ADENO-ASSOCIATED VIRUS (AAV) PRODUCER CELL LINE AND RELATED METHODS

FIELD OF THE INVENTION

[0001] The present disclosure relates to mammalian cell lines for producing adeno-associated virus (AAV). The cells suitably include nucleic acids encoding helper genes and AAV genes, under the control of derepressible promoters. The disclosure also relates to isolated nucleic acid molecules that encode such genes, as well as methods of using the mammalian cells for producing AAVs.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 17, 2019, is named 0132-0049WO1_SL.txt and is 364,567 bytes in size.

BACKGROUND OF THE INVENTION

[0003] The safety profile and long-term expression capacity make adeno-associated virus (AAV) an excellent viral vector for gene therapy in humans. The wildtype AAV genome is composed of a 4.7 kb single-stranded DNA that includes regulatory genes for replication (Rep) and structural genes for Capsid (Cap), flanked by inverted terminal repeats (ITR) for virus replication and packaging. As a dependent virus, AAV replication in host cells requires the coinfection of helper viruses, such as Adenovirus (Ad) and Herpes Simplex virus. Alternatively, the expression of cloned helper genes can also support AAV replication. For instance, recombinant AAV can be produced in HEK293 cells by the co-transfection of three plasmids: pHelper plasmids expressing E2A, E4Orf6 and VA from Adenovirus, pRep-Cap plasmids for Rep and Cap proteins, and AAV transfer plasmids carrying the desired gene of interest (GOI).

[0004] Currently, AAV manufacturing relies on several bridging platforms. Besides the triple trasnfection in HEK293 cells noted above, AAV can be produced by co-infection of two baculoviruses expressing Rep-Cap and the GOI, respectively, into insect cells. However, these baculoviruses are unstable at higher passage and are time-consuming to prepare (*see, e.g.*, Urabe

et al., "Insect Cells as a Factory to Produce Adeno-Associated Virus Type 2 Vectors," *Human Gene Therapy 13*:1935-1943 (2002)). HeLa packaging cells with stably integrated Rep-Cap and GOI have also been developed. These systems, nevertheless, still require the wildtype Adenovirus as a helper virus, which poses risks of contamination of replicative adenovirus in AAV products (*see, e.g.*, Robert *et al.*, "Manufacturing of recombinant adeno-associated viruses using mammalian expression platforms," *Biotechnology Journal 12*:1600193 (1-16) (2017).

[0005] What are needed, are cell lines and related methods for production of AAV that are easily scalable to large volume production, to provide reproducible and stable results, while limiting contamination and reducing cost.

SUMMARY OF THE INVENTION

[0006] In some embodiments, provided herein is a mammalian cell for producing an adeno-associated virus (AAV), comprising a nucleic acid molecule encoding a viral helper gene under control of a first derepressible promoter, a nucleic acid molecule encoding an AAV gene under control of a second derepressible promoter, and a nucleic acid molecule encoding a repressor element of the first and the second derepressible promoters.

[0007] In additional embodiments, provided herein is a mammalian cell for producing an adeno-associated virus (AAV), comprising a nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters.

[0008] In further embodiments, provided herein is an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters.

[0009] In still further embodiments, provided herein is a method of producing an adeno-associated virus (AAV) in a mammalian cell comprising transfecting the mammalian cell with an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters, treating the mammalian cell with a binding partner of the repressor element, activating the first, second and third derepressible promoters, producing the AAV, and harvesting the AAV.

[0010] In further embodiments, provided herein is a method of treatment with an adeno-associated virus (AAV) comprising: transfecting the mammalian cell with an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters, treating the mammalian cell with a binding partner of the repressor element, activating the first, second and third derepressible promoters, producing the AAV, harvesting the AAV and administering the AAV to a mammalian patient.

[0011] In still further embodiments, provided herein is a method of producing an adeno-associated virus (AAV) comprising: transfecting a mammalian cell stably expressing one or more nucleic acids encoding TetR and/or TetR-KRAB with a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA under control of a first derepressible promoter, a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, and, optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter, treating the mammalian cell with a binding partner of the TetR and/or TetR-KRAB, activating the first, second, and third derepressible promoters, producing the AAV, and harvesting the AAV.

[0012] In still further embodiments, provided herein is a method for producing an adeno-associated virus (AAV) , comprising: stably transfecting a mammalian cell with a nucleic acid

encoding a TetR and/or TetR-KRAB repressor, chicken hypersensitive site-4 (cHS4) sequences flanking the TetR and/or TetR-KRAB repressor, and a selection gene, transfecting the stably transfected mammalian cell with: a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA, under control of a first derepressible promoter; a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter; and optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter; treating the mammalian cell with a binding partner of the TetR; activating the first, second and third derepressible promoters; producing the AAV; and harvesting the AAV.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIG. 1 shows a schematic of the use of derepressible promoters to control the expression of helper and VA genes in accordance with embodiments hereof.

[0014] FIGS. 2A and 2B show exemplary nucleic acid molecules for production of helper and VA genes in accordance with embodiments hereof.

[0015] FIG. 3 shows the results of induction of helper and AAV genes in accordance with embodiments hereof.

[0016] FIGS. 4A-4C shows schematics of derepressible constructs for expression of AAV genes in accordance with embodiments hereof.

[0017] FIG. 5 shows exemplary derepressible p5 promoters in accordance with embodiments hereof.

[0018] FIG. 6 shows exemplary derepressible p19 promoters in accordance with embodiments hereof.

[0019] FIG. 7A-7B shows exemplary derepressible p19 promoters, including an artificial intron, in accordance with embodiments hereof.

[0020] FIGS. 8A-8B show the results of Rep-Cap expression and AAV titers using the Rep-Cap vectors, in accordance with embodiments hereof.

[0021] FIGS. 9A-9B show additional results of Rep-Cap expression and AAV titers using the Helpers and Rep-Cap vectors, in accordance with embodiments hereof.

[0022] FIGS. 10A-10D show exemplary nucleic acid constructs encoding helper, AAV and VA genes, in accordance with embodiments hereof.

[0023] FIGS. 11A-11E show exemplary nucleic acid constructs encoding helper, AAV, gene of interest, and Rep-Cap vectors, in accordance with embodiments hereof.

[0024] FIGS. 12A-12B show exemplary nucleic acid constructs encoding TetR and TetR-KRAB, in accordance with embodiments hereof.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[0026] Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the method/device being employed to determine the value. Typically the term is meant to encompass approximately or less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% variability depending on the situation.

[0027] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer only to alternatives or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

[0028] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited, elements or method steps. It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method, system, host cells, expression vectors, and/or composition of the invention. Furthermore,

compositions, systems, cells, and/or nucleic acids of the invention can be used to achieve any of the methods as described herein.

[0029] Adeno-associated virus (AAV) has emerged as the vector of choice for gene therapy in over 120 clinical trials worldwide. The fast-growing demand of recombinant AAV requires highly efficient and robust manufacturing platforms. However, current methods for AAV production, including transient transfection and helper virus systems, are extremely costly and lab-intensive. Described herein is a plasmid/helper virus-free AAV producer cell line, and methods of use thereof, that provides efficient AAV manufacturing for a long-term solution at significantly reduced cost. The AAV producer cell line described herein represents a next generation platform for both clinical and commercial AAV manufacturing.

[0030] Thus, in embodiments, provided herein is a mammalian cell for producing an adeno-associated virus (AAV).

[0031] As used herein, the term "mammalian cell" includes cells from any member of the order Mammalia, such as, for example, human cells, mouse cells, rat cells, monkey cells, hamster cells, and the like. In some embodiments, the cell is a mouse cell, a human cell, a Chinese hamster ovary (CHO) cell, a CHOK1 cell, a CHO-DXB11 cell, a CHO-DG44 cell, a CHOK1SV cell including all variants (e.g. POTELLIGENT®, Lonza, Slough, UK), a CHOK1SV GS-KO (glutamine synthetase knockout) cell including all variants (e.g., XCEED™ Lonza, Slough, UK). Exemplary human cells include human embryonic kidney (HEK) cells, such as HEK293, a HeLa cell, or a HT1080 cell.

[0032] Mammalian cells include mammalian cell cultures which can be either adherent cultures or suspension cultures. Adherent cultures refer to cells that are grown on a substrate surface, for example a plastic plate, dish or other suitable cell culture growth platform, and may be anchorage dependent. Suspension cultures refer to cells that can be maintained in, for example, culture flasks or large suspension vats, which allows for a large surface area for gas and nutrient exchange. Suspension cell cultures often utilize a stirring or agitation mechanism to provide appropriate mixing. Media and conditions for maintaining cells in suspension are generally known in the art. An exemplary suspension cell culture includes human HEK293 clonal cells.

[0033] As used herein, the term "adeno-associated virus (AAV)" refers to a small sized, replicative-defective nonenveloped virus containing a single stranded DNA of the family Parvoviridae and the genus Dependoparvovirus. Over 10 adeno-associated virus serotypes have been identified so far, with serotype AAV2 being the best characterized. Other non-limiting examples of AAV serotypes are ANC80, AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, and AAV11. In addition to these serotypes, AAV pseudotypes have been developed. An AAV pseudotype contains the capsid of a first serotype and the genome of a second serotype (e.g. the pseudotype AAV2/5 would correspond to an AAV with the genome of serotype AAV2 and the capsid of AAV5).

[0034] As referred to herein, the term "adenovirus" refers to a nonenveloped virus with an icosahedral nucleocapsid containing a double stranded DNA of the family Adenoviridae. Over 50 adenoviral subtypes have been isolated from humans and many additional subtypes have been isolated from other mammals and birds. Birds. See, e.g., Ishibashi et al., "Adenoviruses of animals," In *The Adenoviruses*, Ginsberg, ed., Plenum Press, New York, N.Y., pp. 497-562 (1984); Strauss, "Adenovirus infections in humans," In *The Adenoviruses*, Ginsberg, ed., Plenum Press, New York, N.Y., pp. 451-596 (1984). These subtypes belong to the family Adenoviridae, which is currently divided into two genera, namely Mastadenovirus and Aviadenovirus. All adenoviruses are morphologically and structurally similar. In humans, however, adenoviruses show diverging immunological properties and are, therefore, divided into serotypes. Two human serotypes of adenovirus, namely AV2 and AV5, have been studied intensively and have provided the majority of general information about adenoviruses.

[0035] In embodiments, the mammalian cell provided herein suitably includes a nucleic acid molecule encoding a viral helper gene under control of a first derepressible promoter, a nucleic acid molecule encoding an AAV gene under control of a second derepressible promoter, and a nucleic acid molecule encoding a repressor element of the first and the second derepressible promoters.

[0036] In exemplary embodiments, the nucleic acid molecules encoding the various components for producing an AAV are contained within the mammalian cell in separate nucleic acid molecules, for example separate plasmids or vectors. In other embodiments, the nucleic acid

molecules encoding the various components for producing an AAV are included on the same plasmid or vector. In further embodiments, certain of the components are contained on the same nucleic acid molecule (e.g., helper genes and AAV gens), while other genes are contained on separate nucleic acid molecules (e.g., gene encoding the repressor element).

[0037] A "nucleic acid," "nucleic acid molecule," or "oligonucleotide" means a polymeric compound comprising covalently linked nucleotides. The term "nucleic acid" includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single- or double-stranded. DNA includes, but is not limited to, complimentary DNA (cDNA), genomic DNA, plasmid or vector DNA, and synthetic DNA. RNA includes, but is not limited to, mRNA, tRNA, rRNA, snRNA, microRNA, miRNA, or MIRNA.

[0038] In the various embodiments described herein, the nucleic acid molecules are capable of encoding the various genes. That is the nucleic acid molecules, when transcribed, produce mRNA for the genes described herein, which is then translated to the desired or required proteins.

[0039] As described herein, suitably the mammalian cells include a nucleic acid molecule encoding a viral helper gene. Viral helper genes include various adenoviral virus genes, herpes virus genes and bocavirus genes (see, e.g., Guido *et al.*, "Human bocavirus: Current knowledge and future challenges," *World J. Gateroenterol 22*:8684-8697, the disclosure of which is incorporated by reference herein in its entirety). In exemplary embodiments, the viral helper gene is an adenovirus helper gene. As referred to herein, the term "adenovirus helper gene" or "AV helper gene" refers to a gene that is composed of one or more nucleic acid sequences derived from one or more adenovirus subtypes or serotypes that contributes to Adeno-associated virus replication and packaging. In some embodiments, the Adenovirus helper gene is E1A, E1B, E2A, E4 (including E4Orf6), VA, or a combination thereof or any other adenovirus helper gene. In exemplary embodiments, the adenovirus helper gene comprises both E2A and E4Orf6 genes. Suitably, an internal ribosome entry site (IRES) element is included between the E2A and E4Orf6 genes. The IRES element initiates translation of the E4Orf6 gene after the E2A gene in a single expression cassette, providing stability to the construct.

[0040] The various nucleic acid molecules encoding the various genes described herein are suitably under control of a derepressible promoter. As used herein "under control" refers to a gene

being regulated by a "promoter," "promoter sequence," or "promoter region," which refers to a DNA regulatory region/sequence capable of binding RNA polymerase and initiating transcription of a downstream coding or non-coding gene sequence. In other words, the promoter and the gene are in operable combination or operably linked. As referred to herein, the terms "in operable combination", "in operable order" and "operably linked" refer to the linkage of nucleic acid sequences in such a manner that a promoter capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

[0041] In some examples of the present disclosure, the promoter sequence includes the transcription initiation site and extends upstream to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. In some embodiments, the promoter sequence includes a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the gene expression, e.g., in the host cell or vectors of the present disclosure. In some embodiments, the promoter is not a leaky promoter, i.e., the promoter is not constitutively expressing any of the gene products as described herein. In other embodiments as described herein, the promoter is a constitutive promoter, which initiates mRNA synthesis independent of the influence of an external regulation.

[0042] Suitably, the promoters used to control the transcription of the various genes for producing the AAVs described herein are derepressible promoters. As used herein, a "derepressible promoter" refers to a structure that includes a functional promoter and additional elements or sequences capable of binding to a repressor element to cause repression of the functional promoter. "Repression" refers to the decrease or inhibition of the initiation of transcription of a downstream coding or non-coding gene sequence by a promoter. A "repressor element" refers to a protein or polypeptide that is capable of binding to a promoter (or near a promoter) so as to decrease or inhibit the activity of the promoter. A repressor element can interact with a substrate or binding partner of the repressor element, such that the repressor element undergoes a conformation change. This conformation change in the repressor element takes away the ability of the repressor element to decrease or inhibit the promoter, resulting in the

"derepression" of the promoter, thereby allowing the promoter to proceed with the initiation of transcription. A "functional promoter" refers to a promoter, that absent the action of the repressor element, would be capable of initiation transcription. Various functional promoters that can be used in the practice of the present invention are known in the art, and include for example, P_{CMV}, P_{H1}, P19, P5, P40 and promoters of Adenovirus helper genes (e.g., E1A, E1B, E2A, E4Orf6, and VA).

