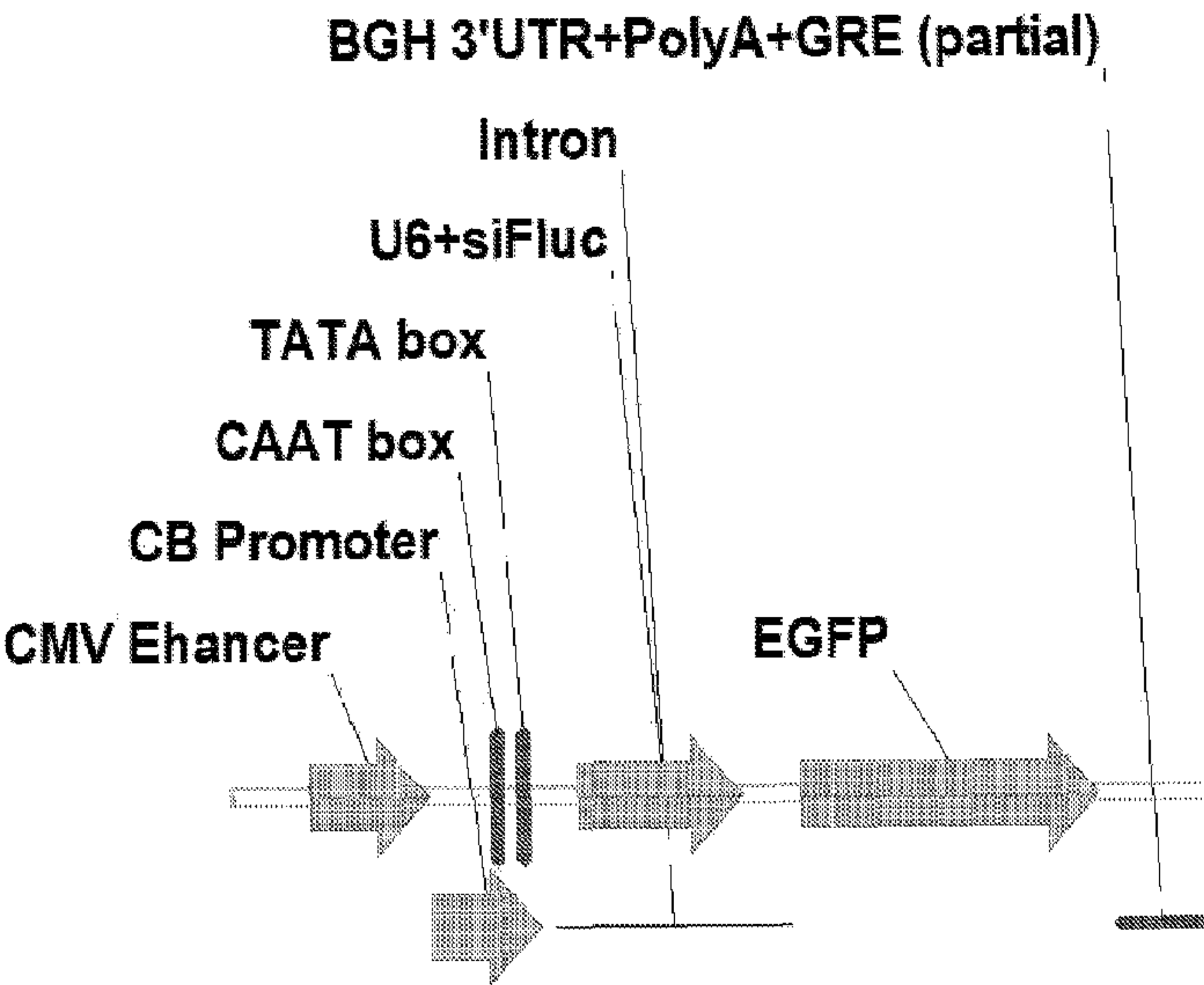




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(54) Titre : CONSTRUCTIONS D'EXPRESSION A CISTRONS MULTIPLES
(54) Title: MULTICISTRONIC EXPRESSION CONSTRUCTS



(57) **Abrégé/Abstract:**
Some aspect of this invention provide nucleic acid constructs for transgene expression. Some aspects of this invention provide multicistronic nucleic acid constructs, for example, comprising an expression cassette encoding a hairpin RNA and a reporter expression cassette. Some aspects of this invention provide nucleic acid constructs comprising two or more self- complementary nucleic acid sequences, for example, hairpin RNA encoding nucleic acid sequences and AAV inverse terminal repeats. Methods for the use of the constructs in therapy and research are also provided.

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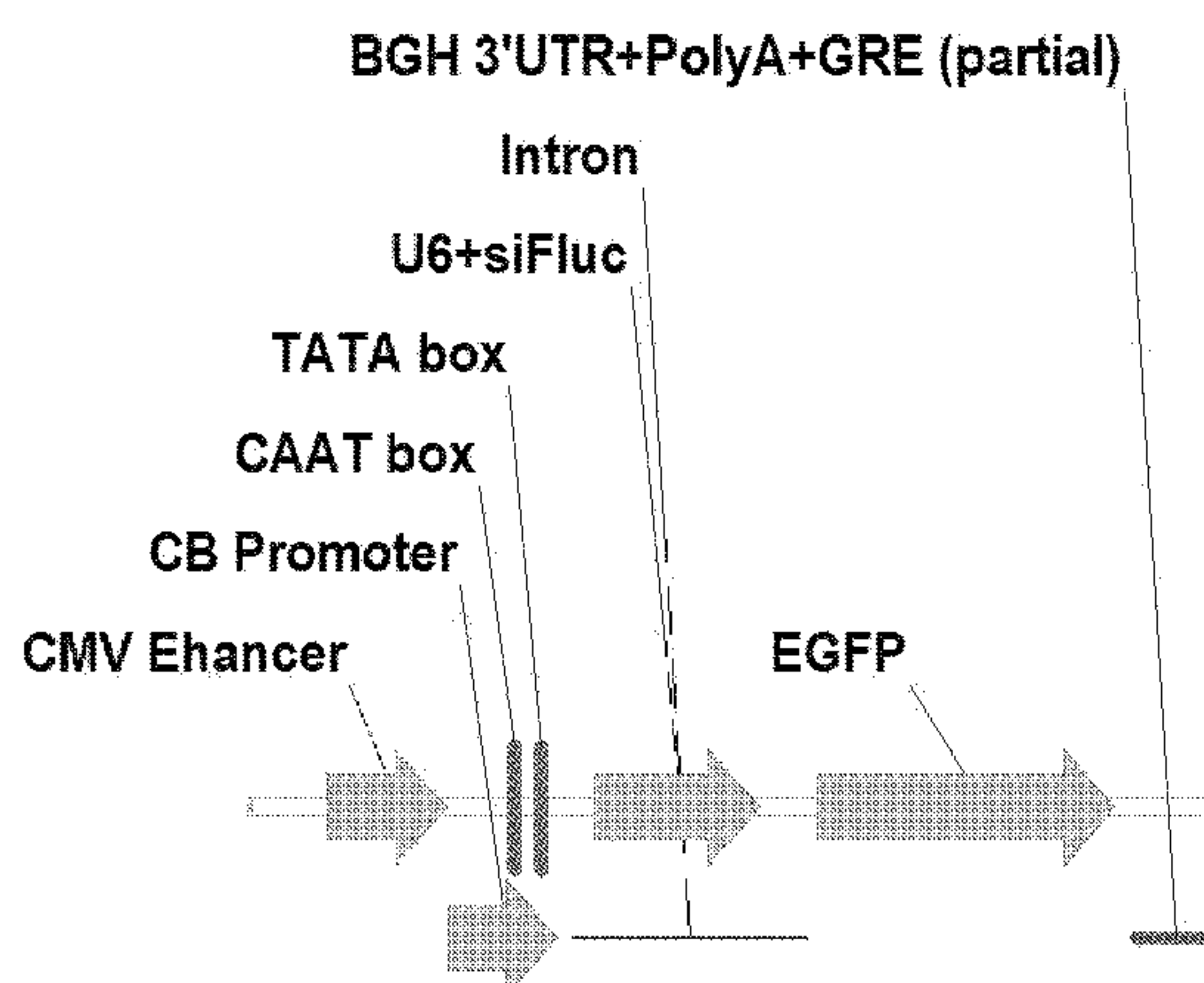


