow them to concatamerize *in vivo* to form a single vector genome. In such an embodiment, a first AAV may carry an expression cassette which expresses a single transgene and a second AAV may carry an expression cassette which expresses a different transgene for co-expression in the host cell. However, the selected transgene may encode any biologically active product or other product, *e.g.*, a product desirable for study. A single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three heterologous genes *(e.g.,* the third and fourth transcription units, and where applicable, the first transcription unit encoding the therapeutic transgene) separated from one another by sequences encoding a self-cleavage peptide *(e.g.,* 2A peptide, T2A) or a protease recognition site *(e.g.,* furin). The ORF thus encodes a single polyprotein, which, either during (in the case of T2A) or after translation, is cleaved into the individual proteins. These IRES and polyprotein systems can be used to save AAV packaging space, they can only be used for expression of components that can be driven by the same promoter.

The invention also relates to DNA constructs used to engineer cell lines for the production of the replication-defective virus compositions; methods for producing and manufacturing the replication-defective virus compositions; expression in a variety of cell types and systems, including plants, bacteria, mammalian cells, etc., and methods of treatment using the replication-defective virus compositions for gene transfer, including veterinary treatment (e.g., in livestock and other mammals), and for *in vivo* or *ex vivo* therapy, including gene therapy in human subjects.

5.1. Transgene Ablation System

The present invention provides a Pharmacologically Induced Transgene Ablation (PITA) System designed for the delivery of a transgene (encoding a therapeutic product - protein or RNA) using replication-defective virus compositions engineered with a built-in safety mechanism for ablating the therapeutic gene product, either permanently or temporarily, in response to a pharmacological agent - preferably an oral formulation, e.g., a pill containing a small molecule that induces expression of the ablator specific for the transgene or its transcription product. However, other routes of delivery for the pharmacologic agent may be selected.

In the PITA system, one or more replication-defective viruses are used in which the viral genome(s) have been engineered to contain a transgene unit (described in Section 5.1.1 herein) and an ablation unit (described in Section 5.1.2 herein). In particular, one or more replication-defective viruses are used in which the viral genome(s) have been engineered to contain (a) a first transcription unit that encodes a therapeutic product in operative association with a promoter that controls transcription, said unit containing at least one ablation recognition site (a transgene unit); and (b) a second transcription unit that encodes an ablator specific for the ablation recognition site in operative association with a promoter that induces transcription in response to a pharmacological agent (an ablation unit).

In one embodiment, the PITA system is designed such that the viral genome(s) of the replication-defective viruses are further engineered to contain a dimerizable domain unit (described in Section 5.1.3). In one embodiment, by delivering a dimerizable TF domain unit, target cells are modified to co- express two fusion proteins: one containing a DNA-binding domain (DBD) of the transcription factor that binds the inducible promoter controlling the ablator and the other containing a transcriptional activation domain (AD) of the transcription factor that activates the inducible promoter controlling the ablator, each fused to dimerizer binding domains (described in Section 5.1.3). Addition of a pharmacological agent, or "dimerizer" (described in Section 5 .1.4) that can simultaneously interact with the dimerizer binding domains present in both fusion proteins results in recruitment of the AD fusion protein to the regulated promoter, initiating transcription of the ablator. *See, e.g.,* the Ariad ARGENT® system described in U.S. Patent No. 5,834,266 and

U.S. Patent No. 7,109,317, each of which is incorporated by reference herein in its entirety. By using dimerizer binding domains that have no affinity for one another in the absence of ligand and an appropriate minimal promoter, transcription is made absolutely dependent on the addition of the dimerizer.

To this end, the viral genome(s) of the replication-defective viruses can be further engineered to contain a third and a fourth transcription unit (a dimerizable TF domain unit), each encoding a dimerizable domain of a transcription factor that regulates the inducible promoter of the ablator in second transcription unit, in which: (c) the third transcription unit encodes the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a constitutive promoter; and (d) the fourth transcription unit encodes the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a promoter. In one embodiment, each component of the dimerizable TF domain is expressed under constitutive promoter. In another embodiment, at least one component of the dimerizable TF domain unit is expressed under an inducible promoter.

One embodiment of the PITA system is illustrated in Figure 24, which shows a transgene unit encoding a therapeutic antibody that is in operative association with a constitutive promoter, an ablation unit encoding an endonuclease that is in operative association with a transcription factor inducible promoter, and a dimerizable TF domain unit, with each transcription factor domain fusion sequence in operative association with a constitutive promoter. Prior to administration of rapamycin or a rapalog, there is baseline expression of the therapeutic antibody and of the two transcription factor domain fusion proteins. Upon rapamycin administration, the dimerized transcription factor induces expression of the endonuclease, which cleaves the endonuclease recognition domain in the transgene unit, thereby ablating transgene expression.

In one embodiment, the replication-defective virus used in the PITA system is an adeno-associated virus ("AAV") (described in Section 5.1.5). AAV1, AAV6, AAV7, AAV8, AAV9 or rh10 are particularly attractive for use in human subjects. Due to size constraints of the AAV genome for packaging, the transcription units can be engineered and packaged in two or more AAV stocks. For example, the first transcription

unit can be packaged in one AAV stock, and the second, third and fourth transcription units packaged in a second AAV stock. Alternatively, the second transcription unit can be packaged in one AAV stock, and the first, third and fourth transcription units packaged in a second AAV stock.

5.1.1. Transgene Unit

In the PITA system, one or more replication-defective viruses are used in which the viral genome(s) have been engineered to contain a transgene unit. As used herein, the term "transgene unit" refers to a DNA that comprises: (1) a DNA sequence that encodes a transgene; (2) at least one ablation recognition site (ARS) contained in a location which disrupts transgene expression, including, within or flanking the transgene or its expression control elements (e.g., upstream or downstream of the promoter and/or upstream of the polyA signal); and (3) a promoter sequence that regulates expression of the transgene. The DNA encoding the transgene can be genomic DNA, cDNA, or a cDNA that includes one or more introns which *e.g.,* may enhance expression of the transgene. In systems designed for removal of the transgene, the ARS used is one recognized by the ablator (described in Section 5.1.2) that ablates or excises the transgene, *e.g.,* an endonuclease recognition sequence including but not limited to a recombinase *(e.g.,* the Cre/loxP system, the FLP/FRT system), a meganuclease *(e.g.,* I-SceI system), an artificial restriction enzyme system or another artificial restriction enzyme system, such as the zinc finger nuclease, or a restriction enzyme specific for a restriction site that occurs rarely in the human genome, and the like. To repress expression of the transgene, the ARS can encode an ablation recognition RNA sequence (ARRS), *i.e.,* an RNA sequence recognized by the ablator that ablates the transcription product of the transgene or translation of its mRNA, *e.g.,* a ribozyme recognition sequence, an RNAi recognition sequence, or an antisense recognition sequence.

Examples of transgenes that can be engineered in the transgene units of the present invention includes, but is not limited to a transgene that encodes: an antibody or antibody fragment that neutralizes HIV infectivity, a therapeutic antibody such as VEGF antibody, TNF-α antibody *(e.g.,* infliximab, adalimumab), an EGF-R antibody, basiliximab, cetuximab, infliximab, rituxumab, alemtuzumab-CLL, daclizumab, efalizumab, omalizumab, pavilizumab, trastuzumab, gemtuzumab, adalimumab, or an antibody

fragment of any of the foregoing therapeutic antibodies; soluble vascular endothelial growth factor receptor-1 (sFIt-1), soluble TNF-a receptor *(e.g.,* etanercept), Factor VIII, Factor IX, insulin, insulin like growth factor (lGF), hepatocyte growth factor (RGF), heme oxygenase-1 (RO-1), nerve growth factor (NGF), beta-IFN, IL-6, anti-EGFR antibody, interferon (IFN), IFN beta-1 a , anti-CD20 antibody, glucagon-like peptide-1 (GLP-1), anti-cellular adhesion molecule, a4-integrin antibody, glial cell line-derived neurotrophic factor (GDNF), aromatic L-amino acid decarboxylase (ADCC), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), galanin, neuropeptide Y (NPY), a TNF antagonist, chemokines from the IL-8 family, BCl2, IL-10, a therapeutic siRNA, a therapeutic u6 protein, endostatin, plasminogen or a fragment thereof, TIMP3, VEGF-A, RIFI alpha, PEDF, or IL-1 receptor antagonist.

The transgene can be under the control of a constitutive promoter, an inducible promoter, a tissue-specific promoter, or a promoter regulated by physiological cues.

Examples of constitutive promoters suitable for controlling expression of the therapeutic products include, but are not limited to human cytomegalovirus (CMV) promoter, the early and late promoters of simian virus 40 (SV40), U6 promoter, metallothionein promoters, EFla promoter, ubiquitin promoter, hypoxanthine phosphoribosyl transferase (HPRT) promoter, dihydrofolate reductase (DHFR) promoter (Scharfmann et al., Proc. Natl. Acad. Sci. USA 88:4626-4630 (1991), adenosine deaminase promoter, phosphoglycerol kinase (PGK) promoter, pyruvate kinase promoter phosphoglycerol mutase promoter, the β-actin promoter (Lai et al., Proc. Natl. Acad. Sci. USA 86: 10006-10010 (1989»), the long terminal repeats (LTR) of Moloney Leukemia Virus and other retroviruses, the thymidine kinase promoter of Herpes Simplex Virus and other constitutive promoters known to those of skill in the art.

Inducible promoters suitable for controlling expression of the therapeutic product include promoters responsive to exogenous agents (e.g., pharmacological agents) or to physiological cues. These response elements include, but are not limited to a hypoxia response element (HRE) that binds HIF-Iα and *β,* tetracycline response element (such as described by Gossen & Bujard (1992, Proc. Natl. Acad. Sci. USA 89:5547-551); an ecdysone-inducible response element (No D et al., 1996, Proc. Natl. Acad. Sci. USA. 93 :3346-3351) a metal-ion response element such as described by Mayo et al. (1982, Cell

29:99-108); Brinster et al. (1982, Nature 296:39-42) and Searle et al. (1985, Mol. Cell. Biol. 5:1480-1489); a heat shock response element such as described by Nouer et al. (in: Heat Shock Response, ed. Nouer, L., CRC, Boca Raton, Fla., ppI67-220, 1991); or a hormone response element such as described by Lee et al. (1981, Nature 294:228-232); Hynes et al. (Proc. Natl. Acad. Sci. USA 78:2038-2042, 1981); Klock et al. (Nature 329:734-736, 1987); and Israel and Kaufman (1989, Nucl. Acids Res. 17:2589-2604) and other inducible promoters known in the art. Preferably the response element is an ecdysone-inducible response element, more preferably the response element is a tetracycline response element.

Examples of tissue-specific promoters suitable for use in the present invention include, but are not limited to those listed in Table 1 and other tissue-specific promoters known in the art.

Table 1: Tissue-specific promoters

Tissue	**Promoter**
Liver	TBG,A1AT
Heart	Troponin T (TnT)
Lung	CC10, SPC, FoxJ1
Central Nervous System/Brain	Synapsin, Tyrosine Hydroxylase, CaMKII (Ca2+/calmodulin-dependent protein kinase)
Pancreas	Insulin, Elastase-I
Adipocyte	Ap2, Adiponectin
Muscle	Desmin,MHC
Endothelial cells	Endothelin-I (ET -I), Flt-I
Retina	VMD

For example, and not by way of limitation, the replication-defective virus compositions of the invention can be used to deliver a VEGF antagonist for treating accelerated macular degeneration in a human subject; Factor VIII for treating hemophilia A in a human subject; Factor IX for treating hemophilia B in a human subject; insulin like

growth factor (IGF) or hepatocyte growth factor (HGF) for treating congestive heart failure in a human subject; nerve growth factor (NGF) for treating a central nervous system disorder in a human subject; or a neutralizing antibody against HIV for treating HIV infection in a human subject.

Still other useful therapeutic products include hormones and growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor α superfamily, including TGFα, activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregluin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

Other useful transgene products include proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-25 (including, *e.g.*, IL-2, IL-4, IL-12 and IL-18), monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors α and β, interferons α, β, and γ, stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the invention. These include, without limitations, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors, class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement regulatory proteins such as complement regulatory

proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

Still other useful gene products include any one of the receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. The invention encompasses receptors for cholesterol regulation and/or lipid modulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and scavenger receptors. The invention also encompasses gene products such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful gene products include transcription factors such as *jun*, *fos*, max, mad, serum response factor (SRF), AP-1, AP2, *myb*, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, *e.g.*, GATA-3, and the forkhead family of winged helix proteins.

Other useful gene products include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin gene product [*e.g.*, a mini- or micro-dystrophin]. Still other useful gene products include enzymes such as may be useful in enzyme replacement therapy, which is useful in a variety of conditions resulting from deficient activity of enzyme. For example, enzymes that contain mannose-6-phosphate may be utilized in therapies for lysosomal storage diseases (*e.g.*, a suitable gene includes that encoding β-glucuronidase (GUSB)).

5.1.2. Ablation Unit

The viral genome(s) of one or more replication-defective viruses used in the

PITA system are engineered to further contain an ablation unit or coding sequences for an ablator, as defined here.

For permanent shut down of transgene expression, the ablator can be an endonuclease, including but not limited to a recombinase, a meganuclease, a zinc finger endonuclease or any restriction enzyme with a restriction site that rarely occurs in the human genome, that binds to the ARS of the transgene unit and ablates or excises the transgene. Examples of such ablators include, but are not limited to the Cre/loxP system (Groth *et al.,* 2000, Proc. Natl. Acad. Sci. USA 97,5995-6000); the FLP/FRT system (Sorrell *et al.,* 2005, Biotechnol. Adv. 23, 431-469); meganucleases such as I-SceI which recognizes a specific asymmetric 18bp element (T AGGGAT AACAGGGT AAT (SEQ ID NO: 25)), a rare sequence in the mammalian genome, and creates double strand breaks (Jasin, M., 1996, Trends Genet., 12,224-228); and artificial restriction enzymes (e.g., a zinc finger nucleases generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain that can be engineered to target ARS sequences unique to the mammalian genome (Miller *et al.,* 2008, Proc. Natl. Acad. Sci. USA, 105: 5809-5814)). In one embodiment, the ablator is a chimeric enzyme, which may be based on a homodimer or a heterodimer fusion protein.

Where temporary shutdown of the transgene is desired, an ablator should be chosen that binds to the ARRS of the RNA transcript of the transgene unit and ablates the transcript, or inhibits its translation. Examples of such ablators include, but are not limited to interfering RNAs (RNAi), ribozymes such as riboswitch (Bayer *et al.,* 2005, Nat Biotechnol. 23(3):337-43), or antisense oligonucleotides that recognize an ARRS. RNAi, ribozymes, and antisense oligonucleotides that recognize an ARRS can be designed and constructed using any method known to those of skill in the art. This system is particularly desirable if the therapeutic transgene is administered to treat cancer or to mediate host immune response.

In one embodiment, expression of the ablator must be controlled by an inducible promoter that provides tight control over the transcription of the ablator gene *e.g.,* a pharmacological agent, or transcription factors activated by a pharmacological agent or in alternative embodiments, physiological cues. Promoter systems that are non-leaky and that can be tightly controlled are preferred. Inducible promoters suitable for controlling

expression of the ablator are *e.g.,* response elements including but not limited to a tetracycline (tet) response element (such as described by Gossen & Bujard (1992, Proc. Natl. Acad. Sci. USA 89:5547-551); an ecdysone-inducible response element (No D et al., 1996, Proc. Natl. Acad. Sci. USA. 93:3346-3351) a metal-ion response element such as described by Mayo et al. (1982, Cell. 29:99-108); Brinster et al. (1982, Nature 296:39-42) and Searle et al. (1985, Mol. Cell. Biol. 5: 1480-1489); a heat shock response element such as described by Nouer et al. *(in:* Heat Shock Response, ed. Nouer, L., CRC, Boca Raton, Fla., ppl67-220, 1991); or a hormone response element such as described by Lee et al. (1981, Nature 294:228-232); Hynes et al. (1981, Proc. Natl. Acad. Sci. USA 78:2038-2042); Klock et al. (1987, Nature 329:734-736); and Israel & Kaufman (1989, Nucl. Acids Res. 17:2589-2604) and other inducible promoters known in the art. Using such promoters, expression of the ablator can be controlled, for example, by the Tet-on/off system (Gossen et al., 1995, Science 268:1766-9; Gossen et al., 1992, Proc. Nati. Acad. Sci. USA., 89(12):5547-51); the TetR-KRAB system (Urrutia R., 2003, Genome Bioi., 4(10):231; Deuschle U et al., 1995, Mol Cell Biol. (4):1907-14); the mifepristone (RU486) regulatable system (Geneswitch; Wang Y et al., 1994, Proc. Natl. Acad. Sci. USA., 91(17):8180-4; Schillinger et al., 2005, Proc. Natl. Acad. Sci. U S A.102(39):13789-94); the humanized tamoxifen-dep regulatable system (Roscilli et al., 2002, Mol. Ther. 6(5):653-63); and the ecdysone-dep regulatable system (Rheoswitch; Karns et al., 2001, BMC Biotechnol. 1: 11; Palli et al., 2003, Eur J Biochem. 270(6):1308-15) to name but a few.

A chimeric enzyme may be controlled by a constitutive or an inducible promoter. In one embodiment, the system utilizes a chimeric endonuclease, wherein the nuclease has at least two domains, *i.e.*, a catalytic domain and a sequence specific DNA binding domain, each of which are expressed under separately controlled promoters and which are operatively linked. When the two domains are expressed at the same time, the products of the two domains form a chimeric endonuclease. Typically, separate transcription units containing each of domains linked to a DNA binding domain are provided. Such DNA binding domains include, for example, zinc finger motifs, homeo domain motifs, HMG-box domains, STAT proteins, B3, helix-loop-helix, winged helix-turn-helix, leucine zipper, helix-turn-helix, winged helix, POU domains, DNA binding domains of repressors, DNA binding

domains of oncogenes and naturally occurring sequence-specific DNA binding proteins that recognize >6 base pairs. [US 5,436,150, issued July 25, 1995].

In one embodiment, the expression of the ablator is under the control of an inducible promoter that is regulated by the dimerizable transcription factor domains described in Section 5.1.3. An example of such an inducible promoter includes, but is not limited to a GAL4 binding site minimum promoter, which is responsive to a GAL4 transcription factor. A GAL4 DNA binding domain or transactivation domain can also be fused to a steroid receptor, such as the ecdysone receptor (EcR). Still other suitable inducible promoters, such as are described herein, may be selected.

5.1.3. Dimerizable Transcription Factor Domain Unit

In one embodiment, the PITA system is designed such that the viral genome(s) of the replication-defective viruses are further engineered to contain a dimerizable units which are heterodimer fusion proteins. These units may be a dimerizable TF unit as defined herein or another dimerizable fusion protein unit (*e.g.,* part of a chimeric enzyme). In such an instance, a dimerizer is used (see Section 5.1.4), which binds to the dimerizer binding domains and dimerizes (reversibly cross-links) the DNA binding domain fusion protein and the activation domain fusion protein, forming a bifunctional transcription factor. See, *e.g.,* the Ariad ARGENT™ system, which is described in U.S. Publication No. 2002/0173474, U.S. Publication No. 200910100535, U.S. Patent No. 5,834,266, U.S. Patent No. 7,109,317, U.S. Patent No. 7,485,441, U.S.Patent No. 5,830,462, U.S. Patent No. 5,869,337, U.S. Patent No. 5,871,753, U.S. Patent No. 6,011,018, U.S. Patent No. 6,043,082, U.S. Patent No. 6,046,047, U.S. Patent No. 6,063,625, U.S. Patent No. 6,140,120, U.S. Patent No. 6,165,787, U.S. Patent No. 6,972,193, U.S. Patent No. 6,326,166, U.S. Patent No. 7,008,780, U.S. Patent No. 6,133,456, U.S. Patent No. 6,150,527, U.S. Patent No. 6,506,379, U.S. Patent No. 6,258,823, U.S. Patent No. 6,693,189, U.S. Patent No. 6,127,521, U.S. Patent No. 6,150,137, U.S. Patent No. 6,464,974, U.S. Patent No. 6,509,152, U.S. Patent No. 6,015,709, U.S. Patent No. 6,117,680, U.S. Patent No. 6,479,653, U.S. Patent No. 6,187,757, U.S. Patent No. 6,649,595, U.S. Patent No. 6,984,635, U.S. Patent No. 7,067,526, U.S. Patent No. 7,196,192, U.S. Patent No. 6,476,200, U.S. Patent No. 6,492,106, WO 94/18347, WO 96/20951, WO 96/06097, WO 97/31898, WO 96/41865, WO 98/02441, WO 95/33052, WO 99110508, WO 99110510, WO 99/36553, WO 99/41258,WO 01114387, ARGENT™ Regulated

Transcription Retrovirus Kit, Version 2.0 (9109102), and ARGENT™ Regulated Transcription Plasmid Kit, Version 2.0 (9109/02), each of which is incorporated herein by reference in its entirety.

In one embodiment, by delivering a dimerizable unit, target cells are modified to co-express two fusion proteins that are dimerized by the pharmacologic agent used: one containing a DNA-binding domain (DBD) of the transcription factor that binds the inducible promoter controlling the ablator and the other containing a transcriptional activation domain (AD) of the transcription factor that activates the inducible promoter controlling the ablator, each fused to dimerizer binding domains. Expression of the two fusion proteins may be constitutive, or as an added safety feature, inducible. Where an inducible promoter is selected for expression of one of the fusion proteins, the promoter may regulatable, but different from any other inducible or regulatable promoters in the viral composition. Addition of a pharmacological agent, or "dimerizer" (described in Section 5 .1.4) that can simultaneously interact with the dimerizer binding domains present in both fusion proteins results in recruitment of the AD fusion protein to the regulated promoter, initiating transcription of the ablator. By using dimerizer binding domains that have no affinity for one another in the absence of ligand and an appropriate minimal promoter, transcription is made absolutely dependent on the addition of the dimerizer. Suitably, a replication-defective virus composition of the invention may contain more than one dimerizable domain. The various replication-defective viruses in a composition may be of different stock, which provide different transcription units (*e.g.*, a fusion protein to form a dimerable unit in situ) and/or additional ablators.

Fusion proteins containing one or more transcription factor domains are disclosed in WO 94/18317, PCT/US94/08008, Spencer et al, supra and Blau et al. (PNAS 1997 94:3076) which are incorporated by reference herein in their entireties. The design and use of such fusion proteins for ligand-mediated gene-knock out and for ligand-mediated blockade of gene expression or inhibition of gene product function are disclosed in PCT/US95/10591. Novel DNA binding domains and DNA sequences to which they bind which are useful in embodiments involving regulated transcription of a target gene are disclosed, *e.g.*, in Pomeranz et al, 1995, Science 267:93 96. Those references provide substantial information, guidance and examples relating to the design, construction and use

of DNA constructs encoding analogous fusion proteins, target gene constructs, and other aspects which may also be useful to the practitioner of the subject invention.

Preferably the DNA binding domain, and a fusion protein containing it, binds to its recognized DNA sequence with sufficient selectivity so that binding to the selected DNA sequence can be detected (directly or indirectly as measured *in vitro)* despite the presence of other, often numerous other, DNA sequences. Preferably, binding of the fusion protein comprising the DNA-binding domain to the selected DNA sequence is at least two, more preferably three and even more preferably more than four orders of magnitude greater than binding to anyone alternative DNA sequence, as measured by binding studies *in vitro* or by measuring relative rates or levels of transcription of genes associated with the selected DNA sequence as compared to any alternative DNA sequences. The dimerizable transcription factor (TF) domain units of the invention can encode DNA binding domains and activation domains of any transcription factor known in the art. Examples of such transcription factors include but are not limited to GAL4, ZFHD1, VPI6, and NF-KB (p65).

The dimerizer binding domain encoded by a dimerizable unit of the invention can be any dimerizer binding domain described in U.S. Publication No. 2002/0173474, U.S. Publication No. 200910100535, U.S. Patent No. 5,834,266, U.S. Patent No. 7,109,317, U.S. Patent No. 7,485,441, U.S. Patent No. 5,830,462, U.S. Patent No. 5,869,337, U.S. Patent No. 5,871,753, U.S. Patent No. 6,011,018, U.S. Patent No. 6,043,082, U.S. Patent No. 6,046,047, U.S. Patent No. 6,063,625, U.S. Patent No. 6,140,120, U.S. Patent No. 6,165,787, U.S. Patent No. 6,972,193, U.S. Patent No. 6,326,166, U.S. Patent No. 7,008,780, U.S. Patent No. 6,133,456, U.S. Patent No. 6,150,527, U.S. Patent No. 6,506,379, U.S. Patent No. 6,258,823, U.S. Patent No. 6,693,189, U.S. Patent No. 6,127,521, U.S. Patent No. 6,150,137, U.S. Patent No. 6,464,974, U.S. Patent No. 6,509,152, U.S. Patent No. 6,015,709, U.S. Patent No. 6,117,680, U.S. Patent No. 6,479,653, U.S. Patent No. 6,187,757, U.S. Patent No. 6,649,595, U.S. Patent No. 6,984,635, U.S. Patent No. 7,067,526, U.S. Patent No. 7,196,192, U.S. Patent No. 6,476,200, U.S. Patent No. 6,492,106, WO 94118347, WO 96/20951, WO 96/06097, WO 97/31898, WO 96/41865, WO 98/02441, WO 95/33052, WO 99/10508, WO 99110510, WO 99/36553, WO 99/41258, WO 01114387, ARGENT™ Regulated Transcription Retrovirus Kit, Version 2.0 (9/09/02), and ARGENT™ Regulated

Transcription Plasmid Kit, Version 2.0 (9/09/02), each of which is incorporated herein by reference in its entirety.

A dimerizer binding domain that can be used in the PITA system is the immunophilin FKBP (FK506-binding protein). FKBP is an abundant 12 kDa cytoplasmic protein that acts as the intracellular receptor for the immunosuppressive drugs FK506 and rapamycin. Regulated transcription can be achieved by fusing multiple copies of FKBP to a DNA binding domain of a transcription factor and an activation domain of a transcription factor, followed by the addition of FK1012 (a homodimer ofFK506; Ho, S.N., et al., 1996, Nature, 382(6594): 822-6); or simpler synthetic analogs such as AP1510 (Amara, J.F., et aI., 1997, Proc. Natl. Acad. Sci. USA, 94(20): 10618-23). The potency of these systems can be improved by using synthetic dimerizers, such as AP1889, with designed 'bumps' that minimize interactions with endogenous FKBP (Pollock *et al.,* 1999, Methods Enzymol, 1999.306: p. 263-81). Improved approaches based on heterodimerization, exploiting the discovery that FK506 and rapamycin naturally function by bringing together FKBP with a second target protein. This allows the natural products themselves, or analogs thereof, to be used directly as dimerizers to control gene expression.

The structure of FKBP-FK506 complexed to calcineurin phosphatase (Griffith et aI., Cell, 82:507 522, 1995) has been reported. Calcineurin A (residues 12 394) was shown to be effective as a dimerizer binding domain using a three hybrid system in yeast using three FKBPs fused to Gal4 and residues 12 to 394 of murine calcineurin A fused C-terminally to the Gal4 activation domain (Ho, 1996 Nature. 382:822 826). Addition of FK506 activated transcription of a reporter gene in these cells. A "minimal" calcineurin domain termed a CAB, which is a smaller, more manipulatable domain can be used as a dimerizer binding domain.

The DNA binding domain fusion protein and activation domain fusion protein encoded by the dimerizable fusion protein units of the invention may contain one or more copies of one or more different dimerizer binding domains. The dimerizer binding domains may be N-terminal, C-terminal, or interspersed with respect to the DNA binding domain and activation domain. Embodiments involving multiple copies of a dimerizer binding domains usually have 2, 3 or 4 such copies. The various domains of the fusion proteins are optionally

separated by linking peptide regions which may be derived from one of the adjacent domains or may be heterologous.

As used herein, the term "variants" in the context of variants of dimerizer binding domains refers to dimerizer binding domains that contain deletions, insertions, substitutions, or other modifications relative to native dimerizer binding domains, but that retain their specificity to bind to dimerizers. The variants of dimerizer binding domains preferably have deletions, insertions, substitutions, and/or other modifications of not more than 10,9,8, 7, 6, 5,4,3,2, or 1 amino acid residues. In a specific embodiment, the variant of a dimerizer binding domain has the native sequence of a dimerizer binding domain as specified above, except that 1 to 5 amino acids are added or deleted from the carboxy and or the amino end of the dimerizer binding domains (where the added amino acids are the flanking amino acid(s) present in the native dimerizer binding domains).

In order to conserve space within the viral genome(s), bicistronic transcription units can be engineered. For example, the third and fourth transcription units can be engineered as a bicistronic unit containing an IRES (internal ribosome entry site), which allows coexpression of heterologous gene products by a message from a single promoter. Altenatively, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three heterologous genes (e.g., the third and fourth transcription units) separated from one another by sequences encoding a self-cleavage peptide *(e.g.,* T2A) or a protease recognition site *(e.g.,* furin). The ORF thus encodes a single polyprotein, which, either during (in the case ofT2A) or after translation, is cleaved into the individual proteins. It should be noted, however, that although these IRES and polyprotein systems can be used to save AAV packaging space, they can only be used for expression of components that can be driven by the same promoter.

As illustrated in the examples below, various components of the invention may include:

ITR: inverted terminal repeats (ITR) of AAV serotype 2 (168 bp). In one embodiment, the AAV2 ITRs are selected to generate a pseudotyped AAV, i.e., an AAV having a capsid from a different AAV than that the AAV from which the ITRs are derived.

CMV: full cytomegalovirus (CMV) promoter; including enhancer. CMV: minimal CMV promoter, not including enhancer. In one embodiment, the human CMV promoter and/or enhancer are selected.

FRB-TA fusion: fusion of dimerizer binding domain and an activation domain of a transcription factor. The FRB fragment corresponds to amino acids 2021-2113 of FRAP (FKBP rapamycin-associated protein, also known as mTOR [mammalian target of rapamycin]), a phosphoinositide 3-kinase homolog that controls cell growth and division. The FRAP sequence incorporates the single point-mutation Thr2098Leu ($FRAP_L$) to allow use of certain non-immunosuppressive rapamycin analogs (rapalogs). FRAP binds to rapamycin (or its analogs) and FKBP and is fused to a portion of human NF-KB p65 (190 amino acids) as transcription activator.

ZFHD-FKBP fusion: fusion of a DNA binding domain and 1 copy of a Dimerizer binding domain, 2 copies of drug binding domain (2xFKBP, or 3 (3xFKBP) copies of drug binding domain. Immunophilin FKBP (FK506-binding protein) is an abundant 12 kDa cytoplasmic protein that acts as the intracellular receptor for the immunosuppressive drugs FK506 and rapamycin. ZFHD is DNA binding domains composed of a zinc finger pair and a homeodomain. In another alternative, various other copy numbers of a selected drug binding domain may be selected. Such fusion proteins may contain N-terminal nuclear localization sequence from human c-Myc at the 5' and/or 3' end.

Z8I: contains 8 copies of the binding site for ZFHD (Z8) followed by minimal promoter from the human interleukin-2 (lL-2) gene (SEQ ID NO: 32). Variants of this may be used, *e.g.*, which contain from 1 to about 20 copies of the binding site for ZFHD followed by a promoter, *e.g.*, the minimal promoter from IL-2 or another selected promoter.

Cre: Cre recombinase. Cre is a type I topoisomerase isolated from bacteriophage P1. Cre mediates site specific recombination in DNA between two loxP sites leading to deletion or gene conversion (1029 bp, SEQ ID NO: 33).

I-SceI: a member of intron endonuclease or homing endonuclease which is a large class of meganuclease (708 bp, SEQ ID NO: 34). They are encoded by mobile genetic elements such as introns found in bacteria and plants. I-SceI is a yeast endonuclease involved in an intron homing process. I-SceI recognizes a specific asymmetric 18bp element, a rare

sequence in mammalian genome, and creates double strand breaks. See, Jasin, M. (1996) Trends Genet., 12,224-228 .

hGH poly A: minimal poly adenylation signal from human GH (SEQ ID NO: 35).

IRES: internal ribosome entry site sequence from ECMV (encephalomyocarditis virus) (SEQ ID NO: 36).

5.1.4. Dimerizers and Pharmacologic Agents

As used herein, the term "dimerizer" is a compound that can bind to dimerizer binding domains of the TF domain fusion proteins (described in Section 5.1.3) and induce dimerization of the fusion proteins. Any pharmacological agent that dimerizes the domains of the transcription factor, as assayed *in vitro* can be used. Preferably, rapamycin and its analogs referred to as "rapalogs" can be used. Any of the dimerizers described in following can be used: U.S. Publication No. 2002/0173474, U.S. Publication No. 2009/0100535, U.S. Patent No. 5,834,266, U.S. Patent No. 7,109,317, U.S. Patent No. 7,485,441, U.S. Patent No. 5,830,462, U.S. Patent No. 5,869,337, U.S. Patent No. 5,871,753, U.S. Patent No. 6,011,018, U.S. Patent No. 6,043,082, U.S. Patent No. 6,046,047, U.S. Patent No. 6,063,625, U.S. Patent No. 6,140,120, U.S. Patent No. 6,165,787, U.S. Patent No. 6,972,193, U.S. Patent No. 6,326,166, U.S. Patent No. 7,008,780, U.S. Patent No. 6,133,456, U.S. Patent No. 6,150,527, U.S. Patent No. 6,506,379, U.S. Patent No. 6,258,823, U.S. Patent No. 6,693,189, U.S. Patent No. 6,127,521, U.S. Patent No. 6,150,137, U.S. Patent No. 6,464,974, U.S. Patent No. 6,509,152, U.S. Patent No. 6,015,709, U.S. Patent No. 6,117,680, U.S. Patent No. 6,479,653, U.S. Patent No. 6,187,757, U.S. Patent No. 6,649,595, U.S. Patent No. 6,984,635, U.S. Patent No. 7,067,526, U.S. Patent No. 7,196,192, U.S. Patent No. 6,476,200, U.S. Patent No. 6,492,106, WO 94118347, WO 96/20951, WO 96/06097, WO 97/31898, WO 96/41865, WO 98/02441, WO 95/33052, WO 99/10508, WO 99/10510, WO 99/36553, WO 99/41258, WO 01114387, ARGENT™ Regulated Transcription Retrovirus Kit, Version 2.0 (9109/02), and ARGENT™ Regulated Transcription Plasmid Kit, Version 2.0 (9/09/02), each of which is incorporated herein by reference in its entirety.

Examples of dimerizers that can be used in the present invention include, but are not limited to rapamycin, FK506, FKI012 (a homodimer of FK506), rapamycin analogs ("rapalogs") which are readily prepared by chemical modifications of the natural product to add a "bump" that reduces or eliminates affinity for endogenous FKBP and/or FRAP.

Examples of rapalogs include, but are not limited to such as AP26113 (Ariad), AP1510 (Amara, J.F., et al.,1997, Proc Natl Acad Sci USA, 94(20): 10618-23) AP22660, AP22594, AP21370, AP22594, AP23054, AP1855, AP1856, AP1701, AP1861, AP1692 and AP1889, with designed 'bumps' that minimize interactions with endogenous FKBP.

Other dimerizers capable of binding to dimerizer binding domains or to other endogenous constituents may be readily identified using a variety of approaches, including phage display and other biological approaches for identifying peptidyl binding compounds; synthetic diversity or combinatorial approaches (see e.g. Gordon et al, 1994, J Med Chern 37(9):1233-1251 and 37(10):1385-1401); and DeWitt et al, 1993, PNAS USA 90:6909-6913) and conventional screening or synthetic programs. Dimerizers capable of binding to dimerizer binding domains of interest may be identified by various methods of affinity purification or by direct or competitive binding assays, including assays involving the binding of the protein to compounds immobilized on solid supports such as pins, beads, chips, etc.). See e.g. Gordon *et al.,* supra.

Generally speaking, the dimerizer is capable of binding to two (or more) protein molecules, in either order or simultaneously, preferably with a Kd value below about 10^{-6} more preferably below about 10^{-7}, even more preferably below about 10^{-8}, and in some embodiments below about 10^{-9} M. The dimerizer preferably is a non-protein and has a molecular weight of less than about 5 kDa. The proteins so oligomerized may be the same or different.

Various dimerizers are hydrophobic or can be made so by appropriate modification with lipophilic groups. Particularly, dimerizers containing linking moieties can be modified to enhance lipophilicity by including one or more aliphatic side chains of from about 12 to 24 carbon atoms in the linker moiety.

5.1.5. Generating Replication-Defective Virus Compositions

Any virus suitable for gene transfer *(e.g.,* gene therapy) may be used for packaging the transcription units into one or more stocks of replication-defective virus, including but not limited to adeno-associated virus ("AAV"); adenovirus; alphavirus; herpesvirus; retrovirus *(e.g.,* lentivirus); vaccinia virus; *etc.* Methods well known in the art for packaging foreign genes into replication-defective viruses can be used to prepare the replication-defective viruses containing the therapeutic transgene unit, the ablation unit, and

optionally (but preferably) the dimerizable transcription factor domain unit. *See,* for example, Gray & Samulski, 2008, "Optimizing gene delivery vectors for the treatment of heart disease," *Expert Opin. Biol. Ther.* 8:911-922; Murphy & High, 2008, "Gene therapy for haemophilia," Br. J. Haematology 140:479-487; Hu, 2008, "Baculoviral vectors for gene delivery: A review," Current Gene Therapy 8:54-65; Gomez *et al.,* 2008, "The poxvirus vectors MV A and NYV AC as gene delivery systems for vaccination against infectious diseases and cancer," Current Gene Therapy 8:97-120.

In preferred embodiments, the replication-deficient virus compositions for therapeutic use are generated using an AAV. Methods for generating and isolating AAVs suitable for gene therapy are known in the art. *See* generally, *e.g.,* Grieger & Samulski, 2005, "Adeno-associated virus as a gene therapy vector: Vector development, production and clinical applications," *Adv. Biochem. Engin/Biotechnol.* 99: 119-145; Buning *et al.,* 2008, "Recent developments in adeno-associated virus vector technology," *J. Gene Med.* 10:717-733; and the references cited below, each of which is incorporated herein by reference in its entirety.

Adeno-associated virus (genus Dependovirus, family Parvoviridae) is a small (approximately 20-26 nm), non-enveloped single-stranded (ss) DNA virus that infects humans and other primates. Adeno-associated virus is not currently known to cause disease. Adeno-associated virus can infect both dividing and non-dividing cells. In the absence of functional helper virus (for example, adenovirus or herpesvirus) AAV is replication-defective. Adeno-associated viruses form episomal concatamers in the host cell nucleus. In non-dividing cells, these concatamers remain intact for the life of the host cell. In dividing cells, AAV DNA is lost through cell division, since the episomal DNA is not replicated along with the host cell DNA. However, AAV DNA may also integrate at low levels into the host genome.

The AAV genome is built of a ssDNA, either positive- or negative-sense, which is about 4.7 kilobases long. The genome of AAV as it occurs in nature comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames (ORFs): rep and cap. The former is composed of four overlapping genes encoding the Rep proteins that are required for the AAV life cycle, and the latter contains overlapping sequences that encode the capsid proteins (Cap): VP1, VP2, and VP3, which interact to

form a capsid of an icosahedral symmetry.

The ITRs are 145 bases each, and form a hairpin that contributes to so-called "self-priming" that allows primase-independent synthesis of the second DNA strand. The ITRs also appear to be required for AAV DNA integration into the host cell genome *(e.g.,* into the 19th chromosome in humans) and rescue from it, as well as for efficient encapsidation of the AAV DNA and assembly of AAV particles.

For packaging a transgene into virions, the ITRs are the only AAV components required in *cis* in the same construct as the transgene. The cap and rep genes can be supplied in *trans.* Accordingly, DNA constructs can be designed so that the AAV ITRs flank one or more of the transcription units *(i.e.,* the transgene unit, the ablator unit, and the dimerizable transcription factor unit), thus defining the region to be amplified and packaged - the only design constraint being the upper limit of the size of the DNA to be packaged (approximately 4.5 kb). Adeno-associated virus engineering and design choices that can be used to save space are described below.

Compositions and System utilizing 10x Zinc Finger Constructs

The invention provides a method for vector-mediated delivery of a therapeutic product having a controlled gene expression ablation system. For convenience, the method is described in the context of an AAV vector. However, one of skill in the art can select another suitable vector (*e.g.*, a replication-defective adenovirus, replication-defective lentivirus, or other viral or genetic element) for use with the compositions and methods described herein. Examples of viral and other vectors are described earlier and incorporated herein by reference. In one embodiment, an AAV vector containing a nucleic acid molecule comprising a nucleic acid sequence encoding a therapeutic or vaccinal product (or another gene or sequence of interest) operably linked to a promoter that controls transcription and at least one endonuclease ablation site which comprises a sequence of at least 30 nucleic acid base pairs which are specifically and selectively recognized by a construct of at least ten (10X) zinc fingers. The at least one endonuclease ablation site is located at least 5' to the sequence encoding the gene of interest (*e.g.,* a transgene such as DNA, RNA, ribozyme, siRNA, shRNA, miRNA or protein, peptide, system's biology engineered pathways, *etc.*). The method further involves

use of at least one ablator which comprises a chimeric endonuclease comprising at least ten zinc fingers linked to a functional endonuclease catalytic domain in operative association with a promoter, wherein transcription and/or ablation activity is induced in response to a pharmacological agent. The at least ten (10X) zinc fingers specifically and selectively recognizes the at least about 30 base pair sequence in the at least one endonuclease ablation site and contains at least 10 independently selected recognition helices.

In one embodiment, the present invention utilizes chimeric endonucleases as a safety measure necessary to destroy a gene of interest in gene delivery applications *in vivo* [including gene therapy, vaccines *etc*], for example when a transgene product is no longer required or exhibits toxic/off target effects inside the organism. Thus, the chimeric endonucleases of the invention have one or more of the following features. They intentionally target sequences that are not present in mammalian (*e.g.,* human) genomes (thus the term the "at least 30bp unique sequence"), they have high *in vivo* selectivity to a target location only, and they have minimal off-target and cytotoxic effects. Due to the fact that the endonucleases described previously in the literature were used for a different purpose, *i.e.,* for *in situ* engineering of mammalian genomes to allow knock-out or correction (knock-in) of specific genes of interest, the inventors were required to develop a new methodology to design a chimeric endonuclease with the features required for the present invention, including designing long stretches, (*i.e.*, in excess of 7, 8, 9, 10, 11, 12, or more zinc fingers) and for selecting unique sequences that are not present within a target genome (*e.g.*, a human or other mammalian subject).

In one embodiment, the zinc finger proteins are engineered to specifically target a DNA sequence which is not part of the target genome in order to mitigate off-target effects and cytotoxicity associated with customarily used designs.

For example, for delivery to humans, random sequences of at least 32 base pair are generated in order to accommodate frame shifts, although smaller sequences (*e.g.,* at least 30 base pairs), could be selected. This size was selected because there are about $3.4x10^9$ base pairs in human genome. The number of all possible 15 base pair-long sequences with permutation of 4 nucleotides is 4^{15}, which is same as approximately 10^9. Which means that for any given DNA sequence which is equal to or less than 15 base pairs, an identical

subsequence within the human genome can be found. Conversely, for any short DNA sequence, there will be at least 15 positions that are identical to a piece of human DNA sequence. During initial assessment, no short sequences whose maximal sequence-identity with any human DNA sequence is less than 65% were identified.

Surprisingly, the first unique sequence randomly generated for use as an ablation recognition site and tested was found to meet the desired parameters: SEQ ID NO: 806-GGTCGATGTTCGCAACGTCGATCGTACGTGCA. The examples below illustrate the sequence being engineered into an expression plasmid vector carrying the gene of interest and being specifically recognized by a chimeric endonuclease of the invention which comprises at least ten zinc fingers. The sequences encoding the ablation recognition site and the sequences of the chimeric endonuclease can be engineered into suitable viral vectors (*e.g.,* AAV vectors) using conventional techniques in order to obtain the AAV vectors, compositions and to perform the methods of the invention.