[0043] Exemplary repressor elements and their corresponding binding partners that can be used as derepressible promoters are known in the art, and include systems such as the cumate gene-switch system (CuO operator, CymR repressor and cumate binding partner) (*see, e.g.*, Mullick *et al.*, "The cumate gene-switch: a system for regulated expression in mammalian cells," *BMC Biotechnology 6*:43 (1-18) (2006), the disclosure of which is incorporated by reference herein in its entirety, including the disclosure of the derepressible promoter system described therein) and the TetO/TetR system described herein (*see, e.g.*, Yao *et al.*, "Tetracycline Repressor, tetR, rather than the tetR-Mammalian Cell Transcription Factor Fusion Derivatives, Regulates Inducible Gene Expression in Mammalian Cells," *Human Gene Therapy 9*:1939-1950 (1998), the disclosure of which is incorporated by reference herein in its entirety).

[0044] In exemplary embodiments, the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$). A schematic showing an exemplary depressible promoter system is provided in FIG. 1. A derepressible promoter including the P_{CMV} promoter, and a derepressible promoter including the P_{H1} promoter, are shown, both including two TetO sequences ($TetO_2$). As illustrated schematically, upon binding of two tetracycline repressor proteins (TetR - the repressor elements for the $TetO_2$ sequences), to the $TetO_2$ sequences, both the P_{CMV} promoter and the P_{H1} promoter are repressed. That is, little or no transcription takes place from these promoters. Upon binding of a binding partner for TetR (suitably Doxycycline (Dox), the TetR proteins change conformation, release from the $TetO_2$ sequences, and the functional promoters begin their normal transcription processes, as they would naturally. As illustrated schematically in FIG. 1, this results in the change of the overall system from an "off" position (where suitably no transcription is taking place from the P_{CMV} promoter and the P_{H1} promoter), to an "on" position when Dox is added, allowing the P_{CMV} promoter and the P_{H1} promoter to return to their natural state of transcribing the genes under their control.

[0045] For example, as shown in FIG. 1, the P_{CMV} promoter with $TetO_2$ sequences (suitably the pcDNA4/TO promotor; INVITROGEN®), is in an "off" position when bound by TetR. When Dox is added, the TetR changes conformation, release from the $TetO_2$ sequences of the depressible promoter, and the P_{CMV} promotor proceeds to transcribe the adenoviral helper genes (e.g., E2A and E4).

[0046] As described herein, and as illustrated in FIG. 1, the mammalian cell can further comprise a nucleic acid encoding a viral-associated (VA), non-coding RNA under control of a fourth derepressible promoter. As shown in FIG. 1, this derepressible promoter can include the functional promoter P_{H1}, and the $TetO_2$ sequences controlling the expression of the non-coding RNA (see, e.g., Wiederschain *et al.*, "Single-vector inducible lentiviral RNAi system for oncology target validation, *Cell Cycle 8*:498-504 (2009), the disclosure of which is incorporated by reference herein in its entirety, including for the disclosure of the promoter system and sequence). As shown in FIG. 1, the P_{H1} promoter with $TetO_2$ sequences, is in an "off" position when bound by TetR. When Dox is added, the TetR changes conformation, release from the $TetO_2$ sequences of the depressible promoter, and the P_{H1} promotor proceeds to transcribe the VA 1 non-coding RNA.

[0047] FIG. 2A shows an exemplary nucleic acid molecule that can be utilized in the various mammalian cells and methods described herein. As illustrated, a CMV promoter is used upstream of both E2A and E4Orf6 genes, linked via a IRES element. The CMV promoter includes the CMV enhancer and the tet operator ($TetO_2$), for control, via derepression. Also illustrated in FIG. 2A is an exemplary location for the H1 promotor, also including $TetO_2$ sequences, controlling the expression of the VA non-coding RNA.

[0048] FIG. 1 and FIG. 2A also illustrate that, in embodiments, the mammalian cells can include the repressor element under control of a constitutive promoter. As described herein, suitably the repressor element that is encoded is a tetracycline repressor protein (TetR). As illustrated in FIG. 1 and FIG. 2A, a suitable promotor for expression of the repressor element is an hPGK promotor. By placing the repressor element under the control of a constitutive promotor, production of the repressor element, suitably TetR, is always active. That is, TetR is being produced upon introduction of the nucleic acid molecule into the mammalian cell. This provides

tight control of the various derepressible promoters that are repressed by the TetR binding to the $TetO_2$ sequences.

[0049] As shown in FIG. 2A, in exemplary embodiments, a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein, can be included. In FIG. 2A, this transcriptional repression domain is a Krueppel-associated box (KRAB) sequence, fused in frame to the C-terminus of TetR (*see, e.g.*, Szulc *et al.*, "A versatile tool for conditional gene expression and knockdown," *Nature Methods 3:*109-116 (2006)). The use of a KRAB sequence, or other transcriptional repression domain, improves the repressive activity of the TetR when binding to $TetO_2$, thereby minimizing the amount of leakage or basal gene expression prior to derepression (i.e., prior to the addition of Dox). FIG. 2B and FIG. 12A shows an exemplary nucleic acid molecule that lacks the KRAB sequence.

[0050] As shown in FIG. 12A, in exemplary embodiments, a nucleic acid encoding the tetracycline repressor protein, can be included or stably expressed within a mammalian cell. In FIG. 2A and FIG.12B, this transcriptional repression domain is a Krueppel-associated box (KRAB) sequence, fused in frame to the C-terminus of TetR (*see, e.g.*, Szulc *et al.*, "A versatile tool for conditional gene expression and knockdown," *Nature Methods 3:*109-116 (2006)). The use of a KRAB sequence, or other transcriptional repression domain, improves the repressive activity of the TetR when binding to $TetO_2$, thereby minimizing the amount of leakage or basal gene expression prior to derepression (i.e., prior to the addition of Dox). FIG. 2B and FIG. 12A show an exemplary nucleic acid molecule that lacks the KRAB sequence.

[0051] In embodiments, the AAV gene that is encoded by the nucleic acid molecule comprises *Rep* and *Cap* genes. Other AAV genes that can be encoded by the nucleic acid molecules include any gene from any AAV serotype. In some embodiments, the AAV gene is *Rep78*, *Rep68*, *Rep52*, *Rep40*, *VP1*, *VP2*, *VP3*, or a combination thereof. In some embodiments, the AAV gene is from adeno-associated virus type 2. In some embodiments, the AAV gene is from the adeno-associated virus Anc80.

[0052] As referred to herein, the term "*Rep*" gene refers to the art-recognized region of the AAV genome which encodes the replication proteins of the virus which are collectively required for replicating the viral genome, or functional homologues thereof such as the human herpesvirus

6 (HHV-6) rep gene which is also known to mediate AAV-2 DNA replication. Thus, the rep coding region can include the genes encoding for AAV *Rep78* and *Rep68* (the "long forms of Rep"), and *Rep52* and *Rep40* (the "short forms of Rep"), or functional homologues thereof. The rep coding region, as used herein, can be derived from any viral serotype, such as the AAV serotypes described herein. The region need not include all wild-type genes but may be altered, (e.g., by insertion, deletion or substitution of nucleotides), so long as the rep genes present provide for sufficient integration functions when expressed in a suitable target cell. *See, e.g.* Muzyczka, N., *Current Topics in Microbiol. and Immunol. 158*:97-129 (1992); and Kotin, R. M., *Human Gene Therapy 5*:793-801 (1994).

[0053] As referred to herein, the term "*Cap*" gene refers to the art-recognized region of the AAV genome which encodes the capsid proteins of the virus. Illustrative (non-limiting) examples of these capsid proteins are the AAV capsid proteins VP1, VP2, and VP3. Cap genes used in this disclosure can come from any AAV serotype or a combination of AAV serotypes.

[0054] FIG. 4A shows the natural location and promoter drivers of *Rep* and *Cap* genes. As known in the art, for successful production of AAV, the ratio of *Rep78* and *Rep52* genes must be maintained at optimum levels. For example, as discussed in Li *et al.*, "Role for Highly Regulated *rep* Gene Expression in Adeno-Associated Virus Vector Production," *Journal of Virology 71*:5236-5243 (1997) (the disclosure of which is incorporated by reference herein in its entirety), control of the amount of Rep78 production can interference with DNA replication. In addition, *Rep78* can be toxic if overly produced in mammalian cells. *See, e.g.,* Clark *et al.*, "Cell Lines for the Production of Recombinant Adeno-Associated Virus," *Human Gene Therapy 6*:1329-1341 (1995), the disclosure of which is incorporated by reference herein in its entirety, discussing that elevate *rep* protein levels can be associated with cytotoxicity. Also, the location of the promoter for *Rep52* expression (p19) is located within the coding region for *Rep78*. As described herein, various modifications have been made to the natural locations of the Rep genes and promoters to overcome these challenges.

[0055] In exemplary embodiments, the mammalian cell can include a nucleic acid encoding a *Rep78* gene under control of the second derepressible promoter and a *Rep52* gene under control of a third derepressible promoter. As shown in FIG. 4B, one way to achieve this arrangement is to

remove the *Rep52* gene from within the *Rep78* gene, and place it downstream of the *Rep78* and Cap genes. The *Rep78* gene can be under the control of a derepressible promoter (p5), that includes the $TetO_2$ sequences. In such embodiments, the natural p19 promoter within *Rep78* is modified or mutated to be silenced. The removed Rep52 gene is also placed under the control of a derepressible promoter (p19) that includes $TetO_2$ sequences.

[0056] FIG. 5 shows three potential locations for each of the TetO sequences, relative to the TATA box, rep-binding element (RBE) and initiator element (INR), for modification to the p5 promotor of Rep78. The wild-type p5 promotor is also illustrated schematically. FIG. 6 shows three potential locations for each of the TetO sequences, relative to the TATA boxes and the Sp1 transcription factor, of the p19 promoter for Rep52. The wild-type p19 promotor is also illustrated schematically. Additional locations of the TetO sequences are also encompassed herein and can readily be envisioned by those of ordinary skill in the art.

[0057] In still further embodiments, the Rep78 gene can be under control of a derepressible promoter, and the Rep52 gene can be under control of a derepressible promoter that is contained within an artificial intron. Such an embodiment is represented schematically in FIG. 4C. As illustrated, a derepressible p5 promoter (e.g., including $TetO_2$ sequences as described herein) is placed upstream of a Rep78 gene. A derepressible p19 promoter (e.g., including $TetO_2$ sequences) contained within an intron (designated In-i-p19), controls the expression of Rep52. A schematic of this embodiment is provided in FIG. 7A, where the two tet operator sequences are illustrated within a chimeric intron. This chimeric intron can be inserted at various locations relative to the components of the p19 promoter. For example, as shown in FIG. 7B, the intron sequence is suitably placed downstream of the TATA-2 of the p19 promoter. Spacing can be, for example, between about 1 and 25 base pairs downstream from the TATA-2 sequence.

[0058] As described herein, in exemplary embodiments, the Cap gene that is encoded by the nucleic acid molecules is suitably under the control of a native promoter. That is, the Cap gene does not necessarily have to be under the control of a derepressible promoter, though a derepressible promoter can be used if desired. In suitable embodiments, the Cap gene is under the control of a p40 promoter.

[0059] In exemplary embodiments, the nucleic acid molecules include two inverted terminal repeat (ITR) sequences. As known in the art, these ITR sequences (i.e., AAV2 ITR) are single stranded sequence of nucleotides, followed downstream by its reverse compliment. ITR sequences represent the minimal sequence required for replication, rescue, packaging and integration of the AAV genome. Suitably, these ITR sequences flank a gene of interest. Thus, in embodiments, the nucleic acid molecules further encode a gene of interest. This gene of interest can be, for example, a reporter gene, a selection gene, or a gene of therapeutic interest, for example.

[0060] For example, as illustrated in FIG. 10C, a gene of interest, such as the gene encoding green fluorescent protein (EGFP) is flanked by two ITR sequences.

[0061] A "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acid molecules. "Gene" also refers to a nucleic acid fragment that can act as a regulatory sequence preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. In some embodiments, genes are integrated with multiple copies. In some embodiments, genes are integrated at predefined copy numbers.

[0062] As referred to herein, the term "gene of interest" or "GOI" is used to describe a heterologous gene. As referred to herein, the term "heterologous gene" or "HG" as it relates to nucleic acid sequences such as a coding sequence or a control sequence, denotes a nucleic acid sequence, e.g. a gene, that is not normally joined together, and/or are not normally associated with a particular cell. In some embodiments, a heterologous gene is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

[0063] As referred to herein, a "reporter gene" is a gene whose expression confers a phenotype upon a cell that can be easily identified and measured. In some embodiments, the reporter gene comprises a fluorescent protein gene. In some embodiments, the reporter gene comprises a selection gene.

[0064] As referred to herein, the term "selection gene" refers to the use of a gene which encodes an enzymatic activity that confers the ability to grow in medium lacking what would

otherwise be an essential nutrient; in addition, a selection gene may confer resistance to an antibiotic or drug upon the cell in which the selection gene is expressed. A selection gene may be used to confer a particular phenotype upon a host cell. When a host cell must express a selection gene to grow in selective medium, the gene is said to be a positive selection gene. A selection gene can also be used to select against host cells containing a particular gene; a selection gene used in this manner is referred to as a negative selection gene.

[0065] As referred to herein, the term "gene of therapeutic interest" refers to any functionally relevant nucleotide sequence. Thus, the gene of therapeutic interest of the present disclosure can comprise any desired gene that encodes a protein that is defective or missing from a target cell genome or that encodes a non-native protein having a desired biological or therapeutic effect (e.g., an antiviral function), or the sequence can correspond to a molecule having an antisense or ribozyme function. Representative (non-limiting) examples of suitable genes of therapeutic interest include those used for the treatment of inflammatory diseases, autoimmune, chronic and infectious diseases, including such disorders as AIDS, cancer, neurological diseases, cardiovascular disease, hypercholestemia; various blood disorders including various anemias, thalassemias and hemophilia; genetic defects such as cystic fibrosis, Gaucher's Disease, adenosine deaminase (ADA) deficiency, emphysema, etc. Several antisense oligonucleotides (e.g., short oligonucleotides complementary to sequences around the translational initiation site (AUG codon) of an mRNA) that are useful in antisense therapy for cancer and for viral diseases have been described in the art and are also examples of suitable genes of therapeutic interest.

[0066] In some embodiments, the mammalian cells provided herein are substantially free of helper virus. As referred to herein, a "helper virus" is any non-AAV virus that is added to enable the replication and packaging of adeno-associated virus. Representative (non-limiting) examples of helper viruses are adenovirus and herpes virus. In some embodiments, the term substantially free of helper virus refers to a cell that has fewer than 100, fewer than 10, or fewer than 1 helper virus per cell. In some embodiments, the term substantially free of helper virus refers to a cell in which no helper viruses are present or to a population of cells in which no helper viruses are present using detection methods known to those skilled in the art. In some embodiments, no wild-type helper virus is in the cell. In some embodiments, the term wild-type virus refers to any complete-non-AAV virus that can replicate in the cell independently of any other virus.