FIG. 1

(57) **Abstract:** Some aspect of this invention provide nucleic acid constructs for transgene expression. Some aspects of this invention provide multicistronic nucleic acid constructs, for example, comprising an expression cassette encoding a hairpin RNA and a reporter expression cassette. Some aspects of this invention provide nucleic acid constructs comprising two or more self-complementary nucleic acid sequences, for example, hairpin RNA encoding nucleic acid sequences and AAV inverse terminal repeats. Methods for the use of the constructs in therapy and research are also provided.

WO 2011/133874 A1

MULTICISTRONIC EXPRESSION CONSTRUCTS

RELATED APPLICATIONS

5 This application claims the benefit under 35 U.S.C. § 119 of U.S. provisional application USSN 61/327,404, filed April 23, 2010.

FIELD OF THE INVENTION

10 Some aspects of the invention relate to the field of gene expression constructs. Some aspects of the invention relate to viral expression constructs, for example, adeno-associated virus (AAV)-related expression constructs. Some aspects of the invention relate to the field of RNAi.

BACKGROUND OF THE INVENTION

15 Expression constructs are useful to effect transgene expression in a target cell. Since many useful transgene products are not easily detected in a target cell, expression constructs harboring a reporter cassette are commonly used to monitor transgene delivery and expression. Multicistronic expression constructs, for example, constructs harboring a first expression cassette, e.g. comprising a first promoter and a first encoding nucleic acid
20 sequence, and a second expression cassette, e.g. comprising a second promoter and a second encoding nucleic acid sequence, are particularly useful in the delivery of transgenes encoding non-translated gene products, such as hairpin RNAs, together with a reporter transgene, for example, a fluorescent protein. However, multicistronic expression constructs may be
25 burdened with reduced expression levels of one or more of the included transgenes, for example, because of promoter interference or the presence of incompatible nucleic acid elements in close proximity. If a multicistronic expression construct is part of a viral vector, the inclusion of a hairpin RNA expression cassette may pose additional problems, for example, because the presence of a self-complementary nucleic acid sequence may interfere with the formation of structures necessary for viral reproduction or packaging.

SUMMARY OF THE INVENTION

Some aspects of the invention relate to isolated nucleic acid constructs. Some aspects of this invention relate to isolated gene expression constructs. Some aspects of this invention relate to multicistronic expression constructs, for example, to bicistronic expression constructs. Some aspects of this invention relate to expression constructs comprising an expression cassette containing a self-complementary nucleic acid sequence, for example, a nucleic acid sequence encoding a hairpin-forming RNA. Some aspects of this invention relate to expression constructs comprising an expression cassette containing a self-complementary nucleic acid sequence positioned in proximity to a second self-complementary nucleic acid sequence. Some aspects of this invention relate to methods of engineering nucleic acid constructs comprising a self-complementary nucleic acid sequence and a second expression cassette and/or a second self-complementary nucleic acid sequence, for example, a terminal repeat sequence. Some aspects of this invention relate to viral gene expression constructs. Some aspects of this invention relate to parvovirus-derived expression constructs, for example, to adeno-associated virus (AAV)-derived expression constructs. Some aspects of this invention relate to compositions and kits comprising an expression construct as provided herein. Some aspects of this invention relate to methods of using an expression construct as provided herein to express a nucleic acid comprising a self-complementary sequence in a target cell. Some aspects of this invention relate to the use of constructs as provided herein in methods of inhibiting the expression of a gene product in the target cell by expressing a nucleic acid comprising a self-complementary sequence corresponding to a nucleic acid sequence encoding the gene product. Some aspects of this invention relate to methods of determining a phenotypic change of a cell effected by contacting the cell with an expression construct as described herein. Some aspects of this invention relate to methods involving administering a recombinant AAV (rAAV) expression construct as provided herein to a subject, wherein the rAAV infects a cell of a target tissue of the subject. Some aspects relate to methods of using a nucleic acid construct as provided herein to inhibit expression of a gene in a target cell, for example, a target cell in a subject, by expressing a hairpin RNA in the target cell that comprises a sequence corresponding to a sequence of a nucleic acid encoding a gene product of the gene.

Some aspects of this invention relate to the surprising discovery that an expression cassette positioned within an intron of another expression cassette is efficiently expressed in a target cell. Further, some aspects of this invention relate to the surprising discovery that an expression cassette positioned within an intron of another expression cassette is efficiently

expressed in a target cell independent of whether the expression cassettes are in the same orientation or in opposite orientation to each other.

Some aspects of this invention relate to the surprising discovery that an expression cassette comprising a nucleic acid sequence encoding a self-complementary RNA positioned
5 in proximity to an AAV-ITR, can efficiently be expressed if the ITR is a Δ TRS ITR, independent of the orientation of the expression cassette.

Some aspects of this invention relate to the surprising discovery that an expression cassette comprising a nucleic acid sequence encoding a self-complementary RNA positioned in proximity to a functional AAV-ITR only if the expression construct comprises at least
10 about 150 nucleotides between the functional ITR and the nucleic acid sequence encoding the self-complementary RNA.