With reference to Figure 218, a streamlined method for selecting a unique sequence of the invention is provided. This involves randomly generate 2 million short DNA sequences and selecting those with a sequence identity of less than (<) 20% to one another. The resulting sequences are compared via BLAST using conventional parameters against the target genome (*e.g.,* human) and those with an E-value of greater than 100 are selected. [An E-value is an Expect value. The statistical significance threshold for reporting matches against database sequences; the default value is 10, meaning that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the Expect threshold, the match will not be reported. Lower Expect thresholds are more stringent, leading to fewer chance matches being reported. Increasing the threshold shows less stringent matches. Fractional values are acceptable.] An exemplary sequence is compared for similarity against the human genome with a slide window. [For sliding windows a window of 32 base pair slides over the nucleotides of the human genome. The window moves 1 nucleotide for each step. for every position of the window identity levels are calculated.] Any sequence with a similarity greater than 70% is discarded. If the sequence has a similarity of less than 70%, then it is analyzed against the target genome for the presence of any 8 consecutive base pairs

and any having 8 or more consecutive base pairs in common with the target sequence are discarded. The remainder may be selected for use in an ablation recognition site.

Thus, a unique sequence for an ablation recognition site of the invention, has less than 70% identity with any subsequence in the human genome, has no more than 8 adjacent identical positions with any subsequence in the human genome. A zinc finger is thereafter designed which specifically targets this sequence as described herein. The unique sequence can be read by different zinc finger designs which recognize different reading frames such that it is possible for two distinct zinc fingers to be specific for a single unique at least 32 bp sequence. The sequences may be 32, 33, 34, 35, 36, 38, 41, 44, or more sequences in length,

In addition to the unique sequence identified above, other unique sequences for use in the ablation recognition site were also generated:

SEQ ID NO: 801:SEQ ID NO: GGTCGGCGACGCGAATCGTCGATTGGCGTAC

SEQ ID NO: 802: SEQ ID NO: GGTCGGCGACGCGTATCGATTGGCGTAC

and

SEQ ID NO: 803: ACTATTCGCACGCCGTACGATAGTCGGCGCGA.

Once the specific ablation site sequence is selected, the ablator can be engineered to contain the zinc finger protein which will specifically recognize this unique at least 30 bp sequence within the ablation site of the vector carrying the gene of interest.

In one embodiment, a chimeric endonuclease of the invention is engineered to contain at least the catalytic domain of an endonuclease fused to a zinc finger protein, optionally via a linker. The catalytic domain may be located on the N-terminus of the zinc finger, the C-terminus of the zinc finger, or it may be located within the zinc finger (*i.e.,* the catalytic domain is flanked on both its N- and C-terminus by zinc finger modules). Optionally, nuclear localization signals are included at the N- or C-terminus of the chimeric endonuclease, or the N- or C-terminus of the zinc finger portion of protein. These elements are described elsewhere in the specification, which passages are incorporated herein by reference.

In one embodiment, the FokI catalytic domain is used without any other functional sequence of the FokI endonuclease, *i.e.,* the DNA binding domain is not present. Further, because of the manner in which the chimeric endonuclease described herein has been

engineered, the known FokI recognition site (5'-CATCC-3') is not required. FokI catalytic domain is a non-sequence specific endonuclease, once the ablator recognizes the ablation site, the location in the ablation recognition site where FokI cuts can be altered by the length of the optional linker sequence between the FokI catalytic domain and the zinc fingers.

For example, a linker sequence of five amino acids (*e.g.,* SEQ ID NO: 805, GTSGK) results in the FokI chimeric ablator cutting 6bp directly following the zinc finger binding site. [The linker length is defined using the convention set forth in Yuka Shimizu, *et al, Bioorg Med Chem Lett.* 2009 July 15; 19(14): 3970–3972]. Thus, the location of the cut made by the FokI chimeric ablator can be adjusted by increasing or decreasing the length of the linker. For example, other linkers for the chimeric ablator (amino acids of 0, 1, 2, 3, 4, 5 or 6 amino acids in length) may be used in combination with spacers of 6, 7, 8, 9, or longer on the ablation site.

The zinc finger is a modular protein which binds DNA in reverse orientation (*i.e.,* the N-terminus of the Zn finger binds starting at the 3' end of the sense strand). Typically, each module or finger uses amino acids in positions : -1, 3, and 6 of its α-helix to target a specific 3-bp recognition site on DNA (*i.e.,* a "triplet"). These helices are specific and selected for specific DNA triplets. Thus, for a 10X zinc finger protein, each of 10 zinc fingers (or modules) may be independently selected to target the unique triplets present in the at least 30 nucleic acid sequence of the invention.

The inventors have demonstrated specific and selective ablation according to the invention using a FokI catalytic domain fused to a 10x zinc finger protein specific for the unique sequence of the ablator. Given the description in the art of zinc fingers of significantly smaller sizes (2-3) and a recent publication that zinc fingers larger than 6x are not successful proteins [Yuka Shimizu, *et al,* Adding Fingers to an Engineered Zinc Finger Nuclease Can Reduce Activity, Biochemistry 2011, 50, 5033–5041] , this result was unexpected.

Once the size and sequence of the at least 30 base pair unique nucleic acid sequence of the ablation recognition site are known (*e.g.,* at least 30 base pairs where the vector is to be delivered to a human), one can engineer a zinc finger which provides for at least one module (1X) for each triplet. Each zinc finger contains a recognition helix specific for a given triplet, in which sequence of recognition helix is engineered to fold into a conserved

zinc finger structure in order to present the about seven amino acid sequence in the proper helical form. More particularly, applicants provide in the following table a selection of illustrative conserved sequences which may be used to construct a zinc finger. In this table, the seven dashes represent the location in which one of the seven amino acid helix recognition sequences may be inserted in order to construct a multi- (X) -zinc finger.

Table - Conserved Zinc Finger Sequences

	SEQ ID NO:
CRCNECGKSFS-------HQRTH	706
QFACDICGRKFA-------HTKIH	707
FACEVCGVRFT-------HMRKH	708
FACSWQDCNKKFA-------HYRTH	709
FECKDCGKAFI-------HQRTH	710
FHCGYCEKSFS-------HIRTH	711
FKCPVCGKAFR-------HQRTH	712
FLCQYCAQRFG-------HMKKSH	713
FQCKTCQRKFS-------HTRTH	714
FQCNQCGASFT-------HIKLH	715
FSCSWKGCERRFA-------HRRTH	716
GSQKPFQCRICMRNFS-------HIRTH	717
HKCLECGKCFS-------HQRTH	718
MAERPFQCRICMRKFA-------HTKIH	719
PGEKKFACPECPKRFM-------HIKTH	720
PGEKPFECKDCGKAFI-------HQRTH	721
PGEKPFKCPVCGKAFR-------HQRTH	722
PGEKPFMCTWSYCGKRFT------- HKRTH	723
PGEKPFQCKTCQRKFS-------HTRTH	724
PGEKPFQCNQCGASFT-------HIKLH	725
PGEKPHICHIQGCGKVYG-------HLRWH	726
PGEKPYECDHCGKAFS-------HRRIH	727
PGEKPYECDHCGKSFS-------HKRTH	728
PGEKPYECEKCGKAFN-------HKKSH	729
PGEKPYECHDCGKSFR-------HRRIH	730
PGEKPYECKECGKAFS-------HQRIH	731
PGEKPYECNYCGKTFS-------HQRIH	732

Table - Conserved Zinc Finger Sequences	
	SEQ ID NO:
PGEKPYGCHLCGKAFS-------HEMIH	733
PGEKPYICRKCGRGFS-------HQRTH	734
PGEKPYKCEECGKAFN-------HKIVH	735
PGEKPYKCEECGKAFR-------HKIIH	736
PGEKPYKCEECGKAFT-------HKKIH	737
PGEKPYKCGQCGKFYS-------HQKIH	738
PGEKPYKCHQCGKAFI-------HERTH	739
PGEKPYKCKECGKAFN-------HHRIH	740
PGEKPYKCKECGQAFR-------HHKLH	741
PGEKPYKCKQCGKAFG-------HGRTH	742
PGEKPYKCMECGKAFN-------HQRIH	743
PGEKPYKCPDCGKSFS-------HQRTH	744
PGEKPYKCPECGKSFS-------HQRTH	745
PGEKPYMCSECGRGFS-------HQRTH	746
PGEKPYRCEECGKAFR-------HKRIH	747
PGEKPYRCKYCDRSFS-------HVRNIH	748
PGEKPYTCKQCGKAFS-------HETTH	749
PGEKPYTCSDCGKAFR-------HRRTH	750
PGEKPYVCDVEGCTWKFA-------HKKRH	751
PGEKPYVCRECGRGFR-------HKRTH	752
PGEKPYVCSKCGKAFT-------HQKIH	753
PGERPFMCTWSYCGKRFT-------HKRTH	754
TGEKPFACDICGKKFA-------HTKIH	755
TGEKPFACDICGRKFA-------HTKIH	756
TGEKPFQCRICMRNFS-------HIRTH	757
TGSQKPFQCRICMRNFS-------HIRTH	758
VPERPFQCQICMRNFS-------HIRTH	759
YACHLCAKAFI-------HEKTH	760
YACHLCGKAFT-------HEKTH	761
YECDHCGKAFS-------HRRIH	762
YECDHCGKSFS-------HKRTH	763
YECDVCGKTFT-------HQRTH	764
YECEKCGKAFN-------HKKSH	765
YECHDCGKSFR-------HRRIH	766

Table - Conserved Zinc Finger Sequences	
	SEQ ID NO:
YECKECGKAFS-------HQRIH	767
YECNECGKAFA-------HQRIH	768
YECNECGKFFS-------HRRSH	769
YECNTCRKTFS-------HQRTH	770
YECQDCGRAFN-------HKRTH	771
YECVQCGKGFT-------HQRVH	772
YECVQCGKSYS-------HQRRH	773
YGCHLCGKAFS-------HEMIH	774
YHCDWDGCGWKFA-------HYRKH	775
YICRKCGRGFS-------HQRTH	776
YKCDECGKNFT-------HKRIH	777
YKCEECGKAFN-------HKIVH	778
YKCEECGKAFR-------HKIIH	779
YKCEECGKAFT-------HKKIH	780
YKCGQCGKFYS-------HQKIH	781
YKCHQCGKAFI-------HERTH	782
YKCKECGKAFN-------HHRIH	783
YKCKECGQAFR-------HHKLH	784
YKCKQCGKAFG-------HGRTH	785
YKCMECGKAFN-------HQRIH	786
YKCPDCGKSFS-------HQRTH	787
YMCSECGRGFS-------HQRTH	788
YQCNICGKCFS-------HQRTH	789
YRCEECGKAFR-------HKRIH	790
YRCKYCDRSFS-------HVRNIH	791
YRCSWEGCEWRFA-------HFRKH	792
YSCGICGKSFS-------HCILH	793
YTCKQCGKAFS-------HETTH	794
YTCSDCGKAFR-------HRRTH	795
YTCSYCGKSFT-------HTRIH	796
YVCDVEGCTWKFA-------HKKRH	797
YVCRECGRGFR-------HKRTH	798
YVCRECRRGFS-------HQRTH	799
YVCSKCGKAFT-------HQKIH	800
TGEKPFQCRICMRNFS------- HLRTH	807

An at least 10x zinc finger, or a zinc finger of another length (e.g., selected for a non-human or other application), may contain the zinc finger modules in which each of the recognition helices is inserted into the same conserved sequence. In another embodiment, an ablator may contain zinc finger modules with contain recognition helices inserted into different conserved sequences. In the examples herein, the conserved sequence of (N- terminus) - PGEKPYKCPECGKSFS - XXXXXXX - HQRTH (carboxy terminus), COOH [SEQ ID NO: 745] was used or (N- terminus) - TGEKPFQCRICMRNFS - XXXXXXX - HLRTH (carboxy terminus) - COOH, wherein XXXXXXX [SEQ ID NO: 807] is the zinc finger recognition helix where used. However, the invention is not limited to these sequences.

According to the invention, in one embodiment, the at least 30 nucleic acid sequence may be 32 base pairs in length in order to accommodation alternate reading frames for the zinc finger.

Depending upon the sequence of the unique nucleic acid sequence of the ablation site, specific zinc finger helices are selected for insertion into the zinc finger conserved sequence. In some embodiments, the unique nucleic acid sequence of the ablation site may contain more than one of the same three base pair triplet. In this instance, one may select the same recognition helix or a different recognition helix.

In one embodiment, the zinc finger for the at least 30 base pair sequence : SEQ ID NO: 806: GGTCGATGTTCGCAACGTCGATCGTACGTGCA was engineered, so that the zinc finger comprises a nucleic acid sequence encoding at least ten zinc fingers consisting of : (a) a first N-terminal zinc finger comprising a recognition helix which specifically binds to TGC; (b) second zinc finger comprising a recognition helix which specifically binds to ACG; (c) a third zinc finger comprising a recognition helix which specifically binds to CGT; (d) a fourth zinc finger comprising a recognition helix which specifically binds to GAT; (e) a fifth zinc finger comprising a recognition helix which specifically binds to GTC; (f) a sixth zinc finger comprising a recognition helix which specifically binds to AAC; (g) a seventh zinc finger comprising a recognition helix

which binds to CGC; (h) an eighth zinc finger comprising a recognition helix that specifically binds to GTT; (i) an ninth zinc finger comprising a recognition helix that specifically binds to GAT; and (j) a tenth zinc finger comprising a recognition helix which specifically binds to GTC.

In the examples provided herein, the recognition helix of (a) which specifically binds to TGC is QRRSLGH (SEQ ID NO: 663); the recognition helix of (b) which specifically binds to ACG is KKNDLTR (aa 29-56 of SEQ ID NO: 60); the recognition helix of (c) which specifically binds to CGT is SRRTCRA (SEQ ID NO: 155); the recognition helix of (d) which specifically binds to GAT is VRHNLTR (SEQ ID NO: 270); the recognition helix of (e) which specifically binds to GTC is DRTSLAR (SEQ ID NO: 540); the recognition helix of (f) which specifically binds to AAC is DSGNLRV (SEQ ID NO: 64); the recognition helix of (g) which specifically binds to CGC is HTGHLLE (SEQ ID NO: 151); the recognition helix of (h) which specifically binds to GTT is TNQALGV (aa 197-224 of SEQ ID NO: 60); the recognition helix of (j) which specifically binds to GAT is VRHNLTR (SEQ ID NO: 270); and the recognition helix of (k) which specifically binds to GTC is DRTSLAR (SEQ ID NO: 540). However, other recognition helices exist for many of these triplets. For example, the recognition helix of (a) which specifically binds to TGC may be selected from the group consisting of: ARNTLVH, QRRSLGH, QARSLRA, QQRSLKN, and QNRSLAH, QGRSLRA, RARNLTL, RGRNLEM, RKRNLIM, RMRNLII, RNRNLVL, RRRNLHL, RRRNLTL, RSRNLDI, RSRNLLL, and RSRNLTL (SEQ ID NO: 658-673); the recognition helix of (b) which specifically binds to ACG may be selected form the group consisting of: KNNDLTR; KRIDLQR; RKHDLNM; RRQTLRQ; KGNDLTR; RNITLVR, RSHDLTV, ASADLTR, QNATRKR, QSGDLTR, RSQTLAQ; and RTDTLRD (SEQ ID NO: 104-119); the recognition helix of (c) which specifically binds to CGT may be selected from the group consisting of RSQTRKT (SEQ ID NO: 154) and SRRTCRA (SEQ ID NO: 155); the recognition helix of (d) which specifically binds to GAT may be selected from the group consisting of VRHNLTR, ISHNLAR, ISSNLQR, LGNNLKR, LNSNLAR, LSTNLTR, LTHNLRR, QSSNLAR, RSDALIQ, SKQALAV, TGQQLRV, TKQRLVV, TRQRLRI, TSANLSR, TSGNLVR, TSQMLVV, TSSNLSR, TTSNLRR,

VGHNLSR, VGSNLTR (SEQ ID NO: 251-270); the recognition helix of (e) which specifically binds to GTC may be selected from the group consisting of DRTSLAR, DHSSLKR, APSSLRR, DATQLVR, DPGALVR, DPTSLNR, DRSALAR, DRSALSR, DRSSLRR, DRTPLNR, DRTPLQN, EGGALRR, ESGALRR, NTSLLRR, RSDVLSE, TGAVLRR, TGAVLTR, TKKILTV, TKSLLAR, TMAVLRR, TRAVLRR, TSTILAR, TSTLLKR, and TSTLLNR (SEQ ID NO: 530-553); the recognition helix of (f) which specifically binds to AAC may be selected from the group consisting of DRSNRKT , DSGNLRV , GASALRQ , GASALRS , GGTALRM , GGTALVM , GHTALAL , GHTALRH , GHTALRN , GPTALVN , and HRTNLIA (SEQ ID NO: 63-73); the recognition helix of (g) which specifically binds to CGC may be HTGHLLE (SEQ ID NO: 151); and the recognition helix of (h) which specifically binds to GTT may be selected from the group consisting of HKSSLTR, TNQALGV, AATALRR, HHNSLTR, HSSSLVR, IKAILTR, INHSLRR , IRTSLKR, MNSVLKR, MTSSLRR, QATLLRR, QSSALTR, THTVLAR, TKPVLKI, TNSVLGR, TRHSLGR, TSGALTR, TSGSLTR, TSGSLVR, TSTLLKR, TSTRLDI, TTALLKR, TTSALTR, TTTVLAR, and VGGSLNR (SEQ ID NO: 583-607); the recognition helix of (i) which specifically binds to GAT may be selected from the group consisting of ISHNLAR, VRHNLTR, ISSNLQR, LGNNLKR, LNSNLAR, LSTNLTR, LTHNLRR, QSSNLAR, RSDALIQ, SKQALAV, TGQQLRV, TKQRLVV, TRQRLRI, TSANLSR, TSGNLVR, TSQMLVV, TSSNLSR, TTSNLRR, VGHNLSR, and VGSNLTR (SEQ ID NO: 251-270); and the recognition helix of (j) which specifically binds to GTC may be selected from the group consisting of DRTSLAR, DHSSLRKR, APSSLRR, DATQLVR, DPGALVR, DPTSLNR, DRSALAR, DRSALSR, DRSSLRR, DRTPLNR, DRTPLQN, EGGALRR, ESGALRR, NTSLLRR, RSDVLSE, TGAVLRR, TGAVLTR, TKKILTV, TKSLLAR, TMAVLRR, TRAVLRR, TSTILAR, TSTLLKR, and TSTLLNR (SEQ ID NO: 530-553).

Still other recognition helices for these and other triplets may be utilized in various embodiments of the invention. The following table provides illustrative recognition helices for various three base pair triplets.

Triplet	Recognition Helix (SEQ ID NO:)

Triplet	Recognition Helix (SEQ ID NO:)	
AAA	QRANLRA (SEQ ID NO: 61)	QRSNLKV (SEQ ID NO: 62)
AAC	DRSNRKT (SEQ ID NO: 63)	GGTALVM (SEQ ID NO: 68)
	DSGNLRV (SEQ ID NO: 64)	GHTALAL (SEQ ID NO: 69)
	GASALRQ (SEQ ID NO:65)	GHTALRH (SEQ ID NO: 70)
	GASALRS (SEQ ID NO: 66)	GHTALRN (SEQ ID NO: 71)
	GGTALRM (SEQ ID NO: 67)	GPTALVN (SEQ ID NO: 72)
		HRTNLIA (SEQ ID NO: 73)
AAG	RKDNLKN (SEQ ID NO: 74)	RSDNLSV (SEQ ID NO: 76)
	RSANLSV (SEQ ID NO: 75)	RSDNLTQ (SEQ ID NO: 77)
AAT	TSSNRKT (SEQ ID NO: 78)	VSSNLNV (SEQ ID NO: 80)
	TTGNLTV (SEQ ID NO: 79)	
ACA	QNATRIN (SEQ ID NO: 81)	
	SPADLTR (SEQ ID NO: 82)	
ACC	DKKDLTR (SEQ ID NO: 83)	
ACG	ASADLTR (SEQ ID NO: 84)	RKHDLNM (SEQ ID NO: 90)
	KGNDLTR (SEQ ID NO: 85)	RNITLVR (SEQ ID NO: 91)
	KNNDLTR (SEQ ID NO: 86)	RRQTLRQ (SEQ ID NO: 92)
	KRIDLQR (SEQ ID NO: 87)	RSHDLTV (SEQ ID NO: 93)
	QNATRKR (SEQ ID NO: 88)	RSQTLAQ (SEQ ID NO: 94)
	QSGDLTR (SEQ ID NO: 89)	RTDTLRD (SEQ ID NO: 95)
ACT	ARSTRTN (SEQ ID NO: 96)	THLDLIR (SEQ ID NO: 98)
	HASTRHC (SEQ ID NO: 97)	
AGA	KNWKLQA (SEQ ID NO: 99)	QSSHLTT (SEQ ID NO: 101)
	QLAHLRA (SEQ ID NO: 100)	RSANLAR (SEQ ID NO: 102)
AGC	ERSHLRE (SEQ ID NO: 103)	
AGG	DSAHLTR (SEQ ID NO: 104)	RRAHLRQ (SEQ ID NO: 112)
	QSAHRTK (SEQ ID NO: 105)	RRTHLRV (SEQ ID NO: 113)
	RGNHLVV (SEQ ID NO: 106)	RSDHLKT (SEQ ID NO: 114)
	RMAHLHA (SEQ ID NO: 107)	RSDHLSA (SEQ ID NO: 115)
	RNEHLKV (SEQ ID NO: 108)	RSDHLSQ (SEQ ID NO: 116)
	RPHHLDA (SEQ ID NO: 109)	RSDHLTN (SEQ ID NO: 117)
	RRAHLLN (SEQ ID NO: 110)	RSDHLTQ (SEQ ID NO: 118)
	RRAHLLS (SEQ ID NO: 111)	RSSHLKM (SEQ ID NO: 119)

Triplet	Recognition Helix (SEQ ID NO:)	
AGT	HRTTLTN (SEQ ID NO:120)	QSAHLST (SEQ ID NO:121)
ATA	QKSSLIA (SEQ ID NO: 122)	
ATG	RRDELNV (SEQ ID NO: 123)	RSDSLSV (SEQ ID NO: 124)
ATT	HKNALQN (SEQ ID NO: 125)	
CAA	DRANLST (SEQ ID NO: 126) QKSNLII (SEQ ID NO: 127)	QSGNLTE (SEQ ID NO: 128) QSSNLTV (SEQ ID NO: 129)
CAC	SKKALTE (SEQ ID NO: 130)	
CAG	DSANRTK (SEQ ID NO: 131) RADNLTE (SEQ ID NO: 132) RSDNLRE (SEQ ID NO: 133)	RSDNLSE (SEQ ID NO: 134) RTDYLVD (SEQ ID NO: 135)
CAT	DRSNRIK (SEQ ID NO: 136)	TSGNLTE (SEQ ID NO: 137)
CCA	DRSDLSR (SEQ ID NO: 138) NRTDLIR (SEQ ID NO: 139)	QNSTRIG (SEQ ID NO: 140) TSHSLTE (SEQ ID NO: 141)
CCC	SKKHLAE (SEQ ID NO: 142)	
CCG	DSSSLTR (SEQ ID NO: 143) DYDVRKR (SEQ ID NO: 144)	RNDTLTE (SEQ ID NO: 145) RSDTLSE (SEQID NO: 146)
CCT	TKNSLTE (SEQ ID NO: 147)	
CGA	QSGHLTE (SEQ ID NO: 148) QSSHLNV (SEQ ID NO: 149)	QSTHLTQ (SEQ ID NO: 150)
CGC	HTGHLLE (SEQ ID NO: 151)	
CGG	RSDNLTE (SEQ ID NO: 152)	RSDKLTE (SEQ ID NO: 153)
CGT	RSQTRKT (SEQ ID NO: 154)	SRRTCRA (SEQ ID NO: 155)
CTA	DSSSRTK (SEQ ID NO: 156)	QNSTLTE (SEQ ID NO: 157)
CTC	ASDDLTQ (SEQ ID NO: 158)	
CTG	HNYARDC (SEQ ID NO: 159) RNDALTE (SEQ ID NO: 160) RSDALRE (SEQ ID NO: 161)	RSDALSA (SEQ ID NO: 162) RSDALSN (SEQ ID NO: 163) RSDTLSE (SEQ ID NO: 164)
CTT	TTGALTE (SEQ ID NO: 165)	
GAA	HKPNLHR (SEQ ID NO: 166)	QRNNLGR (SEQ ID NO: 181)

Triplet	Recognition Helix (SEQ ID NO:)	
	HRPNLTR(SEQ ID NO: 167)	QRSNLAR(SEQ ID NO: 182)
	LGENLRR (SEQ ID NO: 168)	QRSNLVR (SEQ ID NO: 183)
	QASNLAR(SEQ ID NO: 169)	QRTNLQR(SEQ ID NO: 184)
	QASNLLR(SEQ ID NO: 170)	QSGNLAR (SEQ ID NO: 185)
	QASNLTR(SEQ ID NO: 171)	QSNNLNR (SEQ ID NO: 186)
	QDGNLGR(SEQ ID NO: 172)	QSSNLTK(SEQ ID NO: 187)
	QDGNLTR(SEQ ID NO: 173)	QSSNLTR(SEQ ID NO: 188)
	QGSNLAR(SEQ ID NO: 174)	QSSNLVR(SEQ ID NO: 189)
	QHPNLTR(SEQ ID NO: 175)	QTNNLGR(SEQ ID NO: 190)
	QKGNLLR(SEQ ID NO: 176)	QTNNLNR(SEQ ID NO: 191)
	QKSNLIR(SEQ ID NO: 177)	QTNNLTR(SEQ ID NO: 192)
	QLSNLTR(SEQ ID NO: 178)	QTVNLDR(SEQ ID NO: 193)
	QQSNLSR (SEQ ID NO: 179)	RKPNLLR(SEQ ID NO: 194)
	QQTNLTR(SEQ ID NO: 180)	TTTNLRR(SEQ ID NO: 195)
GAC	CPSNLRR (SEQ ID NO: 196)	DRANLSR(SEQ ID NO: 207)
		DRGNLTR(SEQ ID NO: 208)
	DDANLRR(SEQ ID NO: 197)	DRSNLTR(SEQ ID NO: 209)
	DEANLRR (SEQ ID NO: 198)	EEANLRR(SEQ ID NO: 210)
	DLSNLKR(SEQ ID NO: 199)	EESNLRR(SEQ ID NO: 211)
	DMGNLGR(SEQ ID NO: 200)	EEVNLRR(SEQ ID NO: 212)
	DPANLRR(SEQ ID NO: 201)	EGGNLMR(SEQ ID NO: 213)
	DPGNLVR(SEQ ID NO: 202)	EKANLTR(SEQ ID NO: 214)
	DPSNLIR(SEQ ID NO: 203)	EQANLRR(SEQ ID NO: 215)
	DPSNLQR(SEQ ID NO: 204)	HSSNFNK(SEQ ID NO: 216)
	DPSNLRR(SEQ ID NO: 205)	RSDNLSE(SEQ ID NO: 217)
	DQGNLIR(SEQ ID NO: 206)	
GAG	KHSNLAR (SEQ ID NO: 218)	RQMNLDR(SEQ ID NO: 234)
	KHSNLTR(SEQ ID NO: 219)	RRDNLLR(SEQ ID NO: 235)
	KKTNLTR(SEQ ID NO: 220)	RRDNLNR(SEQ ID NO: 236)
	KSSNLRR(SEQ ID NO: 221)	RSANLTR(SEQ ID NO: 237)
	QSFNLRR(SEQ ID NO: 222)	RSDHLSR(SEQ ID NO: 238)
	REDNLGR(SEQ ID NO: 223)	RSDNLAR(SEQ ID NO: 239)
	RGDNLKR(SEQ ID NO: 224)	RSDNLSR(SEQ ID NO: 240)
	RGDNLNR(SEQ ID NO: 225)	RSDNLST (SEQ ID NO: 241)
	RHDQLTR(SEQ ID NO: 226)	RSDNLTR (SEQ ID NO: 242)
	RIDNLGR(SEQ ID NO: 227)	RSDNLVR (SEQ ID NO: 243)

Triplet	Recognition Helix (SEQ ID NO:)	
	RKSNLIR(SEQ ID NO: 228)	RSSNLQR(SEQ ID NO: 244)
	RMSNLDR(SEQ ID NO: 229)	RTHNLKR(SEQ ID NO: 245)
	RNTNLTR(SEQ ID NO: 230)	RTHNLTR(SEQ ID NO: 246)
	RPHNLLR(SEQ ID NO: 231)	RVDNLPR(SEQ ID NO: 247)
	RQDNLGR(SEQ ID NO: 232)	SGSNFTR(SEQ ID NO: 248)
	RQDNLQR(SEQ ID NO: 233)	TNNNLAR(SEQ ID NO: 249)
		VHWNLMR(SEQ ID NO: 250)
GAT	ISHNLAR (SEQ ID NO: 251)	TKQRLVV(SEQ ID NO: 261)
	ISSNLQR(SEQ ID NO: 252)	TRQRLRI(SEQ ID NO: 262)
	LGNNLKR(SEQ ID NO: 253)	TSANLSR(SEQ ID NO: 263)
	LNSNLAR(SEQ ID NO: 254)	TSGNLVR(SEQ ID NO: 264)
	LSTNLTR(SEQ ID NO: 255)	TSQMLVV(SEQ ID NO: 265)
	LTHNLRR(SEQ ID NO: 256)	TSSNLSR(SEQ ID NO: 266)
	QSSNLAR(SEQ ID NO: 257)	TTSNLRR(SEQ ID NO: 267)
	RSDALIQ(SEQ ID NO: 258)	VGHNLSR(SEQ ID NO: 268)
	SKQALAV(SEQ ID NO: 259)	VGSNLTR(SEQ ID NO: 269)
	TGQQLRV(SEQ ID NO: 260)	VRHNLTR(SEQ ID NO: 270)
GCA	DKAQLGR(SEQ ID NO: 271)	QPNTLTR(SEQ ID NO: 286)
	DRSALSR(SEQ ID NO: 272)	QRGTLNR(SEQ ID NO: 287)
	DRSQLAR(SEQ ID NO: 273)	QSGDLRR(SEQ ID NO: 288)
	ERGTLAR(SEQ ID NO: 274)	QSGDLTR(SEQ ID NO: 289)
	HNGTLKR(SEQ ID NO: 275)	QSGSLTR(SEQ ID NO: 290)
	KNTRLSV(SEQ ID NO: 276)	QSNVLSR(SEQ ID NO: 291)
	LKHSLLR(SEQ ID NO: 277)	QSTTLKR(SEQ ID NO: 292)
	LNHTLKR(SEQ ID NO: 278)	QTATLKR(SEQ ID NO: 293)
	LRHSLSR(SEQ ID NO: 279)	QTNTLKR(SEQ ID NO: 294)
	QDNTLRR(SEQ ID NO: 280)	RGQELRR(SEQ ID NO: 295)
	QDVSLVR(SEQ ID NO: 281)	RRQELHR(SEQ ID NO: 296)
	QGGTLRR(SEQ ID NO: 282)	RRQELKR(SEQ ID NO: 297)
	QGNTLTR(SEQ ID NO: 283)	RRQELTR(SEQ ID NO: 298)
	QKGTLGR(SEQ ID NO: 284)	RRVDLLR(SEQ ID NO: 299)
	QNGTLTR(SEQ ID NO: 285)	SPEQLAR(SEQ ID NO: 300)
GCC	DCRDLAR(SEQ ID NO: 301)	DSPTLRR(SEQ ID NO: 313)
	DGSTLNR(SEQ ID NO: 302)	DSSVLRR(SEQ ID NO: 314)
	DGSTLRR(SEQ ID NO: 303)	EHRGLKR(SEQ ID NO: 315)
	DHSNLSR(SEQ ID NO: 304)	ERGTLAR(SEQ ID NO: 316)

Triplet	Recognition Helix (SEQ ID NO:)	
	DKSCLNR(SEQ ID NO: 305)	ERRGLAR(SEQ ID NO: 317)
	DKSVLAR(SEQ ID NO: 306)	ERRGLDR(SEQ ID NO: 318)
	DPSNLRR(SEQ ID NO: 307)	KRRDLDR(SEQ ID NO: 319)
	DPSTLRR(SEQ ID NO: 308)	LKKDLLR(SEQ ID NO: 320)
	DRRTLDR(SEQ ID NO: 309)	SHTVLTR(SEQ ID NO: 321)
	DRSDLTR(SEQ ID NO: 310)	SKKSLTR(SEQ ID NO: 322)
	DRSSLTR(SEQ ID NO: 311)	SNKDLTR(SEQ ID NO: 323)
	DRSSRTK(SEQ ID NO: 312)	VRKDLTR(SEQ ID NO: 324)
GCG	KADTLVR(SEQ ID NO: 325)	RRHTLTR(SEQ ID NO: 343) RRLTLLR(SEQ ID NO: 344)
	KHDTLHR(SEQ ID NO: 326)	RSDDLQR(SEQ ID NO: 345)
	KNNDLTR(SEQ ID NO: 327)	RSDDLTR(SEQ ID NO: 346)
	RADTLRR(SEQ ID NO: 328)	RSDDLVR(SEQ ID NO: 347)
	RAHTLRR(SEQ ID NO: 329)	RSDELNR(SEQ ID NO: 348)
	REDSLPR(SEQ ID NO: 330)	RSDELQR(SEQ ID NO: 349)
	RHAALLS(SEQ ID NO: 331)	RSDELSR(SEQ ID NO: 350)
	RKDGLTR(SEQ ID NO: 332)	RSDELTR(SEQ ID NO: 351)
	RKGTLDR(SEQ ID NO: 333)	RSDERKR(SEQ ID NO: 352)
	RKLGLLR(SEQ ID NO: 334)	RSDSLSK(SEQ ID NO: 353)
	RLDMLAR(SEQ ID NO: 335)	RSDTLKK(SEQ ID NO: 354)
	RLRDLPR(SEQ ID NO: 336)	RSDVLTR(SEQ ID NO: 355)
	RNLTLAR(SEQ ID NO: 337)	RSNTLLR(SEQ ID NO: 356)
	RNLTLVR(SEQ ID NO: 338)	RTDLLRR(SEQ ID NO: 357)
	RPDGLAR(SEQ ID NO: 339)	RTDSLPR(SEQ ID NO: 358)
	RRDDLTR(SEQ ID NO: 340)	RTDTLAR(SEQ ID NO: 359)
	RRDGLTR(SEQ ID NO: 341)	RVDDLGR(SEQ ID NO: 360)
	RRHGLDR(SEQ ID NO: 342)	
GCT	ARSTRTT(SEQ ID NO: 361)	QSSDLRR(SEQ ID NO: 377)
	EGSGLKR(SEQ ID NO: 362)	QSSDLSR(SEQ ID NO: 378)
	GATALKR(SEQ ID NO: 363)	QSSDLTR(SEQ ID NO: 379)
	KHQTLQR(SEQ ID NO: 364)	THSMLAR(SEQ ID NO: 380)
	LKHDLRR(SEQ ID NO: 365)	TKPILVR(SEQ ID NO: 381)
	LRASLRR(SEQ ID NO: 366)	TKQILGR(SEQ ID NO: 382)
	LRQTLAR(SEQ ID NO: 367)	TKQVLDR(SEQ ID NO: 383)
	LRTSLVR(SEQ ID NO: 368)	TSGELVR(SEQ ID NO: 384)
	MKNTLTR(SEQ ID NO: 369)	TSSGLTR(SEQ ID NO: 385)

Triplet	Recognition Helix (SEQ ID NO:)	
	NGQGLRR(SEQ ID NO: 370)	TTQALRR(SEQ ID NO: 386)
	NKQALDR(SEQ ID NO: 371)	VGASLKR(SEQ ID NO: 387)
	NRSDRTR(SEQ ID NO: 372)	VGNSLTR(SEQ ID NO: 388)
	QRQALDR(SEQ ID NO: 373)	VKNTLTR(SEQ ID NO: 389)
	QRSDLHR(SEQ ID NO: 374)	VRQGLTR(SEQ ID NO: 390)
	QRSDLTR(SEQ ID NO: 375)	VSNSLAR(SEQ ID NO: 391)
	QSSDLQR(SEQ ID NO: 376)	VSNTLTR(SEQ ID NO: 392)
GGA	DKTKLNV(SEQ ID NO: 393)	QSQHLVR(SEQ ID NO: 409)
	DKTKLRV(SEQ ID NO: 394)	QSTHLTR(SEQ ID NO: 410)
	DNAHLAR(SEQ ID NO: 395)	QTTHLRR(SEQ ID NO: 411)
	QANHLSR(SEQ ID NO: 396)	QTTHLSR(SEQ ID NO: 412)
	QGGHLKR(SEQ ID NO: 397)	QTTHLSR(SEQ ID NO: 413)
	QHSHLVR(SEQ ID NO: 398)	QVSHLTR(SEQ ID NO: 414)
	QKPHLSR(SEQ ID NO: 399)	RMERLDR(SEQ ID NO: 415)
	QMSHLKR(SEQ ID NO: 400)	RPAKLVL(SEQ ID NO: 416)
	QNSHLRR(SEQ ID NO: 401)	RPSKLVL(SEQ ID NO: 417)
	QNSHLRR(SEQ ID NO: 402)	RRDHRTT(SEQ ID NO: 418)
	QRAHLER(SEQ ID NO: 403)	RSTHLRV(SEQ ID NO: 419)
	QRAHLIR(SEQ ID NO: 404)	RTDRLIR(SEQ ID NO: 420)
	QSAHLKR(SEQ ID NO: 405)	THAHLTR(SEQ ID NO: 421)
	QSGHLAR(SEQ ID NO: 406)	TSAHLAR(SEQ ID NO: 422)
	QSGHLQR(SEQ ID NO: 407)	YNTHLTR(SEQ ID NO: 423)
	QSGHLSR(SEQ ID NO: 408)	
GGC	AKSKLDR(SEQ ID NO: 424)	KNHSLNN(SEQ ID NO: 437)
	APSKLDR(SEQ ID NO: 425)	KNVSLTH(SEQ ID NO: 438)
	DGGHLTR(SEQ ID NO: 426)	LKEHLTR(SEQ ID NO: 439)
	DKSHLPR(SEQ ID NO: 427)	QSSHLAR(SEQ ID NO: 440)
	DPGHLVR(SEQ ID NO: 428)	SKHKLER(SEQ ID NO: 441)
	DRSHLAR(SEQ ID NO: 429)	SPSKLAR(SEQ ID NO: 442)
	DRSHLSR(SEQ ID NO: 430)	SPSKLVR(SEQ ID NO: 443)
	DRSHLTR(SEQ ID NO: 431)	TNSKLTR(SEQ ID NO: 444)
	EKSHLKR(SEQ ID NO: 432)	TPSKLDR(SEQ ID NO: 445)
	EKSHLTR(SEQ ID NO: 433)	TRAKLHI(SEQ ID NO: 446)
	ENSKLNR(SEQ ID NO: 434)	VPSKLAR(SEQ ID NO: 447)
	ESGHLKR(SEQ ID NO: 435)	VPSKLKR(SEQ ID NO: 448)
	ESGHLRR(SEQ ID NO: 436)	VPSKLLR(SEQ ID NO: 449)

Triplet	Recognition Helix (SEQ ID NO:)	
GGG	KGDHLRR(SEQ ID NO: 450)	RNTHLAR(SEQ ID NO: 464)
	KKDHLHR(SEQ ID NO: 451)	RQGHLKR(SEQ ID NO: 465)
	KRERLDR(SEQ ID NO: 452)	RRAHLQN(SEQ ID NO: 466)
	KRERLER(SEQ ID NO: 453)	RREHLVR(SEQ ID NO: 467)
	KSNHLHV(SEQ ID NO: 454)	RRSHLTR(SEQ ID NO: 468)
	KTSHLRA(SEQ ID NO: 455)	RSAHLAR(SEQ ID NO: 469)
	RGDKLAL(SEQ ID NO: 456)	RSAHLSR(SEQ ID NO: 470)
	RGDKLGP(SEQ ID NO: 457)	RSDHLAR(SEQ ID NO: 471)
	RGNHLRR(SEQ ID NO: 458)	RSDHLSK(SEQ ID NO: 472)
	RIDKLGG(SEQ ID NO: 459)	RSDHLSR(SEQ ID NO: 473)
	RKHHLGR(SEQ ID NO: 460)	RSDHLTR(SEQ ID NO: 474)
	RKHRLDG(SEQ ID NO: 461)	RSDKLNR(SEQ ID NO: 475)
	RNDKLVP(SEQ ID NO: 462)	RSDKLVR(SEQ ID NO: 476)
	RNHGLVR(SEQ ID NO: 463)	RTEHLAR(SEQ ID NO: 477)
GGT	DSSKLSR(SEQ ID NO: 478)	RRQKLTI(SEQ ID NO: 493)
		RRSRLVR(SEQ ID NO: 494)
		RSDHLST(SEQ ID NO: 495)
	EAHHLSR(SEQ ID NO: 479)	RSDHLTT(SEQ ID NO: 496)
	HGHRLKT(SEQ ID NO: 480)	TKQKLQT(SEQ ID NO: 497)
	IPNHLAR(SEQ ID NO: 481)	TKQRLEV(SEQ ID NO: 498)
	IRHHLKR(SEQ ID NO: 482)	TRQKLET(SEQ ID NO: 499)
	LTQGLRR(SEQ ID NO: 483)	TRTRLVI(SEQ ID NO: 500)
	MGHHLKR(SEQ ID NO: 484)	TSGHLSR(SEQ ID NO:501)
	MKHHLAR(SEQ ID NO: 485)	TSGHLVR(SEQ ID NO:502)
	MKHHLDA(SEQ ID NO: 486)	TTTKLAI(SEQ ID NO:502)
	MSDHLSR(SEQ ID NO: 487)	VDHHLRR(SEQ ID NO:504)
	MSHHLSR(SEQ ID NO: 488)	VKHGLGR(SEQ ID NO:505)
	QPHHLPR(SEQ ID NO: 489)	VKHGLTR(SEQ ID NO:506)
	QSSHLAR (SEQ ID NO: 490)	WPSNLTR(SEQ ID NO:507)
	QSSHLTR(SEQ ID NO: 491)	YNWHLQR(SEQ ID NO:508)
	RQSRLQR(SEQ ID NO: 492)	
GTA	QGGALQR (SEQ ID NO: 509)	QRSSLVR(SEQ ID NO: 520)
	QGTSLAR(SEQ ID NO: 510)	QSGALAR(SEQ ID NO: 521)
	QKQALDR(SEQ ID NO: 511)	QSGALTR(SEQ ID NO: 522)
	QKQALTR(SEQ ID NO: 512)	QSGSLTR(SEQ ID NO: 523)
	QKVSLKR(SEQ ID NO: 513)	QSGTLTR(SEQ ID NO: 524)

Triplet	Recognition Helix (SEQ ID NO:)	
	QMNALQR(SEQ ID NO: 514)	QSSSLIR(SEQ ID NO: 525)
	QQQALKR(SEQ ID NO: 515)	QSSSLVR(SEQ ID NO: 526)
	QQQALTR(SEQ ID NO: 516)	QSSTLTR(SEQ ID NO: 527)
	QQQALVR(SEQ ID NO: 517)	QSTSLQR(SEQ ID NO: 528)
	QQSSLLR(SEQ ID NO: 518)	TSSARTT(SEQ ID NO: 529)
	QRASLTR(SEQ ID NO: 519)	
GTC	APSSLRR(SEQ ID NO: 530)	ESGALRR(SEQ ID NO: 542)
	DATQLVR(SEQ ID NO: 531)	NTSLLRR(SEQ ID NO: 543)
	DHSSLKR(SEQ ID NO: 532)	RSDVLSE(SEQ ID NO: 544)
	DPGALVR(SEQ ID NO: 533)	TGAVLRR(SEQ ID NO: 545)
	DPTSLNR(SEQ ID NO: 534)	TGAVLTR(SEQ ID NO: 546)
	DRSALAR(SEQ ID NO: 535)	TKKILTV(SEQ ID NO: 547)
	DRSALSR(SEQ ID NO: 536)	TKSLLAR(SEQ ID NO: 548)
	DRSSLRR(SEQ ID NO: 537)	TMAVLRR(SEQ ID NO: 549)
	DRTPLNR(SEQ ID NO: 538)	TRAVLRR(SEQ ID NO: 550)
	DRTPLQN(SEQ ID NO: 539)	TSTILAR(SEQ ID NO: 551)
	DRTSLAR(SEQ ID NO: 540)	TSTLLKR(SEQ ID NO: 552)
	EGGALRR(SEQ ID NO: 541)	TSTLLNR(SEQ ID NO: 553)
GTG	RASVLDI (SEQ ID NO: 554)	RRFILSR(SEQ ID NO: 569)
	RGDALAR(SEQ ID NO: 555)	RRHILDR(SEQ ID NO: 570)
	RHTSLTR(SEQ ID NO: 556)	RSAALSR(SEQ ID NO: 571)
	RKDALHV(SEQ ID NO: 557)	RSDALAR(SEQ ID NO: 572)
	RKHILIH(SEQ ID NO: 558)	RSDALRT(SEQ ID NO: 573)
	RKTALNR(SEQ ID NO: 559)	RSDALSR(SEQ ID NO: 574)
	RNFILAR(SEQ ID NO: 560)	RSDALTR(SEQ ID NO: 575)
	RNFILQR(SEQ ID NO: 561)	RSDELVR(SEQ ID NO: 576)
	RNFVLAR(SEQ ID NO: 562)	RSHILTN(SEQ ID NO: 577)
	RNTALQH(SEQ ID NO: 563)	RTSSLKR(SEQ ID NO: 578)
	RNVALGN(SEQ ID NO: 564)	RTVALNR(SEQ ID NO: 579)
	RNVNLVT(SEQ ID NO: 565)	SRFTLGR(SEQ ID NO: 580)
	RPDALPR(SEQ ID NO: 566)	SRFTLGR(SEQ ID NO: 581)
	RRAALGP(SEQ ID NO: 567)	VSSSLRR(SEQ ID NO: 582)
	RREVLEN(SEQ ID NO: 568)	
GTT	AATALRR(SEQ ID NO: 583)	TKPVLKI(SEQ ID NO: 595)
	HHNSLTR(SEQ ID NO: 584)	TRHSLGR(SEQ ID NO: 598)
	HKSSLTR(SEQ ID NO: 585)	TSGALTR(SEQ ID NO: 599)