[0067] The AAV producer cells described herein provide a long-term and cost-efffective solution for large scale AAV manufracturing. As constitutive expression of either helper or Rep proteins can be cytotoxic, the stratagies described herein allow for control of their expression by engineered, derepressible promoters.

[0068] In still further embodiments, provided herein is a mammalian cell for producing an adeno-associated virus (AAV), comprising, in a single nucleic acid molecule, sequences encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters. In such embodiments, this single nucleic acid molecule includes all of the various sequences, along with other required elements, to enable the production of an AAV within the cell.

[0069] FIG. 10A and 10B show exemplary nucleic acid molecules including these various sequences that can be utilized in mammalian cells to produce AAVs.

[0070] As described herein, suitably the mammalian cells are mammalian cell cultures, and in embodiments, can be suspension cultures. As described herein, the use of suspension cell cultures allows for increased scalability and production of AAV.

[0071] As described herein and as shown in FIGS. 1, 2A-2B, and 10A-10B, suitably the single nucleic acid molecule includes an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[0072] Various constructs are described herein for encoding the Rep and Cap genes, including Rep78 and Rep52 genes. In embodiments, a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter (e.g., as shown in FIG. 4B, the Rep52 gene is separate from the Rep78 gene). In additional embodiments, for example as described herein with reference to FIGS. 4C and 7A-7B, a Rep78 gene can be placed under control of the second derepressible promoter and a Rep52 gene can be

placed under control of a fourth derepressible promoter contained within an artificial intron. Suitably, the Cap gene is under control of a native promoter.

[0073] Various derepressible promoters are described herein, and in embodiments, the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$). In embodiments, the functional promoter of the first derepressible promoter (i.e., controlling the expression of the adenovirus helper gene comprising E2A and E4Orf6 genes) is a cytomegalovirus (CMV) promoter.

[0074] As described herein, in embodiments and as illustrated in FIGS. 1, 2A-2B, and 10A-10D, suitably the repressor element of the derepressible promoter is under control of a constitutive promoter, such that it is produced at all times to limit expression of other genes under the control of the derepressible promoters. Suitably, the repressor element that is encoded is a tetracycline repressor protein, for binding to $TetO_2$ sequences to act as the derepressible promoter. In embodiments, for example as shown in FIG. 2A, 10A and 10C, a nucleic acid encoding a transcriptional repression domain (e.g., a KRAB sequence) is included in frame with the nucleic acid encoding the tetracycline repressor protein. This transcriptional repression domain provides for improved repressive activity of the TetR when binding to $TetO_2$, thereby minimizing the amount of leakage or basal gene expression prior to derepression.

[0075] Exemplary mammalian cells that can be used in the embodiments and methods described herein are described throughout, and include for example, Chinese hamster ovary (CHO) cells, as well as human cells, including human embryonic kidney (HEK, such as HEK293) cells.

[0076] As described herein, suitably the mammalian cells further include a nucleic acid molecule encoding a gene of interest (GOI). As shown in FIGS. 10C-10D, suitably the GOI is included between two ITR sequences.

[0077] Also provided herein is an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted

terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters.

[0078] As used herein an "isolated nucleic acid molecule" includes vectors and plasmids that can contain the isolated nucleic acid molecule, as well as similar structures where the isolated nucleic acid molecule can be manipulated, stored, shipped, and ultimately utilized in various cell transfection systems. The isolated nucleic acid molecules described herein can be used for production of AAVs as described herein, but can also be utilized in various non-AAV producing cell lines (including transient transfection systems). The isolated nucleic acid molecules described herein suitably further include various additional elements and sequences as required to allow for use in the cellular systems, including mammalian cells, described herein.

[0079] For example, as shown in FIGS. 10A-10B, two plasmid constructs are shown. As indicated, in embodiments, an internal ribosome entry site (IRES) element can be included between the E2A and E4Orf6 genes. As described herein with reference to FIG. 4B in embodiments, a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter (i.e., separated from the Rep52 gene).

[0080] In further embodiments, as shown with reference to FIGS. 10A-10B, a Rep78 gene is under control of a derepressible promoter (ip5 promoter as shown, and various iterations described in FIG. 5) and a Rep52 gene is under control of a fourth derepressible promoter contained within an artificial intron, illustrated as ip19, and described with reference to FIGS. 7A-7B. The isolated nucleic acid further suitably includes the Cap gene under control of a native promoter (i.e., p40).

[0081] As described herein, various derepressible promoters can be included in the isolated nucleic acid molecules, and suitably include a functional promoter and two tetracycline operator sequences ($TetO_2$). As shown in FIG. 10A and 10B, with regard to the helper genes E2 and E4, suitably this functional promoter is a CMV promoter, which includes the $TetO_2$ sequences. The derepressible promoter for use with the viral-associated, non-coding RNA suitably includes an H1 promoter, as well as the $TetO_2$ sequences.

[0082] As described herein and as shown in FIGS. 10A and 10B, the repressor element (e.g., a tetracycline repressor protein), is suitably under the control of a constitutive promoter, for

example, a hPGK promoter. As shown in FIG. 10A and 12B, the isolated nucleic acid molecules can further include a nucleic acid encoding a transcriptional repression domain (e.g., a KRAB sequence) in frame with the nucleic acid encoding the tetracycline repressor protein. As shown in FIGS. 10C-10D and FIG. 11B, the isolated nucleic acid molecules can further include a gene of interest (GOI, e.g., a GFP), suitably between the two ITR sequences.

[0083] As described herein and as shown in FIGS. 12A-12B, the repressor element is suitably flanked by an insulator, for example, a chicken hypersensitive site-4 (cHS4) sequence.

[0084] In embodiments, a nucleic acid encoding a transcriptional repression domain (such as KRAB) is included in frame with the nucleic acid encoding the tetracycline repressor protein (e.g., TetR-KRAB).

[0085] Additional genetic and sequence elements for inclusion in the isolated nucleic acid molecules described herein are known in the art and can be found illustrated schematically in FIGS. 10A-10D and FIGS. 11A-11E.

[0086] The terms "sequence identity" or "% identity" in the context of nucleic acid sequences described herein refers to the percentage of residues in the compared sequences that are the same when the sequences are aligned over a specified comparison window. A comparison window can be a segment of at least 10 to over 1000 residues in which the sequences can be aligned and compared. Methods of alignment for determination of sequence identity are well-known can be performed using publicly available databases such as BLAST (blast.ncbi.nlm.nih.gov/Blast. CGI.).

[0087] In some embodiments, nucleic acid molecules have at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, at least about 98%, at least about 99% or about 100% sequence identity with a reference nucleic acid molecule, respectively (or a fragment of the reference polypeptide or nucleic acid molecule). In certain embodiments of the disclosure, polypeptides or nucleic acid molecules have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% or 100% sequence identity with a reference nucleic acid molecule, respectively (or a fragment of the reference nucleic acid molecule). In some embodiments, nucleic

acid molecules have about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% sequence identity with a reference nucleic acid molecule, respectively.

[0088] A "vector" or "expression vector" is a replicon, such as a plasmid, phage, virus, or cosmid, to which a nucleic acid molecule described herein may be attached to bring about the replication and/or expression of the attached nucleic acid molecule in a cell. "Vector" includes episomal (*e.g.,* plasmids) and non-episomal vectors. The term "vector" includes both viral and nonviral means for introducing a nucleic acid molecule into a cell *in vitro*, *in vivo*, or *ex vivo*. The term vector may include synthetic vectors. Vectors may be introduced into the desired host cells by well-known methods, including, but not limited to, transfection, transduction, cell fusion, and lipofection. Vectors can comprise various regulatory elements including promoters.

[0089] Also provided herein is a method of producing an adeno-associated virus (AAV) in a mammalian cell. Suitably, the methods described herein include transfecting the mammalian cell with an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters.

[0090] "Transfection" as used herein means the introduction of an exogenous nucleic acid molecule, including a vector, into a cell. A "transfected" cell comprises an exogenous nucleic acid molecule inside the cell and a "transformed" cell is one in which the exogenous nucleic acid molecule within the cell induces a phenotypic change in the cell. The transfected nucleic acid molecule can be integrated into the host cell's genomic DNA and/or can be maintained by the cell, temporarily or for a prolonged period of time, extra-chromosomally. Host cells or organisms that express exogenous nucleic acid molecules or fragments are referred to as "recombinant," "transformed," or "transgenic" organisms. A number of transfection techniques are generally known in the art. *See, e.g.,* Graham et al., *Virology, 52*:456 (1973); Sambrook et al., Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York (1989); Davis et al., Basic Methods in Molecular Biology, Elsevier (1986); and Chu et al., *Gene 13*:197 (1981). Such

techniques can be used to introduce one or more exogenous DNA moieties, such as an AAV vector cassette, AAV helper constructs, and other nucleic acid molecules, into suitable host cells.

[0091] Various methods of transfecting the mammalian cells with the isolated nucleic acid molecules described herein (i.e., vectors), are known in the art and include various chemical and physical methods, for example, electroporation, cell injection, calcium phosphate exposure, liposome or polymer-based carrier systems, etc.

[0092] In exemplary embodiments, a vectors such as the PIGGYBAC™ transposon can be used for the stable integration of these nucleic acid molecules, which allows one-step insertion of large nucleic acid sequences in multiple copies randomly in a cell genome. The system consists of a PIGGYBAC™ Vector and the Super PIGGYBAC™ Transposase which recognizes transposon-specific inverted terminal repeats (ITRs) and efficiently integrates the ITRs and intervening DNA into the genome at TTAA sites. The Super PIGGYBAC™ Transposase is delivered to the cell via the Super PIGGYBAC™ Transposase Expression Vector, which is co-transfected with one or more PIGGYBAC™ Vectors.

[0093] The methods further include treating the mammalian cell with a binding partner of the repressor element. As described herein, in the presence of a repressor element, the functional promoters of the derepressible promoters controlling the transcription of the various genes encoded by the nucleic acid molecules, are repressed. That is, the genes are not actively being transcribed, and are instead awaiting derepression. As noted herein, the repressor element of the derepressible promoters is suitably under control of constitutive promoter, such that the repressor element is being produced soon after transfection of the nucleic acid molecule into the mammalian cell. Upon treatment with the binding partner of the repressor element, the repressor element binds to the binding partner, changes conformation, and no longer represses the derepressible promoter. This results in the activating of the first, second and third (and additional as needed) derepressible promoters (i.e., the functional promoters of the derepressible promoters) within the mammalian cell.

[0094] Following the activation, the various elements are transcribed and translated within the mammalian cell, resulting in the production of the AAV. The AAV is then harvested using methods known in the art.

[0095] While the methods described herein can be utilized in any mammalian cell, including a mammalian cell culture, suitably the mammalian cell culture is a suspension culture, including a human cell such as an HEK suspension cell culture.

[0096] As described throughout the nucleic acid molecules can further include an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes. Exemplary constructs related to the Rep78 and Rep 52 genes are described herein, including where a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter. In additional embodiments of the methods, a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter contained within an artificial intron.

[0097] As described herein, the use of an artificial intron allows for removal of the fourth derepressible promoter following activating the derepressible promoters and prior to the producing the AAV. As described herein, the derepressible promoter within the intron ensures the repression of Rep52 gene expression before activation, while still allowing for the expression of the Rep78 protein following the removal of the intron during mRNA splicing. In exemplary embodiments of the methods, the Cap gene is under control of a native promoter, such as p40.

[0098] In embodiments, the functional promoter of the derepressible promoter controlling the expression of the helper genes is a cytomegalovirus (CMV) promoter. Suitably, the repressor element that is encoded in the methods described herein is a tetracycline repressor protein, and suitably the derepressible promoters comprise a functional promoter and two tetracycline operator sequences (TetO2). In embodiments, the repressor element is under control of a constitutive promoter, such as hPGK, for example when the repressor element that is encoded is a tetracycline repressor protein.

[0099] As described herein, in embodiments utilizing the TetR and/or TetR-KRAB repressor element, treating the cells with doxycycline changes the conformation of the TetR and activates the transcription of the various genes.

[00100] Various mammalian cells can be utilized in the methods described herein, including human cells such as human embryonic kidney (HEK) cells, or other mammalian cells including Chinese hamster ovary (CHO) cells.

[00101] As described throughout, in embodiments, the AAV comprises a nucleic acid molecule encoding a gene of interest. This GOI can be a reporter gene, a selection gene, or any other gene of interest, including a gene of therapeutic interest.

[00102] The methods of producing the AAVs can be used in a continuous manufacturing system. In exemplary embodiments, the use of a suspension cell culture allows for the production of large volumes of AAV, with high productivity and prolonged culture conditions to allow for multiple harvests of AAV for each batch of starting cells.

[00103] Production methods can utilize any suitable reactor(s) including but not limited to stirred tank, airlift, fiber, microfiber, hollow fiber, ceramic matrix, fluidized bed, fixed bed, and/or spouted bed bioreactors. As used herein, "reactor" can include a fermenter or fermentation unit, or any other reaction vessel and the term "reactor" is used interchangeably with "fermenter." The term fermenter or fermentation refers to both microbial and mammalian cultures. For example, in some aspects, an example bioreactor unit can perform one or more, or all, of the following: feeding of nutrients and/or carbon sources, injection of suitable gas (e.g., oxygen), inlet and outlet flow of fermentation or cell culture medium, separation of gas and liquid phases, maintenance of temperature, maintenance of oxygen and CO_2 levels, maintenance of pH level, agitation (e.g., stirring), and/or cleaning/sterilizing. Example reactor units, such as a fermentation unit, may contain multiple reactors within the unit, for example the unit can have 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100, or more bioreactors in each unit and/or a facility may contain multiple units having a single or multiple reactors within the facility. In various embodiments, the bioreactor can be suitable for batch, semi fed-batch, fed-batch, perfusion, and/or a continuous fermentation processes. Any suitable reactor diameter can be used. In embodiments, the bioreactor can have a volume between about 100 mL and about 50,000 L. Non-limiting examples include a volume of 100 mL, 250 mL, 500 mL, 750 mL, 1 liter, 2 liters, 3 liters, 4 liters, 5 liters, 6 liters, 7 liters, 8 liters, 9 liters, 10 liters, 15 liters, 20 liters, 25 liters, 30 liters, 40 liters, 50 liters, 60 liters, 70 liters, 80 liters, 90 liters, 100 liters, 150 liters, 200 liters, 250 liters, 300 liters,

350 liters, 400 liters, 450 liters, 500 liters, 550 liters, 600 liters, 650 liters, 700 liters, 750 liters, 800 liters, 850 liters, 900 liters, 950 liters, 1000 liters, 1500 liters, 2000 liters, 2500 liters, 3000 liters, 3500 liters, 4000 liters, 4500 liters, 5000 liters, 6000 liters, 7000 liters, 8000 liters, 9000 liters, 10,000 liters, 15,000 liters, 20,000 liters, and/or 50,000 liters. Additionally, suitable reactors can be multi-use, single-use, disposable, or non-disposable and can be formed of any suitable material including metal alloys such as stainless steel (e.g., 316L or any other suitable stainless steel) and Inconel, plastics, and/or glass.