In some embodiments, the expression construct is a linear DNA or RNA construct. In some embodiments, the expression construct is a circular DNA construct. In some embodiments, the expression construct is a viral expression construct. In some embodiments,
15 the expression construct is a parvovirus-derived construct, for example, an AAV-derived construct. In some embodiments, the expression construct comprises an inverted terminal repeat (ITR). In some embodiments, the expression construct comprises an ITR lacking a functional terminal resolution site (TRS), also referred to as Δ TRS ITR or Δ ITR. In some embodiments, the expression construct comprises a nucleic acid sequence encoding a hairpin
20 RNA, for example, a small hairpin RNA (shRNA) or a micro RNA (miRNA). In some embodiments, the expression construct comprises a plurality of expression cassettes. In some embodiments, the expression construct comprises a first and a second expression cassette in the same orientation, with an additional expression cassette optionally present. In some embodiments, the expression construct comprises a first and a second expression cassette in
25 opposite orientation to each other, with an additional expression cassette optionally present. In some embodiments, the expression construct comprises a first expression cassette harboring an intron and the second expression cassette is positioned in the intron of the first expression cassette, either in the same or in opposite orientation to the first expression cassette, with an additional expression cassette optionally present.

30 Some aspects of this invention provide an isolated nucleic acid construct, comprising a first expression cassette, comprising a nucleic acid encoding a gene product under the control of a first promoter, and an intron, and a second expression cassette, comprising a self-complementary nucleic acid sequence under the control of a second promoter, wherein the

second expression cassette is positioned within the intron of the first expression cassette. In some embodiments, the gene product is a reporter. In some embodiments, the reporter is a protein. In some embodiments, the protein is a fluorescent protein, an enzyme catalyzing a reaction yielding a detectable product, or a surface antigen. In some embodiments, the enzyme is a luciferase, a beta-glucuronidase, a chloramphenicol acetyltransferase, an aminoglycoside phosphotransferase, an aminocyclitol phosphotransferase, or a Puromycin N-acetyl-transferase. In some embodiments, the self-complementary sequence encodes a hairpin RNA. In some embodiments, the hairpin RNA is a small hairpin RNA or a microRNA. In some embodiments, the first promoter is an RNA polymerase II promoter. In some
10 embodiments, the second promoter is an RNA polymerase III promoter. In some embodiments, the second promoter is a U6 or an H1 promoter. In some embodiments, the nucleic acid construct comprises the structure of AAV construct A1 or A2. In some embodiments, the first and the second expression cassette are in the same orientation. In some embodiments, the first and the second expression cassette are in opposite orientations. In
15 some embodiments, the nucleic acid construct is comprised in an expression vector. In some embodiments, the expression vector is a viral vector. In some embodiments, the viral vector is a parvovirus vector, an adenovirus vector, or a retrovirus vector. In some embodiments, the viral vector is an adeno-associated virus vector, a lentivirus vector, or a Moloney murine leukemia virus vector. In some embodiments, the viral construct is an AAV construct. In
20 some embodiments, the AAV construct is a self-complementary AAV construct. In some embodiments, the nucleic acid construct is integrated into the genome of a cell expressing a target gene. In some embodiments, the nucleic acid construct comprises a nucleic acid encoding a hairpin RNA comprising a sequence complementary or corresponding to a sequence of an RNA transcribed from the target gene. In some embodiments, the target gene
25 is a gene is an oncogene, a tumor suppressor gene, a gene involved in neovascularization of tissue, a viral gene, or a gene encoding a receptor mediating uptake of viral particles.

Some aspects of this invention provide a nucleic acid construct, comprising (i) an inverted terminal repeat lacking a functional terminal resolution site (Δ TRS ITR), (ii) a first expression cassette, comprising a nucleic acid encoding a gene product under the control of a
30 first promoter, and (iii) a second expression cassette, comprising a self-complementary nucleic acid sequence under the control of a second promoter, wherein the second expression cassette is positioned between the Δ TRS ITR and the first expression cassette, and wherein (a) the first and the second expression cassette are in opposite orientations, or (b) the first and the second expression cassette are in the same orientation and the nucleic acid construct

comprises less than 500 nucleotides between the Δ TRS ITR and the second expression cassette. In some embodiments, the nucleic acid construct is an AAV construct. In some embodiments, the AAV construct is a self-complementary AAV construct. In some embodiments, the gene product is a reporter. In some embodiments, the reporter is a protein.

- 5 In some embodiments, the protein is a fluorescent protein, an enzyme catalyzing a reaction yielding a detectable product, or a surface antigen. In some embodiments, the enzyme is a luciferase, a beta-glucuronidase, a chloramphenicol acetyltransferase, an aminoglycoside phosphotransferase, an aminocyclitol phosphotransferase, or a Puromycin N-acetyl-transferase. In some embodiments, the self-complementary nucleic acid sequence encodes a hairpin RNA.
- 10 In some embodiments, the hairpin RNA is an shRNA or a microRNA. In some embodiments, the first promoter is an RNA polymerase II promoter. In some embodiments, the second promoter is an RNA polymerase III promoter. In some embodiments, the second promoter is a U6 or H1 promoter. In some embodiments, the nucleic acid construct comprises the structure of AAV construct B1 or B2. In some embodiments, the nucleic acid construct is
- 15 integrated into the genome of a cell expressing a target gene. In some embodiments, the nucleic acid construct comprises a nucleic acid encoding a hairpin RNA comprising a sequence complementary or corresponding to a sequence of an RNA transcribed from the target gene. In some embodiments, the target gene is a gene is an oncogene, a tumor suppressor gene, a gene involved in neovascularization of tissue, a viral gene, or a gene
- 20 encoding a receptor mediating uptake of viral particles. In some embodiments, the nucleic acid construct comprises less than about 50, less than about 100, less than about 200, less than about 250, less than about 300, less than about 400, less than about 500, less than about 600, less than about 700, less than about 800, less than about 900, or less than about 1000 nucleotides between the Δ TRS ITR and the second expression cassette.