Triplet	Recognition Helix (SEQ ID NO:)	
	HSSSLVR(SEQ ID NO: 586)	TSGSLTR(SEQ ID NO: 600)
	IKAILTR(SEQ ID NO: 587)	TSGSLVR(SEQ ID NO: 601)
	INHSLRR(SEQ ID NO: 588)	TSTLLKR(SEQ ID NO: 602)
	IRTSLKR(SEQ ID NO: 589)	TSTRLDI(SEQ ID NO: 603)
	MNSVLKR (SEQ ID NO: 590)	TTALLKR(SEQ ID NO: 604)
	MTSSLRR(SEQ ID NO: 591)	TTSALTR(SEQ ID NO: 605)
	QATLLRR(SEQ ID NO: 592)	TTTVLAR(SEQ ID NO: 606)
	QSSALTR(SEQ ID NO: 593)	VGGSLNR(SEQ ID NO: 607)
	THTVLAR(SEQ ID NO: 594)	
TAA	QGGNLAL(SEQ ID NO: 608)	QQGNLRN(SEQ ID NO: 611)
	QGGNLTL(SEQ ID NO: 609)	QRGNLNM(SEQ ID NO: 612)
	QQGNLQL (SEQ ID NO: 610)	QSGNLHT(SEQ ID NO: 613)
TAC	NSDHLTN (SEQ ID NO: 614)	
TAG	QGYNLAG (SEQ ID NO: 615)	RPESLAP (SEQ ID NO: 625)
	RAHNLLL (SEQ ID NO: 616)	RPESLRP (SEQ ID NO: 626)
	REDNLHT (SEQ ID NO: 617)	RRDGLAG (SEQ ID NO: 627)
	RGHNLLV (SEQ ID NO: 618)	RRDHLSL(SEQ ID NO: 628)
	RGTNLRT (SEQ ID NO: 619)	RRDHLSP (SEQ ID NO: 629)
	RHDGLAG(SEQ ID NO: 620)	RRDNLPK (SEQ ID NO: 630)
	RIDHLVP (SEQ ID NO: 621)	RRRNLQI (SEQ ID NO: 631)
	RKTGLLI (SEQ ID NO: 622)	RSHNLKL (SEQ ID NO: 632)
	RLDGLAG (SEQ ID NO: 623)	RSHNLRL (SEQ ID NO: 633)
	RPEGLST (SEQ ID NO: 624)	TSSNRKK (SEQ ID NO: 634)
TCA	QSADRTK (SEQ ID NO: 635)	
TCC	DKRSLPH (SEQ ID NO: 636)	
TCG	ASSTRTK (SEQ ID NO: 637)	RMDSLGG (SEQ ID NO: 644)
	HSSDLTR (SEQ ID NO: 638)	RRDGLSG (SEQ ID NO: 645)
	KNNDLLK (SEQ ID NO: 639)	RSDELRT (SEQ ID NO: 646)
	NRSDLSR (SEQ ID NO: 640)	RSDGLRG (SEQ ID NO: 647)
	QSSDLSK (SEQ ID NO: 641)	RSDTLPA (SEQ ID NO: 648)
	RADGLQL (SEQ ID NO: 642)	RSDTLPL (SEQ ID NO: 649)
	RGDSLKK (SEQ ID NO: 643)	RSSDLSR (SEQ ID NO: 650)
		RTDSLQP (SEQ ID NO: 651)
TCT	NNRDRTK (SEQ ID NO: 652)	SKPNLKM (SEQ ID NO: 654)

Triplet	Recognition Helix (SEQ ID NO:)	
	QRNTLKG (SEQ ID NO: 653)	
TGA	QAGHLAS (SEQ ID NO: 655)	QSGHLTK (SEQ ID NO: 657)
	QREHLTT (SEQ ID NO: 656)	
TGC	ANRTLVH (SEQ ID NO: 658)	RRRNLHL (SEQ ID NO: 669)
	QARSLRA (SEQ ID NO: 659)	RRRNLTL (SEQ ID NO: 670)
	QGRSLRA (SEQ ID NO: 660)	RSRNLDI (SEQ ID NO: 671)
	QNRSLAH (SEQ ID NO: 661)	RSRNLLL (SEQ ID NO: 672)
	QQRSLKN (SEQ ID NO: 662)	RSRNLTL (SEQ ID NO: 673)
	QRRSLGH (SEQ ID NO: 663)	
	RARNLTL (SEQ ID NO: 664)	
	RGRNLEM (SEQ ID NO: 665)	
	RKRNLIM (SEQ ID NO: 666)	
	RMRNLII (SEQ ID NO: 667)	
	RNRNLVL (SEQ ID NO: 668)	
TGG	RMDHLAG (SEQ ID NO: 674)	RSDHLSL (SEQ ID NO: 679)
	RNAHRIN (SEQ ID NO: 675)	RSDHLST (SEQ ID NO: 680)
	RRDHLSL (SEQ ID NO: 676)	RSDHLTT (SEQ ID NO: 681)
	RREHLTI (SEQ ID NO: 677)	RTESLHI (SEQ ID NO: 682)
	RSDHLRE (SEQ ID NO: 678)	
TGT	KRQHLEY (SEQ ID NO: 683)	QQHGLRH (SEQ ID NO: 691)
	QAHGLTA (SEQ ID NO: 684)	QRHGLSS (SEQ ID NO: 692)
	QAHGLTG (SEQ ID NO: 685)	RHQHLKL (SEQ ID NO: 693)
	QPGHLTA (SEQ ID NO: 686)	RKQHLQL (SEQ ID NO: 694)
	QPHGLAH (SEQ ID NO: 687)	RKQHLTL (SEQ ID NO: 695)
	QPHGLGA (SEQ ID NO: 688)	RKQHLVL (SEQ ID NO: 696)
	QPHGLRA (SEQ ID NO: 689)	RRQALEY (SEQ ID NO: 697)
	QPHGLRH (SEQ ID NO: 690)	RRQHLQY (SEQ ID NO: 698)
TTA	QQTGLNV (SEQ ID NO: 699)	
TTC	QRNALRG (SEQ ID NO: 700)	RANHLTI (SEQ ID NO: 701)
TTG	RADALMV (SEQ ID NO: 702)	RSDSLSA (SEQ ID NO:703)
TTT	HSNARKT (SEQ ID NO: 704)	QRNALSG(SEQ ID NO:705)

In one embodiment, the endonuclease catalytic domain is linked to a zinc finger in which the at least 10 independently selected recognition helices are all different. In certain embodiments, it will be desirable to include different recognition helices to a repeated triplet of base pairs in the ablation site. In other embodiments, recognition helices to any repeated triplets are the same. For example, 0, 1, 2, or 3 of the recognition helices selected are the same.

In certain embodiments, an AAV vector contains two or more endonuclease ablation sites, which may the same or different from one another. These endonuclease ablation sites may be engineered into the vector in a variety of configurations. More particularly, an AAV vector carrying a gene of interest (GOI) is engineered to contain at least one ablation site 5' to the coding strand of the gene of interest. Where the vector contains two or more ablation sites, the second ablation site may be located: 3' to the gene of interest in sense orientation; 3' to the gene of interest in inverted orientation; two ablation sites can be located 5' to the gene of interest, with one of sense orientation and the other in inverted orientation, or both in sense orientation; or the vector may contain one or two ablation sites 5' to the gene of interest (one in sense and one in inverted orientation or both in sense orientation) and two ablation sites 3' to the gene of interest (one in sense and one in inverted orientation or both in sense orientation).

Where two ablation sites are located such that there is no intervening gene of interest, *i.e.,* two 5' or two 3' ablation sites, the ablation sites may be separated by a spacer. Any suitable spacer may be selected and the spacer sequence may be a non-coding sequence. In another embodiment, the spacer sequence may be a reporter gene, transgene, or gene of interest.

In one embodiment, when a composition of the invention contains more than one ablation site, the ablation sites are the same. In another embodiment, when a composition or vector system of the invention contains more than one ablation site, each site differs from the other and each is specifically and selectively targeted by a different chimeric endonuclease.

In the working examples below, an ablator encoded by the sequence: 10xZF-FokI_Cat nucleotide sequence: SEQ ID NO: 59, is illustrated:

ATGGGCGAGAAGCCCTACAAGTGCCCTGAGTGCGGCAAGAGCTTCAGCCAG
AGAAGAAGCCTGGGCCACCACCAGCGTACGCACCCCGGCGAGAAACCTTAT
AAGTGTCCCGAATGTGGCAAGTCCTTCAGCAAGAAGAACGACCTGACCCGG
CACCAGCGGACACACCCCGGGGAAAAGCCATACAAATGTCCAGAGTGTGGG
AAGTCTTTCTCCAGCCGGCGGACCTGCAGAGCCCATCAGAGAACACATACCG
GGGAGAAGCCTTTCCAGTGCCGGATCTGCATGAGAAACTTCAGCGTGCGGCA
CAACCTGACCAGACACCTGAGGACCCATACCGGCGAAAAACCCTTTCAGTGC
AGAATCTGTATGCGGAACTTCTCCGACCGGACCAGCCTGGCCCGGCATCTGA
GAACTCATCCTGGGGAAAAGCCCTATAAGTGTCCAGAATGCGGGAAATCCTT
TAGCGACAGCGGCAACCTGCGGGTGCACCAGAGGACTCATCCAGGCGAGAA
ACCCTACAAATGCCCCGAATGCGGAAAGTCATTCTCCCACACCGGCCATCTG
CTCGAGCATCAGCGGACCCACACTGGGGAGAAACCATTTCAGTGTCGCATCT
GTATGAGGAATTTCAGCACCAACCAGGCCCTGGGCGTGCACCTGAGAACAC
ACCCAGGCGAGAAGCCTTACAAGTGTCCAGAGTGCGGAAAGTCATTTTCCGT
GCGCCACAATCTGACACGGCATCAGCGCACCCATCCCGGCGAGAAGCCTTAC
AAATGCCCCGAGTGTGGCAAATCTTTCAGTGACCGGACCTCTCTGGCCAGAC
ATCAGAGGACACACGGCACTAGTGGCAAGCAGCTGGTGAAAAGCGAGCTGG
AAGAGAAGAAGTCCGAGCTGCGGCACAAGCTGAAATACGTGCCCCACGAGT
ACATCGAGCTGATCGAGATCGCCCGGAACCCCACCCAGGACAGAATCCTGG
AAATGAAGGTCATGGAATTTTTCATGAAGGTGTACGGCTACCGGGGCGAGCA
CCTGGGCGGCAGCAGAAAACCCGACGGCGCCATCTACACCGTGGGCAGCCC
CATCGACTACGGCGTGATCGTGGACACCAAGGCCTACAGCGGCGGCTACAA
CCTGCCCATCGGACAGGCCGACGAGATGCAGAGATACGTGGAAGAGAACCA
GACCCGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGTGTACCCCAG
CAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGGGCAAC
TACAAGGCCCAGCTGACCCGGCTGAACCACATCACCAACTGCAACGGCGCTG
TGCTGAGCGTGGAAGAACTGCTGATCGGCGGCGAGATGATCAAGGCCGGCA
CCCTGACCCTGGAAGAAGTGCGGCGGAAGTTCAACAACGGCGAGATCAACT
TCTGATAG.

The transcribed illustrative ablator has the sequence: 10xZF-FokI_Cat amino acid sequence: SEQ ID NO: 60:

MGEKPYKCPECGKSFSQRRSLGHHQRTHPGEKPYKCPECGKSFSKKNDLTRHQR
THPGEKPYKCPECGKSFSSRRTCRAHQRTHTGEKPFQCRICMRNFSVRHNLTRH
LRTHTGEKPFQCRICMRNFSDRTSLARHLRTHPGEKPYKCPECGKSFSDSGNLRV
HQRTHPGEKPYKCPECGKSFSHTGHLLEHQRTHTGEKPFQCRICMRNFSTNQAL
GVHLRTHPGEKPYKCPECGKSFSVRHNLTRHQRTHPGEKPYKCPECGKSFSDRT
SLARHQRTH - GTSGKQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNPTQDR I
LEMKVMEFFMKVYGYRGEHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYN
LPIGQADEMQRYVEENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYK
AQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF.

In an additional embodiment, the ablator is engineered such that it is controlled by a cassette that is activated by a transcription factor following being dimerized by a pharmacologic agent, such as described in detail in this specification and incorporated herein by reference. Typically, this cassette is comprises two transcription units. One of the two transcription units encodes the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a first promoter; and a second of the two transcription units encoding the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a second promoter. As described in more detail elsewhere in the specification and incorporated herein, the first promoter and the second promoter are independently selected from a constitutive promoter and an inducible promoter. In one embodiment, the first and second promoters are both constitutive promoters and the pharmacological agent is a dimerizer that dimerizes the domains of the transcription factor. In another embodiment, one of the first promoter and the second promoters is an inducible promoter. Optionally, the two transcription units are a bicistronic unit containing an IRES or furin-2A. Various genetic elements and methods suitable for vector construction are described elsewhere in the specification and incorporated herein by reference.

In a further embodiment, the promoter is controlled by a rapamycin - regulatable system and the pharmacological agent is rapamycin or a rapalog.

This embodiment, using the unique (at least 30 bp) nucleic acid sequence in the ablation recognition site and the chimeric endonuclease containing at least 10x zinc fingers, can be generated as described herein and other techniques known to those of skill in the art.

Methods for Generating The Replication-Defective Virus Compositions

Many methods have been established for the efficient production of recombinant AAVs (rAAVs) that package a transgene - these can be used or adapted to generate the replication-defective virus compositions of the invention. In a one system, a producer cell line is transiently transfected with a construct that encodes the transgene flanked by ITRs and a construct(s) that encodes rep and cap. In a second system, a packaging cell line that stably supplies rep and cap is transiently transfected with a construct encoding the transgene flanked by ITRs. In a third system, a stable cell line that supplies the transgene flanked by ITRs and rep/cap is used. One method for minimizing the possibility of generating replication competent AAV (rcAAV) using these systems is by eliminating regions of homology between regions flanking the rep/cap cassette and the ITRs that flank the transgene. However, in each of these systems, AAV virions are produced in response to infection with helper adenovirus or herpesvirus, requiring the separation of the rAAVs from contaminating virus.

More recently, systems have been developed that do not require infection with helper virus to recover the AAV - the required helper functions *(i.e.,* adenovirus E1, E2a, VA, and E4 or herpesvirus UL5, UL8, UL52, and UL29, and herpesvirus polymerase) are also supplied, in *trans,* by the system. In these newer systems, the helper functions can be supplied by transient transfection of the cells with constructs that encode the required helper functions, or the cells can be engineered to stably contain genes encoding the helper functions, the expression of which can be controlled at the transcriptional or posttranscriptional level. In yet another system, the transgene flanked by ITRs and rep/cap genes are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, *e.g.,* Grieger & Samulski, 2005; and Btining *et aI.,* 2008; Zhang *et ai.,* 2009, "Adenovirus-adeno-associated virus hybrid for large-scale recombinant adeno-associated virus production," *Human Gene Therapy 20:922-*929, the contents of each of which is incorporated herein by reference in its entirety.

Methods of making and using these and other AAV production systems are also described in the following U.S. patents, the contents of each of which is incorporated herein by reference in its entirety: 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065. See also the paragraphs below, which describe methods for scaling up AAV production using these systems and variants thereof.

Due to size constraints of AAV for packaging (tolerating a transgene of approximately 4.5 kb), the transcription unites) *(i.e.,* the transgene unit, the ablator unit, and the dimerizable transcription factor unit) described may need to be engineered and packaged into two or more replication-deficient AAV stocks. This may be preferable, because there is evidence that exceeding the packaging capacity may lead to the generation of a greater number of "empty" AAV particles.

Alternatively, the available space for packaging may be conserved by combining more than one transcription unit into a single construct, thus reducing the amount of required regulatory sequence space. For example, a single promoter may direct expression of a single RNA that encodes two or three or more genes of interest, and translation of the downstream genes are driven by IRES sequences. In another example, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three or more genes of interest separated from one another by sequences encoding a self-cleavage peptide *(e.g.,* T2A) or a protease recognition site *(e.g.,* furin). The ORF thus encodes a single polyprotein, which, either during (in the case of T2A) or after translation, is cleaved into the individual proteins (such as, *e.g.,* transgene and dimerizable transcription factor). It should be noted, however, that although these IRES and polyprotein systems can be used to save AAV packaging space, they can only be used for expression of components that can be driven by the same promoter.

In another alternative, the transgene capacity of AAV can be increased by providing AAV ITRs of two genomes that can anneal to form head to tail concatamers. Generally, upon entry of the AAV into the host cell, the single-stranded DNA containing the transgene is converted by host cell DNA polymerase complexes into double-stranded DNA, after which the ITRs aid in concatamer formation in the nucleus. As an alternative, the AAV may be engineered to be a self-complementary (sc) AAV, which enables the virus

to bypass the step of second-strand synthesis upon entry into a target cell, providing an scAAV virus with faster and, potentially, higher (e.g., up to 100-fold) transgene expression. For example, the AAV may be engineered to have a genome comprising two connected single-stranded DNAs that encode, respectively, a transgene unit and its complement, which can snap together following delivery into a target cell, yielding a double-stranded DNA encoding the transgene unit of interest. Self-complementary AAV s are described in, *e.g.,* U.S. Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

The transcription units(s) in the replication-deficient rAAVs may be packaged with any AAV capsid protein (Cap) described herein, known in the art, or to be discovered. Caps from serotypes AAV1, AAV6, AAV7, AAV8, AAV9 or rh10 are particularly preferred for generating rAAVs for use in human subjects. In a preferred embodiment, an rAAV Cap is based on serotype AAV8. In another embodiment, an rAAV Cap is based on Caps from two or three or more AAV serotypes. For example, in one embodiment, an rAAV Cap is based on AAV6 and AAV9.

Cap proteins have been reported to have effects on host tropism, cell, tissue, or organ specificity, receptor usage, infection efficiency, and immunogenicity of AAV viruses. See, *e.g.,* Grieger & Samulski, 2005; Buning et al., 2008; and the references cited below in this sub-section; all of which are incorporated herein by reference in their entirety. Accordingly, an AAV Cap for use in an rAAV may be selected based on consideration of, for example, the subject to be treated *(e.g.,* human or non-human, the subject's immunological state, the subject's suitability for long or short-term treatment, etc.) or a particular therapeutic application *(e.g.,* treatment of a particular disease or disorder, or delivery to particular cells, tissues, or organs).

In some embodiments, an rAAV Cap is selected for its ability to efficiently transduce a particular cell, tissue, or organ, for example, to which a particular therapy is targeted. In some embodiments, an rAAV Cap is selected for its ability to cross a tight endothelial cell barrier, for example, the blood-brain barrier, the blood-eye barrier, the blood-testes barrier, the blood-ovary barrier, the endothelial cell barrier surrounding the heart, or the blood-placenta barrier.

Tissue specificity of adeno-associated viruses (AAV) serotypes is determined by the serotype of the capsid, and viral vector based on different AAV capsids may generated taking into consideration their ability to infect different tissues. AAV2 presents a natural tropism towards skeletal muscles, neurons of the central nervous system, vascular smooth muscle cells. AAV1 has been described as being more efficient than AAV2 in transducing muscle, arthritic joints, pancreatic islets, heart, vascular endothelium, central nervous system (CNS) and liver cells, whereas AAV3 appears to be well suited for the transduction of cochlear inner hair cells, AAV4 for brain, AAV5 for CNS, lung, eye, arthritic joints and liver cells, AAV6 for muscle, heart and airway epithelium, AAV7 for muscle, AAV8 for muscle, pancreas, heart and liver, and AAV9 for heart. See, e.g., Buning *et at.,* 2008. Any serotype of AAV known in the art, *e.g.,* serotypes AAV1, AAV2, AAV3A, AAV3B, AAV4, AAV5, AAV6, AAV7 [see, WO 2003/042397], AAV8 [see, e.g., US Patent 7790449; US Patent 7282199], AAV9 [see, WO 2005/033321], AAV10, AAV11, AAV12, rh10, modified AAV [see, e.g., WO 2006/110689], or yet to be discovered, or a recombinant AAV based thereon, may be used as a source for the rAAV capsid.

Various naturally occurring and recombinant AAVs, their encoding nucleic acids, AAV Cap and Rep proteins and their sequences, as well as methods for isolating or generating, propagating, and purifying such AAVs, and in particular, their capsids, suitable for use in producing rAAV s are described in Gao *et al.,* 2004, "Clades of adeno-associated viruses are widely disseminated in human tissues," J. Virol. 78:6381-6388; U.S. Patent Nos. 7,319,002; 7,056,502; 7,282,199; 7,198,951; 7,235,393; 6,156,303; and 7,220,577; U.S. Patent Application Publication Nos. US 2003-0138772; US 2004-0052764; US 2007-0036760; US 2008-0075737; and US 2008-0075740; and International Patent Application Publication Nos. WO 20031014367; WO 20011083692; WO 2003/042397 (AAV7 and various simian AAV); WO 2003/052052; WO 2005/033321; WO 20061110689; WO 2008/027084; and WO 2007/127264; each of which is incorporated herein by reference in its entirety.

In some embodiments, an AAV Cap for use in the rAAV can be generated by mutagenesis *(i.e.,* by insertions, deletions, or substitutions) of one of the aforementioned AAV Caps or its encoding nucleic acid. In some embodiments, the AAV Cap is at least 70% identical, 75 % identical, 80% identical, 85% identical, 90% identical, 95% identical,

98% identical, or 99% or more identical to one or more of the aforementioned AAV Caps.

In some embodiments, the AAV Cap is chimeric, comprising domains from two or three or four or more of the aforementioned AAV Caps. In some embodiments, the AAV Cap is a mosaic of Vp1, Vp2, and Vp3 monomers from two or three different AAVs or recombinant AAVs. In some embodiments, an rAAV composition comprises more than one of the aforementioned Caps.

In some embodiments, an AAV Cap for use in an rAAV composition is engineered to contain a heterologous sequence or other modification. For example, a peptide or protein sequence that confers selective targeting or immune evasion may be engineered into a Cap protein. Alternatively or in addition, the Cap may be chemically modified so that the surface of the rAAV is polyethylene glycolated (PEGylated), which may facilitate immune evasion. The Cap protein may also be mutagenized, *e.g.,* to remove its natural receptor binding, or to mask an immunogenic epitope.

Methods for Scalable Manufacture of AAV

Methods for the scalable *(e.g.,* for production at commercial scale) manufacture of AAV, which may be adapted in order to generate rAAV compositions that are suitably homogeneous and free of contaminants for use in clinical applications, are also known in the art, and are summarized briefly below.

Adeno-associated viruses can be manufactured at scale using a mammalian cell line-based approach, such as the approach using stable producer cell lines described in Thome *et al.,* 2009, "Manufacturing recombinant adeno-associated viral vectors from producer cell clones," *Human Gene Therapy* 20:707-714, which is incorporated herein by reference in its entirety. In the approach described by Thorpe and colleagues, producer cell lines stably containing all the components needed to generate an rAAV - the transgene construct (transgene flanked by ITRs) and AAV rep and cap genes - are engineered, which are induced to make virus by infection with a helper virus, such as a live adenovirus type 5 (Ad5) (methods of scalable production of which are also well known in the art). Producer cell lines are stably transfected with construct(s) containing (i) a packaging cassette (rep and cap genes of the desired serotype and regulatory elements required for their expression), (ii) the transgene flanked by ITRs, (iii) a selection marker for mammalian cells,

and (iv) components necessary for plasmid propagation in bacteria. Stable producer cell lines are obtained by transfecting the packaging construct(s), selecting drug-resistant cells, and replica-plating to ensure production of the recombinant AAV in the presence of helper virus, which are then screened for performance and quality. Once appropriate clones are chosen, growth of the cell lines is scaled up, the cells are infected with the adenovirus helper, and resulting rAAVs are harvested from the cells.

In an alternative to the methods described in Thorpe *et al.,* a packaging cell line is stably transfected with the AAV rep and cap genes, and the transgene construct is introduced separately when production of the rAAV is desired. Although Thorpe and colleagues use HeLa cells for the producer cell line, any cell line *(e.g.,* Vero, A549, HEK 293) that is susceptible to infection with helper virus, able to maintain stably integrated copies of the rep gene and, preferably, able to grow well in suspension for expansion and production in a bioreactor may be used in accordance with the methods described in Thorpe *et al.*

In the foregoing methods, rAAVs are produced using adenovirus as a helper virus. In a modification of these methods, rAAV s can be generated using producer cells stably transfected with one or more constructs containing adenovirus helper functions, avoiding the requirement to infect the cells with adenovirus. In a variation, one or more of the adenovirus helper functions are contained within the same construct as the rep and cap genes. In these methods, expression of the adenovirus helper functions may be placed under transcriptional or post-transcriptional control to avoid adenovirus-associated cytotoxicity.

In an alternative to producing stable cell lines, AAV s may also be produced at scale using transient transfection methods, such as described by Wright, 2009, "Transient transfection methods for clinical adeno-associated viral vector production," *Human Gene Therapy* 20:698-706, which is incorporated herein by reference in its entirety. Wright's approach involves transfection of cells with constructs that contain (i) the transgene of interest flanked by ITRs; (ii) the AAV rep and cap genes; and (iii) helper virus *(e.g.,* adenovirus) genes required to support genome replication and packaging (or alternatively, a helper virus, as described in Thorpe *et al.),* Alternatively, the adenovirus helper functions may be contained within the same construct as the rep and cap genes. Thus, rAAV s are

produced without having to ensure stable transfection of the transgene and rep/cap constructs. This provides a flexible and quick method for generating AAVs, and is thus ideal for pre-clinical and early-phase clinical development. Recombinant AAVs can be generated by transiently transfecting mammalian cell lines with the constructs using transient transfection methods known in the art. For example, transfection methods most suited for large-scale production include DNA co-precipitation with calcium phosphate, the use of poly-cations such as polyethylenimine (PE), and cationic lipids.

The effectiveness of adenovirus as a helper has also been exploited to develop alternative methods for large-scale recombinant AAV production, for example using hybrid viruses based on adenovirus and AAV (an "Ad-AAV hybrid"). This production method has the advantage that it does not require transfection - all that is required for rAAV production is infection of the rep/cap packaging cells by adenoviruses. In this process, a stable rep/cap cell line is infected with a helper adenovirus possessing functional E 1 genes and, subsequently, a recombinant Ad-AAV hybrid virus in which the AAV transgene plus ITRs sequence is inserted into the adenovirus El region. Methods for generating Ad-AAV hybrids and their use in recombinant AAV production are described in Zhang *et al., 2009,* which is incorporated by reference herein in its entirety.

In another variation, rAAVs can be generated using hybrid viruses based on AAV and herpes simplex virus type 1 (HSV) (an "HSV / AAV hybrid"), such as described in Clement *et al.,* 2009, "Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies," *Human Gene Therapy* 20:796-806, which is incorporated herein by reference in its entirety. This method expands on the possibility of using HSV as a helper virus for AAV production (well known in the art, and also reviewed in Clement et al.). Briefly, HSV/AAV hybrids comprise an AAV transgene construct within an HSV backbone. These hybrids can be used to infect producer cells that supply the rep/cap and herpesvirus helper functions, or can be used in co-infections with recombinant HSV s that supply the helper functions, resulting in generation of rAAV s encapsidating the transgene of interest.

In another method, rAAV compositions may produced at scale using recombinant baculovirus-mediated expression of AAV components in insect cells, for example, as described in Virag *et al.,* 2009, "Producing recombinant adeno-associated virus

in foster cells: Overcoming production limitations using a baculovirus-insect cell expression strategy," *Human Gene Therapy* 20:807-817, which is incorporated herein by reference in its entirety. In this system, the well-known baculovirus expression vector (BEV) system is adapted to produce recombinant AAVs. For example, the system described by Virag *et al.* comprises the infection of Sf9 insect cells with two (or three) different BEVs that provide (i) AAV rep and cap (either in one or two BEVs) and (ii) the transgene construct. Alternatively, the Sf9 cells can be stably engineered to express rep and cap, allowing production of recombinant AAV s following infection with only a single BEV containing the transgene construct. In order to ensure stoichiometric production of the Rep and Cap proteins, the latter of which is required for efficient packaging, the BEV s can be engineered to include features that enable pre- and post-transcriptional regulation of gene expression. The Sf9 cells then package the transgene construct into AAV capsids, and the resulting rAAV can be harvested from the culture supernatant or by lysing the cells.

Each of the foregoing methods permit the scalable production of rAAV compositions. The manufacturing process for an rAAV composition suitable for commercial use (including use in the clinic) must also comprise steps for removal of contaminating cells; removing and inactivating helper virus (and any other contaminating virus, such as endogenous retrovirus-like particles); removing and inactivating any rcAAV; minimizing production of, quantitating, and removing empty (transgene-less) AAV particles *(e.g.,* by centrifugation); purifying the rAAV *(e.g.,* by filtration or chromatography based on size and/or affinity); and testing the rAAV composition for purity and safety. These methods are also provided in the references cited in the foregoing paragraphs and are incorporated herein for this purpose.

One disadvantage of the foregoing methods of scalable rAAV production is that much of the rAAV is obtained by lysing the producer cells, which requires significant effort to not only obtain the virus but also to isolate it from cellular contaminants. To minimize these requirements, scalable methods of rAAV production that do not entail cell lysis may be used, such as provided in International Patent Application Publication No. WO 2007/127264, the contents of which is incorporated by reference herein in its entirety. In the example of Section 6 *infra,* a new scalable method obtaining rAAV from cell culture supernatants is provided, which may also be adapted for the preparation of rAAV

composition for use in accordance with the methods described herein.

In still another embodiment, the invention provides human or non-human cells which contain one or more of the DNA constructs and/or virus compositions of the invention. Such cells may be genetically engineered and may include, e.g., plant, bacterial, non-human mammalian or mammalian cells. Selection of the cell types is not a limitation of the invention.

5.2. Compositions

The present invention provides replication-defective virus compositions suitable for use in therapy (*in vivo* or *ex vivo*)in which the genome of the virus (or the collective genomes of two or more replication-defective virus stocks used in combination) comprise the therapeutic transgene unit and the ablator unit defined in Section 3.1, and described *supra;* and may further comprise dimerizable fusion protein or TF domain units(s) (referred to for purposes of convenience as dimerizable unit(s)). Any virus suitable for gene therapy may be used in the compositions of the invention, including but not limited to adeno-associated virus ("AAV"), adenovirus, herpes simplex virus, lentivirus, or a retrovirus. In a preferred embodiment, the compositions are replication-defective AAV s, which are described in more detail in Section 5.2.1 herein.

The compositions of the invention comprise a replication-defective virus(es) suitable for therapy (*in vivo* or *ex vivo*) in which the genome of the virus(es) comprises a transgene unit, an ablation unit, and/or a dimerizable unit. In one embodiment, a composition of the invention comprises a virus suitable for gene therapy in which the genome of the virus comprises a transgene unit. In another embodiment, a composition of the invention comprises a virus suitable for gene therapy in which the genome of the virus comprises an ablation unit. In another embodiment, a composition of the invention comprises a virus suitable for gene therapy in which the genome of the virus comprises a dimerizable unit. In another embodiment, a composition of the invention comprises a virus suitable for gene therapy in which the genome of the virus comprises a transgene unit and an ablation unit. In another embodiment, a composition of the invention comprises a virus suitable for gene therapy in which the genome of the virus comprises a transgene unit and a dimerizable unit. In another embodiment, a composition of the invention comprises a virus suitable for gene therapy in which the genome of the virus comprises an ablation unit and a dimerizable unit.

In another embodiment, a composition of the invention comprises viruses suitable for gene therapy in which the genome of the virus comprises a transgene unit, an ablation unit and a dimerizable unit.

The invention also provides compositions comprising recombinant DNA constructs that comprise one or more transcriptional units described herein. Compositions comprising recombinant DNA constructs are described in more detail in Section 5.2.2.

5.2.1. Replication-Defective Virus Compositions for Gene Therapy

The invention provides compositions comprising a replication-defective virus stock(s) and formulations of the replication-defective virus(es) in a physiologically acceptable carrier. These formulations can be used for gene transfer and/or gene therapy. The viral genome of the compositions comprises: (a) a first transcription unit that encodes a therapeutic product in operative association with a promoter that controls transcription, said unit containing at least one ablation recognition site (transgene unit); and (b) a second transcription unit that encodes an ablator specific for the ablation recognition site, or a fragment thereof, in operative association with a promoter. In one embodiment, the viral genome of the replication-defective virus. The ablator is as defined elsewhere in this specification.

AAV Stocks

In a preferred embodiment, the replication-defective virus of a composition of the invention is an AAV, preferably AAV1, AAV6, AAV6.2, AAV7, AAV8, AAV9 or rh10. In one embodiment, the AAV of the composition is AAV8. Due to the packaging constraints of AAV (approximately 4.5 kb) in most cases, for ease of manufacture, the transgene unit, the ablation unit, and the dimerizable unit will be divided between two or more viral vectors and packaged in a separate AAV stock. In one embodiment, the replication-defective virus composition comprises the first transcription unit (a transgene unit) packaged in one AAV stock, and the second (an ablator unit), third and fourth transcription units (dimerizable TF domain unit) packaged in a second AAV stock. In another embodiment, the replication-defective virus composition comprises the second transcription unit (an ablator unit)

packaged in one AAV stock, and the first (a transgene unit), third and fourth transcription units (dimerizable TF domain unit) packaged in a second AAV stock. In another embodiment, all four units can be packaged in one AAV stock, but this imposes limits on the size of the DNAs that can be packaged. For example, when using Cre as the ablator and FRB/FKB as the dimerizable TF domains (as shown in the examples, *infra),* in order to package all four units into one AAV stock, the size of the DNA encoding the therapeutic transgene should be less than about 900 base pairs in length; this would accommodate DNAs encoding cytokines, RNAi therapeutics, and the like.

Due to size constraints of the AAV genome for packaging, the transcription units can be engineered and packaged in two or more AAV stocks. Whether packaged in one viral stock which is used as a virus composition according to the invention, or in two or more viral stocks which form a virus composition of the invention, the viral genome used for treatment must collectively contain the first and second transcription units encoding the therapeutic transgene and the ablator; and may further comprise additional transcription units (e.g., the third and fourth transcription units encoding the dimerizable TF domains). For example, the first transcription unit can be packaged in one viral stock, and second, third and fourth transcription units packaged in a second viral stock. Alternatively, the second transcription unit can be packaged in one viral stock, and the first, third and fourth transcription units packaged in a second viral stock. While useful for AAV due to size contains in packaging the AAV genome, other viruses may be used to prepare a virus composition according to the invention. In another embodiment, the viral compositions of the invention, where they contain multiple viruses, may contain different replication-defective viruses (e.g., AAV and adenovirus).

In one embodiment, a virus composition according to the invention contains two or more different AAV (or another viral) stock, in such combinations as are described above. For example, a virus composition may contain a first viral stock comprising the therapeutic gene with ablator recognition sites and a first ablator and a second viral stock containing an additional ablator(s). Another viral composition may contain a first virus stock comprising a therapeutic gene and a fragment of an ablator and a second virus stock comprising another fragment of an ablator. Various other combinations of two or

more viral stocks in a virus composition of the invention will be apparent from the description of the components of the present system.

Viral Formulations

Compositions of the invention may be formulated for delivery to animals for veterinary purposes (*e.g.,* livestock (cattle, pigs, etc), and other non-human mammalian subjects, as well as to human subjects. The replication-defective viruses can be formulated with a physiologically acceptable carrier for use in gene transfer and gene therapy applications. Because the viruses are replication-defective, the dosage of the formulation cannot be measured or calculated as a PFU (plaque forming unit). Instead, quantification of the genome copies ("GC") may be used as the measure of the dose contained in the formulation.

Any method known in the art can be used to determine the genome copy (GC) number of the replication-defective virus compositions of the invention. One method for performing AAV GC number titration is as follows: Purified AAV vector samples are first treated with DNase to eliminate un-encapsidated AAV genome DNA or contaminating plasmid DNA from the production process. The DNase resistant particles are then subjected to heat treatment to release the genome from the capsid. The released genomes are then quantitated by real-time PCR using primer/probe sets targeting specific region of the viral genome (usually poly A signal).

Also, the replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0 x 10^9 GC to about 1.0 x 10^{15} GC (to treat an average subject of 70 kg in body weight), and preferably 1.0 x 10^{12} GC to 1.0 x 10^{14} GC for a human patient. Preferably, the dose of replication-defective virus in the formulation is 1.0 x 10^9 GC, 5.0 X 10^9 GC, 1.0 X 10^{10} GC, 5.0 X 10^{10} GC, 1.0 X 10^{11} GC, 5.0 X 10^{11} GC, 1.0 X 10^{12} GC, 5.0 X 10^{12} GC, or 1.0 x 10^{13} GC, 5.0 X 10^{13} GC, 1.0 X 10^{14} GC, 5.0 X 10^{14} GC, or 1.0 x 10^{15} GC.

The replication-defective viruses can be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients. The replication-defective viruses may be formulated for parenteral administration by injection, *e.g.,* by bolus injection or continuous infusion. Formulations for injection may be presented in unit

dosage form, *e.g.,* in ampoules or in multi-dose containers, with an added preservative. The replication-defective virus compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Liquid preparations of the replication-defective virus formulations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents *(e.g.,* sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents *(e.g.,* lecithin or acacia); non-aqueous vehicles *(e.g.,* almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives *(e.g.,* methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts. Alternatively, the compositions may be in powder form for constitution with a suitable vehicle, *e.g.,* sterile pyrogen-free water, before use.

Also encompassed is the use of adjuvants in combination with or in admixture with the replication-defective viruses of the invention. Adjuvants contemplated include but are not limited to mineral salt adjuvants or mineral salt gel adjuvants, particulate adjuvants, microparticulate adjuvants, mucosal adjuvants, and immunostimulatory adjuvants. Adjuvants can be administered to a subject as a mixture with replication-defective viruses of the invention, or used in combination with the replication-defective viruses of the invention.

5.2.2. Recombinant DNA Construct Compositions For Production of Replication-Defective Viral Vectors Useful for Therapeutic Purposes

The invention provides recombinant DNA construct compositions comprising a transgene unit, an ablation unit, and/or one or two dimerizable domain units flanked by viral signals that define the region to be amplified and packaged into replication-defective viral particles. These DNA constructs can be used to generate the replication-defective virus compositions and stocks.

In one embodiment, the recombinant DNA construct comprises a transgene unit flanked by packaging signals of a viral genome. In another embodiment, a composition of the invention comprises a recombinant DNA construct comprising an ablation unit flanked by packaging signals of a viral genome. In another embodiment, the recombinant DNA construct comprises a dimerizable unit flanked by packaging signals of a viral genome. In another embodiment, the recombinant DNA construct comprises a transgene

unit and an ablation unit flanked by packaging signals of a viral genome. In another embodiment, the recombinant DNA construct comprises a transgene unit and a dimerizable unit flanked by packaging signals of a viral genome. In another embodiment, the recombinant DNA construct comprises an ablation unit and a dimerizable unit flanked by packaging signals of a viral genome. In another embodiment, the recombinant DNA construct comprises a transgene unit, an ablation unit and a dimerizable unit flanked by packaging signals of a viral genome.

The first transcription unit encodes a therapeutic product in operative association with a promoter that controls transcription, said unit containing at least one ablation recognition site (transgene unit); and (b) the second transcription unit that encodes an ablator specific for the ablation recognition site, or a fragment thereof fused to a binding domain, in operative association with a promoter that induces transcription in response to a pharmacological agent (ablation unit). In another embodiment, the recombinant DNA construct comprises a dimerizable TF domain unit flanked by packaging signals of a viral genome.

In a preferred embodiment, the recombinant DNA construct composition further comprises a dimerizable unit nested within the viral packaging signals. In one embodiment, each unit encodes a dimerizable domain of a transcription factor that regulates the inducible promoter of the second transcription unit, in which (c) a third transcription unit encodes the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a constitutive promoter; and (d) a fourth transcription unit encodes the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a constitutive promoter. In another embodiment, at least one of (c) or (d) is expressed under an inducible promoter. In a specific embodiment, the pharmacological agent that induces transcription of the promoter that is in operative association with the second unit of the recombinant DNA construct composition is a dimerizer that dimerizes the domains of the transcription factor as measured *in vitro*. In yet another specific embodiment, the pharmacological agent that induces transcription of the promoter that is in operative association with the second unit of the recombinant DNA construct composition is rapamycin. In still a further embodiment, the recombinant DNA construct comprises a dimerizable fusion protein unit. For example, the

dimerizable fusion protein unit may be encode (a) a binding domain of an enzyme fused to a binding domain and (b) a catalytic domain of the enzyme fused to a binding domain, where the binding domains are either DNA binding domains or the binding domains for a dimerizer.

In order to conserve space within the viral genome(s), bicistronic transcription units can be engineered. For example, the third and fourth transcription units can be engineered as a bicistronic unit containing an IRES (internal ribosome entry site), which allows coexpression of heterologous gene products by a message from a single promoter. Altenatively, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three heterologous genes (e.g., the third and fourth transcription units) separated from one another by sequences encoding a self-cleavage peptide *(e.g.,* T2A) or a protease recognition site *(e.g.,* furin). The ORF thus encodes a single polyprotein, which, either during (in the case of T2A) or after translation, is cleaved into the individual proteins. It should be noted, however, that although these IRES and polyprotein systems can be used to save AAV packaging space, they can only be used for expression of components that can be driven by the same promoter.

In a specific embodiment, a recombinant DNA construct composition that comprises a dimerizable unit comprises an IRES. In another specific embodiment, a recombinant DNA construct composition that comprises a third and fourth transcription unit (a dimerizable TF domain unit) comprises and IRES In another specific embodiment, a recombinant DNA construct composition that comprises a transgene unit comprises an IRES. In another specific embodiment, a recombinant DNA construct composition that comprises an ablation unit comprises an IRES. In another specific embodiment, a recombinant DNA construct composition that comprises a dimerizable unit comprises an IRES.

In a specific embodiment, a recombinant DNA construct composition that comprises a third and a fourth transcription unit (a dimerizable TF domain unit) comprises T2A sequence. In another specific embodiment, a recombinant DNA construct composition that comprises a transgene unit comprises T2A sequence. In another specific embodiment, a recombinant DNA construct composition that comprises an ablation unit comprises T2A sequence. In another specific embodiment, a recombinant DNA construct composition that comprises a dimerizable TF domain unit comprises T2A sequence.