[00104] In embodiments and unless stated otherwise herein, the devices, facilities, and methods described herein can also include any suitable unit operation and/or equipment not otherwise mentioned, such as operations and/or equipment for separation, purification, and isolation of such products. Any suitable facility and environment can be used, such as traditional stick-built facilities, modular, mobile and temporary facilities, or any other suitable construction, facility, and/or layout. For example, in some embodiments modular clean-rooms can be used. Additionally and unless otherwise stated, the devices, systems, and methods described herein can be housed and/or performed in a single location or facility or alternatively be housed and/or performed at separate or multiple locations and/or facilities.

[00105] In further embodiments, provided herein is a method of treatment with an adeno-associated virus (AAV) comprising: transfecting the mammalian cell with an isolated nucleic acid molecule encoding: an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters, treating the mammalian cell with a binding partner of the repressor element, activating the first, second and third derepressible promoters, producing the AAV, harvesting the AAV, and administering the AAV to a mammalian patient.

[00106] Suitably, the methods are used to treat a human patient with a gene of interest, including a gene of therapeutic interest. Administration to a human patient can include, for example, inhalation, injection, or intravenous administration, as well as other administration methods known in the art.

[00107] The methods of producing the AAV and the use of various derepressible promoters are described herein.

[00108] In further embodiments, a method of producing an adeno-associated virus (AAV) is provided and includes transfecting a mammalian cell stably expressing one or more nucleic acids encoding TetR and/or TetR-KRAB with: a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA, under control of a first derepressible promoter, a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter; treating the mammalian cell with a binding partner of the TetR; activating the first, second and third derepressible promoters; producing the AAV; and harvesting the AAV.

[00109] As described herein, suitably the mammalian cell is a mammalian cell culture, including a suspension culture

[00110] As described herein, suitably the mammalian cell is a Chinese hamster ovary (CHO) cell or a human cell including a human embryonic kidney (HEK) cell.

[00111] As described herein, using 2 or 3 different nucleic acids or transposons to transfect mammalian cells offers advantages over combining the nucleic acids in one plasmid, including the ability to optimize the ratio of separate AAV-producing and packaging components and to exert temporal control over expression of each transposon.

[00112] As described herein, suitably the nucleic acid encoding the E2A and E4Orf genes further comprises an internal ribosome entry site (IRES) element between the E2A and E4Orf genes.

[00113] As described herein, suitably the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

[00114] As described herein, suitably the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00115] In some embodiments, the mammalian cell used to produce adeno-associated virus (AAV) is stably expressing one or more nucleic acids encoding TetR and/or TetR-KRAB under control of a constitutive promoter.

[00116] As described herein, stable expression of a TetR and/or TetR-KRAB in a mammalian cell (prior to insertion of nucleic acids carrying the components of the virus, suitably via transposons) maximizes the repression of potentially cytotoxic AAV genes introduced by transfection and confers increased temporal control over the derepressible promoter elements.

[00117] In some embodiments, a KRAB repressor domain is fused in frame with the TetR.

[00118] As described herein, fusing a KRAB repressor domain in frame with the TetR ensures that "leaky" expression of the potentially cytotoxic AAV-packaging nucleic acids is less likely to occur. A KRAB repressor domain fused in frame with the TetR repressor domain provides an additional mechanism to accomplish high levels of repression of derepressible promoters.

[00119] In some embodiments, the mammalian cell produces a sufficient amounts of TetR. As used herein, a "sufficient" of TetR is defined as the level of amount and/or activity of a repressor (e.g., TetR) to stop expression and/or activity of a repressible and/or derepressible element (i.e., prior to addition of doxycycline).

[00120] As described herein, producing sufficient amounts of TetR is necessary for repression of the derepressible promoters under normal conditions (e.g., before addition of doxycycline). If sufficient amounts of TetR are not produced by the mammalian cell, the cell may prematurely transcribe and translate potentially cytotoxic AAV-packaging nucleic acids.

[00121] In further embodiments, the mammalian cell further comprises stable expression of one or more nucleic acids encoding chicken hypersensitive site-4 (cHS4) flanking the TetR and/or TetR-KRAB repressor sequences. As described herein, expression of cHS4 sequences flanking the TetR and/or TetR-KRAB repressor sequences prevent the silencing of TetR expression and further improve the stability of integrated repressors in the mammalian cell genome.

[00122] In further embodiments, the treating comprises treating with doxycycline to remove repression of derepressible promoters.

[00123] In further embodiments, each of the first, second and third nucleic acids are flanked by transposon-specific inverted terminal repeats (ITRs).

[00124] In further embodiments, a method for producing an adeno-associated virus (AAV) comprises stably transfecting a mammalian cell with: a nucleic acid encoding a TetR and/or TetR-KRAB repressor, chicken hypersensitive site-4 (cHS4) sequences flanking the TetR and/or TetR-KRAB repressor, and a selection gene; transfecting the stably transfected mammalian cell with a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA, under control of a first derepressible promoter, a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, and optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter; treating the mammalian cell with a binding partner of the TetR; activating the first, second and third derepressible promoters; producing the AAV; and harvesting the AAV.

[00125] In some embodiments, the mammalian cell further comprises a selection gene, such asa zeocin resistance gene. Additional selection genes include other antibiotic resistance genes, such as kanamycin and geneticin resistance genes. As described herein, expression of a zeocin resistance gene allows for efficient selection of correctly integrated repressors in the mammalian cell genome.

Additional Exemplary Embodiments

[00126] Embodiment 1 is a mammalian cell for producing an adeno-associated virus (AAV), comprising a nucleic acid molecule encoding a viral helper gene under control of a first derepressible promoter, a nucleic acid molecule encoding an AAV gene under control of a second derepressible promoter, and a nucleic acid molecule encoding a repressor element of the first and the second derepressible promoters.

[00127] Embodiment 2 includes the mammalian cell of embodiment 1, wherein the mammalian cell is a mammalian cell culture.

[00128] Embodiment 3 includes the mammalian cell of embodiment 2, wherein the mammalian cell culture is a suspension culture.

[00129] Embodiment 4 includes the mammalian cell of any one of embodiments 1-3, wherein the viral helper gene is an adenovirus helper gene.

[00130] Embodiment 5 includes the mammalian cell of embodiment 4, wherein the adenovirus helper gene comprises E2A and E4Orf6 genes.

[00131] Embodiment 6 includes the mammalian cell of embodiment 5, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[00132] Embodiment 7 includes the mammalian cell of any one of embodiments 1-6, wherein the AAV gene comprises Rep and Cap genes.

[00133] Embodiment 8 includes the mammalian cell of embodiment 7, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a third derepressible promoter.

[00134] Embodiment 9 includes the mammalian cell of embodiment 7, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a third derepressible promoter contained within an artificial intron.

[00135] Embodiment 10 includes the mammalian cell of any one of embodiments 8 or 9, wherein the Cap gene is under control of a native promoter.

[00136] Embodiment 11 includes the mammalian cell of any one of embodiments 1-10, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences (TetO2).

[00137] Embodiment 12 includes the mammalian cell of embodiment 11, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00138] Embodiment 13 includes the mammalian cell of any one of embodiments 1-12, wherein the repressor element is under control of a constitutive promoter.

[00139] Embodiment 14 includes the mammalian cell of any one of embodiments 1-13, wherein the repressor element is a tetracycline repressor protein.

[00140] Embodiment 15 includes the mammalian cell of embodiment 14, further comprising a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

[00141] Embodiment 16 includes the mammalian cell of any one of embodiments 1-15, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

[00142] Embodiment 17 includes the mammalian cell of any one of embodiments 1-15, wherein the mammalian cell is a human cell.

[00143] Embodiment 18 includes the mammalian cell of embodiment 17, wherein the human cell is a human embryonic kidney (HEK) cell.

[00144] Embodiment 19 includes the mammalian cell of any one of embodiments 1-18, further comprising a nucleic acid molecule including two inverted terminal repeat (ITR) sequences.

[00145] Embodiment 20 includes the mammalian cell of any one of embodiments 1-19, further comprising a nucleic acid molecule encoding a gene of interest.

[00146] Embodiment 21 includes the mammalian cell of any one of embodiments 1-20, further comprising a nucleic acid encoding a viral-associated, non-coding RNA under control of a fourth derepressible promoter.

[00147] Embodiment 22 is a mammalian cell for producing an adeno-associated virus (AAV), comprising a nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters.

[00148] Embodiment 23 includes mammalian cell of embodiment 22, wherein the mammalian cell is a mammalian cell culture.

[00149] Embodiment 24 includes the mammalian cell of embodiment 23, wherein the mammalian cell culture is a suspension culture.

[00150] Embodiment 25 includes the mammalian cell of any one of embodiments 22-24, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[00151] Embodiment 26 includes the mammalian cell of any one of embodiments 22-25, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter.

[00152] Embodiment 27 includes the mammalian cell of any one of embodiments 22-25, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter contained within an artificial intron.

[00153] Embodiment 28 includes the mammalian cell of any one of embodiments 26 or 27, wherein the Cap gene is under control of a native promoter.

[00154] Embodiment 29 includes the mammalian cell of any one of embodiments 22-28, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences (TetO2).

[00155] Embodiment 30 includes the mammalian cell of embodiment 29, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00156] Embodiment 31 includes the mammalian cell of any one of embodiments 22-30, wherein the repressor element is under control of a constitutive promoter.

[00157] Embodiment 32 includes the mammalian cell of any one of embodiments 22-31, wherein the repressor element is a tetracycline repressor protein.

[00158] Embodiment 33 includes the mammalian cell of embodiment 25, further comprising a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

[00159] Embodiment 34 includes the mammalian cell of any one of embodiments 22-33, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

[00160] Embodiment 35 includes the mammalian cell of any one of embodiments 22-33, wherein the mammalian cell is a human cell.

[00161] Embodiment 36 includes the mammalian cell embodiment 35, wherein the human cell is a human embryonic kidney (HEK) cell.

[00162] Embodiment 37 includes the mammalian cell of any one of embodiments 22-36, further comprising a nucleic acid molecule encoding a gene of interest.

[00163] Embodiment 38 is an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters.

[00164] Embodiment 39 includes the isolated nucleic acid of embodiment 38, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[00165] Embodiment 40 includes the isolated nucleic acid of any of embodiments 38-39, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter.

[00166] Embodiment 41 includes the isolated nucleic acid of any of embodiments 38-39 wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter contained within an artificial intron.

[00167] Embodiment 42 includes the isolated nucleic acid of any of embodiments 40 or 41, wherein the Cap gene is under control of a native promoter.

[00168] Embodiment 43 includes the isolated nucleic acid of any of embodiments 38-42, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences (TetO2).

[00169] Embodiment 44 includes the isolated nucleic acid of embodiment 43, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00170] Embodiment 45 includes the isolated nucleic acid of any of embodiments 38-44, wherein the repressor element is under control of a constitutive promoter.

[00171] Embodiment 46 includes the isolated nucleic acid of any of embodiments 38-45, wherein the repressor element is a tetracycline repressor protein.

[00172] Embodiment 47 includes the isolated nucleic acid of embodiment 46, further comprising a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

[00173] Embodiment 48 includes the isolated nucleic acid of any of embodiments 38-47, further comprising a gene of interest.

[00174] Embodiment 49 is a method of producing an adeno-associated virus (AAV) in a mammalian cell comprising transfecting the mammalian cell with an isolated nucleic acid molecule encoding an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences, and a repressor element of the first, second and third derepressible promoters, treating the mammalian cell with a binding partner of the repressor element, activating the first, second and third derepressible promoters, producing the AAV, and harvesting the AAV.

[00175] Embodiment 50 includes the method of embodiment 49, wherein the mammalian cell is a mammalian cell culture.

[00176] Embodiment 51 includes the method of embodiment 50, wherein the mammalian cell culture is a suspension culture.

[00177] Embodiment 52 includes the method of any of embodiments 49-51, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[00178] Embodiment 53 includes the method of any of embodiments 49-52, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter.

[00179] Embodiment 54 includes the method of any of embodiments 49-52, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter contained within an artificial intron.

[00180] Embodiment 55 includes the method of embodiment 54, wherein the fourth derepressible promoter is removed following the activating the derepressible promoters and prior to the producing the AAV.

[00181] Embodiment 56 includes the method of any of embodiments 53 or 54, wherein the Cap gene is under control of a native promoter.

[00182] Embodiment 57 includes the method of any of embodiments 49-56, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences (TetO2).

[00183] Embodiment 58 includes the method of embodiment 57, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00184] Embodiment 59 includes the method of any of embodiments 49-58, wherein the repressor element is under control of a constitutive promoter.

[00185] Embodiment 60 includes the method of any of embodiments 49-59, wherein the repressor element that is a tetracycline repressor protein.

[00186] Embodiment 61 includes the method of embodiment 60, wherein the nucleic acid further comprises a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

[00187] Embodiment 62 includes the method of any of embodiments 60 or 61, wherein the treating comprises treating with doxycycline.

[00188] Embodiment 63 includes the method of any of embodiments 49-62, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

[00189] Embodiment 64 includes the method of any of embodiments 49-63, wherein the mammalian cell is a human cell.

[00190] Embodiment 65 includes the method of embodiment 64, wherein the human cell is a human embryonic kidney (HEK) cell.

[00191] Embodiment 66 includes the method of any of embodiments 49-65, wherein the AAV comprises a nucleic acid molecule encoding a gene of interest.

[00192] Embodiment 67 includes the method of embodiment 66, wherein the AAV comprises a gene of therapeutic interest.

[00193] Embodiment 68 is a method of treatment with an adeno-associated virus (AAV) comprising transfecting the mammalian cell with an isolated nucleic acid molecule encoding, an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter, an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter, a viral-associated, non-coding RNA under control of a third depressible promoter, two inverted terminal repeat (ITR) sequences; and a repressor element of the first, second and third derepressible promoters; treating the mammalian cell with a binding partner of the repressor element; activating the first, second and third derepressible promoters; producing the AAV; harvesting the AAV; and administering the AAV to a mammalian patient.

[00194] Embodiment 69 includes the method of embodiment 68, wherein the mammalian cell is a mammalian cell culture.

[00195] Embodiment 70 includes the method of embodiment 69, wherein the mammalian cell culture is a suspension culture.

[00196] Embodiment 71 includes the method of any of embodiments 68-70, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[00197] Embodiment 72 includes the method of any of embodiments 68-71, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter.

[00198] Embodiment 73 includes the method of any of embodiments 68-72, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter contained within an artificial intron.

[00199] Embodiment 74 includes the method of embodiment 73, wherein the fourth derepressible promoter is removed following the activating the derepressible promoters and prior to the producing the AAV.

[00200] Embodiment 75 includes the method of any of embodiments 73 or 74, wherein the *Cap* gene is under control of a native promoter.