- 25 Some aspects of this invention provide a nucleic acid construct, comprising (i) an inverted terminal repeat (ITR), (ii) a first expression cassette, comprising a nucleic acid encoding a gene product under the control of a first promoter, and (iii) a second expression cassette, comprising a self-complementary nucleic acid sequence under the control of a second promoter, wherein the first and the second expression cassette are in opposite
- 30 orientation, and, optionally, wherein the nucleic acid construct comprises at least 150 nucleotides between the ITR and the second expression cassette. In some embodiments, the nucleic acid construct is an AAV construct. In some embodiments, the AAV construct is a self-complementary AAV construct. In some embodiments, the AAV construct is a non-self-complementary AAV construct, for example, an AAV construct comprising two ITRs with

functional terminal resolution sites. In some embodiments, the gene product is a self-complementary nucleic acid, for example, a hairpin RNA. In some embodiments, the gene product is a reporter. In some embodiments, the reporter is a protein. In some embodiments, the protein is a fluorescent protein, an enzyme catalyzing a reaction yielding a detectable product, or a surface antigen. In some embodiments, the enzyme is a luciferase, a beta-glucuronidase, a chloramphenicol acetyltransferase, an aminoglycoside phosphotransferase, an aminocyclitol phosphotransferase, or a Puromycin N-acetyl-transferase. In some embodiments, the self-complementary nucleic acid sequence encodes a hairpin RNA. In some embodiments, the hairpin RNA is a small hairpin RNA or a microRNA. In some embodiments, the first promoter is an RNA polymerase II promoter. In some embodiments, the second promoter is an RNA polymerase III promoter. In some embodiments, the first and the second promoters are RNA polymerase II promoters. In some embodiments, the first and the second promoters are RNA polymerase III promoters. In some embodiments, the second promoter is a U6 or H1 promoter. In some embodiments, the nucleic acid construct comprises the nucleic acid sequence of AAV constructs D1 or D2. In some embodiments, the nucleic acid construct is integrated into the genome of a cell expressing a target gene. In some embodiments, the nucleic acid construct comprises a nucleic acid encoding a hairpin RNA comprising a sequence complementary or corresponding to a sequence of an RNA transcribed from the target gene. In some embodiments, the target gene is a gene is an oncogene, a tumor suppressor gene, a gene involved in neovascularization of tissue, a viral gene, or a gene encoding a receptor mediating uptake of viral particles. In some embodiments, the nucleic acid construct comprises at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, or at least about 800 nucleotides between the ITR and the second expression cassette.

Some aspects of this invention provide a recombinant AAV construct, comprising any of the nucleic acid constructs or AAV constructs provided herein. Some aspects of this invention provide a plasmid comprising any of the nucleic acid constructs or AAV constructs provided herein. In some embodiments, the plasmid further comprises a bacterial origin of replication and a bacterial selection marker.

Some aspects of this invention provide a composition comprising any of the nucleic acid constructs, AAV constructs, or plasmids provided herein. In some embodiments, the composition further comprises a pharmaceutically acceptable salt.

Some aspects of this invention provide a method, comprising contacting a cell expressing a target gene with any of the nucleic acid constructs, AAV constructs, or plasmids,

or compositions as described herein. In some embodiments, the nucleic acid construct, AAV construct, plasmid, or composition comprises a self-complementary nucleic acid sequence which comprises a sequence complementary or corresponding to a sequence of an RNA encoded by the target gene. In some embodiments, the nucleic acid construct, the AAV construct, the plasmid, or the composition comprises a nucleic acid sequence encoding a reporter, and wherein the method further comprises detecting expression of the reporter in the cell. In some embodiments, the method further comprises determining a change in the phenotype of the cell after the contacting. In some embodiments, the change in the phenotype is a change in proliferation rate, change in cell size, change in cell viability, change in cell sensitivity to a drug, change in modulation of a cellular pathway in response to drug treatment, or a change in a level of expression of a gene of interest. In some embodiments, the cell is a cell in a subject and wherein the contacting comprises administering the nucleic acid construct, the recombinant AAV construct, the plasmid, and/or the composition to the subject in an amount sufficient to inhibit expression of the target gene in the cell. In some embodiments, the method comprises administering a recombinant AAV construct to the subject via an intravenous, intraperitoneal, intraocular, intramuscular, intraarticular, intracranial, intranasal, or endobronchial route. In some embodiments, the subject has been diagnosed with a disease and inhibition of the target gene is known to prevent or alleviate a symptom and/or progression of the disease.

Some aspects of this invention provide a kit, comprising a container housing any of the nucleic acid constructs, the AAV constructs, the plasmids, or the compositions provided herein.

According to one aspect of the present invention, there is provided a recombinant AAV (rAAV) construct, comprising a recombinant AAV (rAAV) vector comprising inverted terminal repeats (ITRs), a first expression cassette, comprising a nucleic acid encoding a gene product under the control of a first promoter positioned between the ITRs of the vector, and an intron, and a second expression cassette, comprising a self-complementary nucleic acid sequence under the control of a second promoter, wherein the second expression cassette is positioned within the intron of the first expression cassette.

According to another aspect of the present invention, there is provided a composition comprising the rAAV construct as described herein and a pharmaceutically acceptable salt.

According to still another aspect of the present invention, there is provided use
5 of the recombinant AAV construct as described herein for inhibiting expression of a target gene in a cell.

According to yet another aspect of the present invention, there is provided a kit, comprising a container housing the rAAV construct as described herein.

According to a further aspect of the present invention, there is provided a cell,
10 comprising the rAAV construct as described herein.

Each of the embodiments of the invention can encompass various recitations made herein. It is, therefore, anticipated that each of the recitations of the invention involving any one element or combinations of elements can, optionally, be included in each aspect of the invention.

15 These and other aspects of the invention, as well as various advantages and utilities will be more apparent with reference to the drawings and detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Map of CMV/CB-intron-U6siFluc-intron-eGFF (A1). Arrows
20 indicate the orientation of the respective nucleic acid construct elements in Figures 1-9.

Figure 2. Map of CMV/CB-intron-U6siFluc^{inv}-intron-eGFP (A2).

Figure 3. Map of AAV 5'ΔITR CMV/CB-intron-eGFP 3'ITR (A). ITR: 5' Δ TSR ITR. Repeat FOR: forward repeat of ΔTRS ITR. Repeat REV: reverse repeat of ΔTRS ITR. 3'ITR: functional ITR.

Figure 4. Map of AAV 5' ΔITR U6shFluc CMV/CB-intron-eGFP 3'ITR(B1).

5 **Figure 5.** Map of AAV 5' ΔITR U6shFluc^{inv} CMV/CB-intron-eGFP 3'ITR(B2).

Figure 6. Map of AAV 5' ΔITR CMV/CB-intron-U6shFluc-intron-eGFP 3'ITR(C1).

Figure 7. Map of AAV 5' ΔITR CMV/CB-intron-U6shFluc^{inv}-intron-eGFP 3'ITR(C2).

Figure 8. Map of AAV 5'ΔITR CMV/CB-intron-eGFP U6shFluc 3' ITR (D1).

10 **Figure 9.** Map of AAV 5'ΔITR CMV/CB-intron-eGFP U6shFluc^{inv} 3' ITR (D2).

Figure 10. Functional assessment of shRNA AAV constructs B1-D2.