In an embodiment, the ablator that is encoded by the second transcription unit of the recombinant DNA construct composition is an endonuclease, a recombinase, a meganuclease, or an artificial zinc finger endonuclease that binds to the ablation recognition site in the first transcription unit and excises or ablates DNA. In a specific embodiment, the ablator is ere and the ablation recognition site is LoxP, or the ablator is FLP and the ablation recognition site is FRT. In another embodiment, the ablator that is encoded by the second .transcription unit of the recombinant DNA construct composition is an interfering RNA, a ribozyme, or an antisense that ablates the RNA transcript of the first transcription unit, or suppresses translation of the RNA transcript of the first transcription unit. In a specific embodiment, transcription of the ablator is controlled by a tet-on/off system, a tetR-KRAB system, a mifepristone (RU486) regulatable system, a tamoxifen-dep regulatable system, or an ecdysone-dep regulatable system.

The recombinant DNA construct composition contains packaging signals flanking the transcription units desired to be amplified and packaged in replication-defective virus vectors. In a specific embodiment, the packaging signals are AAV ITRs. Where a pseudotyped AAV is to be produced, the ITRs are selected from a source which differs from the AAV source of the capsid. For example, AAV2 ITRs may be selected for use with an AAV1, AAV8, or AAV9 capsid, and so on. In another specific embodiment, the AAV ITRs may be from the same source as the capsid, e.g., AAV1, AAV6, AAV7, AAV8, AAV9, rh10 ITRs, etc. In another specific embodiment, a recombinant DNA construct composition comprises a first transcription unit (transgene unit) flanked by AAV ITRs, and the second (ablation unit), and optional third and fourth transcription units (a dimerizable TF domain unit), and/or a dimerizable fusion protein unit(s), flanked by AAV ITRs. In yet another specific embodiment, a recombinant DNA construct composition comprises a second transcription unit (ablation unit) flanked by AAV ITRs, and the first (transgene unit), third and fourth transcription units (a dimerizable TF domain unit) are flanked by AAV ITRs. In a preferred embodiment, the transcription units of a PIT A system are contained in two or more recombinant DNA compositions.

In a specific embodiment, recombinant DNA construct contains a transgene unit that encodes anyone or more of the following therapeutic products: an antibody or antibody fragment that neutralizes HIV infectivity, soluble vascular endothelial growth

factor receptor-1 (sFlt-I), Factor VIII, Factor IX, insulin like growth factor (IGF), hepatocyte growth factor (HGF), heme oxygenase-1 (HO-1), or nerve growth factor (NGF). In a specific embodiment, recombinant DNA construct contains a transgene unit that comprises anyone of the following promoters that controls transcription of the therapeutic gene: a constitutive promoter, a tissue-specific promoter, a cell-specific promoter, an inducible promoter, or a promoter responsive to physiologic cues.

The DNA constructs can be used in any of the methods described in Section 5.1.5 to generate replication-defective virus stocks.

5.2.3. Pharmaceutical Compositions and Formulations of Dimerizers

The present invention provides pharmaceutical compositions comprising the dimerizers of the invention, described in Section 5.1.4. In a preferred embodiment, the pharmaceutical compositions comprise a pharmaceutically acceptable carrier or excipient. Optionally, these pharmaceutical compositions are adapted for veterinary purposes, e.g., for delivery to a non-human mammal (e.g., livestock), such as are described herein.

The pharmaceutical compositions of the invention can be administered to a subject at therapeutically effective doses to ablate or excise the transgene of a transgene unit of the invention or to ablate the transcript of the transgene, or inhibit its translation. A therapeutically effective dose refers to an amount of the pharmaceutical composition sufficient to result in amelioration of symptoms caused by expression of the transgene, *e.g.*, toxicity, or to result in at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% inhibition of expression of the transgene.

In an embodiment, an amount of pharmaceutical composition comprising a dimerizer of the invention is administered that is in the range of about 0.1-5 micrograms (μg)/kilogram (kg). To this end, a pharmaceutical composition comprising a dimerizer of the invention is formulated in doses in the range of about 7 mg to about 350 mg to treat to treat an average subject of 70 kg in body weight. The amount of pharmaceutical composition comprising a dimerizer of the invention administered is: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 or 5.0 mg/kg. The dose of a dimerizer in a formulation is 7, 8, 9, 10, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 90, 95,

100,125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 400, 425, 450, 475, 500, 525, 550, 575, 600, 625, 650, 675, 700, 725, or 750 mg (to treat to treat an average subject of 70 kg in body weight). These doses are preferably administered orally. These doses can be given once or repeatedly, such as daily, every other day, weekly, biweekly, or monthly. Preferably, the pharmaceutical compositions are given once weekly for a period of about 4-6 weeks. In some embodiments, a pharmaceutical composition comprising a dimerizer is administered to a subject in one dose, or in two doses, or in three doses, or in four doses, or in five doses, or in six doses or more. The interval between dosages may be determined based the practitioner's determination that there is a need for inhibition of expression of the transgene, for example, in order to ameliorate symptoms caused by expression of the transgene, *e.g.,* toxicity. For example, in some embodiments when the need for transgene ablation is acute, daily dosages of a pharmaceutical composition comprising a dimerizer may be administered. In other embodiments, *e.g.,* when the need for transgene ablation is less acute, or is not acute, weekly dosages of a pharmaceutical composition comprising a dimerizer may be administered.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Thus, the dimerizers and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) oral, buccal, parenteral, rectal, or transdermal administration. Noninvasive methods of administration are also contemplated.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents *(e.g.,* pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers *(e.g.,* lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants *(e.g.,* magnesium stearate, talc or silica); disintegrants *(e.g.,* potato starch or sodium starch glycolate); or wetting agents *(e.g.,* sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may

be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents *(e.g.,* sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents *(e.g.,* lecithin or acacia); non-aqueous vehicles *(e.g.,* almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives *(e.g.,* methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the dimerizers.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the dimerizers for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.,* dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.,* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the dimerizers and a suitable powder base such as lactose or starch.

The dimerizers may be formulated for parenteral administration by injection, *e.g.,* by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.,* in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.,* sterile pyrogen-free water, before use.

The dimerizers may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.,* containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the dimerizers may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection.

Thus, for example, the dimerizers may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

Also encompassed is the use of adjuvants in combination with or in admixture with the dimerizers of the invention. Adjuvants contemplated include but are not limited to mineral salt adjuvants or mineral salt gel adjuvants, particulate adjuvants, microparticulate adjuvants, mucosal adjuvants, and immunostimulatory adjuvants. Adjuvants can be administered to a subject as a mixture with dimerizers of the invention, or used in combination with the dimerizers of the invention.

5.3. Treatment of Diseases and Disorders

The invention provides methods for treating any disease or disorder that is amenable to gene therapy. In one embodiment, "treatment" or "treating" refers to an amelioration of a disease or disorder, or at least one discernible symptom thereof. In another embodiment, "treatment" or "treating" refers to an amelioration of at least one measurable physical parameter associated with a disease or disorder, not necessarily discernible by the subject. In yet another embodiment, "treatment" or "treating" refers to inhibiting the progression of a disease or disorder, either physically, *e.g.,* stabilization of a discernible symptom, physiologically, *e.g.,* stabilization of a physical parameter, or both. Other conditions, including cancer, immune disorders, and veterinary conditions, may also be treated.

5.3.1. Target Diseases

Types of diseases and disorders that can be treated by methods of the present invention include, but are not limited to age-related macular degeneration; diabetic retinopathy; infectious diseases *e.g.,* HIV pandemic flu, category 1 and 2 agents of biowarfare, or any new emerging viral infection; autoimmune diseases; cancer; multiple myeloma; diabetes; systemic lupus erythematosus (SLE); hepatitis C; multiple sclerosis;

Alzheimer's disease; parkinson's disease; amyotrophic lateral sclerosis (ALS), huntington's disease; epilepsy; chronic obstructive pulmonary disease (COPD); joint inflammation, arthritis; myocardial infarction (MI); congestive heart failure (CHF); hemophilia A; or hemophilia B.

Infectious diseases that can be treated or prevented by the methods of the present invention are caused by infectious agents including, but not limited to, viruses, bacteria, fungi, protozoa, helminths, and parasites. The invention is not limited to treating or preventing infectious diseases caused by intracellular pathogens. Many medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, Medical Microbiology, Bailliere Tindall, Great Britain 1983, the entire contents of which are hereby incorporated herein by reference.

Bacterial infections or diseases that can be treated or prevented by the methods of the present invention are caused by bacteria including, but not limited to, bacteria that have an intracellular stage in its life cycle, such as mycobacteria (e.g., *Mycobacteria tuberculosis, M bovis, M avium, M leprae,* or *M africanum),* rickettsia, mycoplasma, chlamydia, and legionella. Other examples of bacterial infections contemplated include but are not limited to infections caused by Gram positive bacillus (e.g., *Listeria, Bacillus* such as *Bacillus anthracis, Erysipelothrix* species), Gram negative bacillus (e.g., *Bartonella, Brucella, Campylobacter, Enterobacter, Escherichia, Francisella, Hemophilus, Klebsiella, Morganella, Proteus, Providencia, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio,* and *Yersinia* species), spirochete bacteria (e.g., *Borrelia* species including *Borrelia burgdorferi* that causes Lyme disease), anaerobic bacteria (e.g., *Actinomyces* and *Clostridium* species), Gram positive and negative coccal bacteria, *Enterococcus* species, *Streptococcus* species, *Pneumococcus* species, *Staphylococcus* species, *Neisseria* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria tuberculosis, M avium, M intracellulare, M kansaii, M gordonae, Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus viridans, Streptococcus faecalis, Streptococcus bovis, Streptococcus pneumoniae, Haemophilus injluenzae, Bacillus antracis, corynebacterium diphtheriae, Erysipelothrix rhusiopathiae,*

Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida, Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, Rickettsia, and *Actinomyces israelli.*

Infectious virus of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. Examples of virus that have been found in humans include but are not limited to: Retroviridae (e.g. human immunodeficiency viruses, such as HIV-1 (also referred to as HTL V -III, LA V or HTLV -III/LA V, or HIV -III; and other isolates, such as HIV-LP; Picomaviridae (e.g. polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (e.g. strains that cause gastroenteritis); Togaviridae (e.g. equine encephalitis viruses, rubella viruses); Flaviridae (e.g. dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (e.g. coronaviruses); Rhabdoviridae (e.g. vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g. ebola viruses); Paramyxoviridae (e.g. parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g. influenza viruses); Bungaviridae, (e.g. Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (e.g. reoviruses, orbiviurses and rotaviruses); Bimaviridae; Hepadnaviridae (Hepatitis B virus); Parvovirida (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and lridoviridae (e.g. African swine fever virus); and unclassified viruses (e.g. the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (i.e. Hepatitis C); Norwalk and related viruses, and astroviruses).

Parasitic diseases that can be treated or prevented by the methods of the present invention including, but not limited to, amebiasis, malaria, leishmania, coccidia, giardiasis, cryptosporidiosis, toxoplasmosis, and trypanosomiasis. Also encompassed are infections by various worms, such as but not limited to ascariasis, ancylostomiasis, trichuriasis, strongyloidiasis, toxoccariasis, trichinosis, onchocerciasis, filaria, and dirofilariasis. Also

encompassed are infections by various flukes, such as but not limited to schistosomiasis, paragonimiasis, and clonorchiasis. Parasites that cause these diseases can be classified based on whether they are intracellular or extracellular. An "intracellular parasite" as used herein is a parasite whose entire life cycle is intracellular. Examples of human intracellular parasites include *Leishmania* spp., *Plasmodium* spp., *Trypanosoma cruzi, Toxoplasma gondii, Babesia* spp., and *Trichinella spiralis.* An "extracellular parasite" as used herein is a parasite whose entire life cycle is extracellular. Extracellular parasites capable of infecting humans include *Entamoeba histolytica, Giardia lamblia, Enterocytozoon bieneusi, Naegleria* and *Acanthamoeba* as well as most helminths. Yet another class of parasites is defined as being mainly extracellular but with an obligate intracellular existence at a critical stage in their life cycles. Such parasites are referred to herein as "obligate intracellular parasites". These parasites may exist most of their lives or only a small portion of their lives in an extracellular environment, but they all have at least one obligate intracellular stage in their life cycles. This latter category of parasites includes *Trypanosoma rhodesiense* and *Trypanosoma gambiense, Isospora* spp., *Cryptosporidium* spp, *Eimeria* spp., *Neospora* spp., *Sarcocystis* spp., and *Schistosoma* spp.

Types of cancers that can be treated or prevented by the methods of the present invention include, but are not limited to human sarcomas and carcinomas, *e.g.,* fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.,* acute lymphocytic leukemia and acute

myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease.

5.3.2. Dosage and Mode of Administration of Viral Vectors

The replication-defective virus compositions of the invention can be administered to a human subject by any method or regimen known in the art. For example, the replication-defective virus compositions of the invention can be administered to a human subject by any method described in the following patents and patent applications that relate to methods of using AAV vectors in various therapeutic applications: U.S. Patent Nos. 7,282,199; 7,198,951; U.S. Patent Application Publication Nos. US 2008-0075737; US 2008-0075740; International Patent Application Publication Nos. WO *2003/024502;* WO 2004/108922; WO 20051033321, each of which is incorporated by reference in its entirety.

In an embodiment, the replication-defective virus compositions of the invention are delivered systemically via the liver by injection of a mesenteric tributary of portal vein. In another embodiment, the replication-defective virus compositions of the invention are delivered systemically via muscle by intramuscular injection in to *e.g.,* the quadriceps or bicep muscles. In another embodiment, the replication-defective virus compositions of the invention are delivered to the basal forebrain region of the brain containing the nucleus basalis of Meynert (NBM) by bilateral, stereotactic injection. In another embodiment, the replication-defective virus compositions of the invention are delivered to the eNS by bilateral intraputaminal and/or intranigral injection. In another embodiment, the replication-defective virus compositions of the invention are delivered to the joints by intraarticular injection. In another embodiment, the replication-defective virus compositions of the invention are delivered to the heart by intracoronary infusion. In another embodiment, the replication-defective virus compositions of the invention are delivered to the retina by injection into the subretinal space.

In another embodiment, an amount of replication-defective virus composition is administered at an effective dose that is in the range of about 1.0×10^8 genome copies

(GC)/kilogram (kg) to about 1.0×10^{14} GC/kg, and preferably 1.0×10^{11} GC/kg to 1.0×10^{13} GC/kg to a human patient. Preferably, the amount of replication-defective virus composition administered is 1.0×10^{8} GC/kg, 5.0×10^{8} GC/kg, 1.0×10^{9} GC/kg, 5.0×10^{9} GC/kg, 1.0×10^{10} GC/kg, 5.0×10^{10} GC/kg, 1.0×10^{11} GC/kg, 5.0×10^{11} GC/kg, or 1.0×10^{12} GC/kg, 5.0×10^{12} GC/kg, 1.0×10^{13} GC/kg, 5.0×10^{13} GC/kg, 1.0×10^{14} GC/kg

These doses can be given once or repeatedly, such as daily, every other day, weekly, biweekly, or monthly, or until adequate transgene expression is detected in the patient. In an embodiment, replication-defective virus compositions are given once weekly for a period of about 4-6 weeks, and the mode or site of administration is preferably varied with each administration. Repeated injection is most likely required for complete ablation of transgene expression. The same site may be repeated after a gap of one or more injections. Also, split injections may be given. Thus, for example, half the dose may be given in one site and the other half at another site on the same day.

When packaged in two or more viral stocks, the replication-defective virus compositions can be administered simultaneously or sequentially. When two or more viral stocks are delivered sequentially, the later delivered viral stocks can be delivered one, two, three, or four days after the administration of the first viral stock. Preferably, when two viral stocks are delivered sequentially, the second delivered viral stock is delivered one or two days after delivery of the first viral stock.

Any method known in the art can be used to determine the genome copy (GC) number of the replication-defective virus compositions of the invention. One method for performing AAV GC number titration is as follows: Purified AAV vector samples are first treated with DNase to eliminate un-encapsidated AAV genome DNA or contaminating plasmid DNA from the production process. The DNase resistant particles are then subjected to heat treatment to release the genome from the capsid. The released genomes are then quantitated by real-time PCR using primer/probe sets targeting specific region of the viral genome (usually poly A signal).

In one embodiment, the replication-defective virus compositions of the invention are delivered systemically via the liver by injection of a mesenteric tributary of portal vein at a dose of about 3.0×10^{12} GC/kg. In another embodiment, the replication-defective virus compositions of the invention are delivered systemically via muscle by up to twenty

intramuscular injections in to either the quadriceps or bicep muscles at a dose of about 5.0 x 10^{12} GC/kg. In another embodiment, the replication-defective virus compositions of the invention are delivered to the basal forebrain region of the brain containing the nucleus basalis of Meynert (NBM) by bilateral, stereotactic injection at a dose of about 5.0 x 10^{11} GC/kg. In another embodiment, the replication-defective virus compositions of the invention are delivered to the CNS by bilateral intraputaminal and/or intranigral injection at a dose in the range of about 1.0 x 10^{11} GC/kg to about 5.0 x 10^{11} GC/kg. In another embodiment, the replication-defective virus compositions of the invention are delivered to the joints by intra-articular injection at a dose of about 1.0 x 1011 GC/mL of joint volume for the treatment of inflammatory arthritis. In another embodiment, the replication-defective virus compositions of the invention are delivered to the heart by intracoronary infusion injection at a dose in the range of about 1.4 x 10^{11} GC/kg to about 3.0 x 10^{12} GC/kg. In another embodiment, the replication-defective virus compositions of the invention are delivered to the retina by injection into the subretinal space at a dose of about 1.5 x 10^{10} GC/kg.

Table 2 shows examples of transgenes that can be delivered via a particular tissue/organ by the PITA system of the invention to treat a particular disease.

Table 2: Treatment of Diseases

Disease	Examples of transgenes	Target Tissue
Age relation macular degeneration	s-FIt-1, an anti-VEGF antibody such as bevacizumab (Avastin), ranibizumab (Lucentis), or a domain antibody (dAB)	Retina
HIV	a neutralizing antibody against HIV	Muscle and/or liver
Cancer	Antiangiogenic agents (s-Fit-I, an anti-VEGF antibody such as bevacizumab (Avastin), ranibizumab (Lucentis), or a domain antibody (dAB); cytokines that enhance tumor immune responses, anti-EGFR, IFN	Muscle and/or liver
Autoimmune diseases, *e.g.*, arthritis, systemic lupus with T cell activation; erythematosus, psoriasis, cytokines that bias immune multiple sclerosis (MS)	Antibodies that interfere responses *e.g., β-IFN*; adhesion molecule a4-integrin antibody	Muscle and/or liver
Multiple myeloma	anti-CD20 antibody	Muscle and/or liver

Disease	Examples of transgenes	Target Tissue
Diabetes	GLP-1, IL-6	Muscle and/or liver
Hepatitis C	β-IFN, shRNA targeting IRES	Muscle and/or liver
Alzheimer's disease	NGF	Central nervous system (CNS)
Amyotrophic lateral sclerosis (ALS)	IGF-1	CNS
Huntington's disease	NGF, BDNF AND CNTF, shRNA targeting mutant Huntington	CNS
Epilepsy	galanin, neuropeptide Y (NPY), glial cell line derived neurotrophic factor (GDNF)	CNS
COPD	chemokines from IL 8 family, TNF antagonist	Lung
Inflammatory arthritis	TNF antagonist, IL-1, anti-CD 20, IL-6, IL-1r antagonist	Joint
Myocardial infarction	Heme oxygenase-1	Heart

Disease	Examples of transgenes	Target Tissue
Congestive heart failure	insulin like growth factor (IGF), hepatocyte growth factor (HGF)	Heart
Parkinson's Disease	GDNF, aromatic L-amino acid decarboxylase (ADCC), NGF	CNS

In one embodiment a method for treating age-related macular degeneration in a human subject comprises administering an effective amount of a replication-defective virus composition, in which the therapeutic product is a VEGF antagonist.

In another embodiment, a method for treating hemophilia A in a human subject, comprises administering an effective amount of a replication-defective virus composition, in which the therapeutic product is Factor VIII or its variants, such as the light chain and heavy chain of the heterodimer and the B-deleted domain; US Patent No. 6,200,560 and US Patent No. 6,221,349). The Factor VIII gene codes for 2351 amino acids and the protein has six domains, designated from the amino to the terminal carboxy terminus as A1-A2-B-A3-C1-C2 [Wood *et al*, *Nature*, **312**:330 (1984); Vehar *et al.*, *Nature* **312**:337 (1984); and Toole *et al*, *Nature*, **342**:337 (1984)]. Human Factor VIII is processed within the cell to yield a heterodimer primarily comprising a heavy chain containing the A1, A2 and B domains and a light chain containing the A3, C1 and C2 domains. Both the single chain polypeptide and the heterodimer circulate in the plasma as inactive precursors, until activated by thrombin cleavage between the A2 and B domains, which releases the B domain and results in a heavy chain consisting of the A1 and A2 domains. The B domain is deleted in the activated procoagulant form of the protein. Additionally, in the native protein, two polypeptide chains ("a" and "b"), flanking the B domain, are bound to a divalent calcium cation. In some embodiments, the minigene comprises first 57 base pairs of the Factor VIII heavy chain

which encodes the 10 amino acid signal sequence, as well as the human growth hormone (hGH) polyadenylation sequence. In alternative embodiments, the minigene further comprises the A1 and A2 domains, as well as 5 amino acids from the N-terminus of the B domain, and/or 85 amino acids of the C-terminus of the B domain, as well as the A3, C1 and C2 domains. In yet other embodiments, the nucleic acids encoding Factor VIII heavy chain and light chain are provided in a single minigene separated by 42 nucleic acids coding for 14 amino acids of the B domain [US Patent No. 6,200,560]. Examples of naturally occurring and recombinant forms of Factor VII can be found in the patent and scientific literature including, US Patent No. 5,563,045, US Patent No. 5,451,521, US Patent No. 5,422,260, US Patent No. 5,004,803, US Patent No. 4,757,006, US Patent No. 5,661,008, US Patent No. 5,789,203, US Patent No. 5,681,746, US Patent No. 5,595,886, US Patent No. 5,045,455, US Patent No. 5,668,108, US Patent No. 5,633,150, US Patent No. 5,693,499, US Patent No. 5,587,310, US Patent No. 5,171,844, US Patent No. 5,149,637, US Patent No. 5,112,950, US Patent No. 4,886,876; International Patent Publication Nos. WO 94/11503, WO 87/07144, WO 92/16557, WO 91/09122, WO 97/03195, WO 96/21035, and WO 91/07490; European Patent Application Nos. EP 0 672 138, EP 0 270 618, EP 0 182 448, EP 0 162 067, EP 0 786 474, EP 0 533 862, EP 0 506 757, EP 0 874 057,EP 0 795 021, EP 0 670 332, EP 0 500 734, EP 0 232 112, and EP 0 160 457; Sanberg et al., XXth Int. Congress of the World Fed. Of Hemophilia (1992), and Lind *et al.*, *Eur. J. Biochem.*, **232**:19 (1995).

In another embodiment, a method for treating hemophilia B in a human subject, comprises administering an effective amount of a replication-defective virus composition, in which the therapeutic product is Factor IX.

In another embodiment, a method for treating congestive heart failure in a human subject, comprises administering an effective amount of a replication-defective virus composition, in which the therapeutic product is insulin like growth factor or hepatocyte growth factor.

In another embodiment, a method for treating a central nervous system disorder in a human subject, comprises administering an effective amount of a replication-defective virus composition, in which the therapeutic product is nerve growth factor.

5.4. Monitoring Transgene Expression and Undesired Side Effects

5.4.1. Monitoring Transgene Expression

After administration of the replication-defective virus compositions of the invention, transgene expression can be monitored by any method known to one skilled in the art. The expression of the administered transgenes can be readily detected, *e.g.,* by quantifying the protein and/or RNA encoded by said transgene. Many methods standard in the art can be thus employed, including, but not limited to, immunoassays to detect and/or visualize protein expression *(e.g.,* western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunocytochemistry, immunohistochemical staining on sections *etc.)* and/or hybridization assays to detect gene expression by detecting and/or visualizing respectively mRNA encoding a gene *(e.g.,* northern assays, dot blots, *in situ* hybridization, *etc.).* The viral genome and RNA derived from the transgene can also be detected by Quantitative-PCR (Q-PCR). Such assays are routine and well known in the art. Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIP A buffer (1 % NP-40 or Triton x-100, I % sodium deoxycholate, 0.1 % SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1 % Trasylol) supplemented with protein phosphatase and/or protease inhibitors *(e.g.,* EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell lysate, incubating for a period of time *(e.g.,* 1 to 4 hours) at 40° C, adding protein A and/or protein G Sepharose beads to the cell lysate, incubating for about an hour or more at 40° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, *e.g.,* western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to an antigen and decrease the background *(e.g.,* pre-clearing the cell lysate with sepharose beads).

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel *(e.g.,* 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution *(e.g.,* PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer *(e.g.,* PBS-Tween 20), incubating the membrane with primary

antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.,* an anti-human antibody) conjugated to an enzymatic substrate *(e.g.,* horseradish peroxidase or alkaline phosphatase) or radioactive molecule *(e.g.,* 32p or 1251) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise.

ELISAs generally comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable agent such as an enzymatic substrate *(e.g.,* horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable agent; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable agent may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art.

A phenotypic or physiological readout can also be used to assess expression of a transgene. For example, the ability of a transgene product to ameliorate the severity of a disease or a symptom associated therewith can be assessed. Moreover, a positron emission tomography (PET) scan and a neutralizing antibody assay can be performed.

Moreover, the activity a transgene product can be assessed utilizing techniques well-known to one of skill in the art. For example, the activity of a transgene product can be determined by detecting induction of a cellular second messenger *(e.g.,* intracellular Ca2+, diacylglycerol, 1P3, *etc.),* detecting the phosphorylation of a protein, detecting the activation of a transcription factor, or detecting a cellular response, for example, cellular differentiation, or cell proliferation or apoptosis via a cell based assay. The alteration in levels of a cellular second messenger or phosphorylation of a protein can be determined by,

e.g., immunoassays well-known to one of skill in the art and described herein. The activation or inhibition of a transcription factor can be detected by,. *e.g.,* electromobility shift assays, and a cellular response such as cellular proliferation can be detected by, *e.g.,* trypan blue cell counts, ^{3}H-thymidine incorporation, and flow cytometry.

5.4.2. Monitoring Undesirable Side Effects/Toxicity

After administration of a replication-defective virus composition of the invention to a patient, undesired side effects and/or toxicity can be monitored by any method known to one skilled in the art for determination of whether to administer to the patient a pharmaceutical composition comprising a dimerizer (described in Section 5.2.3) in order to ablate or excise a transgene or to ablate the transcript of the transgene, or inhibit its translation.

The invention provides for methods of determining when to administer a pharmacological agent for ablating the therapeutic product to a subject who received a replication-defective virus composition encoding a therapeutic product and an ablator, comprising: (a) detecting expression of the therapeutic product in a tissue sample obtained from the patient, and (b) detecting a side effect associated with the presence of the therapeutic product in said subject, wherein detection of a side effect associated with the presence of the therapeutic product in said subject indicates a need to administer the pharmacological agent that induces expression of the ablator.

The invention also provides methods for determining when to administer a pharmacological agent for ablating the therapeutic product to a subject who received a replication-defective virus composition encoding a therapeutic product and an ablator, comprising: detecting the level of a biochemical marker of toxicity associated with the presence of the therapeutic product in a tissue sample obtained from said subject, wherein the level of said marker reflecting toxicity indicates a need to administer the pharmacological agent that induces expression of the ablator. Biochemical markers of toxicity are known in the art, and include clinical pathology serum measures such as, but not limited to, markers for abnormal kidney function *(e.g.,* elevated blood urea nitrogen (BUN) and creatinine for renal toxicity); increased erythrocyte sedimentation rate as a marker for generalized inflammation; low white blood count, platelets, or red blood cells as a marker for bone marrow toxicity; *etc.* Liver function tests (Ift) can be performed to detect

abnormalities associated with liver toxicity. Examples of such lfts include tests for albumin, alanine transaminase, aspartate transaminase, alkaline phosphatase, bilirubin, and gamma glutamyl transpeptidase.

The invention further comprises methods for determining the presence of DNA encoding the therapeutic gene product, its RNA transcript, or its encoded protein in a tissue sample from the subject subsequent to treatment with the pharmacological agent that induces expression of the ablator, wherein the presence of the DNA encoding the therapeutic gene product, its RNA transcript, or its encoded protein indicates a need for a repeat treatment with the pharmacological agent that induces expression of the ablator.

One undesired side effect that can be monitored in a patient that has received a replication-defective virus composition of the invention is an antibody response to a secreted transgene product. Such an antibody response to a secreted transgene product occurs when an antibody binds the secreted transgene product or to self antigens that share epitopes with the transgene product. When the transgene product is an antibody, the response is referred to as an "anti-idiotype" response. When soluble antigens combine with antibodies in the vascular compartment, they may form circulating immune complexes that are trapped nonspecifically in the vascular beds of various organs, causing so-called immune complex diseases, such as serum sickness, vasculitis, nephritis systemic lupus erythematosus with vasculitis or glomerulonephritis.

In another, more generalized undesirable immune reaction to the secreted transgene product, an antibody response to the transgene product results in a cross reacting immune response to one or more self antigens, causing almost any kind of autoimmunity. Autoimmunity is the failure of an the immune system to recognize its own constituent parts as self, which allows an immune response against its own cells and tissues, giving rise to an autoimmune disease. Autoimmunity to the transgene product of the invention can give rise to any autoimmune disease including, but not limited to, Ankylosing Spondylitis, Crohns Disease, Idiopathic inflammatory bowel disease, Dermatomyositis, Diabetes mellitus type-1, Goodpasture's syndrome, Graves' disease, Guillain-Barre syndrome (GBS), Anti-ganglioside, Hashimoto's disease, Idiopathic thrombocytopenic purpura, Lupus erythematosus, Mixed Connective Tissue Disease, Myasthenia gravis, Narcolepsy, Pemphigus vulgaris, Pernicious anaemia, Psoriasis, Psoriatic Arthritis, Polymyositis,

Primary biliary cirrhosis, Rheumatoid arthritis, Sjogren's syndrome, Temporal arteritis (also known as "giant cell arteritis"), Ulcerative Colitis (one of two types of idiopathic inflammatory bowel disease "IBD"), Vasculitis, and Wegener's granulomatosis.

Immune complex disease and autoimmunity can be detected and/or monitored in patients that have been treated with replication-defective virus compositions of the invention by any method known in the art. For example, a method that can be performed to measure immune complex disease and/or autoimmunity is an immune complex test, the purpose of which is to demonstrate circulating immune complexes in the blood, to estimate the severity of immune complex disease and/or autoimmune disease, and to monitor response after administration of the dimerizer. An immune complex test can be performed by any method known to one of skill in the art. In particular, an immune complex test can be performed using anyone or more of the methods described in U.S. Patent No. 4,141,965, U.S. Patent No. 4,210,622, U.S. Patent No. 4,210,622, U.S. Patent No. 4,331,649, U.S. Patent No. 4,544,640, U.S. Patent No. 4,753,893, and U.S. Patent No. 5,888,834, each of which is incorporated herein by reference in its entirety.

Detection of symptoms caused by or associated with anyone of the following autoimmune diseases using methods known in the art is yet another way of detecting autoimmunity or immune complex disease caused by a secreted transgene product that was encoded by a replication-defective virus composition administered to a human subject: Ankylosing Spondylitis, Crohns Disease, Idiopathic inflammatory bowel disease, Dermatomyositis, Diabetes mellitus type-I, Goodpasture's syndrome, Graves' disease, Guillain-Barre syndrome (GBS), Anti-ganglioside, Hashimoto's disease, Idiopathic thrombocytopenic purpura, Lupus erythematosus, Mixed Connective Tissue Disease, Myasthenia gravis, Narcolepsy,Pemphigus vulgaris, Pernicious anaemia, Psoriasis, Psoriatic Arthritis, Polymyositis, Primary biliary cirrhosis, Rheumatoid arthritis, Sjogren's syndrome, Temporal arteritis (also known as "giant cell arteritis"), Ulcerative Colitis (one of two types of idiopathic inflammatory bowel disease "IBD"), Vasculitis, and Wegener's granulomatosis.

A common disease that arises out of autoimmunity and immune complex disease is vasculitis, which is an inflammation of the blood vessels. Vasculitis causes changes in the walls of blood vessels, including thickening, weakening, narrowing and scarring. Common

tests and procedures that can be used to diagnose vasculitis include, but are not limited to blood tests, such as erythrocyte sedimentation rate, C-reactive protein test, complete blood cell count and anti-neutrophil cytoplasmic antibodies test; urine tests, which may show increased amounts of protein; imaging tests such as X-ray, ultrasound, computerized tomography (CT) and magnetic resonance imaging (MRI) to determine whether larger arteries, such as the aorta and its branches, are affected; X-rays of blood vessels (angiograms); and performing a biopsy of part of a blood vessel. General signs and symptoms of vasculitis that can be observed in patients treated by the methods of the invention include, but are not limited to, fever, fatigue, weight loss, muscle and joint pain, loss of appetite, and nerve problems, such as numbness or weakness.

When administration of a replication-defective virus composition of the invention results in local transgene expression, localized toxicities can be detected and/or monitored for a determination of whether to administer to the patient a pharmaceutical composition comprising a dimerizer (described in Section 5.2.3) in order to ablate or excise a transgene or to ablate the transcript of the transgene, or inhibit its translation. For example, when administering to the retina a replication-defective virus composition that comprises a transgene unit encoding a VEGF inhibitor for treatment of age-related macular degeneration, it is believed that VEGF may be neuroprotective in the retina, and inhibiting it could worsen eye-sight due to drop out of ganglion cells. Thus, after administration of such a replication-defective virus composition, eye-sight can be regularly monitored and ganglion cell drop out can be detected by any method known the art, *e.g.,* noninvasive imaging of retina. Moreover, VEGF inhibition may also depleted necessary micro vasculature in the retina, which can be monitored using fluorescien angiography or any other method known in the art.

In general, side effects that can be detected/monitored in a patient after administration of a replication-defective virus of the invention for a determination of whether to administer a pharmaceutical composition comprising a dimerizer (described in Section 5.2.3) to the patient, include, but are not limited to bleeding of the intestine or any organ, deafness, loss of eye-sight, kidney failure, dementia, depression, diabetes, diarrhea, vomiting, erectile dysfunction, fever, glaucoma, hair loss, headache, hypertension, heart palpitations, insomnia, lactic acidosis, liver damage, melasma, thrombosis, priapism

rhabdomyolysis, seizures, drowsiness, increase in appetite, decrease in appetite, dizziness, stroke, heart failure, or heart attack. Any method commonly used in the art for detecting the foregoing symptoms or any other side effects can be employed.

Ablator Therapy; Once it has been determined that a transgene product that was delivered to a patient by a method of the invention has caused undesirable side effects in a patient, a pharmaceutical composition comprising a dimerizer can be administered to a patient using any of the regimens, modes of administrations, or doses described in Section 5.2.3 herein.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

6. EXAMPLE 1: MANUFACTURING OF RECOMBINANT AAV VECTORS AT SCALE

A high yielding, recombinant AAV production process based upon poly-ethylenimine (PEI)-mediated transfection of mammalian cells and iodixanol gradient centrifugation of concentrated culture supernatant. AAV vectors produced with the new process demonstrate equivalent or better transduction both *in vitro* and *in vivo* when compared to small scale, cesium chloride (CsCl) gradient-purified vectors. In addition, the iodixanol gradient purification process described effectively separates functional vector particles from empty capsids, a desirable property for reducing toxicity and unwanted immune responses during pre-clinical studies.

Recently it was observed that, in contrast to AAV2, most other AAV serotypes are primarily released into the media of calcium phosphate-transfected production cultures and not retained in the cell lysate (Vandenberghe, L.H., Lock, M., Xiao, R., Lin, J., Korn, M., and Wilson, J.M. 2010. Heparin-dependent release of AAV into the supernatant simplifies manufacturing. Submitted, and now published as "Efficient serotype-dependent

Release of Functional Vector into the Culture Medium During Adeno-Associated Virus Manufacturing", Hu Gene Ther, 21:1251-1257 (October 2010).

A scaled rAAV production method suitable for large animal studies, which is based upon PEI transfection and supernatant harvest can be utilized. The method is high yielding, versatile for the production of vectors with different serotypes and transgenes, and simple enough that it may be performed in most laboratories with a minimum of specialized techniques and equipment. This method is now published in Lock et al, Hum Gen Ther, 2010 Oct; 21 (10): 1259-71, which is incorporated herein by reference.

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7. EXAMPLE 2: CESIUM PURIFICATION OF AAV VECTORS

This example describes a new procedure for cesium chloride (CsCl) purification of AAV vectors from transfected cell pellets.

Day 1 - Pellet Processing and CsCl Spin

1) Lysate preparation

• Thaw cells from -80° C freezer for 15 minutes at 37° C.

• Resuspend the cell pellet in ~ 20 mL of Resuspension Buffer 1(50 mM Tris, pH 8.0, 2 mM MgCl) for 40 plates of cells and for a final volume of 20 mL, and place on ice.

• Freeze/thaw 3 times (dry ice and ethanol bath/37° C water bath).

• Add 100 µL of Benzonase (250 U/mL) per prep and invert gently, incubate the samples at 37° C for 20 minutes, inverting the tube every 5 min.

• Add 6 mL of 5M NaCl to bring the final salt concentration to 1 M. Mix.

• Spin at 8,000 rpm for 15 min at 4°C in Sorval centrifuge. Note: Ensure the Sorval is clean. After centrifugation, sterilize tube with 70% before proceeding further. Transfer supernatant to a new tube.

• Spin again at 8,000 rpm for 15 min at 4° C in Sorval. Note: Ensure the Sorval is clean. After centrifugation, sterilize tube with 70% before proceeding further.

• Add 1.8 mL of 10% OGP for a final concentration of 0.5%, and mix gently by inversion.

2) Cesium Chloride Step Gradient Purification

• For each preparation, prepare two 2-tier gradients consisting of7.5 mL of 1.5 g/mL CsCl and 15 mL 1.3 g/mL CsCl in Beckman SW-28 tubes (do not use ultraclear tubes). Load the less dense CsCl first and then bottom load the heavier CsCl.

• Add 15 mL of sample to the top of each gradient. Add sample slowly to the side of the tube so as not to disturb the gradient. Label the tubes with lot #.

• Spin at 25,000 rpm at 15° C for 20 hours minimum.

Day 2 - Collect AAV band from 1st CsCl Spin and set up 2nd CsCl spin

1) Collect band from CsCl spin

• Carefully remove the centrifuge tubes (A & B) out of the bucket, taking care not to disturb the gradient. Secure the first tube (A) on a tube holder.

• Take a pre-sterilized 2 ft length of tygon-silicone tubing (1.6 mm inner diameter; Fisher NC9422080) fitted with two 1/16th inch male luers (Fisher NC9507090) and insert 18G 1" needles into the luers.

• Pierce the tube at a right angle as close to the bottom as possible with one of the 18G 1" needles (bevel facing up), and clamp the tubing into the easy load rollers of the masterflex pump. Gently increase the speed to ~ 1 mL/min. Collect the first 4.5 mL into a 15 mL falcon tube and then start to collect fractions (250 μL) into a 96 well plate (from tube A). Collect 48 fractions.

• Run the rest of the gradient into a beaker containing a 20% bleach solution and discard the needle/tubing assembly.

• Take another pre-sterilized 2 ft length of tygon-silicone tubing (1.6 mm inner diameter; Fisher NC9422080) fitted with two 1/16th inch male luers (Fisher NC9507090) and insert 18G 1" needles into the luers for collecting fractions from second tube (from tube B).

• Repeat the entire harvest for the tube B. Discard the needle/tubing assembly after use.

2) Read refraction index (RJ)

• Using a multichannel pipetter, transfer 10 μL of each fraction (of the 48 collected, first from 96-well plate A) to a fresh plate (label with 1 to 48) and leave the remainder of the fractions in the biosafety cabinet.

• Take 5 μL of each fraction and read the RI using a refractometer. The fractions containing AAV should have a refractive index of 1.3740-1.3660. Read the RI down to 1.3650 and then pool the fractions in the biosafety cabinet with RI in the 1.3740 to 1.3660 range. (Measure the total volume after pooling both the 96-well plates belonging to tube 1 and 2. In case there is still some space for adding more, add from wells with RI of 1.375.)

• Repeat this process for the second 96-well plate (from tube B).

3) Load the second gradient

• The total pooled volume from each gradient (from tubes A and B) should be 5-6 mL. Pool the two gradient harvests in a 50 mL falcon tube and bring the volume to 13 mL with a 1.41 g/mL solution of CsCl. Mix well with a pipette.

• Using a 10 mL syringe and 18G needle, add the pooled first gradient harvest to a 13 mL sealable centrifuge tube. The solution should be added to the line on the neck of the tube with no bubbles.

• Seal the tube using the portable sealer, metal tube caps and heat sink.

• Squeeze the tube to test for leaks and then place in a Ti70.1 rotor with the appropriate balance. Insert the rotor caps and lid and then spin at 60,000 rpm, 15° C for 20 hours.

Day 3 - Collect AAV band from 2nd CsCl Spin and Desalt

1) Collect band from CsCl spin

Carefully remove the centrifuge tube out of the bucket, taking care not to disturb the gradient. Secure the tube on a tube holder. At this point a single band should be visible after bottom illumination about halfway up the tube.

• Take a pre-sterilized 2 ft length of tygon-silicone tubing (1.6 mm inner diameter; Fisher NC9422080) fitted with two 1/16th inch male luers (Fisher NC9507090) and insert 18G 1" needles into the luers. Use 1 length of tubing per prep.

• Pierce the tube at a right angle as close to the bottom as possible with one of the 18G 1" needles (bevel facing up) and clamp the tubing into the easy load rollers of the masterflex pump. Pierce the tube again at the top with a second 18G needle. Gently increase the speed to ~ 1 mL/min and then start to collect fractions (250 µL) into a 96 well plate. Collect the whole gradient (~45 fractions).

2) Read refractive index (RI):

• Using a multichannel pipetter, transfer 10 µL of each fraction to a fresh plate and leave the remainder of the fractions in the biosafety cabinet.

• Take 5 µL of each fraction and read the RI using a refractometer. The fractions containing AAV should have a refractive index of 1.3750-1.3660. Read the RI down to 1.3650, and then pool fractions with RI in range of 1.3750 to 1.3660.

3) Desalting: Amicon Ultra-I 5 centrifugal concentrators

In this procedure the vector is diluted with PBS and spun at low speed through the 100 kDa MWCO filter device. Because of the large molecular weight of AAV Particles (~5000 kDa), the vector is retained by the membrane and the salt passes through. Vector can build up on the membranes, so rinsing is required at the final stage.

• Aliquot 50 mL PBS + 35 mM NaCl into a 50 mL tube.

• Dilute the pooled fractions from step 2 above with the PBS + 35 mM NaCl to 15 mL total volume. Mix gently and add to Amicon filter device.

• Spin in a bench top Sorvall centrifuge at 2,000 to 4,000 rpm for 2 minutes. Because it is important to keep the level of the liquid above the top of the filter surface (~1.8 mL) at all times so that the vector does not dry onto the membrane, it is recommended that the lower speed spin is attempted first to determine the flow rate of the sample. The goal is to reduce the volume of the retentate to ~1.8 mL. An additional short spines) may be necessary to achieve this. If the volume does go below that desired, bring it back to 1.8 mL with PBS + 35 mM NaCl.

• Add a further 13.2 mL PBS + 35 mM NaCl, mix by pipette with the retentate remaining in the device, and repeat the spinning process described above. Continue this process until all the 50 mL PBS + 35 mM NaCl aliquoted previously is spun through the device.

• Rinse the membrane with the final retentate (~1.8 mL) by repeatedly pipetting against the entire surface. Recover the retentate into a suitably-sized sterile centrifuge tube using 1 mL and 200 µL Eppendorf tips (the 200 µL tip is for the final retentate at the bottom of the device that is inaccessible to a 1 mL tip). Rinse the membrane twice using a minimum of 100 µL of PBS + 35 mM NaCl and pool it with your final retentate.

• Determine the exact volume and add glycerol to 5%.

• Aliquot into 5 x 25 µ: aliquots, 1 x 100 µL for archive, and the rest into l05 µL aliquots.

• Freeze immediately at -80 °C.

Reagents used in rAAV purification

• Resuspension buffer 1[50 mM Tris (pH 8.0), 2 mM MgCl]: 50 mL l M Tris (pH 8.0),2 mL/M MgCh to 948 mL MQ water, filter sterilize.

• 1.5 g/mL CsCI solutions: dissolve 675 g of CsCI in 650 mL PBS and adjust final volume to 1000 mL. Weigh 1 mL of the solution to check the density. Filter sterilize the solution.