[00201] Embodiment 76 includes the method of any of embodiments 68-75, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

[00202] Embodiment 77 includes the method of embodiment 76, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00203] Embodiment 78 includes the method of any of embodiments 68-77, wherein the repressor element is under control of a constitutive promoter.

[00204] Embodiment 79 includes the method of any of embodiments 68-78, wherein the repressor element that is a tetracycline repressor protein.

[00205] Embodiment 80 includes the method of embodiment 79, wherein the nucleic acid further comprises a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

[00206] Embodiment 81 includes the method of any of embodiments 79 or 80, wherein the treating comprises treating with doxycycline.

[00207] Embodiment 82 includes the method of any of embodiments 68-81, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

[00208] Embodiment 83 includes the method of any of embodiments 68-82, wherein the mammalian cell is a human cell.

[00209] Embodiment 84 includes the method of embodiment 83, wherein the human cell is a human embryonic kidney (HEK) cell.

[00210] Embodiment 85 includes the method of any of embodiments 68-84, wherein the AAV comprises a nucleic acid molecule encoding a gene of interest.

[00211] Embodiment 86 includes the method of embodiment 87, wherein the AAV comprises a gene of therapeutic interest.

[00212] Embodiment 87 includes the method of any of embodiments 68-86, wherein the administering comprises inhalation, injection or intravenous administration.

[00213] Embodiment 88 is a method of producing an adeno-associated (AAV) virus, comprising: transfecting a mammalian cell stably expressing one or more nucleic acids encoding TetR and/or TetR with a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA under control of a first derepressible promoter, a second nucleic acid encoding an AAV gene comprising *Rep* and *Cap* genes under control of a second derepressible promoter, and, optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter; treating the mammalian cell with a binding partner of the TetR and/or TetR-KRAB; activating the first, second and third derepressible promoters; producing the AAV; and harvesting the AAV.

[00214] Embodiment 89 includes the method of embodiment 88, wherein the mammalian cell is a mammalian cell culture.

[00215] Embodiment 90 includes the method of any of embodiments 88-89, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

[00216] Embodiment 91 includes the method of any of embodiments 88-90, wherein the mammalian cell is a human cell.

[00217] Embodiment 92 includes the method of embodiment 91, wherein the human cell is a human embryonic kidney (HEK) cell.

[00218] Embodiment 93 includes the method of embodiment 89, wherein the mammalian cell culture is a suspension culture.

[00219] Embodiment 94 includes the method of any of embodiments 88-90, wherein the mammalian cell further comprises an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

[00220] Embodiment 95 includes the method of any of embodiments 88-91, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

[00221] Embodiment 96 includes the method of embodiment 92, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

[00222] Embodiment 97 includes the method of any of embodiments 88-96, wherein the stably expressed TetR and/or TetR-KRAB is under control of a constitutive promoter.

[00223] Embodiment 98 includes the method of embodiment 97, wherein the nucleic acid encoding the TetR-KRAB comprises KRAB fused in frame with the TetR.

[00224] Embodiment 99 includes the method of any one of embodiments 88-98, wherein the treating comprises treating with doxycycline.

[00225] Embodiment 100 includes the method of any one of embodiments 88-99, wherein each of the first, second and third nucleic acids are flanked by transposon-specific inverted terminal repeats (ITRs).

[00226] Embodiment 101 includes the method of any of embodiments 88-100, wherein the mammalian cell expresses sufficient amounts of TetR and/or TetR-KRAB.

[00227] Embodiment 102 includes the method of any of embodiments 88-101, wherein the AAV comprises a gene of therapeutic interest.

[00228] Embodiment 103 includes the method of any of embodiments 88-102, wherein the mammalian cell further comprises stable expression of one or more nucleic acids encoding chicken hypersensitive site-4 (cHS4) flanking the TetR and/or TetR-KRAB repressor sequences.

[00229] Embodiment 104 includes the method of any of embodiments 88-103, wherein the mammalian cell further comprises a zeocin resistance gene.

[00230] Embodiment 105 is a method for producing an adeno-associated virus (AAV), comprising: stably transfecting a mammalian cell with: a nucleic acid encoding a TetR and/or TetR-KRAB repressor; chicken hypersensitive site-4 (cHS4) sequences flanking the TetR and/or TetR-KRAB repressor; and a selection gene; transfecting the stably transfected mammalian cell with: a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA, under control of a first derepressible promoter; a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter; and optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter; treating the stably transfected mammalian cell with a binding partner of the TetR; activating the first, second and third derepressible promoters; producing the AAV; and harvesting the AAV.

[00231] Embodiment 106 includes the method of embodiment 105, wherein the stably transfected mammalian cell produces a sufficient amounts of TetR.

[00232] Embodiment 107 includes the method of any of embodiments 106 and 107, wherein the KRAB repressor domain is fused in frame with the TetR.

EXAMPLES

Example 1: Design and Validation of Derepressible Helper Genes

[00233] To drive the expression of E2A and E4Orf6 genes, a derepressible promoter from the pcDNA4/TO vector (INVITROGEN) was utilized. This promoter includes the complete CMV promoter with an insertion of two Tetracycline operator sequences ($TetO_2$) between the TATA box and transcriptional start site (TSS). In the presence of tetracycline repressor protein (TetR), transcription initiation was blocked by the binding of TetR onto the $TetO_2$ sites. When Tetracycline or Doxycycline is added into the medium, it binds to TetR and changes its conformation. This leads to the release of TetR and derepression/activation of the CMV promoter and results in induced gene expression (FIG 1 showing OFF and ON conformations of TetR/$TetO_2$ derepressible promoter system).

[00234] To simplify the design and improve the stability, an Internal Ribosome Entry Site (IRES) element was used to initiate the translation of E4Orf6 after E2A in a single expression cassette, driven by a single inducible CMV promoter (FIG. 1).

[00235] To induce the VA I non-coding RNA, an H1 promoter with $TetO_2$ insertion was applied (*see, e.g.*, Wiederschain *et al.*, "Single-vector inducible lentiviral RNAi system for oncology target validation, *Cell Cycle 8*:498-504 (2009)). Similarly, the addition of Doxycycline releases TetR and turns on VA I expression (FIG. 1).

[00236] As mentioned, a TetR gene expression cassette is included for the control of the derepressible promoters (*see* FIG. 1). A constitutive human PGK promoter is used to drive the expression of TetR, followed by an IRES that directs the expression of puromycin N-acetyltransferase used to select the transposon integrated cells (*see* FIG. 2A).

[00237] To reduce the potential leaky expression from the derepressible promoters, an enhanced version of TetR was also included (*see, e.g.*, Szulc *et al.*, "A versatile tool for conditional gene expression and knockdown," *Nature Methods 3:*109-116 (2006)). Briefly, a strong repressive domain of KRAB was fused in-frame to the C-terminal of original TetR, which improves its repressive activity and minimizes basal gene expression before induction. An SV40 Nuclear

Localization Signal (NLS) was inserted as well to facilitate the nuclear entry of the larger TetR-KRAB fusion protein (FIG. 2A, FIGS. 12A-12B).

[00238] The sequence of the pcDNA3.1-E2A-E4-VA-TetR vector shown in FIG. 2A is provided below:

[00239] iHelper 1/ pcDNA3.1-E2A-E4-VA-TetR (11, 986 bp)

[00240] GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATC
TGCTCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCG
CTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAAT
TGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCC
AGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGG
TCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTT
CCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGG
TAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATT
GACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGG
GACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGC
GGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAA
GTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACT
TTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTAC
GGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACT
GGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAA
ACTTAAGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCCTGCTT
CGCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAG
TAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAA
CTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCA
ATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGG
GTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCA
AGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAG
TACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTA

TTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACT
CACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGAACC
AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATG
GGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCCCTATCAGTGAT
AGAGATCTCCCTATCAGTGATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTCAG
ATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCG
ATCCAGCCTCCGGACTCTAGCGTTTAAACTTAAGCTTGCCACCatggccagtcgggaagaggagc
agcgcgaaaccacccccgagcgcggacgcggtgcggcgcgacgtccccccaaccatggaggacgtgtcgtccccgtccccgtcgccgc
cgcctccccgggcgcccccaaaaaagcggatgaggcggcgtatcgagtccgaggacgaggaagactcatcacaagacgcgctggtgc
cgcgcacacccagcccgcggccatcgacctcggcggcggatttggccattgcgcccaagaagaaaaagaagcgcccttctcccaagcc
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ccggtgctaatcaagcatggcaaaggaggtaagcgcacagtgcggcggctgaatgaagacgacccagtggcgcgtggtatgcggacgc
aagaggaagaggaagagcccagcgaagcggaaagtgaaattacggtgatgaacccgctgagtgtgccgatcgtgtctgcgtgggagaa
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aagctctggcggccgtatgcaagacctggctgaacgaggagcaccgcgggttgcagctgaccttcaccagcaacaagacctttgtgacga
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aacaccgacgcaaggtgctgcgtgcacgacgcggcctgtccggccaatcagttttccggcaagtcttgcggcatgttcttctctgaaggcg
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C (SEQ ID NO:1)

[00241] The sequence of the pcDNA3.1-E2A-E4-VA-TetR-V2 vector shown in FIG. 2B is provided below:

[00242] iHelper2/ pcDNA3.1-E2A-E4-VA-TetR-V2 (11, 641 bp)

[00243] GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATC
TGCTCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCG
CTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAAT
TGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCC
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GACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGC
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CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTC
GGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCG
GTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGT
TCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAG
AATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAG
GAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA
GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA

GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCC
GCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGC
TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTG
CACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAG
TCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT
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ACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCT
TCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT
TTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCT
TTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATT
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AGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGC
TTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCT
GACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTG
CTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACC
AGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATC
CAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTG
CGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATG
GCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTG
TGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCC
GCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGT
GTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCAC
ATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCT
CAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACT
GATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGC
AAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTC
TTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACA
TATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAA
AAGTGCCACCTGACGTC (SEQ ID NO:2)

[00244] The entire transfer plasmid including the derepressible Helper (piHelper1) and TetR expression cassettes was tested for use in AAV production by transient transfection. As shown in FIG. 3, when the plasmids were co-transfected with control pRep-Cap and pAAV-GOI plasmids, the addition of Dox activated the production of AAV to the level of approximately 30% of control Helper vectors that supports constitutive helper gene expression. Therefore, the derepressible helper construct was functional for AAV production upon induction.

Example 2: Design and Validation of Derepressible Rep-Cap Genes

[00245] Challenges for producing Rep proteins are two-fold. First, the ratio of Rep78 and Rep52 needs to be maintained during induction for high titer AAV production. Second, the p19 promoter required for Rep52 expression is located inside the coding region of Rep78, which creates challenges for including a derepressible promoter. To overcome these challenges, two strategies were developed (FIGS. 4A-4C).

[00246] First, to retain the natural regulation of Rep gene expression (*see* FIG. 4A), the original viral promoters were retained but modified by inserting two TetO sites surround the TATA box and TSS. Two copies of TetO sites was inserted into the upstream truncated p5 promoter with core elements including TATA box, Rep Binding Element (RBE), and YY1 site. In addition, a wildtype copy of p5 promoter, serving as an enhancer, was placed downstream of cap gene to support both Rep and Cap expression (FIG. 5) (*See, e.g.*, U.S. Patent No. 5,622,856).

[00247] The sequences for the derepressible p5 promoters set forth in FIG. 5 are as follows

min-p5-i1

[00248] TATTTAATCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGATC GCCCGAGTGAGCACGCAGGGTCTCCATTTTGAAGCGGGAGGTTTGAACGCGCAGCC GCC (SEQ ID NO: 3)

min-p5-i2

[00249] TATTTAAtcTCCCTATCAGTGATAGAGAtcGCCCGAGTGAGCACGCAGGGTC TCCATTTTGATCCCTATCAGTGATAGAGAAGCGGGAGGTTTGAACGCGCAGCCGCC (SEQ ID NO:4)

min-p5-i3

[00250] TCCCTATCAGTGATAGAGAtcTATTTAAGCCCGAGTGAGCACGCAGTCCCT ATCAGTGATAGAGAGGTCTCCATTTTGAAGCGGGAGGTTTGAACGCGCAGCCGCC (SEQ ID NO:5)

[00251] Similarly, two copies of TetO sites were also inserted into the p19 promoter next to TSS site (FIG. 6). In order to minimize the interference of such insertion and maximize the original activity of these viral promoters, three ways of insertion of TetO sites were designed for each promoter for best performance. Thus, a total of 9 variations were examined (iRepCap1 to iRepCap9).

[00252] The sequences for the derepressible promoters including p19 illustrated schematically in FIG. 6 are provided below:

p19-i1

[00253] ccagaaatggcgccggaggcgggaacaaggtggtggatgagtgctacatccccaattacttgctccccaaaacccagcc tgagctccagtgggcgtggactaatatggaacagtatttaagcgcctgTCCCTATCAGTGATAGAGATCTCCCTAT CAGTGATAGAGAtttgaatctcacggag (SEQ ID NO:6)

p19-i2

[00254] ccagaaatggcgccggaggcgggaacaaggtggtggatgagtgctacatccccaattacttgctccccaaaacccagcc tgagTCCCTATCAGTGATAGAGActccagtgggcgtggactaatatggaacagtatttaagcgcctgTCCCTATC AGTGATAGAGAtttgaatctcacggag (SEQ ID NO:7)

p19-i3

[00255] ccagaaatggcgccggaggcgggaacaaggtggtggatgagtgctacatccccaattacttgctccccaaaacccagcc tgagctccagtgggcgtggactaatatggaaTCCCTATCAGTGATAGAGAcagtatttaagcgcctgTCCCTATC AGTGATAGAGAtttgaatctcacggag (SEQ ID NO:8)

[00256] Two methods were developed for the placement of the derepressible p19 promoters. In the first method, a separate expression cassette for Rep52 was generated, driven by the

derepressible p19 promoters. In the first method shown in FIG. 4B, the original p19 promoter in the Rep78 ORF was silenced by changing six nucleotides in three core regulatory elements required for p19 activity (SP1, TATA-1, and TATA-2 sites). These changes did not alter the Rep78 protein sequence. Rep78 was controlled by a derepressible p5 promoter.

[00257] In a second method, an artificial intron was created for the insertion of TetO sites in the Rep78 ORF (FIG. 4C). The chimeric intron between introns from human β-globin and immunoglobulin heavy chain genes was adopted by replacing the non-essential internal sequence with the TetO2 sites (FIG. 7A). The new artificial intron was inserted 1bp or 25bp downstream of TATA-2 of p19 promoter in situ (iRepCap-10 and iRepCap11) (FIG. 4B and FIG. 7B). The new p19 promoter with adjacent TetO-containing intron ensured the repression of Rep52 gene expression before induction, while still allowing for the expression of the Rep78 protein after the removal of the intron during mRNA splicing. The efficiency of splicing is evaluated by PCR analysis for the cDNA.

[00258] The nucleic acid sequences for the intron-based p19 promoters illustrated in FIGS. 7A-7B are provided below.