Figure 11. Effect of shRNA expression cassette on AAV packaging efficiency in different serotypes.

Figure 12. Effect of shRNA positions on AAV production - AAV titer.

15 **Figure 13.** Effect of hairpin RNA expression construct position and orientation on packaging of AAV constructs.

Figure 14. Effect of hairpin RNA expression construct position and orientation on yield of AAV constructs.

20 **Figure 15.** Effect of shRNA position on AAV production - genome copy to particle ratio.

Figure 16. AAV particles produced from different AAV constructs.

Figure 17. Replication of different AAV constructs.

Figure 18. Effect of shRNA position on single-stranded AAV production.

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DETAILED DESCRIPTION

Multicistronic expression constructs allow the expression of a plurality of gene products from a single nucleic acid and are useful in many basic research and therapeutic applications. One beneficial feature of multicistronic expression constructs is the possibility to express two or more gene products from the same nucleic acid construct, achieving simultaneous expression of the two or more gene products in a target cell. For example, some multicistronic expression constructs provided herein allow for expression of a gene product of interest from a first expression cassette of the multicistronic construct, and for monitoring

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that expression by detecting a reporter expressed from a second expression cassette included in the same multicistronic construct. Multicistronic expression/reporter constructs are of high value in therapeutic and research applications in which expression of the gene product of interest is not easily detectable in a target cell. Examples for such hard-to-detect gene products are non-translated RNAs, such as shRNAs, siRNAs, and microRNAs, as well as proteins that cannot be detected by conventional immunostaining methods, for example, for lack of a suitable antibody. Detecting a reporter expressed from the same nucleic acid construct as the gene product of interest can serve as an efficient proxy for identifying cells that do express the gene product of interest, thus facilitating or enabling monitoring, enrichment, purification, positive and/or negative selection, and observation of cells expressing an otherwise hard or impossible to detect gene product of interest.

Some aspects of this invention are based on the surprising discovery that efficient expression of multiple expression cassettes can be effected from multicistronic expression constructs comprising a first expression cassette including an intron, and a second expression cassette positioned within the intron of the first expression cassette, either in the same or in the opposite orientation of the first expression cassette.

Some aspects of this invention are related to the surprising discovery that such nested multicistronic expression constructs can be introduced into an AAV genome, for example, an scAAV genome, and can be efficiently packaged into infectious AAV virus particles.

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Multicistronic nucleic acid constructs

General structure and definitions

Some aspects of this invention provide multicistronic nucleic acid constructs.

The term “cistron”, as used herein, refers to a nucleic acid cassette sufficient for expression of a gene product. In some embodiments, a cistron is an expression cassette. Accordingly, some aspects of this invention provide nucleic acid constructs comprising two or more cistrons, for example, two or more expression cassettes.

The term “nucleic acid construct”, as used herein, refers to an isolated or artificially generated construct comprising a nucleic acid molecule. Non-limiting examples of nucleic acid constructs are plasmids, cosmids, bacterial artificial chromosomes, and nucleic acid vectors. The term “vector” is art recognized and refers to any nucleic acid useful to transfer a nucleic acid into a cell. Examples of vectors are plasmid vectors, gene targeting vectors, and viral vectors, for example parvoviral vectors, such as AAV vectors. A vector may comprise a nucleic acid construct in single-stranded or double-stranded form, and may comprise

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additional molecules, for example, DNA-associated proteins or viral capsid or envelope proteins. Vectors for eukaryotic and prokaryotic cells are well known to those in the art and include, for example, linear and circular DNA or RNA, viral vectors, (e.g., retroviral and parvoviral vectors, such as lentivirus-derived, Moloney murine leukemia virus-derived, adenovirus-derived, and AAV-derived vectors).

The term "isolated", refers to the characteristic of a material as provided herein being removed from its original or native environment (e.g., the natural environment if it is naturally occurring). Therefore, a naturally-occurring polynucleotide or protein or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated by human intervention from some or all of the coexisting materials in the natural system, is isolated. An artificial or engineered material, for example, a non-naturally occurring nucleic acid construct, such as the expression constructs and vectors described herein, are, accordingly, also referred to as isolated. A material does not have to be purified in order to be isolated. Accordingly, a material may be part of a vector and/or part of a composition, and still be isolated in that such vector or composition is not part of the environment in which the material is found in nature.

As used herein, the term "nucleic acid molecule", refers to an isolated or artificially produced polymer of nucleotides. The term includes, but is not limited to, oligonucleotides and polynucleotides, and single-stranded and double-stranded forms, including hybrids, for example, of DNA and RNA strands, or of strands comprising ribonucleotides, deoxyribonucleotides, and/or modified nucleotides. The polymer may include natural nucleosides (*i.e.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, dihydrouridine, methylpseudouridine, 1-methyl adenosine, 1-methyl guanosine, N6-methyl adenosine, and 2-thiocytidine), chemically modified bases, biologically modified bases (*e.g.*, methylated bases), intercalated bases, modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, 2'-O-methylcytidine, arabinose, and hexose), or modified phosphate groups (*e.g.*, phosphorothioates and 5' -N-phosphoramidite linkages).

The term "expression cassette", as used herein, refers to a nucleic acid construct comprising nucleic acid elements sufficient for the expression of a gene product. Typically,

an expression cassette comprises a nucleic acid encoding a gene product operatively linked to a promoter sequence. The term “operatively linked” refers to the association of two or more nucleic acid fragments on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operatively linked with a coding sequence when it is capable of affecting the expression of that coding sequence (e.g., the coding sequence is under the transcriptional control of the promoter). Encoding sequences can be operatively linked to regulatory sequences in sense or antisense orientation. In some embodiments, the promoter is a heterologous promoter. The term “heterologous promoter”, as used herein, refers to a promoter that does is not found to be operatively linked to a given encoding sequence in nature. In some embodiments, an expression cassette may comprise additional elements, for example, an intron, an enhancer, a polyadenylation site, a woodchuck response element (WRE), and/or other elements known to affect expression levels of the encoding sequence. Without wishing to be bound by theory, inclusion of an intron in an expression cassette, for example, between the transcriptional start site and an encoding nucleic acid sequence, for example, a protein-encoding cDNA sequence, is believed to result in increased expression levels of the encoding nucleic acid and the encoded gene product as compared to an expression construct not including an intron.

The term “intron” is art recognized and refers to a nucleic acid sequence in an expression cassette that is removed after transcription of a primary transcript by a cellular process termed splicing. Intron sequences generally comprise a splice donor and a splice acceptor and sequences of such donor and acceptor sites are well known to those of skill in the art. The term “positioned within an intron”, as used herein, refers to a nucleic acid construct, for example, an expression cassette, that is positioned between a splice donor and a splice acceptor sites of an intronic sequence.