• 1.3 g/mL CsCI solutions: dissolve 405 g of CsCI in 906 mL PBS and adjust final volume to 1000 mL. Weigh 1 mL of the solution to check the density. Filter sterilize the solution.

• 10% (W/V) Octyl-PD-glucopyranoside (OGP) (Sigma, 08001-10G): Bring 10 grams to 100 mL with milliQ water. Filter sterilize the solution.

• Final formulation buffer: PBS + 35 mM NaCl. To 1 liter sterile PBS, add 7.05 mL sterile 5 M NaCl.

• Sterile glycerol: Aliquot glycerol into l00 mL glass bottles. Autoclave for 20 minutes on liquid cycle.

8. EXAMPLE 3: DNA CONSTRUCTS FOR PREPARATION OF PITA AAV VECTORS

The invention is illustrated by Examples 3-5 , which demonstrate the tight regulation of ablator expression using rapamycin, to dimerize transcription factor domains that induce expression of Cre recombinase; and the successful inducible ablation of a transgene containing Cre recognition sites (loxP) in cells. The tight regulation of expression of the ablator is demonstrated in animal models.

The following are examples of DNA constructs DNA constructs and their use to generate replication-defective AAV vectors for use in accordance with the PITA system of the invention is illustrated in the examples below.

8.1. Constructs Encoding a Dimerizable Transcription Factor Domain Unit and an Ablation Unit

Figs. 1A-B through Figs. 5B are diagrams of the following DNA constructs that can be used to generate AAV vectors that encode a dimerizable transcription factor domain unit and an ablation unit: (1) pAAV.CMV.TF.FRB-IlRES-1xFKBP.Cre (Figs. 1A-

B); (2) pAAV.CMV.TF.FRB-T2A-2xFKBP.Cre (Figs. 2A-B); (3) pAAV.CMVI73.TF.FRB-T2A-3xFKBP.Cre (Figs. 3A-B); and (4) pAAV.CMV.TF.FRB-T2A-2xFKBP.ISce-I (Figs. 4A-B).

A description of the various domains contained in the DNA constructs follows:

ITR: inverted terminal repeats of AAV serotype 2 (168 bp).[SEQ ID NO: 26]

CMV: full cytomegalovirus (CMV) promoter; including enhancer . [SEQ ID NO 27]

CMV (173 bp): minimal CMV promoter, not including enhancer. [SEQ ID NO: 28]

FRB-TA fusion: fusion of dimerizer binding domain and an activation domain of a transcription factor (900 bp, SEQ ID NO: 29). The protein is provided herein as SEQ ID NO: 30. The FRB fragment corresponds to amino acids 2021-2113 of FRAP (FKBP rapamycin-associated protein, also known as mTOR [mammalian target of rapamycin]), a phosphoinositide 3-kinase homolog that controls cell growth and division. The FRAP sequence incorporates the single point-mutation Thr2098Leu ($FRAP_L$) to allow use of certain non-immunosuppressive rapamycin analogs (rapalogs). FRAP binds to rapamycin (or its analogs) and FKBP and is fused to a portion of human NF-KB p65 (190 amino acids) as transcription activator.

ZFHD-FKBP fusion: fusion of a DNA binding domain and 1 copy of a Dimerizer binding domain (1xFKBP; 732 bp), 2 copies of drug binding domain (2xFKBP; 1059 bp), or 3 (3xFKBP;1389 bp) copies of drug binding domain. Immunophilin FKBP (FK506-binding protein) is an abundant 12 kDa cytoplasmic protein that acts as the intracellular receptor for the immunosuppressive drugs FK506 and rapamycin. ZFHD is DNA binding domains composed of a zinc finger pair and a homeodomain. Both fusion proteins contain N-terminal nuclear localization sequence from human c-Myc at the 5' end. See, SEQ ID NO: 45.

T2A: self cleavage peptide 2A (54 bp) (SEQ ID NO: 31).

Z8I: 8 copies of the binding site for ZFHD (Z8) followed by minimal promoter from the human interleukin-2 (lL-2) gene (SEQ ID NO: 32). Variants of this promoter may be used, *e.g.,* which contain from 1 to about 20 copies of the binding site for ZFHD followed by a promoter, *e.g.,* the minimal promoter from IL-2.

Cre: Cre recombinase. Cre is a type I topoisomerase isolated from bacteriophage P1. Cre mediates site specific recombination in DNA between two loxP sites leading to deletion or gene conversion (1029 bp, SEQ ID NO: 33).

I-SceI: a member of intron endonuclease or homing endonuclease which is a large class of meganuclease (708 bp, SEQ ID NO: 34). They are encoded by mobile genetic elements such as introns found in bacteria and plants. I-SceI is a yeast endonuclease involved in an intron homing process. I-SceI recognizes a specific asymmetric 18bp element, a rare sequence in mammalian genome, and creates double strand breaks. See, Jasin, M. (1996) Trends Genet., 12,224-228 .

hGH poly A: minimal poly adenylation signal from human GH (SEQ ID NO: 35).

IRES: internal ribosome entry site sequence from ECMV (encephalomyocarditis virus) (SEQ ID NO: 36).

8.2. Constructs Encoding Transgene Units

Figures 5A-B and Figs. 6A-B are diagrams of the following DNA constructs for generating an AAV vector encoding a transgene flanked by loxP recognition sites for Cre recombinase:

(1) pENN.CMV.Pl.loxP.Luc.SV40 (Figs. 5A-B); and (2) pENN.CMV.Pl.sce.Luc.SV40 (Figs. 6A-B). A description of the various domains of the constructs follows:

ITR: inverted terminal repeats of AAV serotype 2 (SEQ ID NO: 26).

CMV: cytomegalovirus (CMV) promoter and enhancer regulating immediate early genes expression (832 bp, SEQ ID NO: 27).

loxP: recognition sequences of Cre. It is a 34 bp element comprising of two 13 bp inverted repeat flanking an 8 bp region which confers orientation (34 bp, SEQ ID NO: 37).

Ffluciferase: fire fly luciferase (1656 bp, SEQ ID NO: 38).

SV 40: late polyadenylation signal (239 bp, SEQ ID NO: 39).

I -SceI site: SceI recognition site (18 bp, SEQ ID NO: 25).

8.3. Constructs Encoding a Transgene Unit and a Dimerizable Transcription Factor Domain Unit

Figure 7 is a diagram of DNA construct for generating an AAV vector that contains a transgene unit and a dimerizable transcription factor domain unit. This plasmid provides, on AAV plasmid backbone containing an ampicillin resistance gene, an AAV 5' ITR, a transcription factor (TF) domain unit, a CMV promoter, an FRB (amino acids 2021-2113 of FRAP (FKBP rapamycin-associated protein, also known as mTOR [mammalian target of rapamycin]), a phosphoinositide 3-kinase homolog that controls cell growth and division), a T2A self-cleavage domain, an FKBP domain, and a human growth hormone polyA site, a CMV promoter, a loxP site, an interferon alpha coding sequence, and an SV40 polyA site. The ablation unit (cre expression cassette) can be located on a separate construct. This strategy could minimize any potential background level expression of cre derived from upstream CMV promoter.

9. EXAMPLE 4: *IN VITRO* MODEL FOR PITA

This example demonstrates that the DNA elements (units) engineered into the AAV vectors successfully achieve tightly controlled inducible ablation of the transgene in cells. In particular, this example shows that luciferase transgene expression can be ablated upon dimerizer (rapamycin) treatment of cells transfected with constructs containing a transgene unit (expressing luciferase and containing lox p sites), an ablation unit (expressing Cre), and a dimerizable transcription factor domain unit.

Human embryonic kidney fibroblast 293 cells were seeded onto 12 well plates. Transfection of the cells with various DNA constructs described in section 9.1 herein was carried out the next day when the cell density reached 90% confluency using lipofectamine 2000 purchased from Invitrogen. A vector encoding enhanced green fluorescent protein (EGFP) was added at 10% of total DNA in each well to serve as internal control for transfection. The DNA suspended in DMEM was mixed with lipofectamine 2000 to form DNA-lipid complex and added to 293 cells for transfection following instructions provided by Invitrogen Corporation. At 6 hours post transfection, half of the wells were treated with rapamycin at a final concentration of 50 nM. Culture medium (DMEM supplemented with 10% FBS) was replaced daily with fresh rapamycin. At 48 and 72 hour post transfection, cells were washed once with PBS and then scraped out of the well, resuspended in lysis buffer supplied in Luciferase assay kit purchased from Promega. The cell suspension was vortexed and the debri spun down. The luciferase activity was determined by mixing 10 μL of the lysate with 100 μL of the substrate and light emission per second read from a luminometer.

9.1. CONSTRUCTS

The following constructs, most of which are described in Section 8, Example 1, were used to generate infectious, replication-defective AAV vectors:

1. pENN.AAV.CMV.RBG as a control, containing a CMV promoter and no transgene
2. pENN.CMV.Pl.loxP.Luc.SV40 (Figs. 5A-B)/ pENN.AAV.CMV.RBG (CMV promoter and no transgene)
3. pENN.CMV.Pl.loxP.Luc.SV40(Figs. 5A-B/ pAAV.TF.CMV.FRB-T2A-2xFKBP.Cre

(Figs. 2A-B)

4. pENN.CMV.Pl.loxP.Luc.SV40(Figs. 5A-B/pAAV.TF.CMV.FRB-IRES-FKBP.Cre (Figs. 1A-B)

5. pENN.CMV.Pl.loxP.Luc.SV40(Figs. 5A-B)/ pAAV.CMVI73.FRB-T2A-3xFKBP.Cre (Figs. 3A-B)

6. pENN.CMV.PI.loxP.Luc.SV40(Figs. 5A-B)/pENN.AAV.CMV.PI.Cre.RBG, which expresses the Cre gene from a constitutive promoter

9.2. RESULTS

The results at 48 hours are shown in Figure 8A and the results at 72 hours are shown in Figure 8B. In the control (treatment 6), where Cre is constitutively expressed, luciferase expression was ablated independently of rapamycin compared to the control expression of luciferase without 10xP sites (treatment 2, cells transfected with luciferase construct). In contrast, in cells receiving the 10xP flanking luciferase construct plus one of the constructs carrying cre under the control of PITA system (treatment 3,4 and 5), the level of the reporter gene expression is comparable to the control in the absence of dimerizer, rapamycin, indicating very little or no cre expression is induced. However, upon induction by treatment with rapamycin, the level of reporter gene expression in cells receiving PIT A controlled cre constructs were significantly reduced compared to the control (treatment 2), indicating cre expression was activated. The results confirm that the expression of the ablator is specifically regulated by the dimerizer, rapamycin.

10. EXAMPLE 5: IN VIVO MODEL FOR A DIMERIZER-INDUCIBLE SYSTEM

This example shows tight tissue-specific control of transgene expression using a liver-specific promoter that is regulated by the dimerizer-inducible system described herein. These data serves as a model for tight regulation of the ablator in the PITA system.

Four groups of three mice received IV injection of AAV vectors encoding bicistronic reporter genes (GFP-Luciferase) at doses of 3×10^{10}, 1×10^{11} and 3×10^{11} particles

of virus, respectively: Group 1 (G 1, G2, and G3) received AAV vectors expressing GFP Luciferase under the control of ubiquitous constitutive CMV promoter (see Figure 9A for a diagram of the DNA construct). Group 2 (G4, G5, and G6) received co-injection of the following 2 AAV vectors: (1) AAV vector expressing a dimerizable transcription factor domain unit (FRB fused with p65 activation domain and DNA binding domain ZFHD fused with 3 copies of FKBP) driven by the CMV promoter (the DNA construct shown in Figure 2B; and (2) AAV vector expressing GFP-Luciferase driven by a promoter induced by the dimerized TF (see Figure 12C for a diagram of the DNA constructs). Group 3 (G7, G8, and G9) received AAV vector expressing GFP-Luciferase under the control of a liver constitutive promoter, TBG (see Figure 9C for a diagram of the DNA construct). Group 4 (G10, G11, G12) received co-injection of the following 2 AAV vectors: (1) AAV vector expressing a dimerizable transcription factor domain unit (FRB fused with p65 activation domain and DNA binding domain ZFHD fused with 3 copies of FKBP) driven by the TBG promoter; and (2) AAV vector expressing GFP-Luciferase driven by a promoter induced by the dimerized TF (see Figure 9D for a diagram of the DNA constructs).

About 2 weeks post virus administration, the mice were given IP injection of the dimerizer, rapamycin, at the dose of 2 mg/kg. Starting the next day the luciferase expression was monitored by Xenogen imaging analysis. Approximately 24 hours post rapamycin injection, the mice were IP injected with luciferin, the substrate for luciferase, then anesthetized for imaging.

The mice that received 3×10^{11} particles of virus had images taken 30 min post luciferin injection (Figs. 10A-D). For Group 1 mice that received vectors carrying GFP-Luciferase, expression driven by CMV promoter, the luciferase expression was observed in various tissues and predominantly in lungs, liver and muscle (See Fig 10A). In contrast, luciferase expression was restricted to liver in Group 3 mice, which received luciferase vector in which the expression was controlled by TBG promoter (see Fig 10B). In Group 2 mice, the level of luciferase expression was elevated by more than 2 logs compared to level of pre-induction, and the expression is predominantly in liver and muscle (see Fig. 10C). In Group 4 mice, more than 100 fold of luciferase expression was induced and restricted in the liver, compared to pre-inducement (see Fig. 10D).

The mice that received 1×10^{11} particles of viruses, show results similar to that of

high dose groups but with lower level of expression upon induction, and predominantly in liver (see Figs. 11A-D).

CONCLUSIONS:

1. The dimerizer-inducible system is robust with peak level of luciferase expression more than 2 logs over baseline and back to close to baseline within a week (not shown).

2. Liver is the most efficient tissue to be infected when viruses were given IV.

3. Liver is also the most efficient tissue to be cotransduced with 2 viruses which is critical for the dimerizer-inducible system to work.

4. The luciferase expression regulated by that dimerizer-inducible system with transcription factor expression controlled by CMV promoter is significantly higher in mouse liver than expression coming from CMV promoter without regulation. This indicated that inducible promoter is a stronger promoter in liver once it is activated compared to the CMV promoter.

5. Luciferase expression was detected specifically in liver upon induction by rapamycin in mice receiving vectors carrying the inducible TBG promoter system. Luciferase expression mediated by the liver-specific regulatable vectors was completely dependent upon induction by rapamycin and the peak level of luciferase expression is comparable to that under the control of TBG promoter. This study confirmed that liver specific gene regulation can be achieved by AAV mediated gene delivery of liver specific dimerizer-inducible system.

11. EXAMPLE 6: PITA FOR AGE-RELATED MACULAR DEGENERATION (AMD) THERAPY

Intravitreal administration of a monoclonal antibody has proven to be an effective therapy for AMD to slow down disease progression and improve visual acuity in a subpopulation of patients. A key limitation of this approach, however, is the requirement for repeated intravitreal injections. Gene therapy has the potential to provide long term correction and a single injection should be sufficient to achieve a therapeutic effect. Figures 12 A-C show PITA DNA constructs for treating AMD, containing transgene units

comprising a VEGF antagonist, such as an anti-VEGF antibody (Avastin heavy chain (AvastinH) and Avastin light chain (AvastinL); Figures 12B and 12C) or a soluble VEGF receptor (sFlt-1; Figure 12A). Vectors comprising these DNA constructs can be delivered via subretinal injection at the dose of 0.1-10 mg/kg. Ablation of transgene expression can be achieved by oral dimerizer administration if adverse effects of long term anti-VEGF therapy are observed.

12. EXAMPLE 7: PITA FOR LIVER METABOLIC DISEASE THERAPY

PITA is potentially useful for treating liver metabolic disease such as hepatitis C and hemophilia. Figure 13A shows a PITA construct for treating hemophilia A and/or B, containing a transgene unit comprising Factor IX. Factor VIII can also be delivered for treatment of hemophilia A and B respectively (Factor VIII and IX for hemophilia A and B, respectively). The therapy could be ablated in patients if inhibitor formation occurs. Figure 13B shows a PITA construct for delivery of shRNA targeting the IRES of HCV. A vector comprising this construct could be injected *via* a mesenteric tributary of portal vein at the dose of $3x10^{12}$ GC/kg. The expression of shRNA can be ablated if nonspecific toxicity of RNA interference arises or the therapy is no longer needed.

13. EXAMPLE 8: PITA FOR HEART DISEASE THERAPY

PITA could be utilized for heart disease applications including, but not limited to, congestive heart failure (CHF) and myocardial infarction (MI). The treatment of CHF could involve the delivery of insulin like growth factor (IGF) or hepatocyte growth factor (HGF) using the constructs shown in Figures 14A and 14B. For the treatment of myocardial infarction, delivery of genes in the early stages of MI could protect the heart from the deleterious effects of ischemia but allow ablation of the therapy when no longer required. Therapeutic genes for this approach include heme oxygenase-1 (HO-1) which can function to limit the extent of ischemic injury. Delivery methods for vector-mediated gene delivery to the heart include transcutaneous, intravascular, intramuscular and cardiopulmonary bypass techniques. For the human, the optimal vector-mediated gene delivery protocol would likely utilize retrograde or ante grade trans coronary delivery into the coronary artery or anterior cardiac vein.

14. EXAMPLE 9: PITA FOR CENTRAL NERVOUS SYSTEM (CNS) DISEASE THERAPY

Attractive candidates for the application of PITA in the central nervous system include neurotrophic factors for the treatment of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease and ocular diseases. Figure 15 shows a PITA construct for treating Alzheimer's disease, containing a transgene unit comprising nerve growth factor (NGF). AAV vector-mediated gene delivery of NGF, is currently being studied in a Phase I clinical trial conducted by Ceregene for the treatment of Alzheimer's disease. NGF is a neurotrophic factor, which has been shown to be effective in reducing cholinergic cell loss in animal models of neurodegenerative disease and may be effective in preventing loss of memory and cognitive abilities in patients with AD. The delivery method for the approach consists of bilateral, stereotactic injection to target the basal forebrain region of the brain containing the nucleus basalis of Meynert (NBM). Due to the potential for side-effects resulting in the need to end treatment, further engineering the construct to include PITA is warranted.

The application of PITA in the central nervous system for the treatment of epilepsies could also be of value both due to the potential to ablate gene expression once the issue surrounding the seizures becomes resolved as well as due to the limited alternative approaches available for the treatment of epilepsies that are unresponsive to drug therapy and surgically difficult to treat. In these cases, in particular, delivery methods involving sterotactic injection of vectors expressing therapeutic genes, would be far less invasive than alternative surgical treatments. Candidates for gene expression could include galanin, neuropeptide Y (NPY) and glial cell line-derived neurotrophic factor, GDNF, which have been shown to have therapeutic effects in animal models of epilepsy. Other applications include to deliver nerve growth factor (NGF) for Alzheimer's and aromatic L-amino acid decarboxylase (ADCC) for Parkinson's Disease.

15. EXAMPLE 10: PITA FOR HIV THERAPY

Naturally induced neutralizing antibody against HIV has been identified in the sera of long term infected patients. As an alternative to active vaccine approaches, which

have resulted in inefficient induction but sufficient levels of neutralizing antibody delivered by AAV, PITA is a promising approach to deliver anti-HIV neutralizing antibody for passive immunity therapy. See Fig. 16. The construct design is similar to avastin gene delivery for AMD therapy (see Figures 12B and 12C). A vector comprising a construct encoding an antibody regulated by the liver specific promoter (TBG) could be injected into the liver at a dose of 3×10^{12} GC/kg. Alternatively, a vector comprising a construct carrying a ubiquitous CB7 promoter driving antibody expression could be delivered by intramuscular injection at a dose of $5x\ 10^{12}$ GC/mL for up to 20 injections into the quadriceps or biceps muscle. The therapy can be ablated if it is no longer needed or if toxicity develops due to induction of anti-drug antibody.

16. EXAMPLE 11:

The DNA constructs described in the following example may be used to prepare replication-defective AAV viruses and virus compositions according to the invention.

Open reading frames encoding for various endonucleases were codon optimized and *de novo* synthesized by GeneArt. Ablator expression and target plasmids were produced using standard molecular biological cloning techniques. Transfections were performed in HEK293 cells using Lipofectamine™ 2000 transfection reagent (Life Technologies). All transfections were performed using optimal transfection conditions as defined in transfection reagent protocol. Briefly, 200-250 ng plasmid DNA (excluding transfection control plasmid) was complexed with lipofectamine and added to cells in 96 well plates. DNA quantities were consistent across all conditions by supplementation with an unrelated plasmid containing the same promoter as test plasmids. Transfection complexes were incubated with cells for 4-6 hours as transfection reagent protocol before the addition of FBS supplemented media. Transfected cells were incubated at 37°C for 24-72 hours. Following incubation, cells were assayed for reporter gene expression using Promega Dual Luciferase detection kit according to the manufacturer's instructions on a BioTek Clarity platereader and renilla luciferase was used to control for transfection efficiency. All samples were performed in quadruplicate and standard errors of the mean were calculated.

A. Coexpression of wild-type FokI ablates expression of transgene more effectively than delivery of FokI protein

The amino acid sequence of the FokI enzyme is provided in SEQ ID NO: 12, wherein amino acids 1 to 387 are the DNA binding domain and amino acids 387 to 584 are the catalytic domain. The codon optimized FokI sequence is provided in SEQ ID NO:1.

Fig. 18 illustrates that wild-type FokI effective ablated expression of the luciferase reporter gene following contrasfection into HEK295 cells (Fig. 18A bar 2), while only partial ablation was observed when FokI protein was delivered to the cells (Fig. 18A, bar 3).

In a dose - dependent experiment, the FokI expression vector contained the FokI catalytic domain fused to a zinc finger DNA binding domain (ZFHD). This construct, which is 963 bp, is provided in SEQ ID NO: 21 and is composed of base pairs 1 to 366 bp ZFHD, 367 to 372 bp linker, and 373 to 963 bp FokI catalytic domain. The resulting expression product comprises amino acids 1 to 122 (ZFHD), amino acids 123-124 are a linker and amino acids 125 to 321 are from the FokI catalytic domain. Fig. 18B illustrates that increasing the concentration of FokI resulted in dose dependent ablation of Luc reporter. No ablation sites were required to be engineered into the transcription unit containing the transgene in this illustration, as luciferase contains multiple native FokI sites.

This provides support for the use of the PITA system using a transfected FokI enzyme directed to specific ablation sites in a transcription unit containing a transgene for delivery to the cell.

B. Chimeric engineered FokI tethered to non-cognate recognition site on the DNA by the Zinc Finger - Homeodomain effectively ablates expression of Luc reporter gene

The plasmid constructs in this example contains either the FokI catalytic domain (198 amino acids (SEQ ID NO: 14), corresponding to amino acids 387 to 584 of the full-length protein) (untethered FokI) or a ZFHD-FokI catalytic domain of 963 bp as described in Part A above (tethered FokI). Even at the highest concentration, the catalytic domain of FokI which is un-tethered to DNA does have no effect on expression of Luc reporter gene (Fig. 19A). Chimeric engineered Fok1 tethered to DNA via fusion with ZFHD effectively ablated expression of luciferase reporter in a dose dependent manner when increasing

concentrations of ZF-HD-FokI expression plasmid were cotransfected into HEK293 cells (Fig. 19B).

This supports the use of the PITA system and the additional safety element provided by a chimeric enzyme directed to specific ablation sites in a transcription unit containing a transgene for delivery to the cell.

C. DNA binding specificity of chimeric FokI can be reproducibly changed by fusion with various classes of heterologous DNA binding domains and ablation of target transgene can be further improved by addition of heterologous NLS

This example illustrates that the zinc finger homeodomain (ZFHD) is not the only domain suitable for altering the specificity of ablation mediated by a chimeric engineered enzyme. FokI effectively ablated expression of luciferase reporter in a dose dependent manner when HTH DNA binding domain was fused to FokI catalytic domain (Fig. 20A). In a separate experiment (Fig. 27B), the activity of HTH-FokI was further improved by adding heterologous NLS at the N-terminus of the HTH-FokI coding sequence.

The HTH-FokI Catalytic domain (SEQ ID NO:5), is composed of 1-171 bp HTH from Gin (a serine recombinase), a linker (bp 172-177), and a FokI catalytic domain (178-768 bp) derived from codon-optimized FokI. The resulting chimeric enzyme (SEQ ID NO: 6) contains aa 1-57 of HTH from Gin, a linker (aa 58-59), and a FokI catalytic domain (amino acids 60 - 256).

Figs. 20A-20B are bar charts illustrating that the DNA binding specificity of chimeric FokI can be reproducible changed by fusion with another classes of heterologous DNA binding domains and ablation of target transgene can be further improved by the additional of a heterologous nuclear localiazation signal (NLS). Fig. 20A illustrates the results of co-transfection of pCMV.Luciferase with increasing concentrations of an expression plasmid encoding FokI tethered to DNA via an HTH fusion (6.25, 12.5, 25, 50, and 100 ng). The first bar is a control showing 50 ng pCMV.Luciferase alone. Fig. 20B pCMV.Luciferase with increasing concentrations of an expression plasmid encoding an HTH - FokI fusion, which further has a NLS at its N-terminus.

17. EXAMPLE 12:

Although not illustrated here, other chimeric enzymes have been made using the techniques described herein:

An AAV plasmid containing SV40 T-Ag NLS-Helix-turn-helix (HTH) from Gin (192 bp, SEQ ID NO:7), which includes the nuclear localization signal (1-24 bp) of SV40 T-Ag and HTH from Gin, a serine recombinase (25 - 192 bp). In the resulting enzyme (SEQ ID NO:8), amino acids 1-8 are from the SV40 T-Ag NLS and amino acids 9-64 are the HTH from Gin;

An AAV plasmid containing SV40 T-Ag NLS-HTH-FokI Catalytic domain (789 bp, SEQ ID NO:9), which includes the SV40 T-Ag NLS (bp 1-24), the HTH from Gin (bp 25-192), a linker (bp 193-198), and the catalytic domain of the FokI (bp 199-789). In the resulting chimeric enzyme (SEQ ID NO:10), amino acids 1-8 are from the SV40 T-Ag NLS, amino acids 9-64 are HTH from Gin, amino acids 65-66 are linker residues, and amino acids 67-263 are the FokI catalytic domain.

An AAV plasmid containing a SV40 T-Ag NLS-ZFHD-FokI catalytic domain (984 bp) was prepared (SEQ ID NO: 23), which includes the SV40 T-Ag NLS (bp 1-24), the zinc finger homodomain (bp 25 - 387), a linker (bp 388-393), and the FokI catalytic domain (bp acids 394-984). In the resulting chimeric enzyme (SEQ ID NO: 21, 328 aa), amino acids 1-8 are the SV40 T-Ag NLS, amino acids 9-129 are the ZFHD, amino acids 130-131 are linker residues, and amino acids 132-138 are FokI catalytic domain.

These and other constructs can be used to prepare viruses according the method of the invention for use in a virus composition and the PITA system.

18. EXAMPLE 13: USE OF REPLICATION-DEFECTIVE AAV VIRUS COMPOSTION IN TREATMENT OF HIV

This composition could be potentially used as a safety mechanism in the treatment of HIV. Recently, broadly neutralizing antibodies from long-term non-progressors, individuals which maintain an HIV^+ status for several decades without progression to AIDS, have been identified by several research groups.

All coding regions of the neutralizing antibody to HIV (HIV NAb) are placed between the inverted terminal repeats (ITRs) of the AAV. If the overall size of the

constructs are below 4.7 kb (including the two ITRs), they are packaged into the AAV capsid. The AAV serotype capsid chosen will depend of the level of gene expression, the method of delivery and the extent of biodistribution from the injection site required. In addition, the constitutive promoters used for expression of the HIV NAb (and potentially the parts of the inducible system in the one small molecule situation) would depend on the tissue type targeted. In the following example of a potential clinical study the vector serotype chosen would be AAV8 administered by intravenous injection which would enable utilization of the liver specific promoter TBG.

In HIV^+ patients, administration of AAV vectors expressing one or more of these HIV neutralizing antibodies would lead to long-term, high level expression of one or more broadly HIV NAb and would reduce viral load and potentially prevent acquisition of HIV. In this situation, individuals would receive intravenous injection of two AAV vectors at a dose of $5x10^{12}$ genome copies/kilogram of each vector. Contained within the two AAV vectors would be the HIV neutralizing antibody under control of a constitutive promoter, allowing expression to occur rapidly following administration of the vector.

A. Heterodimer and two small molecules

Following the first signs of potential toxicity to the HIV NAb, the first small molecule drug would be administered to induce expression of the components of the inducible system, in this case the DNA binding domain linked to FKBP and $FRAP_L$ linked to the catalytic domain of a endonuclease enzyme. This would allow the system to be primed for action should further toxicity to the HIV NAb develop. If toxicity levels continue to rise then initiation of endonuclease activity would be induced by administration of a second small molecule drug which would lead to the formation of an active enzyme and ablation of HIV NAb gene expression.

B. Heterodimer and one small molecule

Also under the control of constitutive expression would be the elements of the rapamycin inducible system, FKBP and $FRAP_L$. Following administration of the AAV vectors, patients would be closely monitored at regular intervals for several years. If toxicity to the HIV NAb develops then delivery of rapamycin or a rapalog would be implemented.

IV administration of 1 mg/kg rapamycin/rapalog in the first instance with the potential to increase to repeated dosing would be administered to ablate expression of the HIV antibody.

Toxicity and HIV antibody levels would be closely monitored until expression of the HIV NAb had reached undetectable levels. Therefore, the ablation of gene expression of the HIV NAb would provide a safety switch to ablate gene expression should insurmountable toxicity occur.

In one aspect, the invention provides a replication-defective virus composition suitable for use in human subjects in which the viral genome has been engineered to contain: (a) a first transcription unit that encodes a therapeutic product in operative association with a promoter that controls transcription, said unit containing at least one ablation recognition site; and (b) a second transcription unit that encodes an ablator specific for the at least one ablation recognition site in operative association with a promoter, wherein transcription and/or ablation activity is controlled by a pharmacological agent, e.g., a dimerizer. For example, one suitable pharmacologic agent may be rapamycin or a rapamycin analog. The virus composition may contain two or more different virus stocks.

In one aspect, the invention provides a replication-defective virus composition suitable for use in human subjects in which the viral genome comprises (a) a first transcription unit that encodes a therapeutic product in operative association with a promoter that controls transcription, said first transcription unit containing an ablation recognition site; and a second transcription unit that encodes an ablator specific for the ablation recognition site in operative association with a promoter, wherein transcription and/or ablation activity is controlled by a pharmacological agent. The first transcription unit can contains more than one ablation recognition site. Where the genome comprises more than one ablation recognition site, said more than one ablation recognition site comprising a first ablation recognition site and a second ablation recognition site which differs from said first ablation recognition site, said virus further comprising a first ablator specific for the first ablation recognition site and a second ablator specific for the second recognition site.

In one embodiment, the transcription, bioactivity and/or the DNA binding specificity of the ablator is controlled by a regulatable system. The regulatable system can be selected from a tet-on/off system, a tetR-KRAB system, a mifepristone (RU486) regulatable system,

a tamoxifen-dependent regulatable system, a rapamycin - regulatable system, or an ecdysone-based regulatable system.

In one embodiment, the ablator is selected from the group consisting of: an endonuclease, a recombinase, a meganuclease, or a zinc finger endonuclease that binds to the ablation recognition site in the first transcription unit and excises or ablates DNA and an interfering RNA, a ribozyme, or an antisense that ablates the RNA transcript of the first transcription unit, or suppresses translation of the RNA transcript of the first transcription unit. In one specific embodiment, the ablator is Cre and the ablation recognition site is loxP, or the ablator is FLP and the ablation recognition site is FRT.

In an embodiment, the ablator is a chimeric engineered endonuclease, wherein the virus composition comprises (i) a first sequence comprising the DNA binding domain of the endonuclease fused to a binding domain for a first pharmacological agent; and wherein the virus composition further comprises (ii) a second sequence encoding the nuclease cleavage domain of the endonuclease fused to a binding domain for the first pharmacological agent, wherein the first sequences (i) and the second sequence (ii) are each in operative association with at least one promoter which controls expression thereof. The chimeric engineered endonuclease can be contained within a single bicistronic open reading frame in the second transcription unit, said transcription unit further comprising a linker between (i) and (ii). Optionally, the sequence (ii) has an inducible promoter. In another embodiment, the fusion partners/fragments of the chimeric engineered endonuclease are contained within separate open reading frames. In one embodiment, each of the first sequence and the second sequence are under the control of a constitutive promoter and the ablator is bioactivated by the first pharmacological agent.

The coding sequence for the ablator may further comprise a nuclear localization signal located 5' or 3' to the ablator coding sequence.

In one embodiment, the DNA binding domain is selected from the group consisting of a zinc finger, helix-turn-helix, a HMG-Box, Stat proteins, B3, helix-loop-helix, winged helix-turn-helix, leucine zipper, a winged helix, POU domains, and a homeodomain.

In still another embodiment, the endonuclease is selected from the group consisting of a type II restriction endonuclease, an intron endonuclease, and serine or tyrosine recombinases. In one specific embodiment, the ablator is a chimeric FokI enzyme.

In yet another embodiment, in a replication-defective virus composition of the invention, the viral genome further comprises a third and a fourth transcription unit, each encoding a dimerizable domain of a transcription factor that regulates an inducible promoter for the ablator, in which: (c) the third transcription unit encodes the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a first promoter; and (d) the fourth transcription unit encodes the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a second promoter. The first promoter of (c) and the second promoter of (d) are independently selected from a constitutive promoter and an inducible promoter. In another embodiment, the first and second promoters are both constitutive promoters and the pharmacological agent is a dimerizer that dimerizes the domains of the transcription factor. In still a further embodiment, one of the first promoter and the second promoters is an inducible promoter. The third and fourth transcription units can be a bicistronic unit containing an IRES or furin-2A.

In one embodiment, the pharmacological agent is rapamycin or a rapalog.

In one embodiment, the virus is an AAV. Such an AAV may be selected from among, e.g., AAV1, AAV6, AAV7, AAV8, AAV9 and rh10. Still other viruses may be used to generate the DNA constructs and replication-defective viruses of the invention including, e.g., adenovirus, herpes simplex viruses, and the like.

In one embodiment, the therapeutic product is an antibody or antibody fragment that neutralizes HIV infectivity, soluble vascular endothelial growth factor receptor-1 (sFlt-1), Factor VIII, Factor IX, insulin like growth factor (IGF), hepatocyte growth factor (HGF), heme oxygenase-1 (HO- 1), or nerve growth factor (NGF).

In one embodiment of the replication-defective virus composition, the first transcription unit and the second transcription unit are on different viral stocks in the composition. Optionally, the first transcription unit and the second transcription unit are in a first viral stock and a second viral stock comprises a second ablator(s).

In one embodiment, a recombinant DNA construct comprises a first and second transcription unit flanked by packaging signals of a viral genome, in which: (a) a first transcription unit that encodes a therapeutic product in operative association with a promoter that controls transcription, said first transcription unit containing at least one ablation

recognition site; and (b) a second transcription unit that encodes an ablator specific for the at least one ablation recognition site in operative association with a promoter that induces transcription in response to a pharmacological agent. The packaging signals flanking the transcription units may be an AAV 5' inverted terminal repeats (ITR) and a AAV 3' ITR. Optionally, the AAV ITRs are AAV2, or AAV1, AAV6, AAV7, AAV8, AAV9 or rh10 ITRs. In one embodiment, the first transcription unit is flanked by AAV ITRs, and the second, third and fourth transcription units are flanked by AAV ITRs. Optionally, the transcription units are contained in two or more DNA constructs.

In one embodiment, the therapeutic product is an antibody or antibody fragment that neutralizes HIV infectivity, soluble vascular endothelial growth factor receptor-1 (sFlt-1), Factor VIII, Factor IX, insulin like growth factor (IGF), hepatocyte growth factor (HGF), heme oxygenase-1 (HO-1), or nerve growth factor (NGF).

In one embodiment, the promoter that controls transcription of the therapeutic product is a constitutive promoter, a tissue-specific promoter, a cell-specific promoter, an inducible promoter, or a promoter responsive to physiologic cues.

A method is described for treating age-related macular degeneration in a human subject, comprising administering an effective amount of the replication-defective virus composition as described herein, in which the therapeutic product is a VEGF antagonist.

A method is provided for treating hemophilia A in a human subject, comprising administering an effective amount of the replication-defective virus composition as described herein, in which the therapeutic product is Factor VIII.

A method is provided for treating hemophilia B in a human subject, comprising administering an effective amount of the replication-defective virus composition as described herein, in which the therapeutic product is Factor IX.

A method is provided for treating congestive heart failure in a human subject, comprising administering an effective amount of the replication-defective virus composition as described herein, in which the therapeutic product is insulin like growth factor or hepatocyte growth factor.

A method is provided for treating a central nervous system disorder in a human subject, comprising administering an effective amount of the replication-defective virus composition as described herein, in which the therapeutic product is nerve growth factor.

A method is provided for treating HIV infection in a human subject, comprising administering an effective amount of the replication-defective virus composition as described herein in which the therapeutic product is a neutralizing antibody against HIV.

A replication-defective virus is provided herein for use in controlling delivery of the transgene product. The product may be selected from the group consisting of a VEGF antagonist, Factor IX, Factor VIII, insulin like growth factor, hepatocyte growth factor, nerve growth factor, and a neutralizing antibody against HIV.

A genetically engineered cell is provided which comprises a replication-defective virus or a DNA construct as provided herein. The genetically engineered cell may be selected from a plant, bacterial or non-human mammalian cell.

A method is provided for determining when to administer a pharmacological agent for ablating a therapeutic product to a subject who received the replication-defective virus as provided herein containing a therapeutic product and an ablator, comprising: (a) detecting expression of the therapeutic product in a tissue sample obtained from the patient, and (b) detecting a side effect associated with the presence of the therapeutic product in said subject, wherein detection of a side effect associated with the presence of the therapeutic product in said subject indicates a need to administer the pharmacological agent that induces expression of the ablator.

A method is provided for determining when to administer a pharmacological agent for ablating a therapeutic product to a subject who received the replication-defective virus composition as described herein encoding a therapeutic product and an ablator, comprising: detecting the level of a biochemical marker of toxicity associated with the presence of the therapeutic product in a tissue sample obtained from said subject, wherein the level of said marker reflecting toxicity indicates a need to administer the pharmacological agent that induces expression of the ablator.

These methods may further comprise determining the presence of DNA encoding the therapeutic gene product, its RNA transcript, or its encoded protein in a tissue sample from the subject subsequent to treatment with the pharmacological agent that induces expression of the ablator, wherein the presence of the DNA encoding the therapeutic gene product, its RNA transcript, or its encoded protein indicates a need for a repeat treatment with the pharmacological agent that induces expression of the ablator.

The invention further provides a replication-defective virus as described herein for use in controlling delivery of the transgene product.

In another embodiment, the invention provides a genetically engineered cell, comprising a replication-defective virus or a DNA construct as described herein. Such a cell may be a plant, yeast, fungal, insect, bacterial, non-human mammalian cells, or a human cell.

In yet a further embodiment, the invention provides a method of determining when to administer a pharmacological agent for ablating a therapeutic product to a subject who received the replication-defective virus as described herein encoding a therapeutic product and an ablator, comprising: (a) detecting expression of the therapeutic product in a tissue sample obtained from the patient, and (b) detecting a side effect associated with the presence of the therapeutic product in said subject, wherein detection of a side effect associated with the presence of the therapeutic product in said subject indicates a need to administer the pharmacological agent that induces expression of the ablator. In still a further embodiment, the invention provides a method of determining when to administer a pharmacological agent for ablating a therapeutic product to a subject who received the replication-defective virus composition as described herein encoding a therapeutic product and an ablator, comprising: detecting the level of a biochemical marker of toxicity associated with the presence of the therapeutic product in a tissue sample obtained from said subject, wherein the level of said marker reflecting toxicity indicates a need to administer the pharmacological agent that induces expression of the ablator.

EXAMPLE 14 : Generation of 10xZF Constructs

Open reading frames encoding for various endonucleases were codon optimized and *de novo* synthesized by GeneArt (Invitrogen). Ablator expression and target reporter plasmids were produced using standard molecular biological cloning techniques. Transfections were performed in HEK293 cells using Lipofectamine™ 2000 transfection reagent (Life Technologies). All transfections were performed using optimal transfection conditions as defined in transfection reagent protocol. Briefly, 150-200 ng plasmid DNA (excluding transfection control plasmid) was complexed with lipofectamine and added to cells in 96 well plates. DNA quantities were consistent across all conditions by supplementation with an unrelated plasmid containing the same promoter as test plasmids.

Transfection complexes were incubated with cells for 4 hours as transfection reagent protocol before the addition of FBS supplemented media. Transfected cells were incubated at 37°C for 24-48 hours. Following incubation, cells were assayed for reporter gene expression using Promega Dual Luciferase detection kit according to the manufacturer's instructions on a BioTek Clarity plate reader and renilla luciferase was used to control for transfection efficiency. All samples were performed in quadruplicate and standard errors of the mean were calculated.

A. Generation of 10x ZF expression plasmid

101. Once random sequence 1 was generated and determined to be targetable by standard zinc finger design methodologies, the design of the nucleases was performed using the resources available from The Zinc Finger (ZF) Consortium and the zinc finger database (these are publically available. *See, e.g.,* bindr.gdcb.iastate.edu:8080/ZiFDB/). Based on the breakdown of the 28-32 bp sequence into 10x 3bp ZF binding sites, ZF domains which bind to each of the 3 bp sequences were identified.

The following 28-32 bp sequences were generated:

[SEQ ID NO: 806] GGTCGATGTTCGCAACGTCGATCGTACGTGCA . For this sequence, there are two reading frames which are targetable by different zinc fingers: GGT-CGA-TGT-TCG-CAA-CGT-CGA-TCG-TAC-GTG-CA - targetable G-GTC-GAT-GTT-CGC-AAC-GTC-GAT-CGT-ACG-TGC-A - targetable (the experiments described herein were generated for this sequence)

[SEQ ID NO: 817]: GGTCGGCGACGCGTAATCGTCGATTGGCGTAC. For this sequence, there are two reading frames which are targetable by different zinc fingers. G-GTC-GGC-GAC-GCG-TAA-TCG-TCG-ATT-GGC-GTA-C - targetable GG-TCG-GCG-ACG-CGT-AAT-CGT-CGA-TTG-GCG-TAC - targetable

[SEQ ID NO: 801] GGTCGGCGACGCGAATCGTCGATTGGCGTAC. For this sequence, there are two reading frames which are targetable by different zinc fingers. G-GTC-GGC-GAC-GCG-TAA-TCG-TCG-ATT-GGC-GTA-C - targetable GG-TCG-GCG-ACG-CGT-AAT-CGT-CGA-TTG-GCG-TAC - targetable

[SEQ ID NO: 802] GGTCGGCGACGCGTATCGATTGGCGTAC.

For this sequence, there is one potential targetable by a zinc finger: GGT-CGG-CGA-CGC-GTA-TCG-ATT-GGC-GTA-C - targetable

[SEQ ID NO: 803] ACTATTCGCACGCCGTACGATAGTCGGCGCGA. For this sequence, there are two reading frames targetable by zinc fingers: ACT-ATT-CGC-ACG-CCG-TAC-GAT-AGT-CGG-CGC-GA - targetable and A-CTA-TTC-GCA-CGC-CGT-ACG-ATA-GTC-GGC-GCG-A - targetable.

For any 3 bp sequence for which only one known ZF sequence was available within the consortium, this sequence was chosen for the final protein. This was the case for 3 out of the 10x ZF domains.

Provided within the consortium database was a table of previously tested combination of 2 or 3 ZFs linked together in a particular orientation for binding to DNA. Within our random sequence 1, there are two regions which contain previously tested combinations. One is a 3x 3bp stretch and the other is a 2x 3bp stretch. Where a previous combination of ZFs was reported to work, this was incorporated into the 10x ZF sequence.

For 3 out of 10x ZF sequences, only the recognition helix of the DNA binding region was included in the consortium. For 7 out of the 10x ZF sequences, the full ZF sequence was available in the consortium. Therefore, the full sequence of the ZFs where only the recognition helix was available through the consortium had to be generated. In addition, to prevent high level sequence homology between the 10x ZF protein, the conserved protein sequence of the ZF protein domain was varied between one of the two conserved sequences in the consortium:

P start conserved sequence	PGEKPYKCPECGKSFS-------HQRTH [SEQ ID NO: 745]
T start conserved sequence	TGEKPFQCRICMRNFS-------HLRTH [SEQ ID NO: 807]

The ZFs were linked N terminus to C terminus directly as there innate protein sequence would allow for the correct structure of the final protein.