In-p19-i1

[00259] ccagaaatggcgccggaggcgggaacaaggtggtggatgagtgctacatccccaattacttgctccccaaaacccagcc tgagctccagtgggcgtggactaatatggaacagtatttaaggtaagtTCCCTATCAGTGATAGAGATCTCCCTAT CAGTGATAGAGAtactgacatccactttgcctttctctccacagcgcctgtttgaatctcacggag (SEQ ID NO:9)

In-p19-i2

[00260] ccagaaatggcgccggaggcgggaacaaggtggtggatgagtgctacatccccaattacttgctccccaaaacccagcc tgagctccagtgggcgtggactaatatggaacagtatttaagcgcctgtttgaatctcacggaaaggtaagtTCCCTATCAGTGA TAGAGATCTCCCTATCAGTGATAGAGAtactgacatccactttgcctttctctccacag (SEQ ID NO:10)

[00261] To test the activity of the 11 inducible Rep-Cap designs, HEK293 cells were transfected with one of the vectors plus standard pHelper and pAAV-GFP for AAV production. Three days after transfection, cells were harvested for Rep-Cap protein expression and AAV titer analysis. Western blot analysis revealed various expression levels of Rep and Cap proteins; many

maintained similar ratios of Rep78 vs Rep52 as control RepCap vector (FIG. 8A). qPCR analysis of the AAV titer showed that the designs performed similarly or had higher titers compared to control triple transfection (FIG. 8B).

[00262] To test the performance of both inducible helpers and inducible Rep-Cap designs together, HEK293 cells were transfected with selected iRepCap vectors, iHelper1/2, and pAAV-GFP, and left untreated or treated with Doxycycline for three days. As shown in FIG. 9A, the protein expression of Rep and Cap was only induced by the addition of Dox, and the derepression of the derepressible promoters. Accordingly, the AAV titer was significantly increased over 10 to 25 fold upon derepression (FIG. 9B).

[00263] To stably integrate the iHelper and iRepCap, iHelper1/2 and iRepCap10 were chosen with AAV-GFP to assemble the functional cassettes into a single PIGGYBAC™ transposon transfer vector PB007 (Transposagen, Inc., Lexington, KY). FIGS. 10A-10D show the plasmid constructs used for integration into mammalian cells, suitably HEK293 cells. To facilitate the future addition of specific AAV genes of interest, the AAV-GFP was not included in some of the transfer vectors (FIGS. 10A and 10B). HEK293 cells will be transfected with both the transfer vectors and transposase mRNA and the integrated cell pool will be enriched by puromycin selection. The single cell clones will be isolated and screened for AAV production with and without Dox treatment to activate derepression.

[00264] The nucleic acid sequence of the vector illustrated in FIG. 10 A is provided below:

PB007-iHelper1-iRepCap10/ PBBG7 (18, 281 bp)

[00265] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC
ATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG
TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATC
CCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAAC
AAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA
TCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAG
GTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC

GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG
GCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCC
GCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTG
TAAAACGACGGCCAGTGAGCGCGCCTCGTTCATTCACGTTTTTGAACCCGTGGAGGA
CGGGCAGACTCGCGGTGCAAATGTGTTTTACAGCGTGATGGAGCAGATGAAGATGC
TCGACACGCTGCAGAACACGCAGCTAGATTAACCCTAGAAAGATAATCATATTGTG
ACGTACGTTAAAGATAATCATGCGTAAAATTGACGCATGTGTTTTATCGGTCTGTAT
ATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACA
TACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAA
CAAAAACTCAAAATTTCTTCTATAAAGTAACAAAACTTTTATCGAATTCCTGCAGCC
CGGGGGATCCACTAGTTCTAGAGGGACAGCCCCCCCCCAAAGCCCCCAGGGATGTA
ATTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGT
CCGGCGCTCCCCCCGCATCCCCGAGCCGGCAGCGTGCGGGGACAGCCCGGGCACGG
GGAAGGTGGCACGGGATCGCTTTCCTCTGAACGCTTCTCGCTGCTCTTTGAGCCTGC
AGACACCTGGGGGGATACGGGGAAAAGGCCTCCAAGGCCAGCTTCCCACAATAAGT
TGGGTGAATTTTGGCTCATTCCTCCTTTCTATAGGATTGAGGTCAGAGCTTTGTGATG
GGAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCgcGATCgcTAGcGTTT
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TTCGCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAAT
AGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACAT
AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGT
CAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAAT
GGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGC
CAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCC
AGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGC
TATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGA
CTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGAA
CCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAAT
GGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCCCTATCAGTG

ATAGAGATCTCCCTATCAGTGATAGAGATCGTCGACGAGCTCGTTTAGTGAACCGTC
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CGATCCAGCCTCCGGACTCTAGCGTTTAAACTTAAGCTTGCCACCatggccagtcgggaagagg
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ID NO:11)

[00266] The sequence of the vector illustrated in FIG. 10B is provided below.

PB007-iHelper2-iRepCap10/ PBBG8 (17, 936 bp)

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CAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATG
TTGAATACTCAT (SEQ ID NO:12)

[00268] The sequence of the vector represented in FIG. 10C is provided below.

PB007-iHelper1-iRepCap10-AAV-GFP/ PBBG9 (21, 391 bp)

[00269] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
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ACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAA
TAAGGGCGACACGGAAATGTTGAATACTCAT (SEQ ID NO:13)

[00270] The sequence of the vector illustrated in FIG. 10D is provided below.

PB007-iHelper2-iRepCap10-AAV-GFP/ PBBG10 (21, 046 bp)

[00271] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
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CGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCAT (SEQ ID NO:14)

[00272] Sequences of additional vectors for use in the practice of the present invention:

[00273] iRepCap1/pKan-Anc80-RepCap-p5i1-p19i1 (10,497 bp)

[00274] cctcgaggGCTAGCcattcTATTTAAtcTCCCTATCAGTGATAGAGATCTCCCTATC
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[00293] iRepCap11/ pKan-Anc80-intron-inducibleRepCap-d2 (7,567 bp)

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[00295] The sequence for the PBBG-iHelper-Puro construct illustrated in in FIG. 11A is shown below:

[00296] PBBG-iHelper-Puro (11,801 bp)

[00297] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC ATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATC CCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAAC AAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA TCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAG GTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG GCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCC GCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGT TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTG TAAAACGACGGCCAGTGAGCGCGCCTCGTTCATTCACGTTTTTGAACCCGTGGAGGA CGGGCAGACTCGCGGTGCAAATGTGTTTTACAGCGTGATGGAGCAGATGAAGATGC TCGACACGCTGCAGAACACGCAGCTAGATTAACCCTAGAAAGATAATCATATTGTG ACGTACGTTAAAGATAATCATGCGTAAAATTGACGCATGTGTTTTATCGGTCTGTAT ATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACA TACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAA CAAAAACTCAAAATTTCTTCTATAAAGTAACAAAACTTTTATCGAATTCCTGCAGCC CGGGGGATCCACTAGTTCTAGAGGGACAGCCCCCCCCCAAAGCCCCCAGGGATGTA ATTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGT

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AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGT
CAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAAT
GGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGC
CAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCC
AGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGC
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GCTCGGGGATGCGGGGGGAGCGCCGGACCGGAGCGGAGCCCCGGGCGGCTCGCTG
CTGCCCCCTAGCGGGGGAGGGACGTAATTACATCCCTGGGGGCTTTGGGGGGGGGC
TGTCCCTCTAGGGGATCCTCTAGGGCCTCTGAGCTATTCCAGAAGTAGTGAAGAGGC
TTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTCCGGATCGATCGAGCGGATCCAGCA
CAGTGGCGGCCGCaatatttgcatgtcgctatgtgttctgggaaatcaccataaacgtgaaatccctatcagtgatagagacttata
agttccctatcagtgatagagaaccggtgggcactcttccgtggtctggtggataaattcgcaagggtatcatggcggacgaccggggttcg
agccccgtatccggccgtccgccgtgatccatgcggttaccgcccgcgtgtcgaacccaggtgtgcgacgtcagacaacgggggagtgc
tccttttgaattccactttggccgcggctcgagTGAGCTATTCCAGAAGTAGTGAAGAGGCTTTTTTGGA
GGCCTAGGCTTTTGCAAAAAGCTCCGGATCGATGCCCGGGGGATCCACTAGTTCTAG
AGGGACAGCCCCCCCCCAAAGCCCCCAGGGATGTAATTACGTCCCTCCCCCGCTAG
GGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGTCCGGCGCTCCCCCCGCATCC
CCGAGCCGGCAGCGTGCGGGGACAGCCCGGGCACGGGGAAGGTGGCACGGGATCG
CTTTCCTCTGAACGCTTCTCGCTGCTCTTTGAGCCTGCAGACACCTGGGGGGATACG
GGGAAAAGGCCTCCAAGGCCAGCTTCCCACAATAAGTTGGGTGAATTTTGGCTGAG
CTATTCCAGAAGTAGTGAAGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTC
CGGATCGATCATATATGGCAGATATACGCGTTGACATTGATTATTGACTAGTTATTA
ATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTAC
ATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGAC
GTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATAT
GCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGC
CCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATC
GCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTT
GACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGG
CACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCA
AATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAA
CTATCGTCGACGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACG
CTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGGACTCTAGCGTT
TAAACTTAAGCTTGCCACCatgaccgagtacaagcccacggtgcgcctcgccacccgcgacgacgtccccagggccgt
acgcaccctcgccgccgcgttcgccgactaccccgccacgcgccacaccgtcgatccggaccgccacatcgagcgggtcaccgagctg

caagaactcttcctcacgcgcgtcgggctcgacatcggcaaggtgtgggtcgcggacgacggcgccgcggtggcggtctggaccacgc
cggagagcgtcgaagcgggggcggtgttcgccgagatcggcccgcgcatggccgagttgagcggttcccggctggccgcgcagcaac
agatggaaggcctcctggcgccgcaccggcccaaggagcccgcgtggttcctggccaccgtcggcgtctcgcccgaccaccagggca
agggtctgggcagcgccgtcgtgctccccggagtggaggcggccgagcgcgccggggtgcccgccttcctggagacctccgcgcccc
gcaacctccccttctacgagcggctcggcttcaccgtcaccgccgacgtcgaggtgcccgaaggaccgcgcacctggtgcatgacccgc
aagcccggtgcctgaAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGTTTA
TTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAG
CATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCAT
GTCTGTAGCtGATgtATAcCTAggATCCGGCCGGccTGCAggTGTCCTCACAGGAACGAA
GTCCCTAAAGAAACAGTGGCAGCCAGGTTTAGCCCCGGAATTGACTGGATTCCTTTT
TTAGGGCCCATTGGTATGGCTTTTTCCCCGTATCCCCCCAGGTGTCTGCAGGCTCAAA
GAGCAGCGAGAAGCGTTCAGAGGAAAGCGATCCCGTGCCACCTTCCCCGTGCCCGG
GCTGTCCCCGCACGCTGCCGGCTCGGGGATGCGGGGGGAGCGCCGGACCGGAGCGG
AGCCCCGGGCGGCTCGCTGCTGCCCCCTAGCGGGGGAGGGACGTAATTACATCCCT
GGGGGCTTTGGGGGGGGGCTGTCCCTCTAGAGCGGCCGCCACCGCGGTGGAGCTCC
AGCTTTTGTTCCCTTTAGTGAGGGTTAATTAGATCTTAATACGACTCACTATAGGGCG
AATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCTATA
ACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATA
TAATTATCGTATGAGTTAAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCATTT
TGACTCACGCGGTCGTTATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGCAC
GCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATT
CGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCA
GACTATCTTTCTAGGGTTAATCTAGCTGCATCAGGATCATATCGTCGGGTCTTTTTTC
CGGCTCAGTCATCGCCCAAGCTGGCGCTATCTGGGCATCGGGGAGGAAGAAGCCCG
TGCCTTTTCCCGCGAGGTTGAAGCGGCATGGAAAGAGTTTGCCGAGGATGACTGCTG
CTGCATTGACGTTGAGCGAAAACGCACGTTTACCATGATGATTCGGGAAGGTGTGGC
CATGCACGCCTTTAACGGTGAACTGTTCGTTCAGGCCACCTGGGATACCAGTTCGTC
GCGGCTTTTCCGGACACAGTTCCGGATGGTCAGCCCGAAGCGCATCAGCAACCCGA
ACAATACCGGCGACAGCCGGAACTGCCGTGCCGGTGTGCAGATTAATGACAGCGGT
GCGGCGCTGGGATATTACGTCAGCGAGGACGGGTATCCTGGCTGGATGCCGCAGAA
ATGGACATGGATACCCCGTGAGTTACCCGGCGGGCGCGCTTGGCGTAATCATGGTCA

TAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCC
GGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAAT
TGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTA
ATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTC
CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA
CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACA
TGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGC
GTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA
GAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCT
CCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT
CCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGT
GTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCG
CTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC
GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTG
CTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGAT
CCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTA
CGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACG
CTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGG
ATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATAT
ATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG
CGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTAC
GATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCAC
GCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGC
AGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACA
GGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAAC
GATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGG
CAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGG
TGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG

CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCA
TCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT
CCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATA
AGGGCGACACGGAAATGTTGAATACTCAT (SEQ ID NO: 26)

[00298] The sequence for the PBBG-ITRGFP construct illustrated in in FIG. 11B is shown below:

[00299] PBBG-ITRGFP (7,798 bp)

[00300] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC
ATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG
TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATC
CCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAAC
AAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA
TCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAG
GTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC
GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG
GCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCC
GCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTG
TAAAACGACGGCCAGTGAGCGCGCCTCGTTCATTCACGTTTTTGAACCCGTGGAGGA
CGGGCAGACTCGCGGTGCAAATGTGTTTTACAGCGTGATGGAGCAGATGAAGATGC
TCGACACGCTGCAGAACACGCAGCTAGATTAACCCTAGAAAGATAATCATATTGTG
ACGTACGTTAAAGATAATCATGCGTAAAATTGACGCATGTGTTTTATCGGTCTGTAT
ATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACA
TACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAA
CAAAAACTCAAAATTTCTTCTATAAAGTAACAAAACTTTTATCGAATTCCTGCAGCC
CGGGGGATCCACTAGTTCTAGAGGGACAGCCCCCCCCCAAAGCCCCCAGGGATGTA

ATTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGT
CCGGCGCTCCCCCCGCATCCCCGAGCCGGCAGCGTGCGGGGACAGCCCGGGCACGG
GGAAGGTGGCACGGGATCGCTTTCCTCTGAACGCTTCTCGCTGCTCTTTGAGCCTGC
AGACACCTGGGGGGATACGGGGAAAAGGCCTCCAAGGCCAGCTTCCCACAATAAGT
TGGGTGAATTTTGGCTCATTCCTCCTTTCTATAGGATTGAGGTCAGAGCTTTGTGATG
GGAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCgcGATCgcTAGcAAA
CGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATG
TCCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGG
GCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGG
GAGTGGCCAACTCCATCACTAGGGGTTCCTGCGGCCGCACGCGTGGAGCTAGTTATT
AATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTA
CATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGA
CGTCAATAATGACGTATGTTCCCATAGTAACGTCAATAGGGACTTTCCATTGACGTC
AATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATA
TGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATG
CCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCAT
CGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTT
TGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTG
CACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCA
AATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGA
ACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACC
GGGACCGATCCAGCCTCCGCGGATTCGAATCCCGGCCGGGAACGGTGCATTGGAAC
GCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCA
CAAAAAATGCTTTCTTCTTTTAATATACTTTTTTGTTTATCTTATTTCTAATACTTTCC
CTAATCTCTTTCTTTCAGGGCAATAATGATACAATGTATCATGCCTCTTTGCACCATT
CTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAATATTTCTGCATATA
AATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCAG
CTACAATCCAGCTACCATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATTATTC
TGAGTCCAAGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACA
GCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTGGG
ATTCGAACATCGATTGAATTCTGAATGGTGAGCAAGGGCGAGGAGCTGTTCACCGG

GGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCG
TGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATC
TGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTAC
GGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAG
TCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGG
CAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCA
TCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTG
GAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGG
CATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCG
CCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGAC
AACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGA
TCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGA
GCTGTACAAGTACTCAGATCTCGAGCTCAAGTAGGGATCCTCTAGAGTCGACCTGCA
GAAGCTTGCCTCGAGCAGCGCTGCTCGAGAGATCTACGGGTGGCATCCCTGTGACCC
CTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGT
CCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATG
GGGTGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGG
CCTGCGGGGTCTATTGGGAACCAAGCTGGAGTGCAGTGGCACAATCTTGGCTCACTG
CAATCTCCGCCTCCTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTTGTTGGG
ATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTTTGGTAGAGACGGGG
TTTCACCATATTGGCCAGGCTGGTCTCCAACTCCTAATCTCAGGTGATCTACCCACCT
TGGCCTCCCAAATTGCTGGGATTACAGGCGTGAACCACTGCTCCCTTCCCTGTCCTTC
TGATTTTGTAGGTAACCACGTGCGGACCGAGCGGCCGCAGGAACCCCTAGTGATGG
AGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGG
TCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGC
TGCCTGCAGGGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCAC
ACCGCATACGTCgtaGCtGATcaATTgGCGCGCCGAATTCGTTAACAAGCTtTAATTAaCG
CgtATAcCTAggATCCGGCCGGccTGCAggTGTCCTCACAGGAACGAAGTCCCTAAAGA
AACAGTGGCAGCCAGGTTTAGCCCCGGAATTGACTGGATTCCTTTTTTAGGGCCCAT
TGGTATGGCTTTTTCCCCGTATCCCCCCAGGTGTCTGCAGGCTCAAAGAGCAGCGAG
AAGCGTTCAGAGGAAAGCGATCCCGTGCCACCTTCCCCGTGCCCGGGCTGTCCCCGC

ACGCTGCCGGCTCGGGGATGCGGGGGGAGCGCCGGACCGGAGCGGAGCCCCGGGC
GGCTCGCTGCTGCCCCCTAGCGGGGGAGGGACGTAATTACATCCCTGGGGGCTTTGG
GGGGGGGCTGTCCCTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCC
CTTTAGTGAGGGTTAATTAGATCTTAATACGACTCACTATAGGGCGAATTGGGTACC
GGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCTATAACAAGAAAAT
ATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTA
TGAGTTAAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCATTTTGACTCACGCG
GTCGTTATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCACGGGA
GCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTATTTAG
AAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCT
AGGGTTAATCTAGCTGCATCAGGATCATATCGTCGGGTCTTTTTTCCGGCTCAGTCAT
CGCCCAAGCTGGCGCTATCTGGGCATCGGGGAGGAAGAAGCCCGTGCCTTTTCCCG
CGAGGTTGAAGCGGCATGGAAAGAGTTTGCCGAGGATGACTGCTGCTGCATTGACG
TTGAGCGAAAACGCACGTTTACCATGATGATTCGGGAAGGTGTGGCCATGCACGCCT
TTAACGGTGAACTGTTCGTTCAGGCCACCTGGGATACCAGTTCGTCGCGGCTTTTCC
GGACACAGTTCCGGATGGTCAGCCCGAAGCGCATCAGCAACCCGAACAATACCGGC
GACAGCCGGAACTGCCGTGCCGGTGTGCAGATTAATGACAGCGGTGCGGCGCTGGG
ATATTACGTCAGCGAGGACGGGTATCCTGGCTGGATGCCGCAGAAATGGACATGGA
TACCCCGTGAGTTACCCGGCGGGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCC
TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAA
GTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC
ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCA
ACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGA
CTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT
AATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAA
GGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAG
GCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAA
ACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCT
CTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAG
CGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGC
TCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCC

GGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCA
GCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTT
GAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCT
GCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAA
CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA
AAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACG
AAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGA
TCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTT
GGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTAT
TTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGG
GCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTC
CAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCT
GCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGT
AGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTG
TCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
GTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATC
GTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCAT
AATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAA
CCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAA
TACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAA
CGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATG
TAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTG
GGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACG
GAAATGTTGAATACTCAT (SEQ ID NO: 27)

[00301] The sequence for the PBBG-iRC8 construct illustrated in in FIG. 11C is shown below:

[00302] PBBG-iRC8 (9,399 bp)

[00303] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC
ATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG

TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATC
CCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAAC
AAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA
TCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAG
GTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC
GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG
GCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCC
GCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTG
TAAAACGACGGCCAGTGAGCGCGCCTCGTTCATTCACGTTTTTGAACCCGTGGAGGA
CGGGCAGACTCGCGGTGCAAATGTGTTTTACAGCGTGATGGAGCAGATGAAGATGC
TCGACACGCTGCAGAACACGCAGCTAGATTAACCCTAGAAAGATAATCATATTGTG
ACGTACGTTAAAGATAATCATGCGTAAAATTGACGCATGTGTTTTATCGGTCTGTAT
ATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACA
TACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAA
CAAAAACTCAAAATTTCTTCTATAAAGTAACAAAACTTTTATCGAATTCCTGCAGCC
CGGGGGATCCACTAGTTCTAGAGGGACAGCCCCCCCCCAAAGCCCCCAGGGATGTA
ATTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGT
CCGGCGCTCCCCCCGCATCCCCGAGCCGGCAGCGTGCGGGGACAGCCCGGGCACGG
GGAAGGTGGCACGGGATCGCTTTCCTCTGAACGCTTCTCGCTGCTCTTTGAGCCTGC
AGACACCTGGGGGGGATACGGGGAAAAGGCCTCCAAGGCCAGCTTCCCACAATAAGT
TGGGTGAATTTTGGCTCATTCCTCCTTTCTATAGGATTGAGGTCAGAGCTTTGTGATG
GGAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCgcGATCgcTAGcTTAa
gCGCtGATcaATTgGCGCGCCGAATTCGTTatctgcagaattcggcttggcggctgcgcgttcaaacctcccgcttc
aaaatggagaccctgcgtgctcactcgggcttaaatacccagcgtgaccacatggtgtcgcaaaatgtcgcaaaacactcacgtgacctcta
atacaggacctctagagcatggaaactagataagaaagaaatacgcagagaccaaagttcaactgaaacgaattaaacggtttattgattaa
caagcaaactagtttacagattacgggtgaggtaacgggtgccaatggggcggggttcagagtacacgccttctgtattaacagcaaagtc
cacacttgtagatttgtagtagttggaggtgtactggatctcggggttccagcgcttgctgttttccttctgcagctcccattcaatttccacgctg
acctgtccggtgctgtattgcgtgatgaaagagttcagctttgactggttgaaggtggtcggaggatccgcaggtacaggcgtgttcttgatc
aggatctgaggcggaggatgtttcaggccaaagccgcccatcagcggagacgggtggaagttgccgtccgtgtgaggaatcttggccca

gatgggaccctgcaggtacacgtcccggttctgccagaccataccgggtaaggccccctggctgttgacagttccaatttgaggagccgtg
ttttgctgctgcaagttatctgccacgataccgtattcctctgtagccacagggttagtggttttgatttcttcctcgctggtgagcatgacatcgct
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CGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCA
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GGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAAC

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GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGG
CAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGG
TGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTG
CCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCA
TCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT
CCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCAC
CAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATA
AGGGCGACACGGAAATGTTGAATACTCAT (SEQ ID NO: 28)

[00304] The sequence for the PBBG-iRC9 construct illustrated in in FIG. 11D is shown below:

[00305] PBBG-iRC9 (9,393 bp)

[00306] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC
ATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG
TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATC
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AAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA
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GTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC
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TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
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ACGTACGTTAAAGATAATCATGCGTAAAATTGACGCATGTGTTTTATCGGTCTGTAT
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CCGGCGCTCCCCCCGCATCCCCGAGCCGGCAGCGTGCGGGGACAGCCCGGGCACGG
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AGACACCTGGGGGGATACGGGGAAAAGGCCTCCAAGGCCAGCTTCCCACAATAAGT
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TCTCCACGCTGACTTGGCCAGTAGAATACTGGGTGATGAAAGAGTTCAGCTTGTCCT
TGTTGAAGGCCGTTGGAGGATCCGCAGGTACAGGTGTGTTTTTGATGAGGATCTGAG
GAGGCGGGTGCTTCATTCCAAACCCTCCCATCAGCGGAGAAGGGTGAAAGTTGCCG
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CAAACCATACCCGGAAGTATTCCTTGGTTTTGAACCCAGCCGGTCTGCGCCTGTGCT
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GGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAG

GCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCC
GATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACT
GCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC
TCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCG
TCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGA
AAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCG
ATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGAC
ACGGAAATGTTGAATACTCAT (SEQ ID NO: 29)

[00307] The sequence for the PBBG-Anc80iRC construct illustrated in in FIG. 11E is shown below:

[00308] PBBG-Anc80iRC (9,393 bp)

[00309] ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG
AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCAC
ATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCG
TTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATC
CCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAAC
AAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTA
TCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAG
GTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGAC
GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGG
GCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCC
GCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG
ATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTG
TAAAACGACGGCCAGTGAGCGCGCCTCGTTCATTCACGTTTTTGAACCCGTGGAGGA
CGGGCAGACTCGCGGTGCAAATGTGTTTTACAGCGTGATGGAGCAGATGAAGATGC
TCGACACGCTGCAGAACACGCAGCTAGATTAACCCTAGAAAGATAATCATATTGTG
ACGTACGTTAAAGATAATCATGCGTAAAATTGACGCATGTGTTTTATCGGTCTGTAT

ATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACA
TACTAATAATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAA
CAAAAACTCAAAATTTCTTCTATAAAGTAACAAAACTTTTATCGAATTCCTGCAGCC
CGGGGGATCCACTAGTTCTAGAGGGACAGCCCCCCCCCAAAGCCCCCAGGGATGTA
ATTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGCCGCCCGGGGCTCCGCTCCGGT
CCGGCGCTCCCCCCGCATCCCCGAGCCGGCAGCGTGCGGGGACAGCCCGGGCACGG
GGAAGGTGGCACGGGATCGCTTTCCTCTGAACGCTTCTCGCTGCTCTTTGAGCCTGC
AGACACCTGGGGGGATACGGGGAAAAGGCCTCCAAGGCCAGCTTCCCACAATAAGT
TGGGTGAATTTTGGCTCATTCCTCCTTTCTATAGGATTGAGGTCAGAGCTTTGTGATG
GGAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCgcGATCgcTAGcTTAa
gCGCtGATcaATTgGCGCGCCGAATTCGTTatctgcagaattcggcttggcggctgcgcgttcaaacctcccgcttc
aaaatggagaccctgcgtgctcactcgggcttaaatacccagcgtgaccacatggtgtcgcaaaatgtcgcaaaacactcacgtgacctcta
atacaggacctctagagcatggaaactagataagaaagaaatacgcagagaccaaagttcaactgaaacgaattaaacggtttattgattaa
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aggtacggattgtcacccgctttgagctgctggtcgtaggccttgtcgtgctcgagggccgctgcgtccgccgcgttgacgggctccccctt
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actcccattccttctcggccacccagttcacaaagctgtcagaaatgccgggcagatgctcgtcaaggtcgctggggaccttaatcacaatct
cgtaaaaccccggcatGGCGGCTGCGCGTTCAAACCTCCCGCTTCAAAATGGAGACCCTGCGT
GCTCACTCGGGCgaTCTCTATCACTGATAGGGAGATCTCTATCACTGATAGGGAgaTT
AAATAgaatggCTAggATCCGGCCGGccTGCAggTGTCCTCACAGGAACGAAGTCCCTAA
AGAAACAGTGGCAGCCAGGTTTAGCCCCGGAATTGACTGGATTCCTTTTTTAGGGCC

CATTGGTATGGCTTTTTCCCCGTATCCCCCCAGGTGTCTGCAGGCTCAAAGAGCAGC
GAGAAGCGTTCAGAGGAAAGCGATCCCGTGCCACCTTCCCCGTGCCCGGGCTGTCC
CCGCACGCTGCCGGCTCGGGGATGCGGGGGGAGCGCCGGACCGGAGCGGAGCCCC
GGGCGGCTCGCTGCTGCCCCCTAGCGGGGGAGGGACGTAATTACATCCCTGGGGGC
TTTGGGGGGGGGCTGTCCCTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTT
GTTCCCTTTAGTGAGGGTTAATTAGATCTTAATACGACTCACTATAGGGCGAATTGG
GTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCTATAACAAGA
AAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTAT
CGTATGAGTTAAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCATTTTGACTCA
CGCGGTCGTTATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCAC
GGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTA
TTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATC
TTTCTAGGGTTAATCTAGCTGCATCAGGATCATATCGTCGGGTCTTTTTTCCGGCTCA
GTCATCGCCCAAGCTGGCGCTATCTGGGCATCGGGGAGGAAGAAGCCCGTGCCTTTT
CCCGCGAGGTTGAAGCGGCATGGAAAGAGTTTGCCGAGGATGACTGCTGCTGCATT
GACGTTGAGCGAAAACGCACGTTTACCATGATGATTCGGGAAGGTGTGGCCATGCA
CGCCTTTAACGGTGAACTGTTCGTTCAGGCCACCTGGGATACCAGTTCGTCGCGGCT
TTTCCGGACACAGTTCCGGATGGTCAGCCCGAAGCGCATCAGCAACCCGAACAATA
CCGGCGACAGCCGGAACTGCCGTGCCGGTGTGCAGATTAATGACAGCGGTGCGGCG
CTGGGATATTACGTCAGCGAGGACGGGTATCCTGGCTGGATGCCGCAGAAATGGAC
ATGGATACCCCGTGAGTTACCCGGCGGGCGCGCTTGGCGTAATCATGGTCATAGCTG
TTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGC
ATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTG
CGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATC
GGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTC
ACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAG
GCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGC
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ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG
CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGT
GCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCG

GGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTC
GTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCC
TTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTG
GCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGA
GTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTG
CGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAA
ACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAG
AAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTG
GAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC
CTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTA
AACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTG
TCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGG
GAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACC
GGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTG
GTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGT
AAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGT
GGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAG
GCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCC
GATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACT
GCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC
TCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCG
TCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGA
AAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCG
ATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGAC
ACGGAAATGTTGAATACTCAT (SEQ ID NO: 30)

[00310] The sequence for the pcDNA-TetR-Ins construct illustrated in in FIG. 12A is shown below:

[00311] pcDNA-TetR-Ins (7147 bp)

[00312] gacggatcgggagatctgagctcacggggacagccccccccaaagccccagggatgtaattacgtccctcccccgct
aggggcagcagcgagccgcccggggctccgctccggtccggcgctcccccgcatccccgagccggcagcgtgcggggacagcc
cgggcacggggaaggtggcacgggatcgctttcctctgaacgcttctcgctgctctttgagcctgcagacacctgggggatacggggaa
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ggccagatatacgcgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgtt
acataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgcc
aatagggactttccattgacgtcaatgggtggactatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccc
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attgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggt
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ctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagca

gagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgc
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agaaaaataaacaaataggggttccgcgcacatttccccgaaaagtgccacctgacgtc (SEQ ID NO: 31)

[00313] The sequence for the pcDNA-TetR-KRAB-Ins construct illustrated in in FIG. 12B is shown below:

[00314] pcDNA-TetR-KRAB-Ins (7493 bp)

[00315] gacggatcgggagatctgagctcacggggacagcccccccccaaagcccccagggatgtaattacgtccctcccccgct
agggggcagcagcgagccgcccggggctccgctccggtccggcgctcccccccgcatccccgagccggcagcgtgcggggacagcc
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gggttccgcgcacatttccccgaaaagtgccacctgacgtc (SEQ ID NO: 32)

[00316] It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein can be made without departing from the scope of any of the embodiments.