The term “gene product,” as used herein, refers to any product encoded by a nucleic acid sequence. Accordingly, a gene product may, for example, be a primary transcript, a mature transcript, a processed transcript, or a protein or peptide encoded by a transcript. Examples for gene products, accordingly, include mRNAs, rRNAs, hairpin RNAs (e.g. microRNAs, shRNAs, siRNAs, tRNAs), and peptides and proteins, for example, reporter proteins or therapeutic proteins.

The term “promoter”, as used herein, refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a nucleic acid sequence encoding a gene product is located 3' of a promoter sequence. In some embodiments, a promoter sequence consists of proximal and more distal upstream elements

and can comprise an enhancer element. An “enhancer” is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. In some embodiments, the promoter is derived in its entirety from a native gene. In some
5 embodiments, the promoter is composed of different elements derived from different naturally occurring promoters. In some embodiments, the promoter comprises a synthetic nucleotide sequence. It will be understood by those skilled in the art that different promoters will direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions or to the presence or the
10 absence of a drug or transcriptional co-factor. Ubiquitous, cell-type-specific, tissue-specific, developmental stage-specific, and conditional promoters, for example, drug-responsive promoters (e.g. tetracycline-responsive promoters) are well known to those of skill in the art. In some embodiments, the promoter is a RNA polymerase I promoter. In some embodiments, the promoter is a RNA polymerase II promoter. In some embodiments, the promoter is a
15 RNA polymerase III promoter. Promoters mediating transcription by recruiting RNA polymerase I (e.g. most rRNA gene promoters), II (e.g. U6 and H1 promoters), or III (e.g. most promoters of protein-encoding genes), are well known to those of skill in the art. While protein encoding nucleic acid sequences are typically expressed from RNA pol II promoters and hairpin RNA encoding nucleic acid sequences from RNA pol III promoters, it is possible
20 to express both types of gene products from either promoter type.

 The term “reporter”, as used herein, refers to a gene product, encoded by a nucleic acid comprised in an expression construct as provided herein, that can be detected by an assay or method known in the art, thus “reporting” expression of the construct. Reporters and nucleic acid sequences encoding reporters are well known in the art. Reporters include, for
25 example, fluorescent proteins, such as green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), enhanced fluorescent protein derivatives (e.g. eGFP, eYFP, eRFP, mCherry, etc.), enzymes (e.g. enzymes catalyzing a reaction yielding a detectable product, such as luciferases, beta-glucuronidases, chloramphenicol acetyltransferases, aminoglycoside phosphotransferases,
30 aminocyclitol phosphotransferases, or puromycin N-acetyl-transferases), and surface antigens. Appropriate reporters will be apparent to those of skill in the related arts.

internal ribosomal entry sites, or by separating cistrons comprising their own promoter with transcriptional insulator elements. All suggested strategies to overcome promoter interference are burdened with their own set of problems, though. For example, single-promoter driven expression of multiple cistrons usually results in uneven expression levels of the cistrons.

- 5 Further some promoters cannot efficiently be isolated and isolation elements are not compatible with some gene transfer vectors, for example, some retroviral vectors.

In some embodiments of this invention, a multicistronic expression construct is provided that allows efficient expression of a first encoding nucleic acid sequence driven by a first promoter and of a second encoding nucleic acid sequence driven by a second promoter
10 without the use of transcriptional insulator elements. Various configurations of such multicistronic expression constructs are provided herein, for example, expression constructs harboring a first expression cassette comprising an intron and a second expression cassette positioned within the intron, in either the same or opposite orientation as the first cassette. Other configurations are described in more detail elsewhere herein.

- 15 In some embodiments, multicistronic expression constructs are provided allowing for efficient expression of two or more encoding nucleic acid sequences. In some embodiments, the multicistronic expression construct comprises two expression cassettes. In some embodiments, a first expression cassette of a multicistronic expression construct as provided herein comprises an RNA polymerase II promoter and a second expression cassette
20 comprises an RNA polymerase III promoter.

In some embodiments, the multicistronic expression construct provided is a recombinant AAV (rAAV) construct.

AAV and rAAV

- 25 Adeno-associated virus (AAV) is a small (20 nm) replication-defective, nonenveloped DNA virus, that depends on the presence of a second virus, for example, adenovirus or herpesvirus, for productive infection. AAV is not known to cause disease and induces a very mild immune response. AAV can infect both dividing and non-dividing cells and stably incorporates its genome into that of the host cell. AAV vectors based on serotype 2 provided
30 a proof-of-concept for non-toxic and stable gene transfer in murine and large animal models. AAV vectors having distinct tissue targeting capabilities have been developed for gene therapy and research applications. Various serotypes of AAV are known in the art. AAV serotype affects tissue tropism of the respective viral particles and allows to target specific cell types or tissues, making AAV vectors attractive for in vivo gene delivery applications in

which only a specific cell type or tissue is targeted and/or gene transfer into non-targeted cells or tissues is not desirable.

Wild type AAV particles harbor a single-stranded DNA genome comprising two genes: The AAV rep gene encodes proteins controlling viral replication, structural gene
 5 expression, and integration into the host genome. The AAV cap gene encodes capsid structural proteins. The 5' and 3' termini each comprise an inverted terminal repeat region (ITR), which is involved in multiplication of the AAV genome.. In some embodiments, an AAV ITR sequence comprises 145 nucleotides. In general, an AAV ITR sequence is a self-complementary nucleic acid structure that is able to form a hairpin, which plays a role in
 10 AAV self-priming for synthesis of the second DNA AAV strand during the viral life cycle. An exemplary ITR is described herein as SEQ ID NO: 2. Recombinant AAV (rAAV) vectors are generally produced by replacing the viral genes, or parts thereof, with a heterologous expression cassettes. Typically, rAAV genomes up to about 5kb in length can efficiently be packaged into infectious viral particles useful for gene transfer. In some embodiments, the
 15 rAAV construct is a single-stranded rAAV construct. That is, the rAAV construct contains two ITRs, a 5'ITR and a 3'ITR that comprise a functional TRS each. In some embodiments, one of the ITRs, for example, the 5'ITR is a Δ TRS ITR. In some such embodiments, the AAV construct is a double-stranded, self-complementary AAV (scAAV) construct. For an overview of AAV biology, ITR function, and scAAV constructs, see McCarty DM. *Self-complementary AAV vectors; advances and applications*. Mol Ther. 2008 Oct;16(10): at
 20 pages 1648-51, first full paragraph. An exemplary Δ TRS ITR, also referred to as deltaITR, deltaTRS ITR, or Δ ITR herein, is described in SEQ ID NO: 1. A rAAV vector comprising a Δ TRS ITR cannot correctly be nicked during the replication cycle and, accordingly, produces a self-complementary, double-
 25 stranded AAV (scAAV) genome, which can efficiently be packaged into infectious AAV particles. Various rAAV, ssAAV, and scAAV vectors, as well as the advantages and drawbacks of each class of vector for specific applications and methods of using such vectors in gene transfer applications are well known to those of skill in the art (see, for example, Choi VW, Samulski RJ, McCarty DM. *Effects of adeno-associated virus DNA hairpin structure on recombination*. J Virol. 2005 Jun;79(11):6801-7; McCarty DM, Young SM Jr, Samulski RJ. *Integration of adeno-associated virus (AAV) and recombinant AAV vectors*. Annu Rev Genet. 2004;38:819-45; McCarty DM, Monahan PE, Samulski RJ. *Self-complementary recombinant*

adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. Gene Ther. 2001 Aug;8(16):1248-54; and McCarty DM. *Self-complementary AAV vectors; advances and applications.* Mol Ther. 2008 Oct;16(10):1648-56).

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The term "recombinant AAV construct" refers to an AAV construct comprising an AAV 5' ITR, at least one recombinant expression cassette, and a 3' ITR. In some embodiments, a rAAV vector is packaged into a capsid protein and delivered to a selected target cell. In some embodiments, the transgene is an expression cassette, comprising a nucleic acid sequence heterologous to the AAV sequences, and encoding a gene product. The encoding nucleic acid coding sequence is operatively linked to a regulatory component, for example, a promoter, in a manner permitting transcription, translation, and/or expression of the gene product in a cell of a target tissue. Recombinant AAV based vectors harboring multicistronic expression constructs are provided herein. In some embodiments, rAAV vectors are engineered to target specific cells, cell types, or tissues, for example, liver tissue.

The AAV sequences of a rAAV construct provided herein typically comprise the cis-acting 5' and 3' inverted terminal repeat sequences (See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990)). The ITR sequences are about 145 bp in length. Preferably, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K. Fisher et al., J Virol., 70:520 532 (1996)). AAV ITR sequences may be obtained from any known AAV, including presently identified mammalian AAV types.

In addition to the major elements identified above for the rAAV vectors and constructs, a rAAV vector may also include additional transcriptional control elements. Transcriptional control elements are known to those of skill in the art and exemplary elements include transcription initiation, termination, promoter and enhancer sequences, RNA processing signals such as splicing and polyadenylation (polyA) signals, sequences that stabilize cytoplasmic mRNA, sequences that enhance translation efficiency (e.g., Kozak consensus sequences), sequences that enhance protein stability, and, if appropriate, sequences that enhance secretion of the encoded product. A great number of expression control

sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

In some embodiments, transcriptional control elements or sequences impart tissue-specific gene expression capabilities to a multicistronic expression construct as provided herein. In some embodiments, tissue-specific transcriptional control sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (e.g., promoters, enhancers, etc.) are well known in the art.

10 ***rAAVs: Production Methods***

Methods for obtaining rAAV preparations having a desired capsid protein are well known in the art. (See, for example, US 2003/0138772). Typically the methods involve culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid protein or fragment thereof; a functional *rep* gene; a recombinant AAV vector composed of, AAV inverted terminal repeats (ITRs) and an expression cassette encoding a transgene; and sufficient helper functions to permit packaging of the recombinant AAV vector into the AAV capsid proteins.

The components to be cultured in the host cell to package a rAAV vector in an AAV capsid may be provided to the host cell in *trans*. Alternatively, any one or more of the required components (e.g., recombinant AAV vector, *rep* sequences, *cap* sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art. Most suitably, such a stable host cell will contain the required component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. Examples of suitable inducible and constitutive promoters are provided herein, in the discussion of regulatory elements suitable for use with the transgene. In still another alternative, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contain the *rep* and/or *cap* proteins under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art.

The recombinant AAV vector, rep sequences, cap sequences, and helper functions required for producing the rAAV of the invention may be delivered to the packaging host cell using any appropriate genetic element (vector). The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct any embodiment of this invention are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, *e.g.*, Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present invention. See, *e.g.*, K. Fisher et al, J. Virol., 70:520-532 (1993) and U.S. Pat. No. 5,478,745.

In some embodiments, recombinant AAVs may be produced using the triple transfection method (described in detail in U.S. Pat. No. 6,001,650). Typically, the recombinant AAVs are produced by transfecting a host cell with an recombinant AAV vector (comprising a transgene) to be packaged into AAV particles, an AAV helper function vector, and an accessory function vector. An AAV helper function vector encodes the "AAV helper function" sequences (*i.e.*, rep and cap), which function in *trans* for productive AAV replication and encapsidation. Preferably, the AAV helper function vector supports efficient AAV vector production without generating any detectable wild-type AAV virions (*i.e.*, AAV virions containing functional rep and cap genes). Non-limiting examples of vectors suitable for use with the present invention include pHLP19, described in U.S. Pat. No. 6,001,650 and pRep6cap6 vector, described in U.S. Pat. No. 6,156,303. The accessory function vector encodes nucleotide sequences for non-AAV derived viral and/or cellular functions upon which AAV is dependent for replication (*i.e.*, "accessory functions"). The accessory functions include those functions required for AAV replication, including, without limitation, those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV-DNA replication, synthesis of cap expression products, and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1), and vaccinia virus.

In some aspects, the invention provides transfected host cells. The term "transfection" is used to refer to the uptake of foreign DNA by a cell, and a cell has been "transfected" when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham et al. (1973) Virology, 52:456, Sambrook et al. (1989) Molecular Cloning, a laboratory manual,

Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) Gene 13:197. Such techniques can be used to introduce one or more exogenous nucleic acids, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells. Transfection may be achieved, for example, by infecting a cell with an rAAV harboring an rAAV vector.

A “host cell” refers to any cell that harbors, or is capable of harboring, a substance of interest. Often a host cell is a mammalian cell. A host cell may be used as a recipient of an AAV helper construct, an AAV transgene plasmid, e.g., comprising a promoter operably linked with an miRNA inhibitor, an accessory function vector, or other transfer DNA associated with the production of recombinant AAVs. The term includes the progeny of the original cell which has been transfected. Thus, a “host cell” as used herein may refer to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

As used herein, the term “cell line” refers to a population of cells capable of continuous or prolonged growth and division *in vitro*. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

As used herein, the terms “recombinant cell” refers to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active polypeptide or production of a biologically active nucleic acid such as an RNA, has been introduced.