The 10x ZF sequence was then linked to the catalytic domain of the FokI enzyme (the DNA binding domain was removed from the wild-type sequence) through a standard linker sequence on the C terminal of the 10x ZF protein.

In the working examples below, an ablator encoded by the sequence: 10xZF-FokI_Cat nucleotide sequence: SEQ ID NO: 59, is illustrated:

ATGGGCGAGAAGCCCTACAAGTGCCCTGAGTGCGGCAAGAGCTTCAGCCAG
AGAAGAAGCCTGGGCCACCACCAGCGTACGCACCCCGGCGAGAAACCTTAT
AAGTGTCCCGAATGTGGCAAGTCCTTCAGCAAGAAGAACGACCTGACCCGG
CACCAGCGGACACACCCCGGGGAAAAGCCATACAAATGTCCAGAGTGTGGG
AAGTCTTTCTCCAGCCGGCGGACCTGCAGAGCCCATCAGAGAACACATACCG
GGGAGAAGCCTTTCCAGTGCCGGATCTGCATGAGAAACTTCAGCGTGCGGCA
CAACCTGACCAGACACCTGAGGACCCATACCGGCGAAAAACCCTTTCAGTGC
AGAATCTGTATGCGGAACTTCTCCGACCGGACCAGCCTGGCCCGGCATCTGA
GAACTCATCCTGGGGAAAAGCCCTATAAGTGTCCAGAATGCGGGAAATCCTT
TAGCGACAGCGGCAACCTGCGGGTGCACCAGAGGACTCATCCAGGCGAGAA
ACCCTACAAATGCCCCGAATGCGGAAAGTCATTCTCCCACACCGGCCATCTG
CTCGAGCATCAGCGGACCCACACTGGGGAGAAACCATTTCAGTGTCGCATCT
GTATGAGGAATTTCAGCACCAACCAGGCCCTGGGCGTGCACCTGAGAACAC
ACCCAGGCGAGAAGCCTTACAAGTGTCCAGAGTGCGGAAAGTCATTTTCCGT
GCGCCACAATCTGACACGGCATCAGCGCACCCATCCCGGCGAGAAGCCTTAC
AAATGCCCCGAGTGTGGCAAATCTTTCAGTGACCGGACCTCTCTGGCCAGAC
ATCAGAGGACACACGGCACTAGTGGCAAGCAGCTGGTGAAAAGCGAGCTGG
AAGAGAAGAAGTCCGAGCTGCGGCACAAGCTGAAATACGTGCCCCACGAGT
ACATCGAGCTGATCGAGATCGCCCGGAACCCCACCCAGGACAGAATCCTGG
AAATGAAGGTCATGGAATTTTTCATGAAGGTGTACGGCTACCGGGGCGAGCA
CCTGGGCGGCAGCAGAAAACCCGACGGCGCCATCTACACCGTGGGCAGCCC
CATCGACTACGGCGTGATCGTGGACACCAAGGCCTACAGCGGCGGCTACAA
CCTGCCCATCGGACAGGCCGACGAGATGCAGAGATACGTGGAAGAGAACCA
GACCCGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGTGTACCCCAG
CAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGGGCAAC
TACAAGGCCCAGCTGACCCGGCTGAACCACATCACCAACTGCAACGGCGCTG
TGCTGAGCGTGGAAGAACTGCTGATCGGCGGCGAGATGATCAAGGCCGGCA

CCCTGACCCTGGAAGAAGTGCGGCGGAAGTTCAACAACGGCGAGATCAACT TCTGATAG.

In this construct, bp1-84: zinc finger N1 (recognition helix QRRSLGH, P form, binds to TGC); bp 85-168: zinc finger N2 (recognition helix KKNDLTR, P form, binds to ACG); bp 169-252: zinc finger N3 (recognition helix SRRTCRA, P form, binds to CGT); bp 253-336: zinc finger N4 (recognition helix VRHNLTR, T form, binds to GAT); bp 337-420: zinc finger N5 (recognition helix DRTSLAR, T form, binds to GTC), bp 421-504 bp: zinc finger N6 (recognition helix DSGNLRV, P form, binds to AAC); bp 505-588: zinc finger N7 (recognition helix HTGHLLEM, P form, binds to CGC); bp 589-672: zinc finger N8 (recognition helix TNQALGV, T form, binds to GTT); bp 673-756: zinc finger N9 (recognition helix VRHNLTR, P form, binds to GAT); bp 757-840: zinc finger N10 (recognition helix DRTSLAR, P form, binds to GTC); bp 841-855 : 5 amino acid linker; bp 856-1443: FokI catalytic domain.

The transcribed illustrative ablator has the following sequence in which the odd zinc fingers are underlined in order to illustrate the location of each of the 10 zinc fingers; the zinc fingers are separated by a hyphen only in order to facilitate their identification: 10xZF-FokI_Cat amino acid sequence: SEQ ID NO: 60:

MGEKPYKCPECGKSFSQRRSLGHHQRTH – PGEKPYK
(N1)
CPECGKSFSKKNDLTRHQRTH – PGEKPYKCPECGKSFSSRRTCRAHQRTH
(N2) (N3)
TGEKPFQCRICMRNFSVRHNLTRHLRTH – TGEKPFQCRICMRNFSDRT
(N4)
SLARHLRTH – PGEKPYKCPECGKSFSDSGNLRVHQRTH - PGEKPYKCPECGK
(N5) (N6)

SFSHTGHLLEHQRTH – TGEKPFQCRICMRNFSTNQALGVHLRTH - PGEKPYKC
(N7) (N8)
PECGKSFSVRHNLTRHQRTH – PGEKPYKCPECGKSFSDRTSLARHQRTH
(N9) (N10)

GTSGKQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNPTQDRILEMKVMEFFM
KVYGYRGEHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQR
YVEENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNC
NGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF. 1-28 aa – zinc finger N1 (recognition helix QRRSLGH, P form, binds to TGC); 29-56 aa – zinc finger N2 (recognition helix KKNDLTR, P form, binds to ACG); 57-84 aa – zinc finger N3 (recognition helix SRRTCRA, P form, binds to CGT); 85-112 aa – zinc finger N4 (recognition helix VRHNLTR, T form, binds to GAT); 113-140 aa – zinc finger N5 (recognition helix DRTSLAR, T form, binds to GTC); 141-168 aa – zinc finger N6 (recognition helix DSGNLRV, P form, binds to AAC); 169-196 aa – zinc finger N7 (recognition helix HTGHLLEM, P form, binds to CGC); 197-224 aa – zinc finger N8 (recognition helix TNQALGV, T form, binds to GTT); 225-252 aa – zinc finger N9 (recognition helix VRHNLTR, P form, binds to GAT); 253-280 aa – zinc finger N10 (recognition helix DRTSLAR, P form, binds to GTC); 281-284 aa – 5 amino acid linker; and 285-481 aa – FokI catalytic domain.

In another embodiment, the ablator has the sequence of SEQ ID NO: 60, except that the sequence of the first zinc finger (N1) in the construct above (SEQ ID NO: 15) is replaced by SEQ ID NO: 188, which has a P inserted following the initiation codon.

In another embodiment, the 10x ZF sequence is linked to the catalytic domain of the FokI enzyme (the DNA binding domain was removed from the wild-type sequence) through a linker sequence on the N terminus of the 10x ZF protein.

N-linked FokI_Cat-10xZF nucleotide sequence: SEQ ID NO: 808:

ATGAAGCAGCTGGTGAAAAGCGAGCTGGAAGAGAAGAAGTCCGAGCTGCGGCA
CAAGCTGAAATACGTGCCCCACGAGTACATCGAGCTGATCGAGATCGCCCGGAA
CCCCACCCAGGACAGAATCCTGGAAATGAAGGTCATGGAATTTTTCATGAAGGT
GTACGGCTACCGGGGCGAGCACCTGGGCGGCAGCAGAAAACCCGACGGCGCCA
TCTACACCGTGGGCAGCCCCATCGACTACGGCGTGATCGTGGACACCAAGGCCT
ACAGCGGCGGCTACAACCTGCCCATCGGACAGGCCGACGAGATGCAGAGATAC
GTGGAAGAGAACCAGACCCGGAACAAGCACATCAACCCCAACGAGTGGTGGAA
GGTGTACCCCAGCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTC

AAGGGCAACTACAAGGCCCAGCTGACCCGGCTGAACCACATCACCAACTGCAAC
GGCGCTGTGCTGAGCGTGGAAGAACTGCTGATCGGCGGCGAGATGATCAAGGCC
GGCACCCTGACCCTGGAAGAAGTGCGGCGGAAGTTCAACAACGGCGAGATCAA
CTTCGGCACTAGTGGCGGCGAGAAGCCCTACAAGTGCCCTGAGTGCGGCAAGAG
CTTCAGCCAGAGAAGAAGCCTGGGCCACCACCAGCGTACGCACCCCGGCGAGA
AACCTTATAAGTGTCCCGAATGTGGCAAGTCCTTCAGCAAGAAGAACGACCTGA
CCCGGCACCAGCGGACACACCCCGGGGAAAAGCCATACAAATGTCCAGAGTGT
GGGAAGTCTTTCTCCAGCCGGCGGACCTGCAGAGCCCATCAGAGAACACATACC
GGGGAGAAGCCTTTCCAGTGCCGGATCTGCATGAGAAACTTCAGCGTGCGGCAC
AACCTGACCAGACACCTGAGGACCCATACCGGCGAAAAACCCTTTCAGTGCAGA
ATCTGTATGCGGAACTTCTCCGACCGGACCAGCCTGGCCCGGCATCTGAGAACT
CATCCTGGGGAAAAGCCCTATAAGTGTCCAGAATGCGGGAAATCCTTTAGCGAC
AGCGGCAACCTGCGGGTGCACCAGAGGACTCATCCAGGCGAGAAACCCTACAA
ATGCCCCGAATGCGGAAAGTCATTCTCCCACACCGGCCATCTGCTCGAGCATCA
GCGGACCCACACTGGGGAGAAACCATTTCAGTGTCGCATCTGTATGAGGAATTT
CAGCACCAACCAGGCCCTGGGCGTGCACCTGAGAACACACCCAGGCGAGAAGC
CTTACAAGTGTCCAGAGTGCGGAAAGTCATTTTCCGTGCGCCACAATCTGACAC
GGCATCAGCGCACCCATCCCGGCGAGAAGCCTTACAAATGCCCCGAGTGTGGCA
AATCTTTCAGTGACCGGACCTCTCTGGCCAGACATCAGAGGACACAC.

This nucleic acid sequence encodes, at: FokI catalytic domain (1-594 bp), 4 amino acid linker (595-606 bp), zinc finger N1 (recognition helix QRRSLGH, P form, binds to TGC) (607-687 bp), zinc finger N2 (recognition helix KKNDLTR, P form, binds to ACG) (688-771 bp), zinc finger N3 (recognition helix SRRTCRA, P form, binds to CGT) (772-855 bp), zinc finger N4 (recognition helix VRHNLTR, T form, binds to GAT) (856-939 bp), zinc finger N5 (recognition helix DRTSLAR, T form, binds to GTC) (940-1023 bp), zinc finger N6 (recognition helix DSGNLRV, P form, binds to AAC) (1024-1107 bp), zinc finger N7 (recognition helix HTGHLLEM, P form, binds to CGC) (1108-1191 bp), zinc finger N8 (recognition helix TNQALGV, T form, binds to GTT) (1192-1275 bp), zinc finger N9 (recognition helix VRHNLTR, P form, binds to GAT) (1276-1359 bp), zinc finger N10 (recognition helix DRTSLAR, P form, binds to GTC) (1360-1443 bp).

The transcribed N-linked 10xZF-FokI_Cat amino acid sequence: SEQ ID NO: 809 : is as follows.

MKQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNPTQDRILEMKVMEFFMKVYGY
RGEHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQT
RNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEE
LLIGGEMIKAGTLTLEEVRRKFNNGEINFGTSGGEKPYKCPECGKSFSQRRSLGHHQ
RTHPGEKPYKCPECGKSFSKKNDLTRHQRTHPGEKPYKCPECGKSFSSRRTCRAHQR
THTGEKPFQCRICMRNFSVRHNLTRHLRTHTGEKPFQCRICMRNFSDRTSLARHLRT
HPGEKPYKCPECGKSFSDSGNLRVHQRTHPGEKPYKCPECGKSFSHTGHLLEHQRT
HTGEKPFQCRICMRNFSTNQALGVHLRTHPGEKPYKCPECGKSFSVRHNLTRHQRT
HPGEKPYKCPECGKSFSDRTSLARHQRTH.

This chimeric endonuclease is: FokI catalytic domain (1-198 aa), 5 amino acid linker (199-202 aa), zinc finger N1 (recognition helix QRRSLGH, P form, binds to TGC) (203-229 aa), zinc finger N2 (recognition helix KKNDLTR, P form, binds to ACG) (230-257 aa), zinc finger N3 (recognition helix SRRTCRA, P form, binds to CGT) (258-285 aa), zinc finger N4 (recognition helix VRHNLTR, T form, binds to GAT) (286-313 aa), zinc finger N5 (recognition helix DRTSLAR, T form, binds to GTC) (314-341 aa), zinc finger N6 (recognition helix DSGNLRV, P form, binds to AAC) (342-369 aa), zinc finger N7 (recognition helix HTGHLLEM, P form, binds to CGC) (370-397 aa), zinc finger N8 (recognition helix TNQALGV, T form, binds to GTT) (398-425 aa), zinc finger N9 (recognition helix VRHNLTR, P form, binds to GAT) (426-453 aa), and zinc finger N10 (recognition helix DRTSLAR, P form, binds to GTC) (454-481 aa).

B. Generation of reporter plasmids for 10x ZF-FokI_Cat protein

In order to study the efficiency of the 10x ZF expression plasmid, a series of luciferase reporter plasmids were also designed with the unique 32 bp sequence: [SEQ ID NO: 806] GGTCGATGTTCGCAACGTCGATCGTACGTGCA generated as described in A above. These vectors include:

pITA-030, which contains, from 5' to 3': a cytomegalovirus (CMV) immediate early (IE) enhancer and promoter (bp 1-832), a Promega™ intron (833-1029 bp), the 32 bp sequence in sense orientation (1069-1100 bp), a spacer (1101-1106 bp), the 32 bp sequence

in inverted orientation (1107-1138 bp), a Kozak sequence (1147-1152 bp), the coding sequence for a luciferase reporter gene (1153-2802 bp), and an SV40 polyA sequence (2822-3061 bp), of SEQ ID NO: 811.

pITA-031, which contains, from 5' to 3': a CMV promoter IE enhancer/promoter (1-832 bp), a Promega™ intron (833-1029 bp), the 32 bp sequence in direct/sense orientation (1069-1100 bp), a spacer (1101-1106 bp), the 32 bp sequence in inverted orientation (1107-1138 bp), a Kozak sequence (1147-1152 bp), the coding sequence for a luciferase reporter gene (1153-2802 bp), a 32 bp sequence in sense orientation (2815-2846 bp), a spacer (2847-2852 bp), the 32 bp sequence in inverted orientation (2853-2884 bp), and an SV40 polyA sequence (2893-3131 bp), of SEQ ID NO: 812.

pITA-032, which contains, from 5' to 3': a CMV IE enhancer/promoter (1-832 bp), a Promega™ intron (833-1029 bp), the 32 bp sequence in sense orientation (1069-1100 bp), a Kozak sequence (1115-1120 bp), the coding sequence for a luciferase reporter gene (1121-2770 bp), and an SV40 polyA sequence (2791-3029 bp), of SEQ ID NO: 813.

pITA-033, which contains, from 5' to 3': a CMV IE enhancer/promoter (1-832 bp), a Promega™ intron (833-1029 bp), the 32 bp sequence in sense orientation (1069-1100 bp), a Kozak sequence (1115-1120 bp), the coding sequence for a luciferase reporter gene (1121-2770 bp), the 32 bp sequence in sense orientation (2783-2814 bp), and an SV40 polyA sequence (2829-3067 bp) , of SEQ ID NO: 814.

pITA-034, which contains, from 5' to 3': a CMV IE enhancer/promoter (1-832 bp), a Promega™ intron (833-1029 bp), the 32 bp sequence in sense orientation (1069-1100 bp), a Kozak sequence (1115-1120 bp), the coding sequence for a luciferase reporter gene (1121-2770 bp), the 32 bp sequence in direct orientation (2789-2820 bp), and an SV40 polyA sequence (2829-3067 bp) , of SEQ ID NO: 815 .

pITA-005 (control) contains from 5' to 3': a CMV IE enhancer/promoter (1-832 bp), a Promega™ intron (833-1029 bp), a Kozak sequence (1077-1082 bp), the coding sequence for a luciferase reporter gene (1083-2732 bp), and an SV40 polyA sequence (2752-2991 bp).

These reporter plasmids were studied in dose-dependent studies, of SEQ ID NO: 810.

(A) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected into

293 cells with 50 ng pCMV.32bp-Luciferase (pITA-032, single specific site for 10xZF protein at 5' end of luciferase gene). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

(B) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected into 293 cells with 50 ng pCMV.32bp-Luciferase-32bp (pITA-033, single specific site for 10xZF protein at 5' and at 3' end of luciferase gene in head-to-tail orientation). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

(C) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected into 293 cells with 50 ng pCMV.32bp-Luciferase-32bp (pITA-034, single specific site for 10xZF protein at 5' end of luciferase and a single specific inverted site at the 3' end of luciferase gene). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

(D) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected with 50 ng pCMV.32bpSpacer32bp-Luciferase (pITA-030, single combined specific site for 10xZF containing a 32 bp site with a spacer separating an identical but inverted 32 bp at the 5' end of the luciferase gene). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

(E) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected with 50 ng pCMV.32bpSpacer32bp-Luciferase-32bpSpacer32bp (pITA-031, single combined specific site for 10xZF containing a 32 bp site with a spacer separating an identical but inverted 32 bp at both the 5' end and the 3' end of the luciferase gene). The cells were assayed for reporter gene expression as described above 48 hours post-transfection. (F) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected with 50 ng pCMV. Luciferase (pITA-005, luciferase expression plasmid containing no specific site for 10xZF). The cells were assayed for reporter gene expression as described above 24 hours post-transfection.

(G) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion (1.56, 3.13, 6.25, 12.5, 25, 50 and 100ng) were co-transfected with 50 ng pCMV. Luciferase (pITA-005, luciferase expression plasmid containing no specific site for 10xZF). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

In each of (A) - (E), dose-dependent ablation was observed for all five reporter plasmids, demonstrating that the 10x ZF design provided by this invention requires only one 32 bp sequence to be present within the reporter and, therefore, within the vector in the applications described herein.

Different orientations of the reporter plasmid were studied in dose-dependent studies.

(H) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion with the 10xZF domain at the N-terminus of the protein (1.56, 3.13, 6.25, 12.5, 25 and 50ng) were co-transfected into 293 cells with 50 ng pCMV.32bpSpacer32bpLuciferase (pITA-030, single combined specific site for 10xZF containing a 32 bp site with a spacer separating an identical but inverted 32 bp at the 5' end of the luciferase gene). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

(I) Increasing concentrations of the expression plasmid encoding FokI tethered to DNA via 10xZF fusion with the FokI catalytic domain at the N-terminus of the protein (1.56, 3.13, 6.25, 12.5, 25 and 50ng) were co-transfected into 293 cells with 50 ng pCMV.32bpSpacer32bpLuciferase (pITA-030, single combined specific site for 10xZF containing a 32 bp site with a spacer separating an identical but inverted 32 bp at the 5' end of the luciferase gene). The cells were assayed for reporter gene expression as described above 48 hours post-transfection.

In order to assess the affect targeting a different AAC triplet, a different zinc finger was substituted for the sixth (N6) zinc finger in the expression plasmid encoding FokI tethered to DNA via 10xZF fusion, which was prepared as described in Example 14A herein. Using SOEing PCR strategy, the alternative recognition helix DSGNLRV (SEQ ID NO: 64) was inserted into the place of the GASALRS (SEQ ID NO: 66) in N6 of the expression plasmid of Example 14A. This second generation construct is termed pITA-047 and

contains a 5' ITR, CMV IE-promoter/enhancer, the zinc finger – FokI catalytic domain, poly A, and 3' ITR. In separate studies, the reporter plasmids described above (pITA-30, pITA-031, pITA-032, and pITA-005) were co-transfected (50 ng reporter plasmid) into 293 cells with increasing concentrations of the pITA-047 plasmid and luciferase expression was measured 48 hours post-transfection. Decreased luciferase expression demonstrates higher endonuclease activity. The results showed that the pITA-047 construct with the altered AAC targeting sequence successfully reduced luciferase expression as compared to the first generation plasmid containing the same vector elements but with the wild-type zinc finger domains.

(Sequence Listing Free Text)

The following information is provided for sequences containing free text under numeric identifier <223>.

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SEQ ID NO: (containing free text)	Free text under <223>
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SEQ ID NO: (containing free text)	Free text under <223>
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723, 726, 754,	<221> misc_feature <222> (19)..(25) <223> Xaa can be any naturally occurring amino acid
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717, 719, 720, 721, 722, 724, 725, 727 - 750, 752, 753, 755, 756, 757, 759, 807	<221> misc_feature <222> (17)..(23) <223> Xaa can be any naturally occurring amino acid
808	<220> <221> misc_feature <222> (1)..(594) <223> FokI catalytic domain <220> <221> misc_feature <222> (595)..(606) <223> 4 amino acid linker <220> <221> misc_feature <222> (607)..(687) <223> zinc finger N1 <220>

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812	<220> <221> misc_feature <222> (1)..(832) <223> CMV I.E. enhancer and promoter <220> <221> misc_feature <222> (833)..(1029) <223> Promega intron <220> <221> misc_feature <222> (1069)..(1100) <223> 32 bp sequence in direct orientation <220>

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813	<220>

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SEQ ID NO: (containing free text)	Free text under <223>
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All publications, patents, and patent applications cited in this application, as well as priority applications PCT/US2011/030213, filed March 28, 2011 and US Patent Application No. 61/318,755 and the Sequence Listing, are hereby incorporated by reference in their entireties as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A composition for AAV-mediated delivery of a therapeutic product having a controlled transgene expression ablation system, said composition comprising

(a) an AAV vector containing a nucleic acid molecule comprising:

(i) a nucleic acid sequence encoding a therapeutic product operably linked to a promoter that controls transcription; and

(ii) at least one endonuclease ablation site which comprises a sequence of at least 30 nucleic acid base pairs which are specifically recognized by at least ten (10X) zinc fingers, said at least one endonuclease ablation site being located at least 5' to the sequence encoding the therapeutic product;

(b) at least one ablator which comprises a chimeric endonuclease comprising at least ten copies of a zinc finger domain linked to a functional endonuclease catalytic domain in operative association with a promoter, wherein transcription and/or ablation activity is induced in response to a pharmacological agent, said at least ten (10X) zinc finger domain specifically recognizing said at least about 30 base pair sequence in said at least one endonuclease ablation site and comprising at least 10 independently selected recognition helices.

2. The composition according to claim 1, wherein the endonuclease catalytic domain is a FokI catalytic domain.

3. The composition according to claim 1 or 2, wherein the at least 10 independently selected recognition helices are all different.

4. The composition according to claim 3, wherein the at least 10 independently selected recognition helices include different recognition helices to the same triplet of base pairs in the ablation site.

5. The composition according to claim 1 or claim 2, wherein the at least 10 independently selected recognition helices contain 1 to 3 helices which are the same.

6. The composition according to any one of claims 1 to 5, wherein the at least 30 base pair sequence in the endonuclease ablation site comprise at least 32 base pairs.

7. The composition according to any one of claims 1 to 6, wherein the molecule comprises a first endonuclease ablation site and a second endonuclease ablation site, wherein said first and said second endonuclease ablation site may be the same or different unique sequence.

8. The composition according to claim 7, wherein the first endonuclease ablation site and the second endonuclease ablation site are both located 5' to the coding sequence and are separated by a spacer sequence.

9. The composition according to claim 8, wherein the second endonuclease ablation site is spacer sequence is inverted.

10. The composition according to claim 8, wherein the spacer sequence is a non-coding sequence.

11. The composition according to claim 7, wherein said second endonuclease ablation site is located 3' to the coding sequence.

12. The composition according to claim 1, wherein the catalytic domain of the endonuclease is linked to the zinc finger catalytic domain sequence on the N or C terminus of the zinc finger catalytic domain sequence

13. The composition according to claim 12, wherein the catalytic domain is linked to the zinc finger catalytic domain sequence via a linker sequence.

14. The composition according to claim 1, wherein the catalytic domain of the endonuclease is located within the zinc finger domain sequence.

15. The composition according to any one of claims 1 to 14, wherein the nucleic acid molecule consists of a double-stranded DNA molecule, wherein the at least one endonuclease ablation site is on a first strand of the DNA molecule and at least a second endonuclease ablation is located on the second strand of the DNA molecule, wherein said second endonuclease ablation site is distinct from said endonuclease ablation said on the first strand and is specifically and selectively recognized by a different zinc fingers.

16. The composition according to claim 1, wherein the at least 30 base pair sequence is selected from the group consisting of:

(i) SEQ ID NO: 806: 5' - GGTCGATGTTCGCAACGTCGATCGTACGTGCA - 3';

(ii) SEQ ID NO: 801: 5' - GGTCGGCGACGCGAATCGTCGATTGGCGTAC - 3'

and

(iii) SEQ ID NO: 803: 5' - ACTATTCGCACGCCGTACGATAGTCGGCGCGA - 3'.

17. The composition according to claim 1, wherein said zinc finger domain comprises a nucleic acid sequence encoding at least ten zinc fingers consisting of : (a) a first N-terminal zinc finger comprising a recognition helix which specifically binds to TGC; (b) second zinc finger comprising a recognition helix which specifically binds to ACG; (c) a third zinc finger comprising a recognition helix which specifically binds to CGT; (d) a fourth zinc finger comprising a recognition helix which specifically binds to GAT; (e) a fifth zinc finger comprising a recognition helix which specifically binds to GTC; (f) a sixth zinc finger comprising a recognition helix which specifically binds to AAC; (g) a seventh zinc finger comprising a recognition helix which binds to CGC; (h)

an eighth zinc finger comprising a recognition helix that specifically binds to GTT; (i) an ninth zinc finger comprising a recognition helix that specifically binds to GAT; and (j) a tenth zinc finger comprising a recognition helix which specifically binds to GTC.

18. The composition according to claim 17, wherein:

the recognition helix of (a) which specifically binds to TGC is selected from the group consisting of: ARNTLVH, QRRSLGH, QARSLRA, QQRSLKN, and QNRSLAH, QGRSLRA, RARNLTL, RGRNLEM, RKRNLIM, RMRNLII, RNRNLVL, RRRNLHL, RRRNLTL,RSRNLDI, RSRNLLL, and RSRNLTL (SEQ ID NO: 658-673);

the recognition helix of (b) which specifically binds to ACG is selected form the group consisting of: KNNDLTR; KRIDLQR; RKHDLNM; RRQTLRQ; KGNDLTR; PSQTLAWQ; RNITLVR, RSHDLTV, ASADLTR, QNATRKR, QSGDLTR, RSQTLAQ; and RTDTLRD (SEQ ID NO: 104-119);

the recognition helix of (c) which specifically binds to CGT is selected from the group consisting of RSQTRKT (SEQ ID NO: 154) and SRRTCRA (SEQ ID NO: 155);

the recognition helix of (d) which specifically binds to GAT is selected from the group consisting of VRHNLTR, ISHNLAR,ISSNLQR, LGNNLKR, LNSNLAR, LSTNLTR, LTHNLRR, QSSNLAR, RSDALIQ, SKQALAV, TGQQLRV, TKQRLVV, TRQRLRI, TSANLSR, TSGNLVR, TSQMLVV, TSSNLSR, TTSNLRR, VGHNLSR, VGSNLTR (SEQ ID NO: 251-270);

the recognition helix of (e) which specifically binds to GTC is selected from the group consisting of DRTSLAR, DHSSLKR, APSSLRR, DATQLVR, DPGALVR, DPTSLNR, DRSALAR, DRSALSR, DRSSLRR, DRTPLNR, DRTPLQN, EGGALRR, ESGALRR, NTSLLRR, RSDVLSE, TGAVLRR, TGAVLTR, TKKILTV, TKSLLAR, TMAVLRR, TRAVLRR, TSTILAR, TSTLLKR, and TSTLLNR (SEQ ID NO: 530-553);

the recognition helix of (f) which specifically binds to AAC is selected from the group consisting of DRSNRKT , DSGNLRV , GASALRQ , GASALRS ,

GGTALRM , GGTALVM , GHTALAL , GHTALRH , GHTALRN , GPTALVN , and HRTNLIA (SEQ ID NO: 63-73);

the recognition helix of (g) which specifically binds to CGC is HTGHLLE (SEQ ID NO: 151);

the recognition helix of (h) which specifically binds to GTT is selected from the group consisting of HKSSLTR, TNQALGV, AATALRR, HHNSLTR, HSSSLVR, IKAILTR, INHSLRR , IRTSLKR, MNSVLKR, MTSSLRR, QATLLRR, QSSALTR, THTVLAR, TKPVLKI, TNSVLGR, TRHSLGR, TSGALTR, TSGSLTR, TSGSLVR, TSTLLKR, TSTRLDI, TTALLKR, TTSALTR, TTTVLAR, and VGGSLNR (SEQ ID NO: 583-607);

the recognition helix of (i) which specifically binds to GAT is selected from the group consisting of ISHNLAR, VRHNLTR, ISSNLQR, LGNNLKR, LNSNLAR, LSTNLTR, LTHNLRR, QSSNLAR, RSDALIQ, SKQALAV, TGQQLRV, TKQRLVV, TRQRLRI, TSANLSR, TSGNLVR, TSQMLVV, TSSNLSR, TTSNLRR, VGHNLSR, and VGSNLTR (SEQ ID NO: 251-270);

and

the recognition helix of (j) which specifically binds to GTC is selected from the group consisting of DRTSLAR, DHSSLRKR, APSSLRR, DATQLVR, DPGALVR, DPTSLNR, DRSALAR, DRSALSR, DRSSLRR, DRTPLNR, DRTPLQN, EGGALRR, ESGALRR, NTSLLRR, RSDVLSE, TGAVLRR, TGAVLTR, TKKILTV, TKSLLAR, TMAVLRR, TRAVLRR, TSTILAR, TSTLLKR, and TSTLLNR (SEQ ID NO: 530-553).

19. The composition according to claim 18, wherein each of the zinc fingers (a) - (j) has the selected recognition domain inserted in a zinc finger construct selected from :

SEQ ID NO: 745: (N- terminus) - PGEKPYKCPECGKSFS - XXXXXXX - HQRTH (carboxy terminus), COOH and

SEQ ID NO: 807: (N- terminus) - TGEKPFQCRICMRNFS - XXXXXXX - HLRTH (carboxy terminus), COOH,

wherein XXXXXXX is the zinc finger recognition domain.

20. The composition according to claim 18, wherein:

the recognition helix of (a) which specifically binds to TGC is QRRSLGH (SEQ ID NO: 663);

the recognition helix of (b) which specifically binds to ACG is KKNDLTR (aa 29-56 of SEQ ID NO: 60);

the recognition helix of (c) which specifically binds to CGT is SRRTCRA (SEQ ID NO: 155);

the recognition helix of (d) which specifically binds to GAT is VRHNLTR (SEQ ID NO: 270);

the recognition helix of (e) which specifically binds to GTC is DRTSLAR (SEQ ID NO: 64);

the recognition helix of (f) which specifically binds to AAC is DSGNLRV (SEQ ID NO: 540);

the recognition helix of (g) which specifically binds to CGC is HTGHLLE (SEQ ID NO: 151);

the recognition helix of (h) which specifically binds to GTT is TNQALGV (aa 197-224 of SEQ ID NO: 60);

the recognition helix of (j) which specifically binds to GAT is VRHNLTR (SEQ ID NO: 270);

and

the recognition helix of (k) which specifically binds to GTC is DRTSLAR (SEQ ID NO: 540).

21. The composition according to claim 1, wherein the zinc finger sequence is linked to the sequence encoding a FokI catalytic domain through a sequence encoding five amino acids GTSGK (SEQ ID NO: 805), whereby the resulting chimeric ablator cuts 6bp directly following the zinc finger binding site.

22. The composition according to any one of claims 1 to 21, wherein the transcription of the promoter is controlled by a rapamycin - regulatable system.

23. The composition according to claim 19, wherein which the pharmacological agent is rapamycin or a rapalog.

24. The composition according to any one of claims 1 to 21, wherein the ablator (b) is controlled by a cassette that is activated by a transcription factor following being dimerized by a pharmacologic agent, said cassette comprising two transcription units, wherein:

(c) one of said two transcription units encoding the DNA binding domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a first promoter; and

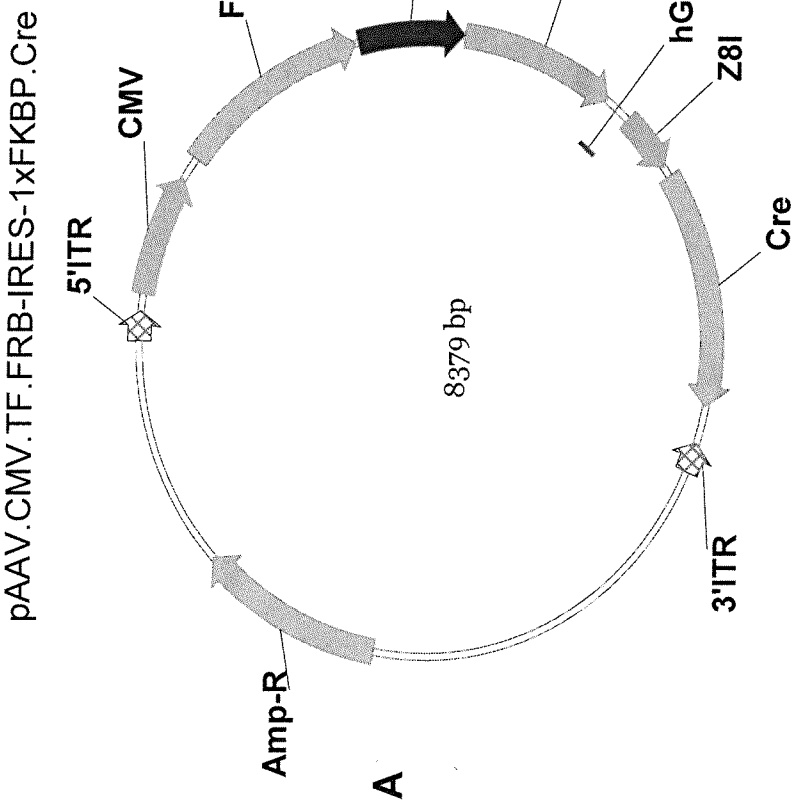
(d) a second of said two transcription units encoding the activation domain of the transcription factor fused to a binding domain for the pharmacological agent in operative association with a second promoter.

25. The composition of claim 24, wherein the first promoter of (c) and the second promoter of (d) are independently selected from a constitutive promoter and an inducible promoter.

26. The composition of claim 25, wherein the first and second promoters are both constitutive promoters and the pharmacological agent is a dimerizer that dimerizes the domains of the transcription factor.

27. The composition of claim 18, wherein one of the first promoter and the second promoters is an inducible promoter.

28. A composition according to any one of claims 1 to 27, which is useful for controlling delivery of a transgene product.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CMV
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IRES
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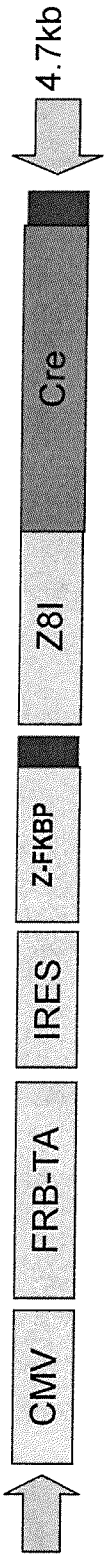
Amp-R
8379 bp

FIG. 1A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CMV
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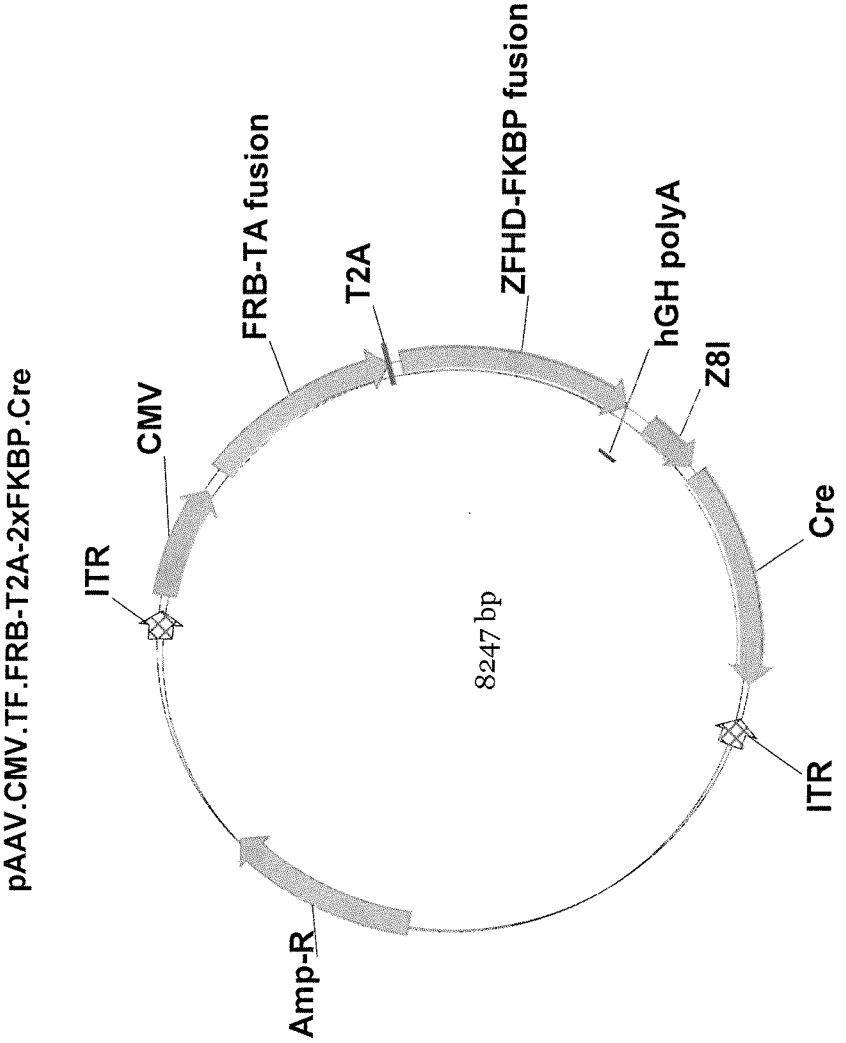
FIG. 1B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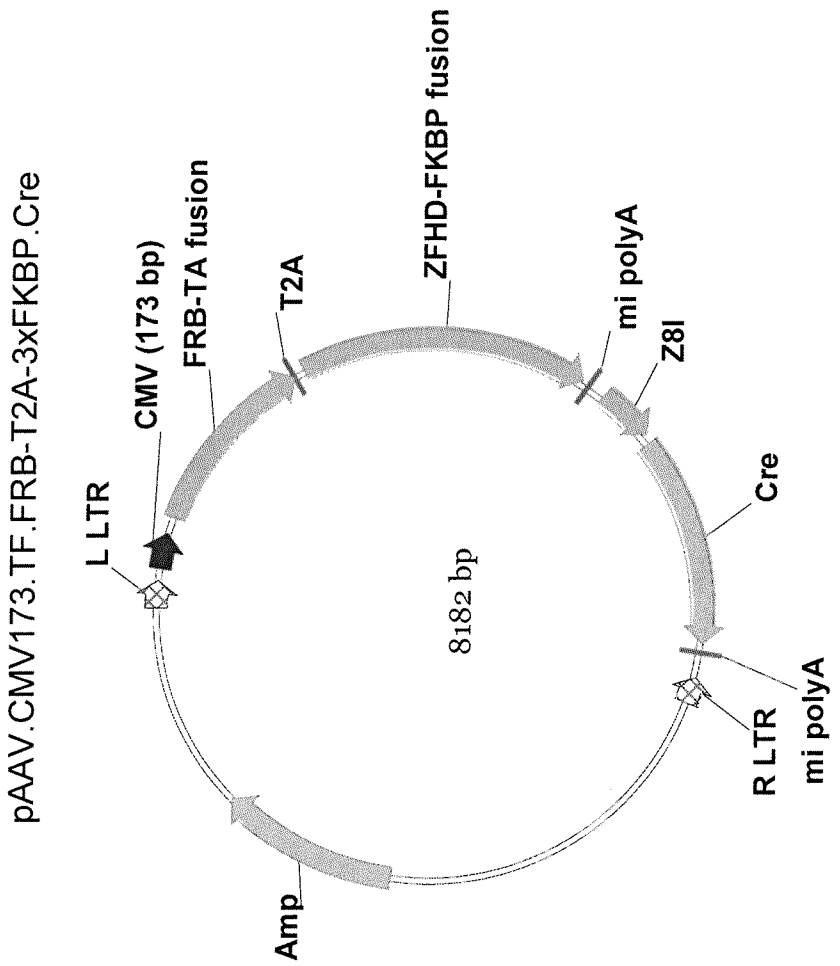
ITR
CMV
FRB-TA fusion
T2A
ZFHD-FKBP fusion
hGH polyA
Z8I
Cre
ITR
Amp-R
8247 bp

FIG. 2A

CMV
FRB-TA
Z-2XFKBP
Z8I
Cre
4.6k

FIG. 2B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CMV (173 bp)
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Z8I
Cre
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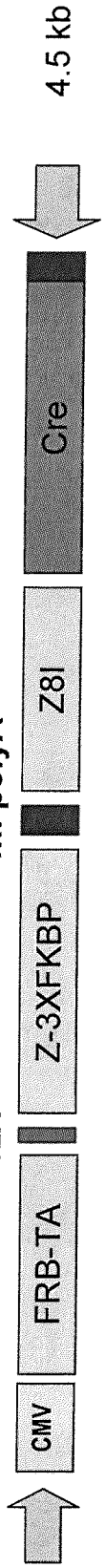
mi polyA
Amp
8182 bp

FIG. 3A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Z8I
mi polyA
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T2A
FRB-TA
CMV

FIG. 3B

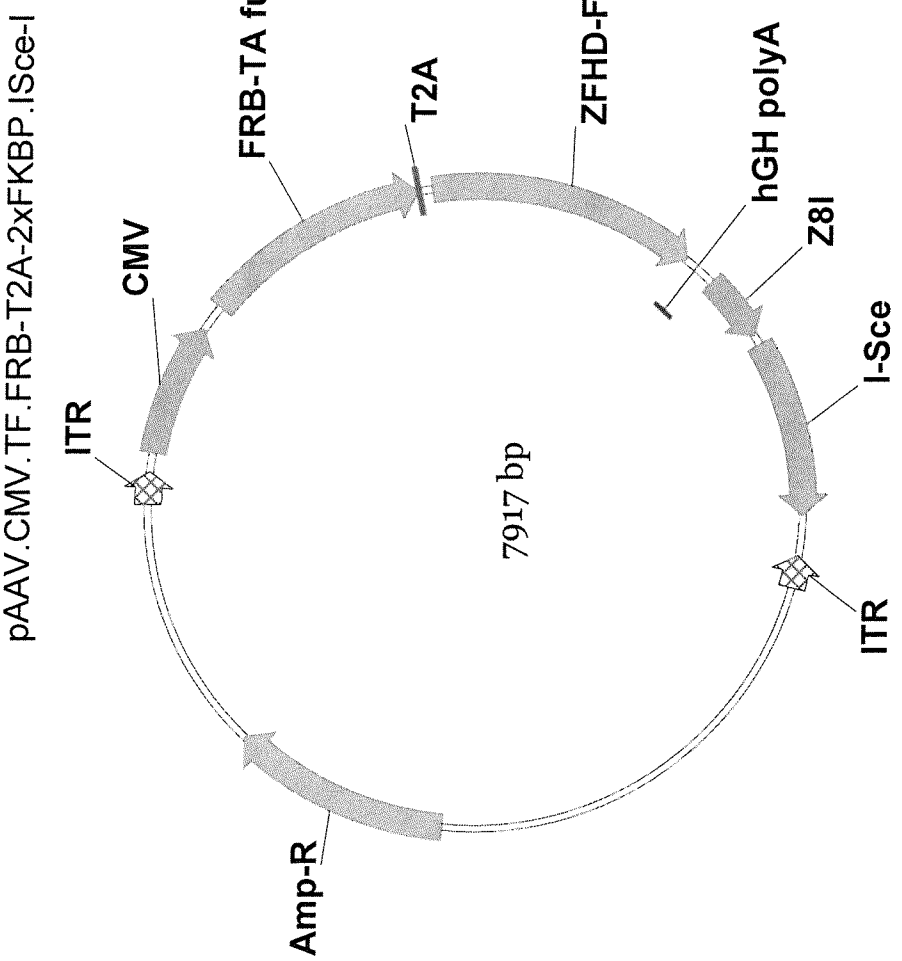

pAAV.CMV.TF.FRB-T2A-2xFKBP.ISce-I
ITR
CMV
FRB-TA fusion
T2A
ZFHD-FKBP fusion
hGH polyA
Z8I
I-Sce
ITR
Amp-R
7917 bp