[00317] It is to be understood that while certain embodiments have been illustrated and described herein, the claims are not to be limited to the specific forms or arrangement of parts described and shown. In the specification, there have been disclosed illustrative embodiments and, although specific terms are employed, they are used in a generic and descriptive sense only and not for purposes of limitation. Modifications and variations of the embodiments are possible in light of the above teachings. It is therefore to be understood that the embodiments may be practiced otherwise than as specifically described.

[00318] All publications, patents and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

What is claimed is:

1. A mammalian cell for producing an adeno-associated virus (AAV), comprising:

    a. a nucleic acid molecule encoding a viral helper gene under control of a first derepressible promoter;

    b. a nucleic acid molecule encoding an AAV gene under control of a second derepressible promoter; and

    c. a nucleic acid molecule encoding a repressor element of the first and the second derepressible promoters.

2. The mammalian cell of claim 1, wherein the mammalian cell is a mammalian cell culture.

3. The mammalian cell of claim 2, wherein the mammalian cell culture is a suspension culture.

4. The mammalian cell of any one of claims 1-3, wherein the viral helper gene is an adenovirus helper gene.

5. The mammalian cell of claim 4, wherein the adenovirus helper gene comprises E2A and E4Orf6 genes.

6. The mammalian cell of claim 5, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

7. The mammalian cell of any one of claims 1-6, wherein the AAV gene comprises *Rep* and *Cap* genes.

8. The mammalian cell of claim 7, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a third derepressible promoter.

9. The mammalian cell of claim 7, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a third derepressible promoter contained within an artificial intron.

10. The mammalian cell of claim 8 or claim 9, wherein the *Cap* gene is under control of a native promoter.

11. The mammalian cell of any one of claims 1-10, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

12. The mammalian cell of claim 11, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

13. The mammalian cell of any one of claims 1-12, wherein the repressor element is under control of a constitutive promoter.

14. The mammalian cell of any one of claims 1-13, wherein the repressor element is a tetracycline repressor protein.

15. The mammalian cell of claim 14, further comprising a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

16. The mammalian cell of anyone of claims 1-15, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

17. The mammalian cell of any one of claims 1-15, wherein the mammalian cell is a human cell.

18. The mammalian cell of claim 17, wherein the human cell is a human embryonic kidney (HEK) cell.

19. The mammalian cell of any one of claims 1-18, further comprising a nucleic acid molecule including two inverted terminal repeat (ITR) sequences.

20. The mammalian cell of any one of claims 1-19, further comprising a nucleic acid molecule encoding a gene of interest.

21. The mammalian cell of any one of claims 1-20, further comprising a nucleic acid encoding a viral-associated, non-coding RNA under control of a fourth derepressible promoter.

22. A mammalian cell for producing an adeno-associated virus (AAV), comprising:

a. a nucleic acid molecule encoding:

i. an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter;

ii. an AAV gene comprising *Rep* and *Cap* genes under control of a second derepressible promoter;

iii. a viral-associated, non-coding RNA under control of a third depressible promoter;

iv. two inverted terminal repeat (ITR) sequences; and

v. a repressor element of the first, second and third derepressible promoters.

23. The mammalian cell of claim 22, wherein the mammalian cell is a mammalian cell culture.

24. The mammalian cell of claim 23, wherein the mammalian cell culture is a suspension culture.

25. The mammalian cell of any one of claims 22-24, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

26. The mammalian cell of any one of claims 22-25, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter.

27. The mammalian cell of any one of claims 22-25, wherein a Rep78 gene is under control of the second derepressible promoter and a Rep52 gene is under control of a fourth derepressible promoter contained within an artificial intron.

28. The mammalian cell of claim 26 or claim 27, wherein the *Cap* gene is under control of a native promoter.

29. The mammalian cell of any one of claims 22-28, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

30. The mammalian cell of claim 29, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

31. The mammalian cell of any one of claims 22-30, wherein the repressor element is under control of a constitutive promoter.

32. The mammalian cell of any one of claims 22-31, wherein the repressor element is a tetracycline repressor protein.

33. The mammalian cell of claim 25, further comprising a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

34. The mammalian cell of any one of claims 22-33, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

35. The mammalian cell of any one of claims 22-33, wherein the mammalian cell is a human cell.

36. The mammalian cell of claim 35, wherein the human cell is a human embryonic kidney (HEK) cell.

37. The mammalian cell of any one of claims 22-36, further comprising a nucleic acid molecule encoding a gene of interest.

38. An isolated nucleic acid molecule encoding:

a. an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter;

b. an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter;

c. a viral-associated, non-coding RNA under control of a third depressible promoter;

d. two inverted terminal repeat (ITR) sequences; and

e. a repressor element of the first, second and third derepressible promoters.

39. The isolated nucleic acid of claim 38, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

40. The isolated nucleic acid of any one of claims 38-39, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter.

41. The isolated nucleic acid of any one of claims 38-39 wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter contained within an artificial intron.

42. The isolated nucleic acid of claim 40 or claim 41, wherein the Cap gene is under control of a native promoter.

43. The isolated nucleic acid of any one of claims 38-42, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences (TetO2).

44. The isolated nucleic acid of claim 43, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

45. The isolated nucleic acid of any one of claims 38-44, wherein the repressor element is under control of a constitutive promoter.

46. The isolated nucleic acid of any one of claims 38-45, wherein the repressor element is a tetracycline repressor protein.

47. The isolated nucleic acid of claim 46, further comprising a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

48. The isolated nucleic acid of any one of claims 38-47, further comprising a gene of interest.

49. A method of producing an adeno-associated virus (AAV) in a mammalian cell comprising:

a. transfecting the mammalian cell with an isolated nucleic acid molecule encoding:

i. an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter;

ii. an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter;

iii. a viral-associated, non-coding RNA under control of a third depressible promoter;

iv. two inverted terminal repeat (ITR) sequences; and

v. a repressor element of the first, second and third derepressible promoters;

b. treating the mammalian cell with a binding partner of the repressor element;

c. activating the first, second and third derepressible promoters;

d. producing the AAV; and

e. harvesting the AAV.

50. The method of claim 49, wherein the mammalian cell is a mammalian cell culture.

51. The method of claim 50, wherein the mammalian cell culture is a suspension culture.

52. The method of any one of claims 49-51, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

53. The method of any one of claims 49-52, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter.

54. The method of any one of claims 49-52, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter contained within an artificial intron.

55. The method of claim 54, wherein the fourth derepressible promoter is removed following the activating the derepressible promoters and prior to the producing the AAV.

56. The method of claim 53 or claim 54, wherein the *Cap* gene is under control of a native promoter.

57. The method of any one of claims 49-56, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

58. The method of claim 57, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

59. The method of any one of claims 49-58, wherein the repressor element is under control of a constitutive promoter.

60. The method of any one of claims 49-59, wherein the repressor element is a tetracycline repressor protein.

61. The method of claim 60, wherein the nucleic acid further comprises a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

62. The method of claim 60 or 61, wherein the treating comprises treating with doxycycline.

63. The method of any one of claims 49-62, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

64. The method of any one of claims 49-63, wherein the mammalian cell is a human cell.

65. The method of claim 64, wherein the human cell is a human embryonic kidney (HEK) cell.

66. The method of any one of claims 49-65, wherein the AAV comprises a nucleic acid molecule encoding a gene of interest.

67. The method of claim 66, wherein the AAV comprises a gene of therapeutic interest.

68. A method of treatment with an adeno-associated virus (AAV) comprising:

a. transfecting the mammalian cell with an isolated nucleic acid molecule encoding:

i. an adenovirus helper gene comprising E2A and E4Orf6 genes under control of a first derepressible promoter;

ii. an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter;

iii. a viral-associated, non-coding RNA under control of a third depressible promoter;

iv. two inverted terminal repeat (ITR) sequences; and

v. a repressor element of the first, second and third derepressible promoters;

b. treating the mammalian cell with a binding partner of the repressor element;

c. activating the first, second and third derepressible promoters;

d. producing the AAV;

e. harvesting the AAV; and

f. administering the AAV to a mammalian patient.

69. The method of claim 68, wherein the mammalian cell is a mammalian cell culture.

70. The method of claim 69, wherein the mammalian cell culture is a suspension culture.

71. The method of any one of claims 68-70, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

72. The method of any one of claims 68-71, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter.

73. The method of any one of claims 68-72, wherein a *Rep78* gene is under control of the second derepressible promoter and a *Rep52* gene is under control of a fourth derepressible promoter contained within an artificial intron.

74. The method of claim 73, wherein the fourth derepressible promoter is removed following the activating the derepressible promoters and prior to the producing the AAV.

75. The method of claim 73 or claim 74, wherein the *Cap* gene is under control of a native promoter.

76. The method of any one of claims 68-75, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

77. The method of claim 76, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

78. The method of any one of claims 68-77, wherein the repressor element is under control of a constitutive promoter.

79. The method of any one of claims 68-78, wherein the repressor element that is a tetracycline repressor protein.

80. The method of claim 79, wherein the nucleic acid further comprises a nucleic acid encoding a transcriptional repression domain in frame with the nucleic acid encoding the tetracycline repressor protein.

81. The method of claim 79 or 80, wherein the treating comprises treating with doxycycline.

82. The method of any one of claims 68-81, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

83. The method of any one of claims 68-82, wherein the mammalian cell is a human cell.

84. The method of claim 83, wherein the human cell is a human embryonic kidney (HEK) cell.

85. The method of any one of claims 68-84, wherein the AAV comprises a nucleic acid molecule encoding a gene of interest.

86. The method of claim 85, wherein the AAV comprises a gene of therapeutic interest.

87. The method of any one of claims 68-86, wherein the administering comprises inhalation, injection or intravenous administration.

88. A method of producing an adeno-associated virus (AAV), comprising:

a. transfecting a mammalian cell stably expressing one or more nucleic acids encoding TetR and/or TetR-KRAB with:

i. a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA, under control of a first derepressible promoter;

ii. a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter; and

iii. optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter;

b. treating the mammalian cell with a binding partner of the TetR;

c. activating the first, second and third derepressible promoters;

d. producing the AAV; and

e. harvesting the AAV.

89. The method of claim 88, wherein the mammalian cell is a mammalian cell culture.

90. The method of any one of claims 88-89, wherein the mammalian cell is a Chinese hamster ovary (CHO) cell.

91. The method of any one of claims 88-90, wherein the mammalian cell is a human cell.

92. The method of claim 91, wherein the human cell is a human embryonic kidney (HEK) cell.

93. The method of claim 89, wherein the mammalian cell culture is a suspension culture.

94. The method of any one of claims 88-90, further comprising an internal ribosome entry site (IRES) element between the E2A and E4Orf6 genes.

95. The method of any one of claims 88-91, wherein each of the derepressible promoters comprise a functional promoter and two tetracycline operator sequences ($TetO_2$).

96. The method of claim 95, wherein the functional promoter of the first derepressible promoter is a cytomegalovirus (CMV) promoter.

97. The method of any one of claims 88-96, wherein the TetR and/or TetR-KRAB is under control of a constitutive promoter.

98. The method of claim 97, wherein the nucleic acid encoding the TetR-KRAB comprises KRAB fused in frame with the TetR.

99. The method of any one of claims 88-98, wherein the treating comprises treating with doxycycline.

100. The method of any one of claims 88-99, wherein each of the first, second and third nucleic acids are flanked by transposon-specific inverted terminal repeats (ITRs).

101. The method of any one of claims 88-100, wherein the mammalian cell expresses sufficient amounts of TetR and/or TetR-KRAB.

102. The method of any one of claims 88-101, wherein the AAV comprises a gene of therapeutic interest.

103. The method of any one of claims 88-102, wherein the mammalian cell further comprises stable expression of one or more nucleic acids encoding chicken hypersensitive site-4 (cHS4) flanking the TetR and/or TetR-KRAB repressor sequences.

104. The method of any one of claims 88-103, wherein the mammalian cell further comprises a selection gene.

105. A method for producing an adeno-associated virus (AAV), comprising:

a. stably transfecting a mammalian cell with:

a nucleic acid encoding a TetR and/or TetR-KRAB repressor;

chicken hypersensitive site-4 (cHS4) sequences flanking the TetR and/or TetR-KRAB repressor; and

a selection gene;

b. transfecting the stably transfected mammalian cell with:

i. a first nucleic acid encoding an adenovirus helper gene comprising an E2A gene, a E4Orf gene and a viral-associated non-coding RNA, under control of a first derepressible promoter;

ii. a second nucleic acid encoding an AAV gene comprising Rep and Cap genes under control of a second derepressible promoter; and

iii. optionally, a third nucleic acid encoding a gene of interest under control of a third derepressible promoter;

c. treating the stably transfected mammalian cell with a binding partner of the TetR;

d. activating the first, second and third derepressible promoters;

e. producing the AAV; and

f. harvesting the AAV.

106. The method of claim 105, wherein the stably transfected mammalian cell produces a sufficient amounts of TetR.

107. The method of any one of claims 105 and 106, wherein the KRAB repressor domain is fused in frame with the TetR.