FIG. 4A

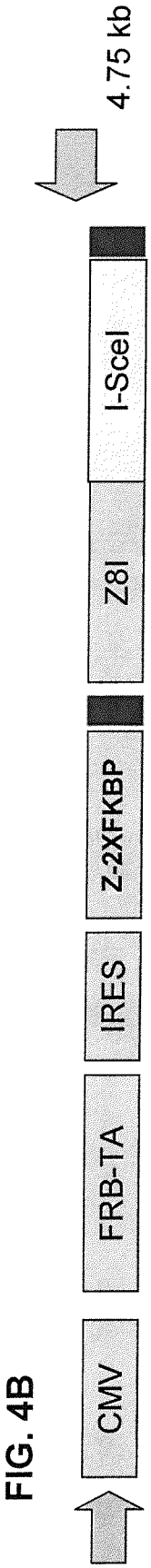

4.75 kb
CMV
FRB-TA
IRES
Z-2XFKBP
Z8I
I-SceI

FIG. 4B

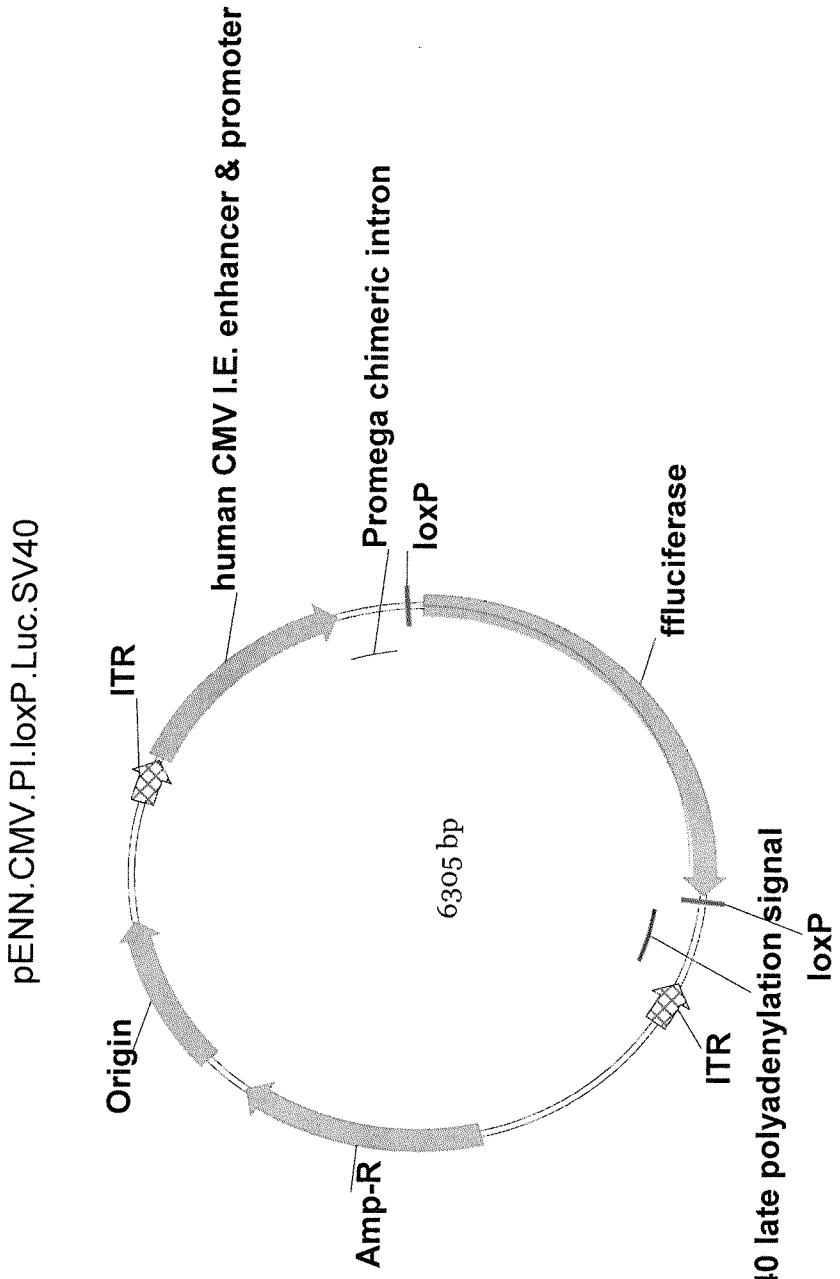

pENN.CMV.PI.loxP.Luc.SV40
human CMV I.E. enhancer & promoter
Promega chimeric intron
loxP
ffluciferase
ITR
6305 bp
Origin
Amp-R
ITR
SV40 late polyadenylation signal
loxP

FIG. 5A


3.5 kb
Poly A
Luciferase
loxP site
CMV

FIG. 5B

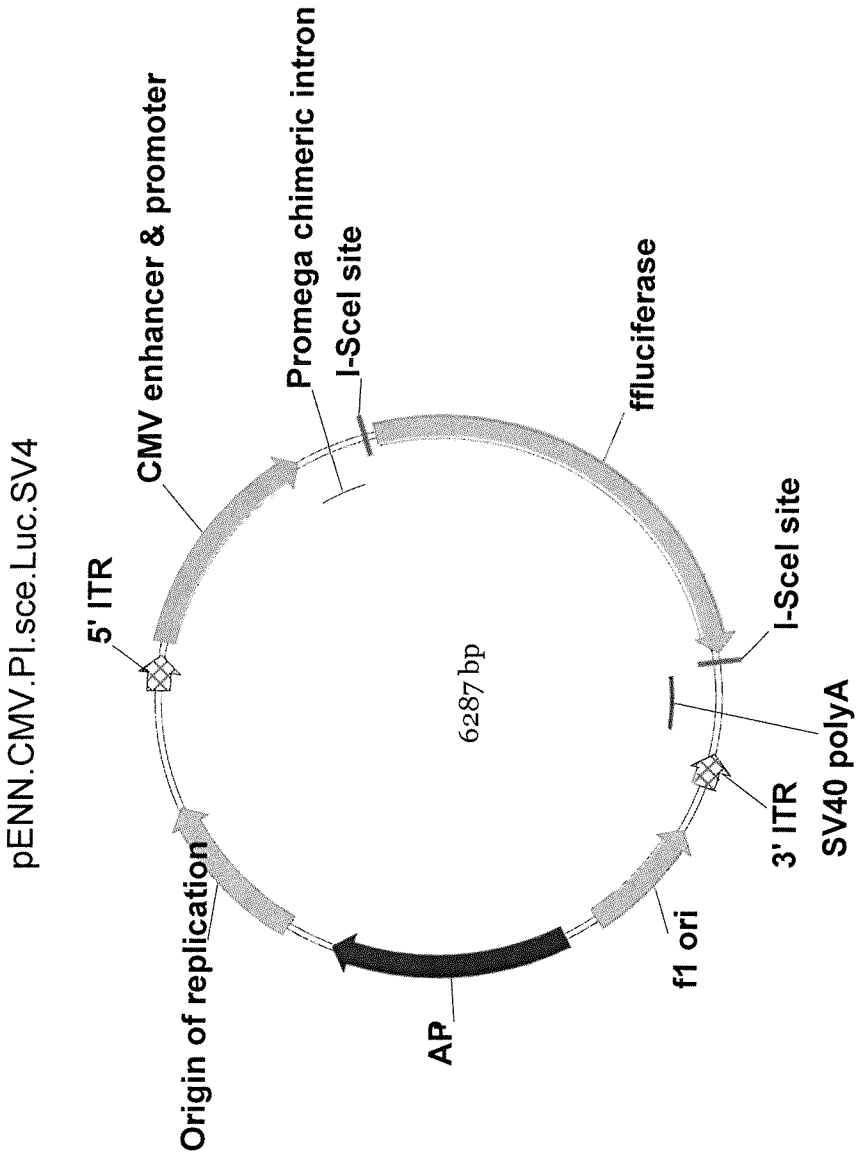

pENN.CMV.PI.sce.Luc.SV4
5' ITR
CMV enhancer & promoter
Promega chimeric intron
I-SceI site
ffluciferase
I-SceI site
SV40 polyA
3' ITR
f1 ori
AP
Origin of replication
6287 bp

FIG. 6A

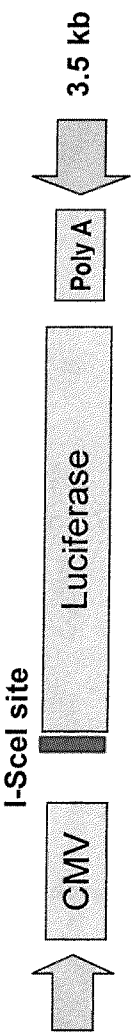

3.5 kb
Poly A
Luciferase
I-SceI site
CMV

FIG. 6B

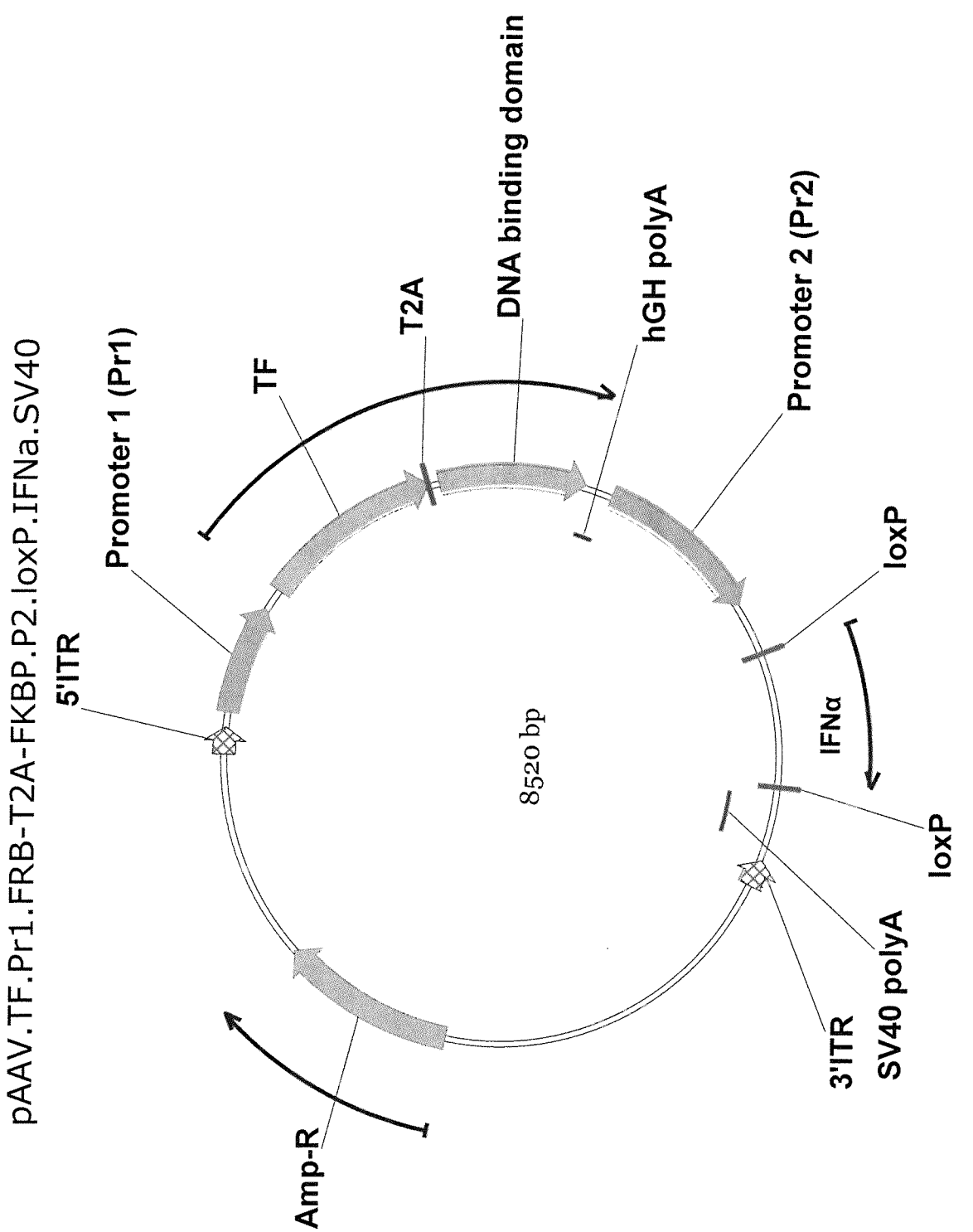

pAAV.TF.Pr1.FRB-T2A-FKBP.P2.loxP.IFNa.SV40
5'ITR
Promoter 1 (Pr1)
TF
T2A
DNA binding domain
hGH polyA
Promoter 2 (Pr2)
loxP
IFNα
loxP
3'ITR
SV40 polyA
Amp-R
8520 bp

FIG. 7

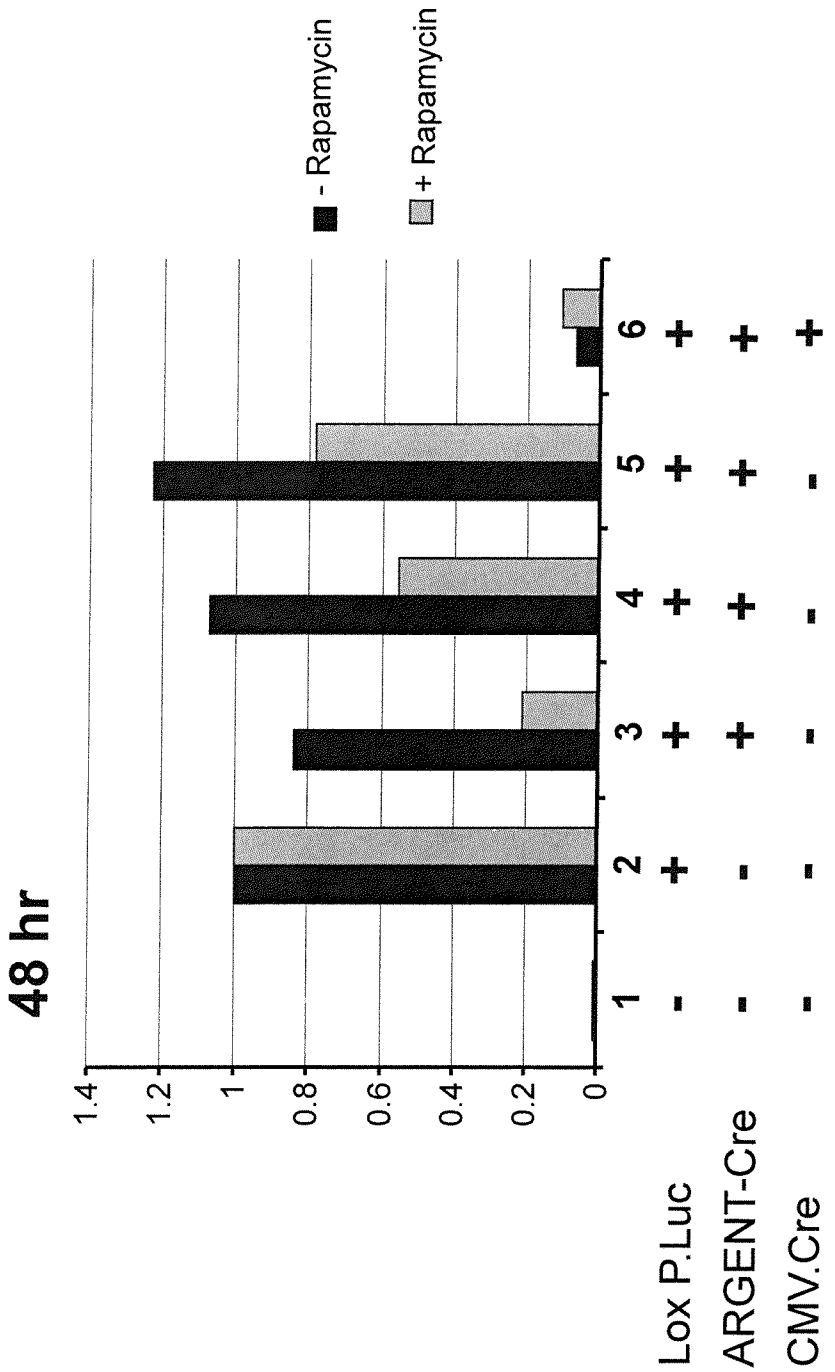

48 hr
- Rapamycin
+ Rapamycin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6
Lox P.Luc - + + + + +
ARGENT-Cre - - + + + +
CMV.Cre - - - - - +

FIG. 8A

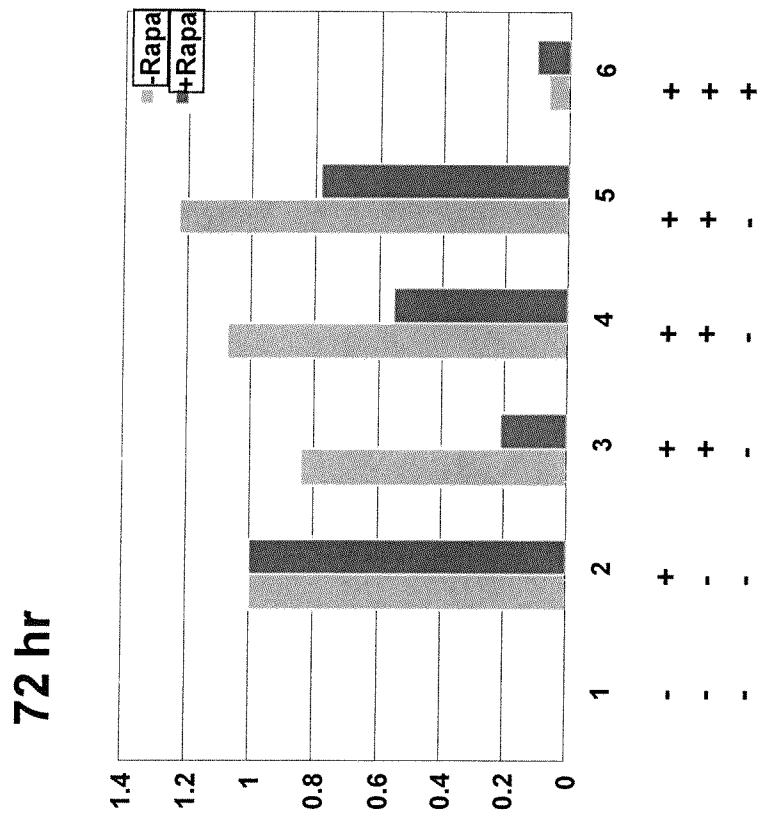


FIG. 8B

FIG. 9A

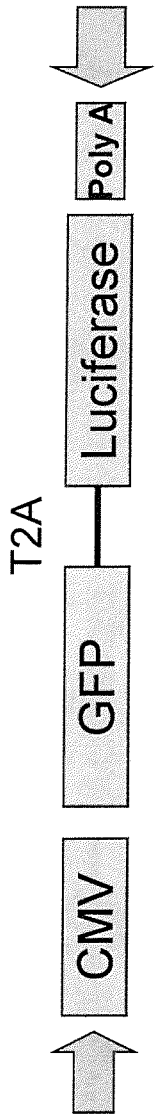
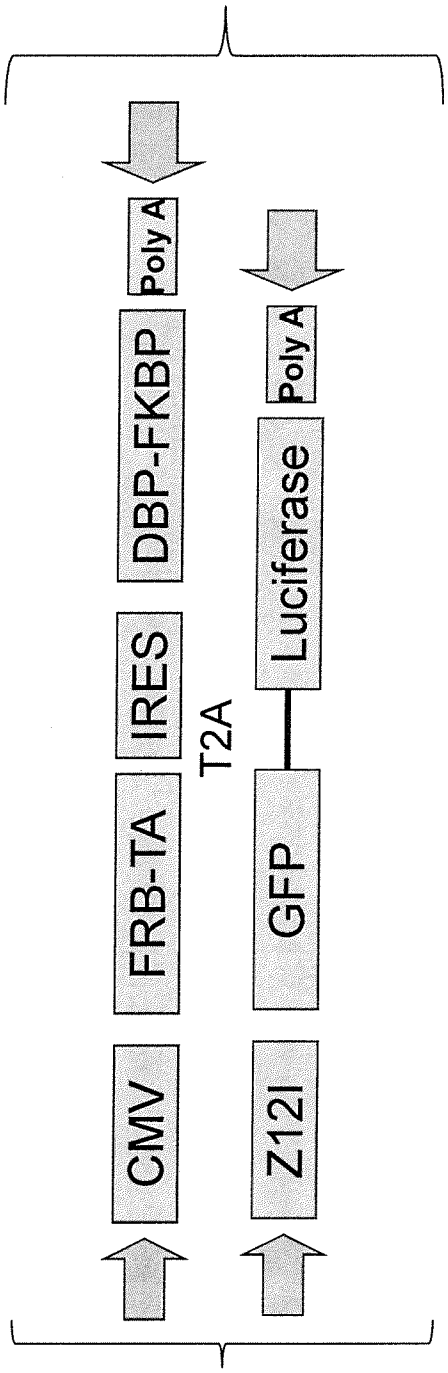

CMV
GFP
T2A
Luciferase
Poly A

FIG 9B


CMV
FRB-TA
IRES
DBP-FKBP
Poly A
Z12I
GFP
T2A
Luciferase
Poly A

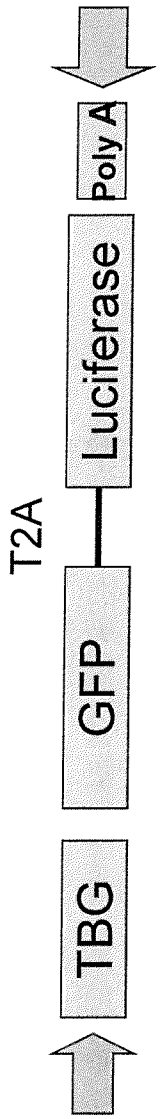

TBG
GFP
T2A
Luciferase
Poly A

FIG. 9C

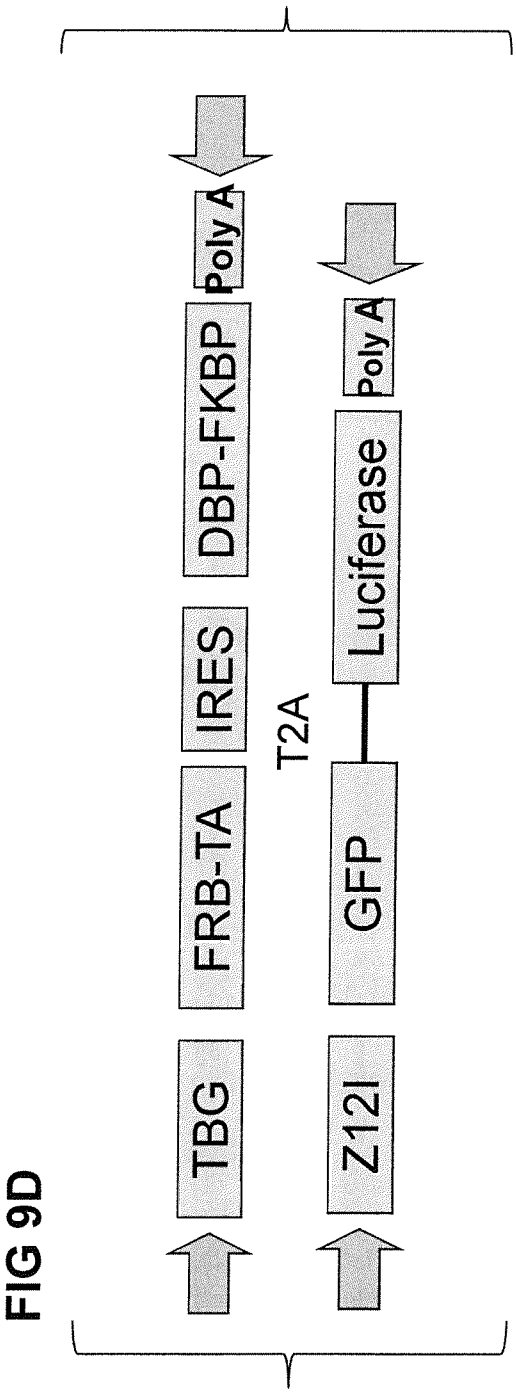

TBG
FRB-TA
IRES
DBP-FKBP
Poly A
Z12I
GFP
T2A
Luciferase
Poly A

FIG 9D

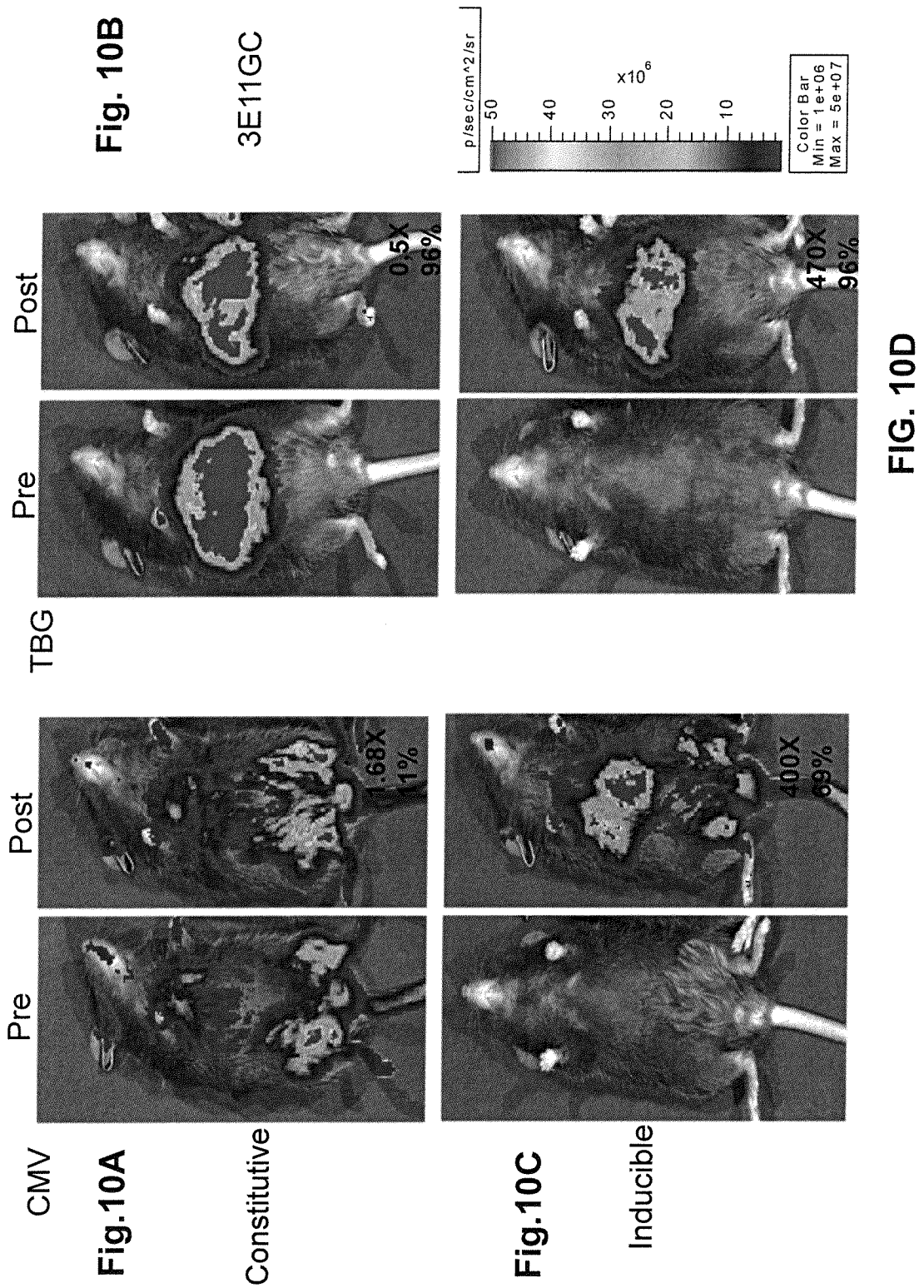


Fig. 10B
3E11GC
p/sec/cm^2/sr
50
40
30
20
10
x10^6
Color Bar
Min = 1e+06
Max = 5e+07
TBG
Pre
Post
0.5X
96%
470X
96%
CMV
Fig.10A
Constitutive
1.68X
1%
Fig.10C
Inducible
400X
99%

FIG. 10D

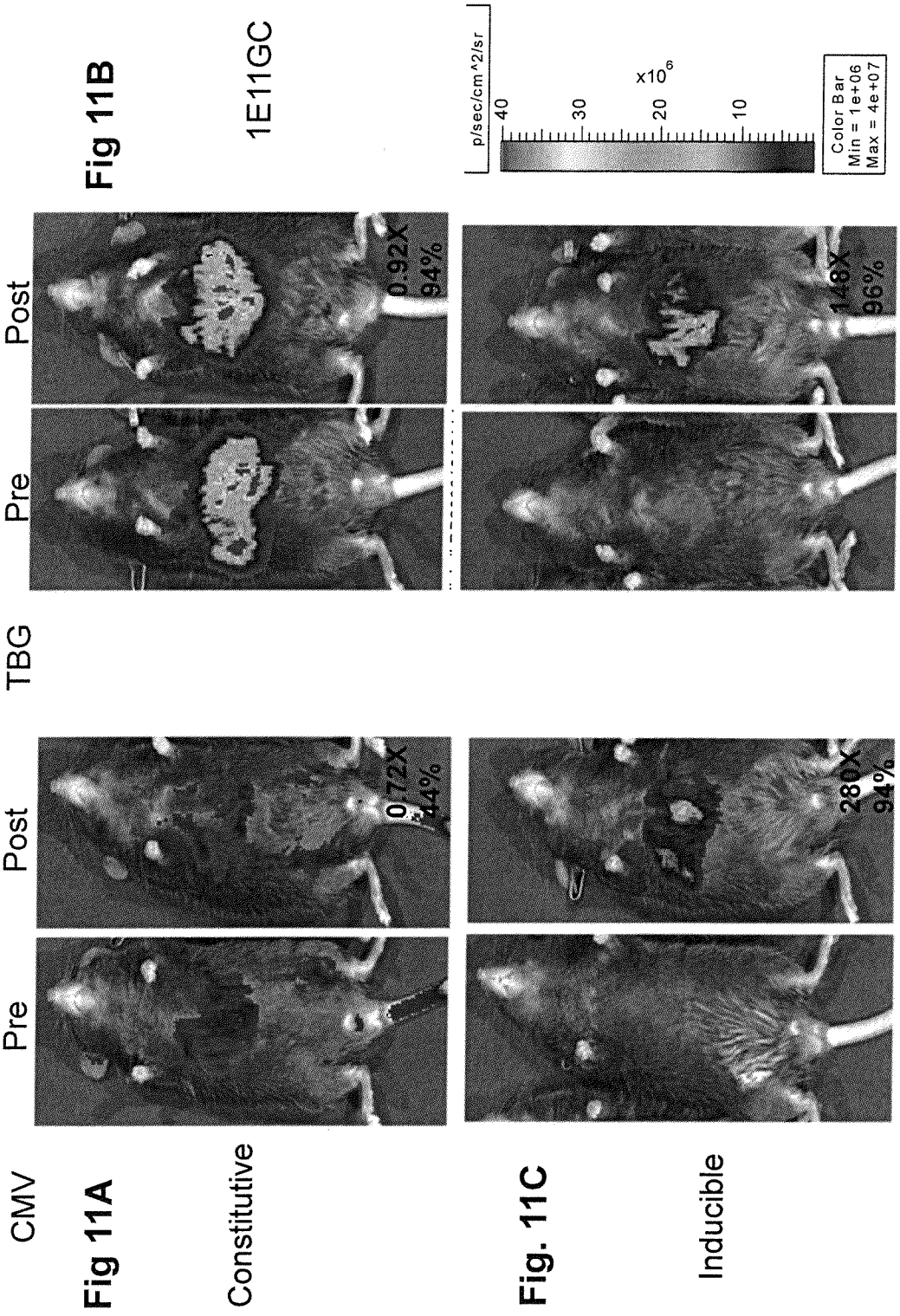

CMV
Fig 11A
Constitutive
Pre
Post
0.72X
44%
TBG
Fig 11B
1E11GC
Pre
Post
0.92X
94%
Fig. 11C
Inducible
280X
94%
p/sec/cm^2/sr
40
30
20
10
x10^6
Color Bar
Min = 1e+06
Max = 4e+07
148X
96%

FIG. 11D

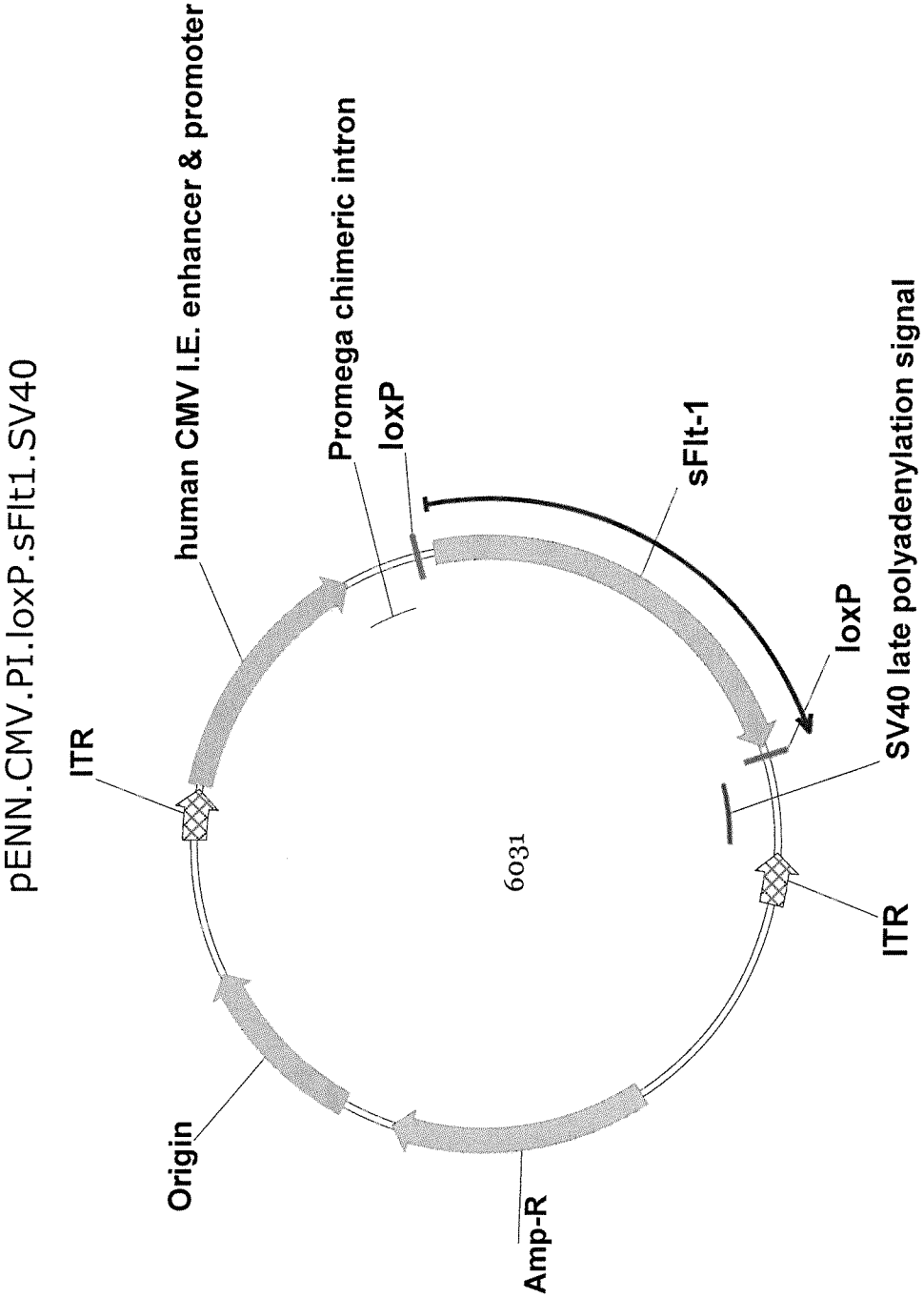

pENN.CMV.PI.loxP.sFlt1.SV40
human CMV I.E. enhancer & promoter
Promega chimeric intron
loxP
sFlt-1
loxP
SV40 late polyadenylation signal
ITR
ITR
Origin
Amp-R
6031

FIG. 12A

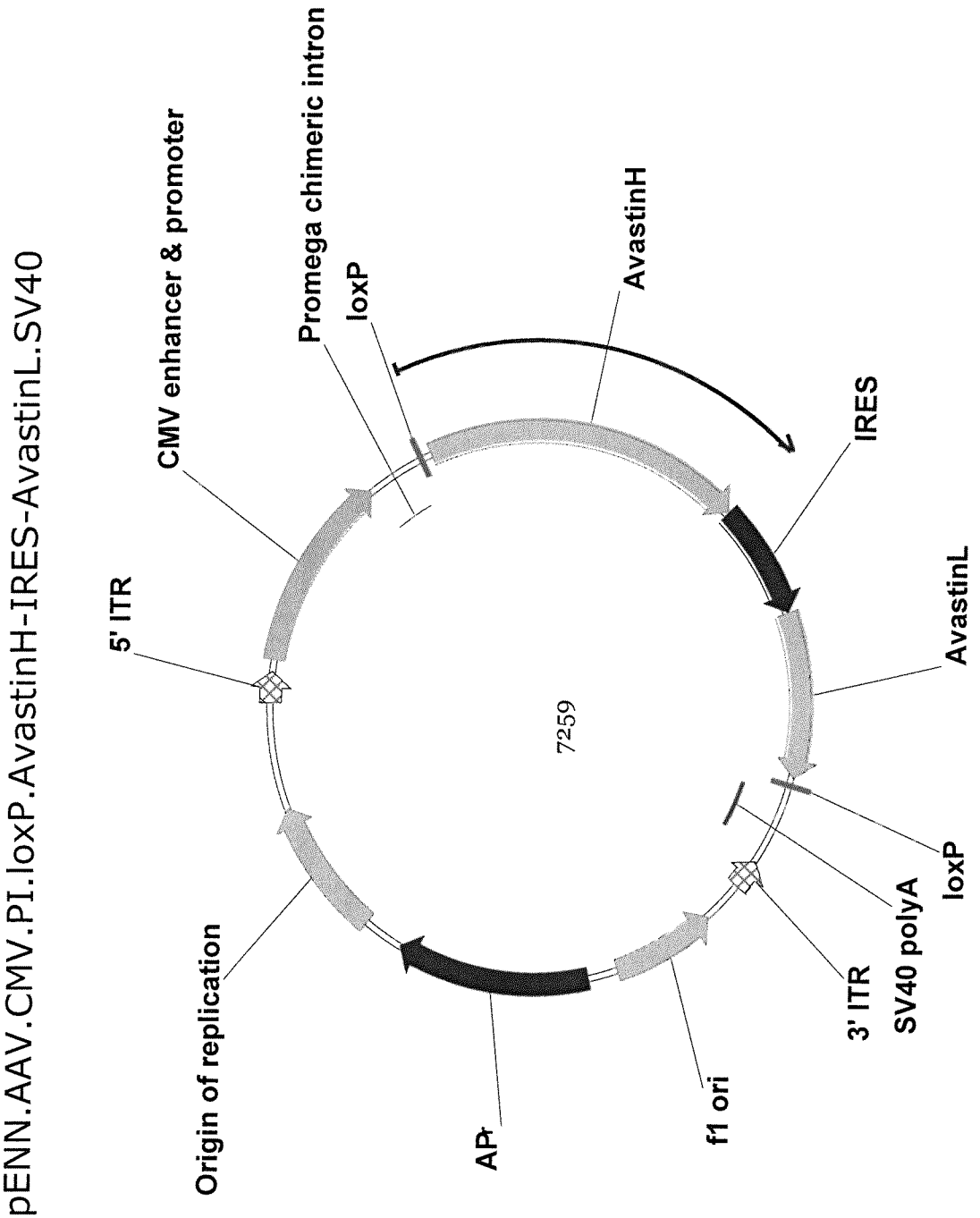

pENN.AAV.CMV.PI.loxP.AvastinH-IRES-AvastinL.SV40
Promega chimeric intron
loxP
AvastinH
CMV enhancer & promoter
IRES
5' ITR
AvastinL
7259
loxP
SV40 polyA
3' ITR
Origin of replication
f1 ori
AP

FIG. 12B

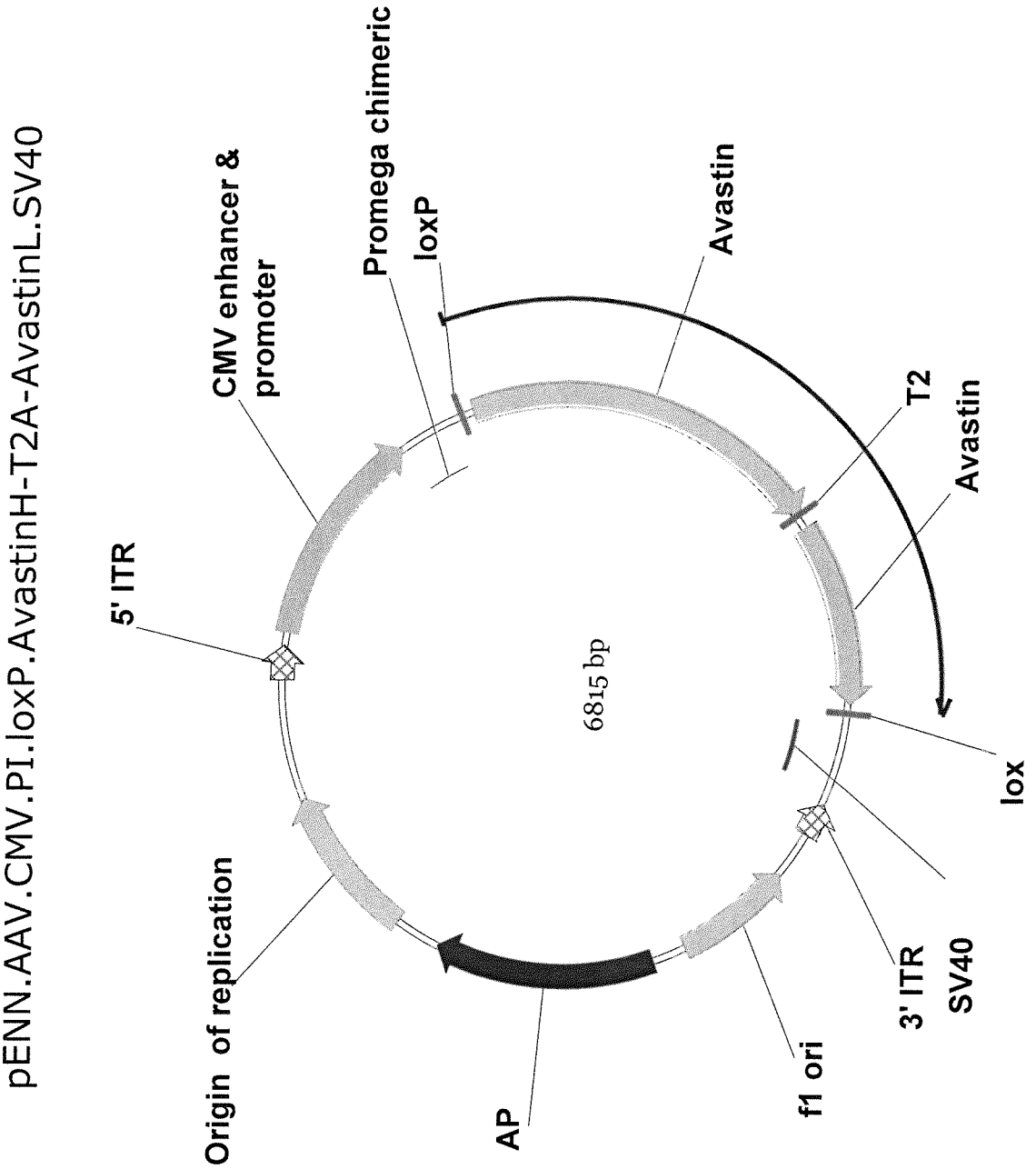

pENN.AAV.CMV.PI.loxP.AvastinH-T2A-AvastinL.SV40
6815 bp
5' ITR
CMV enhancer & promoter
Promega chimeric
loxP
Avastin
T2
Avastin
lox
3' ITR
SV40
f1 ori
AP
Origin of replication

FIG. 12C

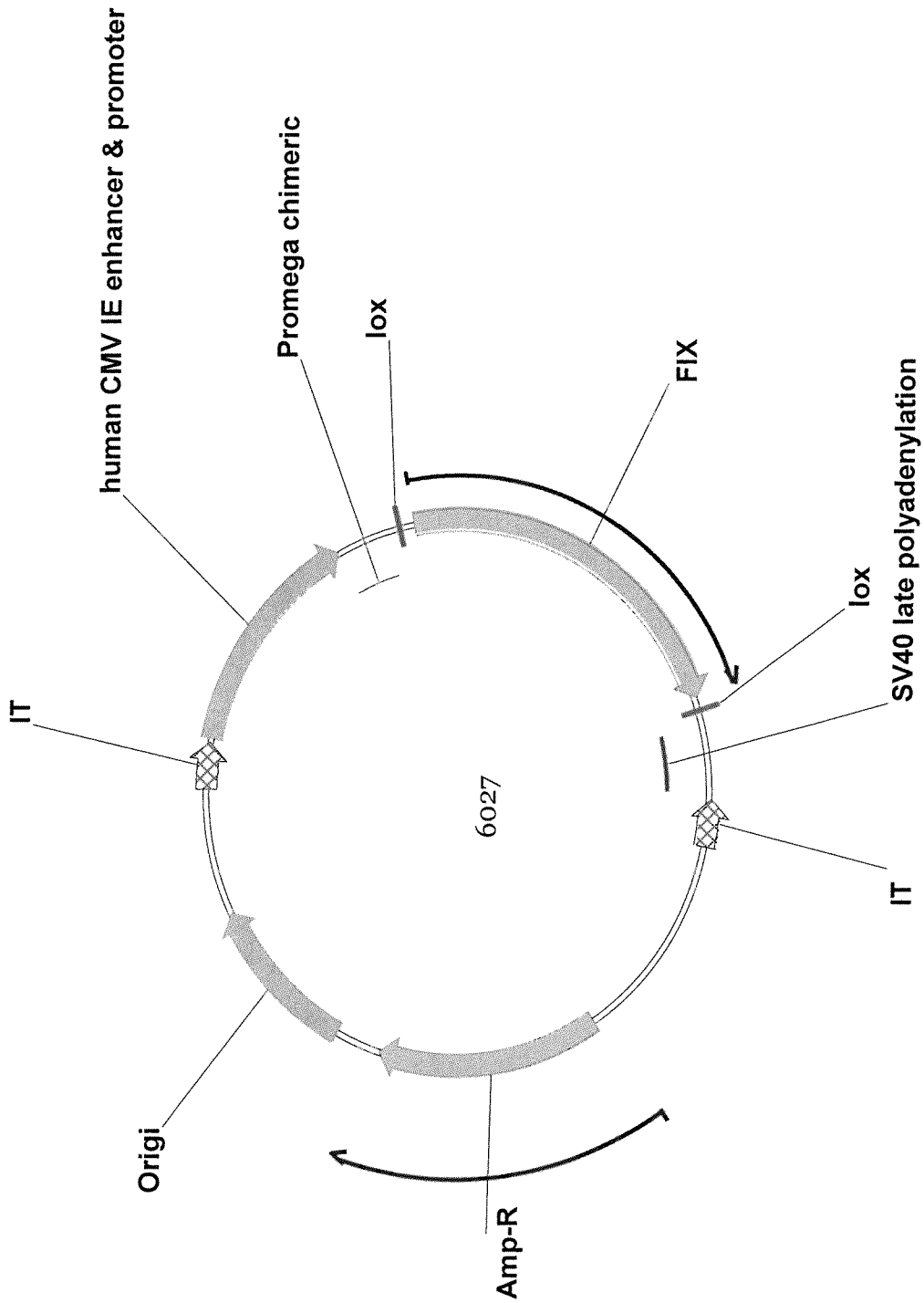

pENN.CMV.PI.loxP.FIX.SV40
human CMV IE enhancer & promoter
Promega chimeric
lox
FIX
lox
SV40 late polyadenylation
IT
IT
6027
Origi
Amp-R

FIG. 13A

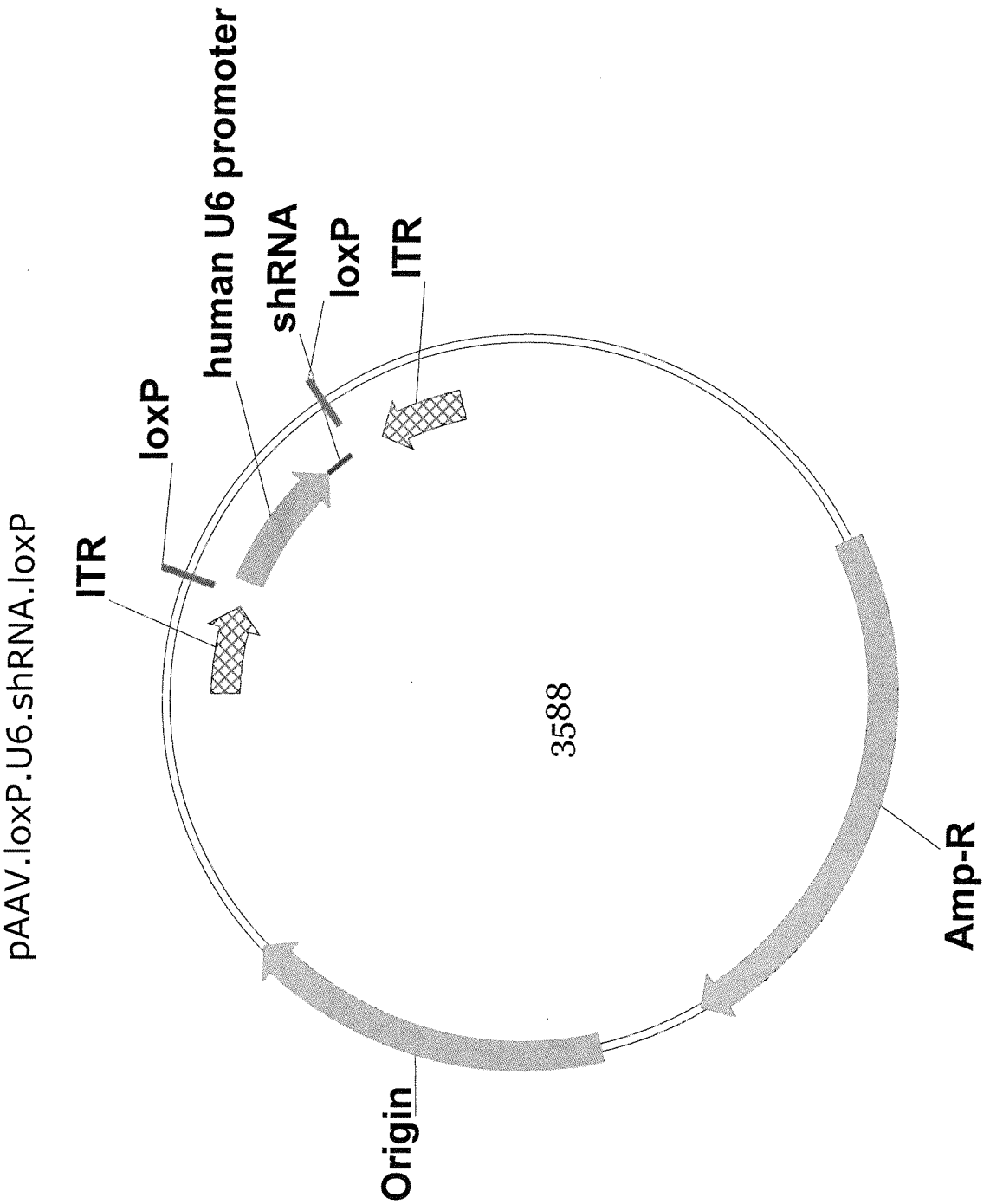

pAAV.loxP.U6.shRNA.loxP
ITR
loxP
human U6 promoter
shRNA
loxP
ITR
3588
Origin
Amp-R

FIG. 13B

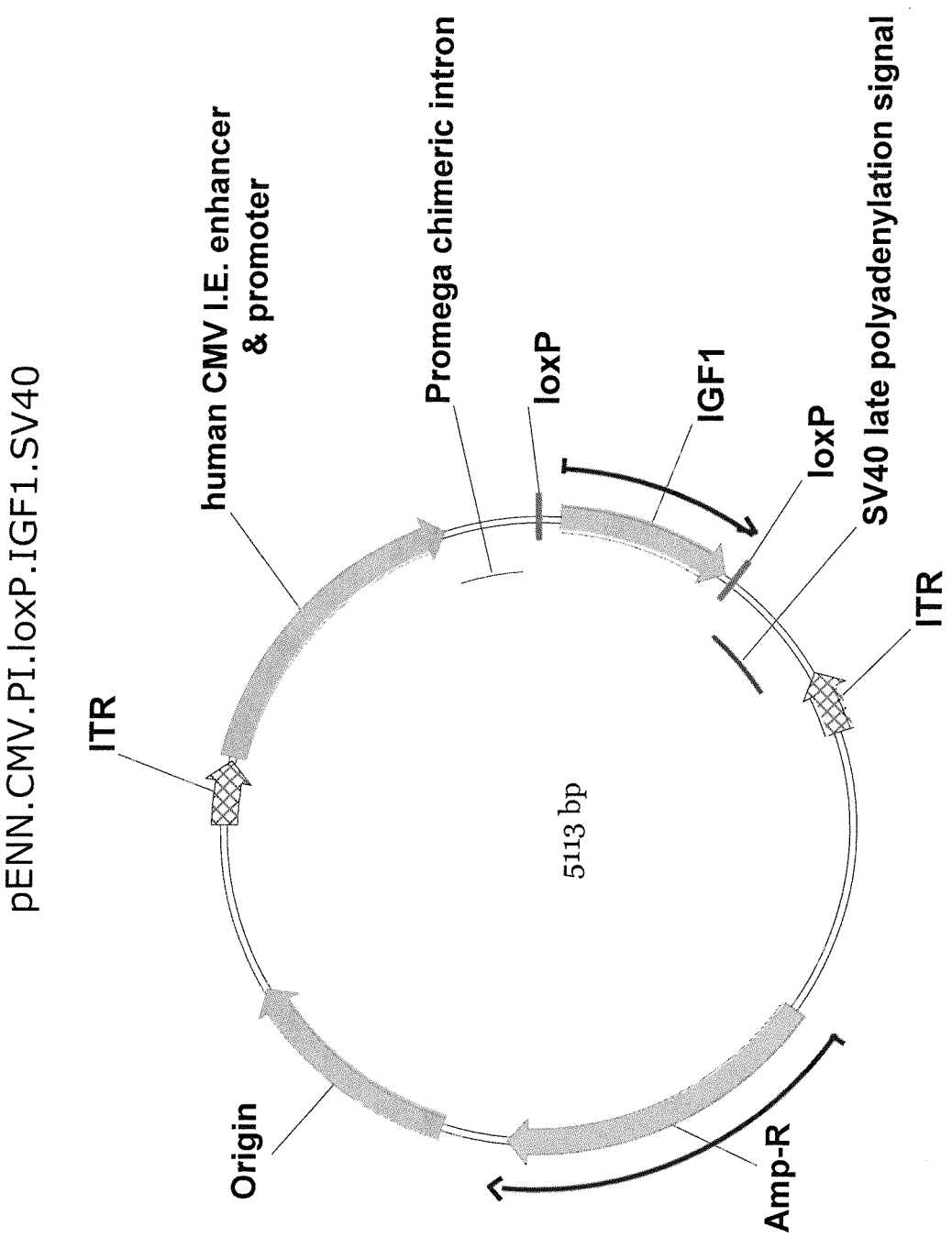

pENN.CMV.PI.loxP.IGF1.SV40
ITR
human CMV I.E. enhancer & promoter
Promega chimeric intron
loxP
IGF1
loxP
SV40 late polyadenylation signal
ITR
5113 bp
Origin
Amp-R

FIG. 14A

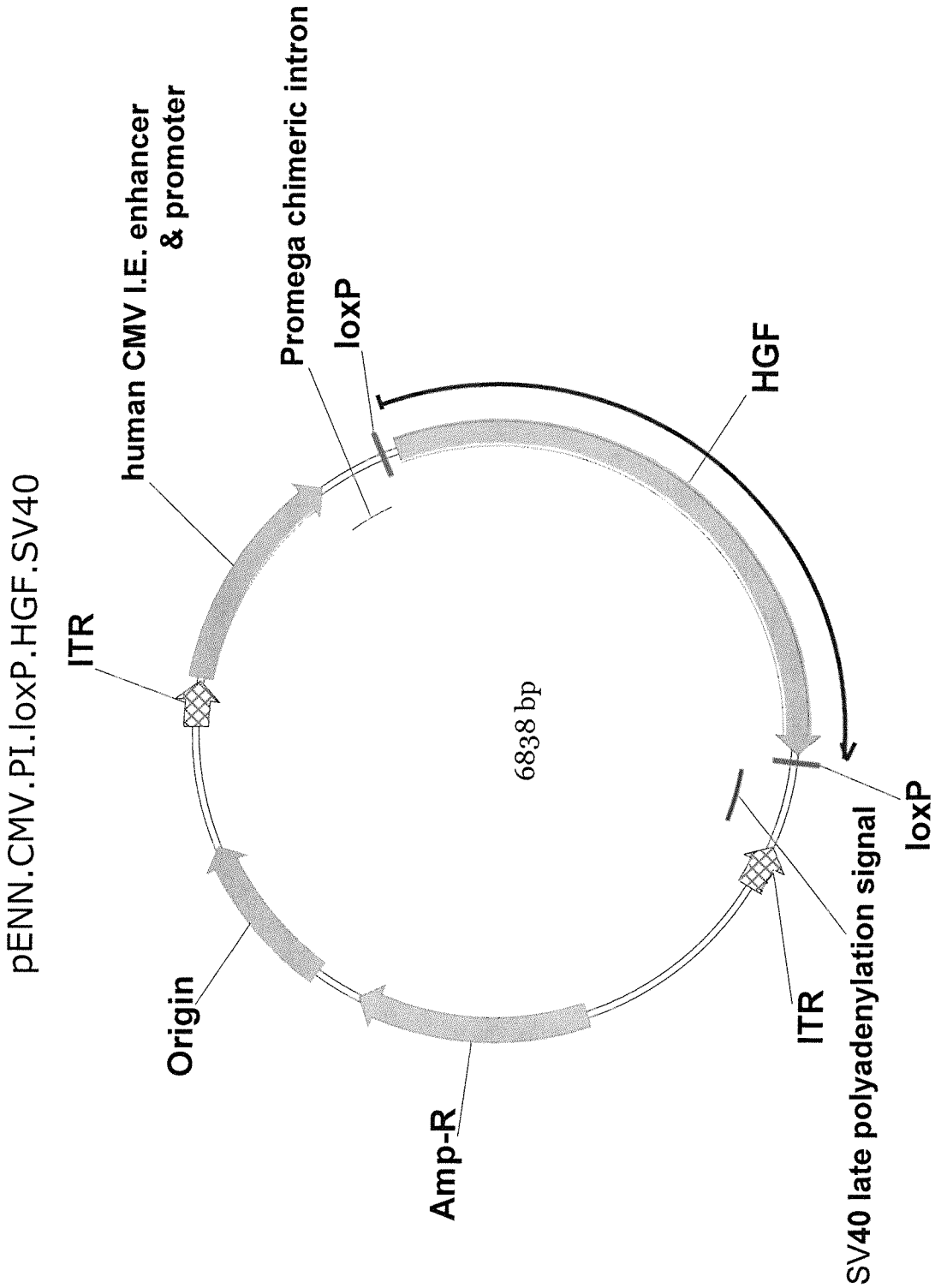

pENN.CMV.PI.loxP.HGF.SV40
human CMV I.E. enhancer & promoter
Promega chimeric intron
loxP
HGF
ITR
6838 bp
Origin
Amp-R
ITR
SV40 late polyadenylation signal
loxP

FIG. 14B

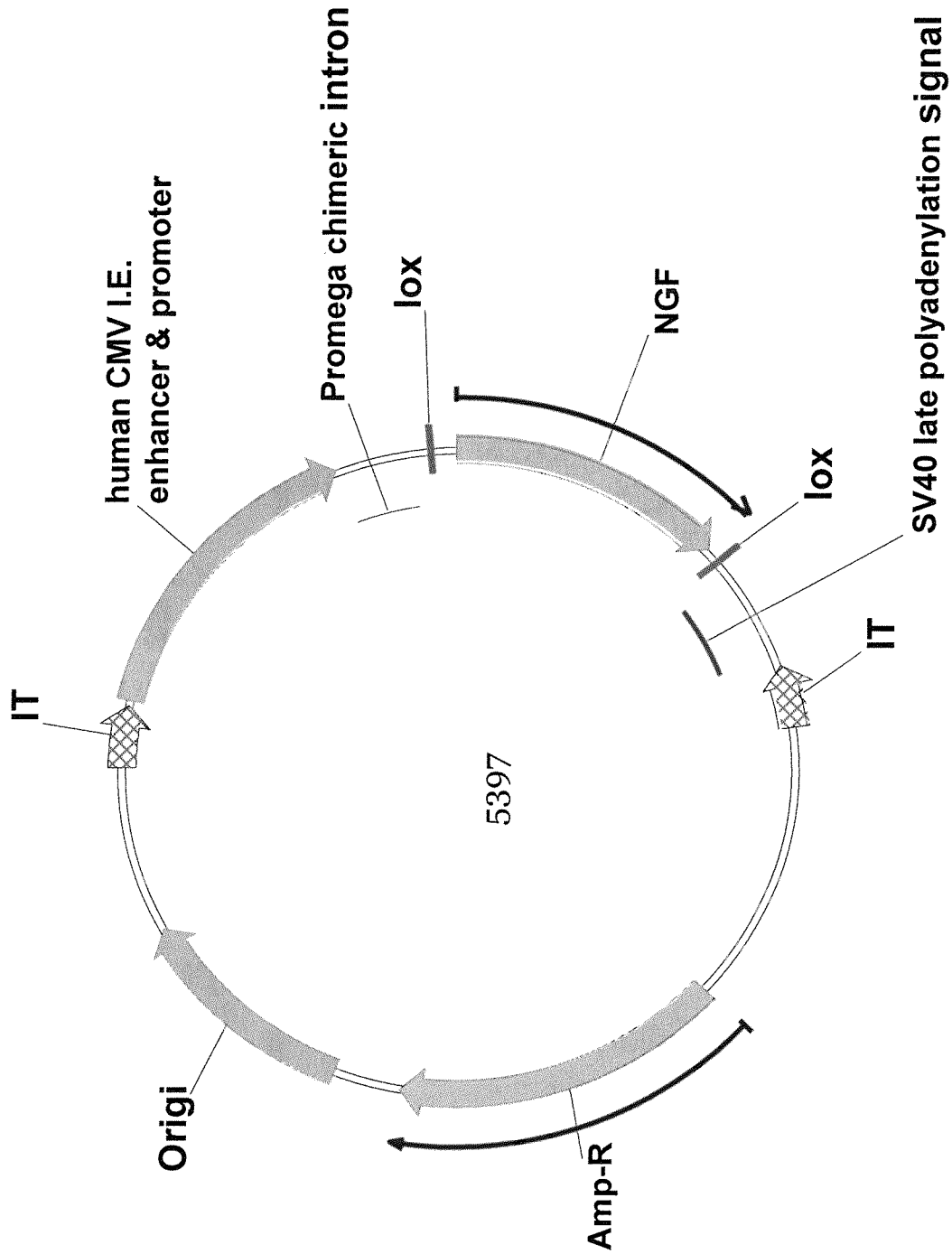

pENN.CMV.PI.loxP.NGF.SV40
IT
human CMV I.E.
enhancer & promoter
Promega chimeric intron
lox
NGF
lox
SV40 late polyadenylation signal
IT
5397
Origi
Amp-R

FIG. 15

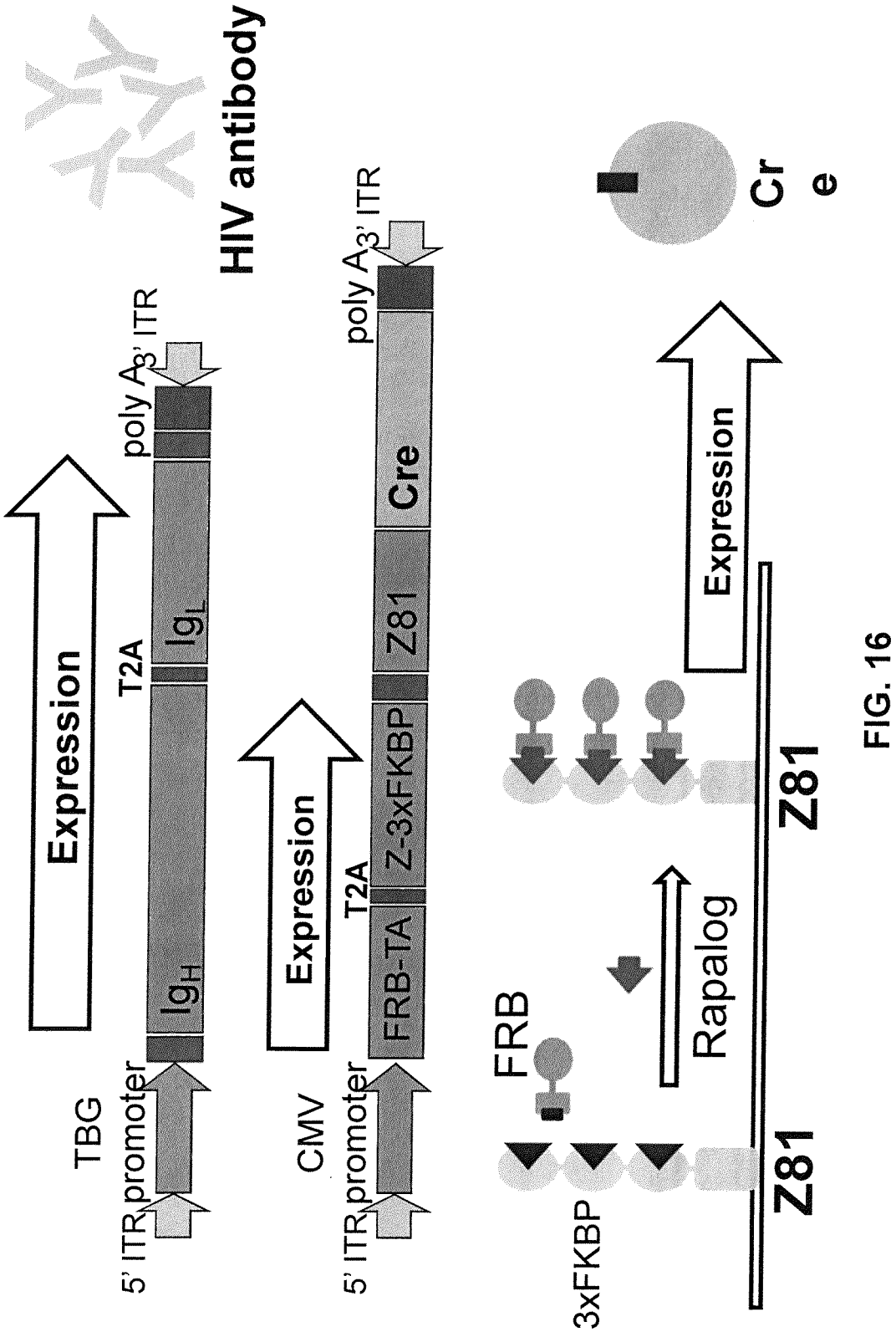

HIV antibody
Expression
TBG
promoter
5' ITR
IgH
T2A
IgL
poly A
3' ITR
Expression
CMV
promoter
5' ITR
FRB-TA
T2A
Z-3xFKBP
Z81
Cre
poly A
3' ITR
Cre
Expression
FRB
Rapalog
3xFKBP
Z81
Z81

FIG. 16

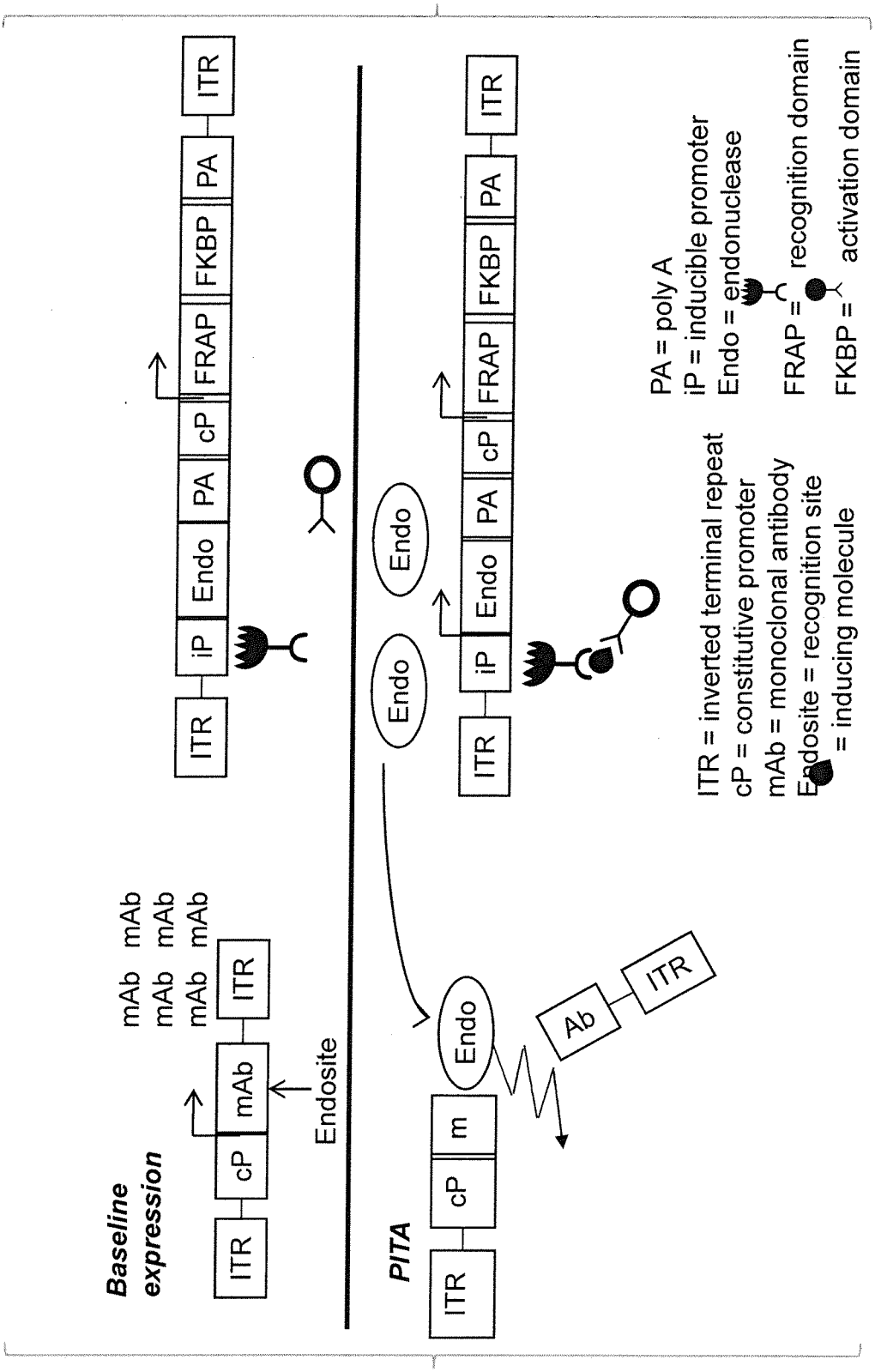

Baseline expression
mAb mAb
mAb mAb
mAb mAb
ITR
mAb
cP
ITR
Endosite
ITR
iP
Endo
PA
cP
FRAP
FKBP
PA
ITR
PITA
ITR
cP
m
Endo
Ab
ITR
Endo
Endo
ITR
iP
Endo
PA
cP
FRAP
FKBP
PA
ITR
ITR = inverted terminal repeat
cP = constitutive promoter
mAb = monoclonal antibody
Endosite = recognition site
= inducing molecule
PA = poly A
iP = inducible promoter
Endo = endonuclease
FRAP = recognition domain
FKBP = activation domain

FIG. 17

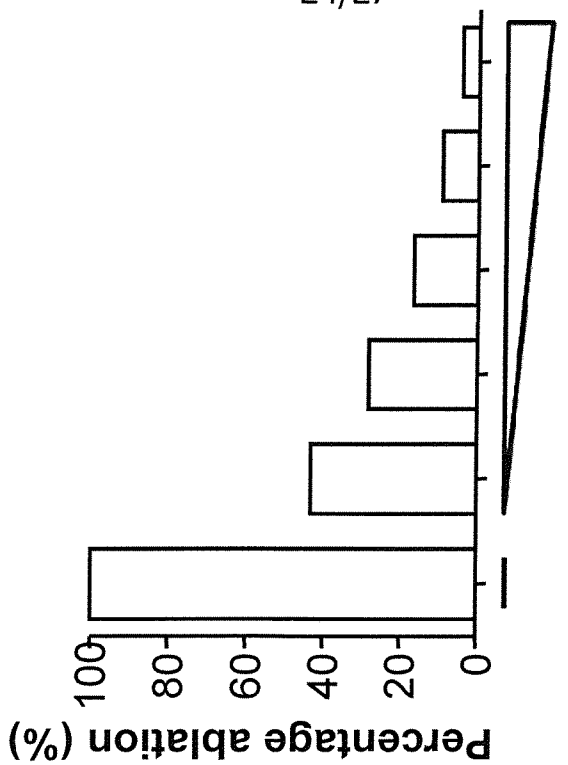


Fig. 18B

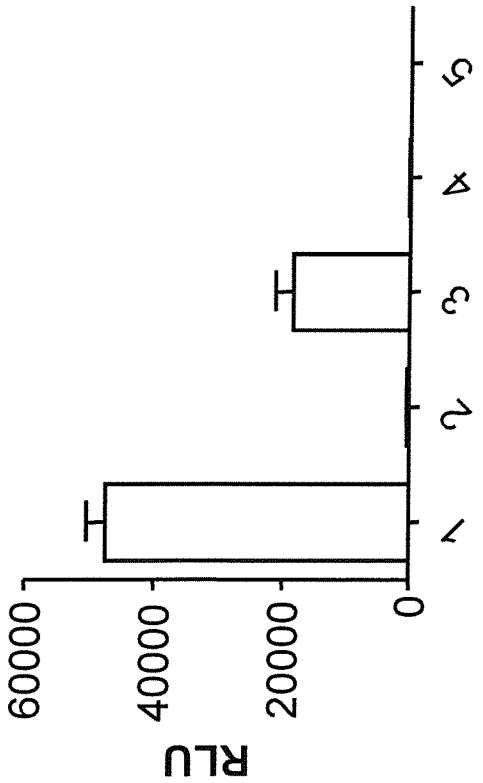


Fig. 18A

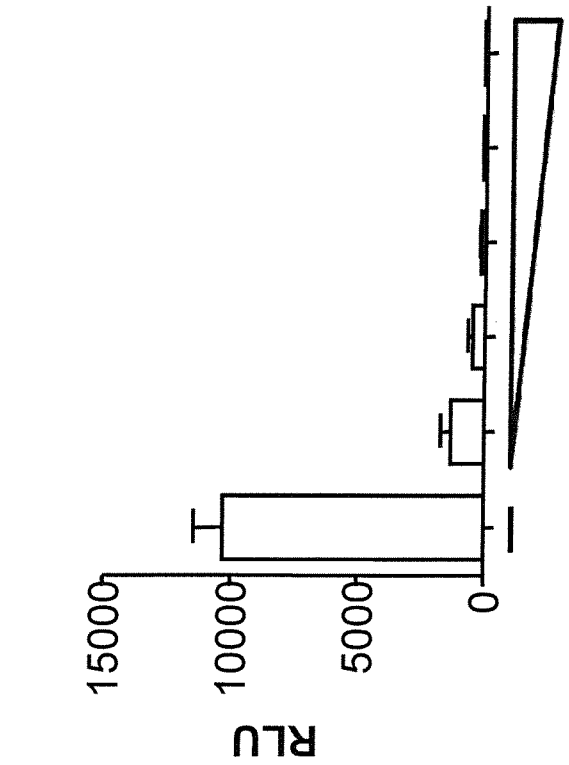


Fig. 19B

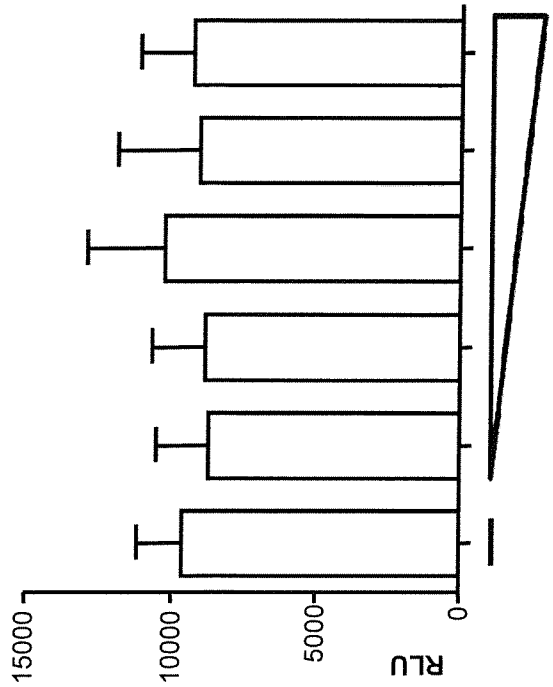


Fig. 19A

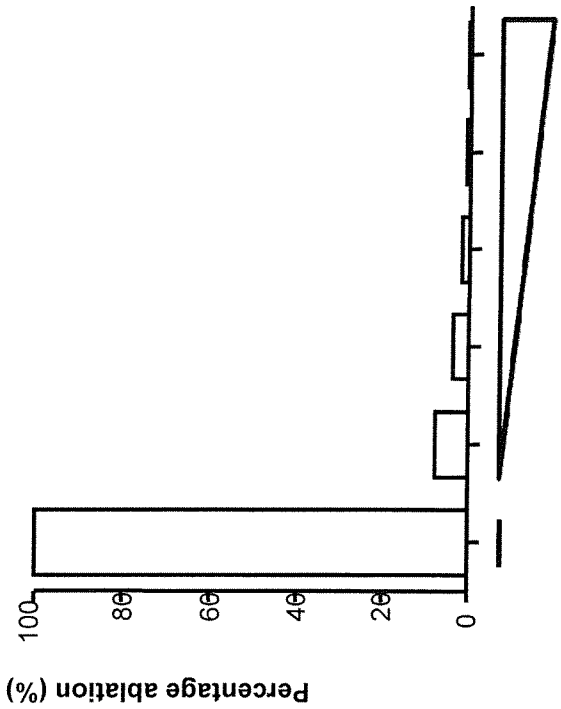


Fig. 20B

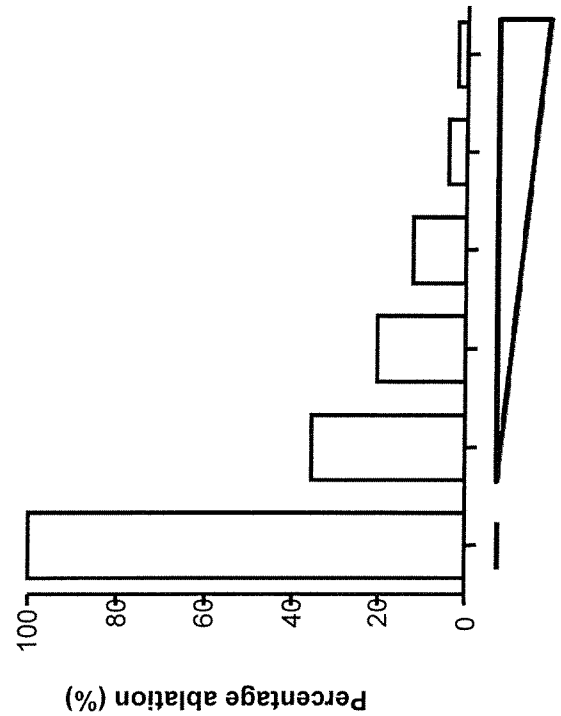


Fig. 20A

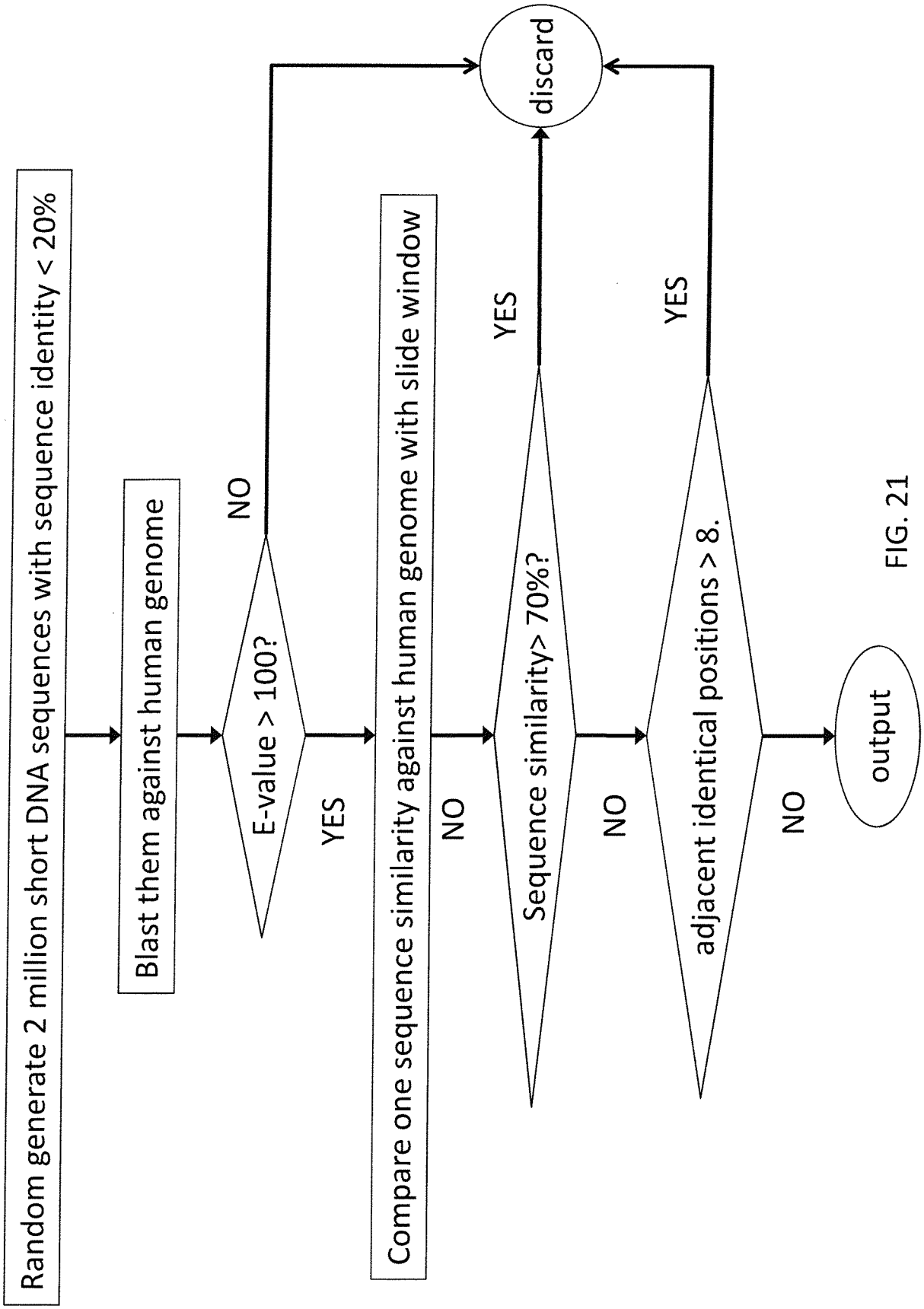

Random generate 2 million short DNA sequences with sequence identity < 20%
Blast them against human genome
E-value > 100?
NO
YES
discard
Compare one sequence similarity against human genome with slide window
Sequence similarity> 70%?
YES
NO
adjacent identical positions > 8.
YES
NO
output

FIG. 21

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/057803

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/86 A61K48/00
ADD.

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)
C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/126798 A2 (CELL GENESYS INC [US]; ARIAD PHARMA INC [US]; HARDING THOMAS C [US]; F) 8 November 2007 (2007-11-08) the whole document	1-29
A	JULIE JOHNSTON ET AL: "Regulated expression of erythropoietin from an AAV vector safely improves the anemia of beta-thalassemia in a mouse model.", MOLECULAR THERAPY, vol. 7, no. 4, 1 April 2003 (2003-04-01), pages 493-497, XP55047164, ISSN: 1525-0016 the whole document	1-29

-/--

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

[X] 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 another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2012	07/01/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Wimmer, Georg

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/057803

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/41566 A1 (SANAGAMO BIOSCIENCES INC [US]; COX GEORGE NORBERT III [US]; CASE CASEY) 20 July 2000 (2000-07-20) the whole document -----	1-29
A	TING LI ET AL: "TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain", NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 39, no. 1, 1 January 2011 (2011-01-01), pages 359-372, XP002632807, ISSN: 0305-1048, DOI: 10.1093/NAR/GKQ704 [retrieved on 2010-08-10] the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/057803

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007126798 A2	08-11-2007	CA 2648064 A1	08-11-2007
		EP 2001517 A2	17-12-2008
		JP 2009532025 A	10-09-2009
		US 2007292922 A1	20-12-2007
		US 2010151523 A1	17-06-2010
		WO 2007126798 A2	08-11-2007
WO 0041566 A1	20-07-2000	AT 304792 T	15-10-2005
		AU 745844 B2	11-04-2002
		AU 2847000 A	01-08-2000
		CA 2323086 A1	20-07-2000
		DE 60022705 D1	27-10-2005
		DE 60022705 T2	14-06-2006
		DK 1061805 T3	09-01-2006
		EP 1061805 A1	27-12-2000
		ES 2250103 T3	16-04-2006
		GB 2348424 A	04-10-2000
		JP 2001231583 A	28-08-2001
		JP 2002534104 A	15-10-2002
		JP 2011207893 A	20-10-2011
		US 6534261 B1	18-03-2003
		US 6607882 B1	19-08-2003
		US 6824978 B1	30-11-2004
		US 2002160940 A1	31-10-2002
		US 2003087817 A1	08-05-2003
		US 2004204345 A1	14-10-2004
		US 2005215502 A1	29-09-2005
		US 2006078878 A1	13-04-2006
		US 2006276427 A1	07-12-2006
		US 2006281704 A1	14-12-2006
		US 2010261271 A1	14-10-2010
		US 2010279406 A1	04-11-2010
		US 2011247087 A1	06-10-2011
		US 2012294838 A1	22-11-2012
		WO 0041566 A1	20-07-2000