Nucleic acid constructs harboring multiple self-complementary nucleic acid sequences

Some aspects of this invention provide rAAV constructs harboring a heterologous self-complementary nucleic acid sequence, for example, as part of a hairpin RNA expression cassette. Some aspects of this invention relate to the surprising discovery that an expression cassette comprising a nucleic acid sequence encoding a self-complementary RNA positioned in proximity to an AAV-ITR, can efficiently be expressed if the ITR is a Δ TRS ITR, independent of the orientation of the expression cassette. In some embodiments, an AAV

expression construct is provided comprising an expression cassette encoding a hairpin RNA in close proximity to a rAAV Δ ITR. In some embodiments, proximal positioning refers to the expression cassette and the Δ ITR being separated by less than about 500, less than about 400, less than about 300, less than about 250, less than about 200, less than about 100, less than about 50, less than about 25, or less than about 10 nucleotides.

In some embodiments, a multicistronic AAV expression construct is provided comprising an expression cassette encoding a hairpin RNA and oriented in opposite orientation to a second expression cassette comprised in the expression construct. In some embodiments, the hairpin expression cassette is positioned in proximity to the Δ ITR.

In some embodiments, a multicistronic AAV expression vector is provided in which a hairpin expression cassette is positioned adjacent to a functional ITR. In some embodiments, the expression cassette and the functional ITR are separated by at least about 150, at least about 200, at least about 300, at least about 400, at least about 500, at least about 600, at least about 700, at least about 800, at least about 900, or at least about 1000 nucleotides.

Methods of use

The multicistronic expression constructs provided herein are useful in various research and therapeutic applications. In some embodiments, a cell is contacted with an expression construct as provided herein. In some embodiments, the cell expresses a transcript comprising a target sequence corresponding or complementary to a hairpin RNA sequence encoded by the expression construct. In some embodiments, expression of the target sequence is decreased by expression of a corresponding hairpin RNA sequence in the cell. In some embodiments, the effect of expression of a multicistronic expression construct as provided herein on a phenotypic parameter of the cell is monitored after the cell has been contacted with the expression construct. Examples for phenotypic parameters of a cell include, but are not limited to, cell viability, cell survival, cell proliferation rate, cell growth rate, cell shape, cell size, cell volume, cell expression profile, cell surface marker expression, resistance to antibiotic drugs, and cell reaction to drug treatment. Methods of determining and monitoring such parameters are well known to those of skill in the relevant arts.

Some aspects of this invention provide a cell contacted with a multicistronic expression construct provided herein. In some embodiments, the cell comprises the multicistronic expression construct, or a fragment thereof, integrated into the cell's genome. In some embodiments, the cell is a non-human mammalian cell. In some embodiments, the cell is a human cell.

Methods for contacting a cell with a nucleic acid construct, for example, a nucleic acid construct provided herein, are well known to those of skill in the art and include, for example, electroporation of naked nucleic acid constructs, transfection of nucleic acid constructs complexed with transfection agents, such as Fugene or Lipofectamine, or
5 transduction of cells with nucleic acid constructs packaged into viral particles, for example, AAV, or retroviral particles. Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In some embodiments, a cell is contacted with an expression construct in vitro. In some embodiments,
10 a cell is contacted in vivo, for example, in a subject. In some embodiments, a cell is obtained from a subject, contacted ex vivo, and subsequently returned to a subject, for example, to the same subject the cell was obtained from.

Recombinant AAV Administration Methods

15 In some embodiments, the multicistronic rAAV expression constructs provided herein are delivered to a subject in compositions according to any appropriate methods known in the art. The rAAV, preferably suspended in a physiologically compatible carrier (*i.e.*, in a composition), may be administered to a subject, such as, for example, a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, or a non-
20 human primate (e.g., Macaque).

In some embodiments, delivery of the rAAV expression constructs provided herein to a subject is effected via intravenous injection, e.g., injection into a portal vein. Administration into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit. In some embodiments, rAAVs are injected into the bloodstream by way of
25 isolated limb perfusion, a technique well known in the surgical arts, the method essentially enabling the artisan to isolate a limb from the systemic circulation prior to administration of the rAAV virions. A variant of the isolated limb perfusion technique, described in U.S. Pat. No. 6,177,403, can also be employed by the skilled artisan to administer the rAAVs into the vasculature of an isolated limb to potentially enhance transduction into muscle cells or tissue.
30 Moreover, in some embodiments, it may be desirable to deliver the virions to the CNS of a subject. "CNS" refers to all cells and tissue of the brain and spinal cord of a vertebrate. Thus, the term includes, but is not limited to, neuronal cells, glial cells, astrocytes, cerebrospinal fluid (CSF), interstitial spaces, bone, cartilage and the like. Recombinant AAVs may be delivered directly to the CNS or brain by injection into, e.g., the ventricular region, as well as

to the striatum (*e.g.*, the caudate nucleus or putamen of the striatum), spinal cord and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection (see, *e.g.*, Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al.,
 5 Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000).

In certain circumstances it will be desirable to deliver the rAAV-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intrapancreatically, intranasally, parenterally, intravenously, intramuscularly,
 10 intrathecally, or orally, intraperitoneally, or by inhalation. In some embodiments, the administration modalities as described in U.S. Pat. Nos. 5,543,158; 5,641,515 and 5,399,363 may be used to deliver rAAVs. In some embodiments, a preferred mode of administration is by portal vein injection.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions
 15 or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In many cases the form is sterile and fluid to the extent that easy syringability exists. It must be stable
 20 under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a
 25 coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged
 30 absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous,

intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example,

5 "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580).

Some variation in dosage will necessarily occur depending on the condition of the host. The person responsible for administration will, in any event, determine the appropriate dose for the individual host.

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

The compositions of the invention may comprise an rAAV alone, or in combination with one or more other viruses (*e.g.*, a second rAAV encoding having one or more different transgenes). In some embodiments, a compositions comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or
20 more different rAAVs each comprising one or more different expression cassettes or configurations.

Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the rAAV is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered
25 saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

Optionally, the compositions of the invention may contain, in addition to the rAAV
30 and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

The rAAVS are administered in sufficient amounts to transfect the cells of a desired tissue and to provide sufficient levels of gene transfer and expression without undue adverse effects. Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the selected organ (*e.g.*, intraportal delivery to the liver),
5 oral, inhalation (including intranasal and intratracheal delivery), intraocular, intravenous, intramuscular, subcutaneous, intradermal, intratumoral, and other parental routes of administration. Routes of administration may be combined, if desired.

The dose of rAAV virions required to achieve a particular "therapeutic effect," *e.g.*, the units of dose in vector genomes/per kilogram of body weight (vg/kg), will vary based on
10 several factors including, but not limited to: the route of rAAV virion administration, the level of gene or RNA expression required to achieve a therapeutic effect, the specific disease or disorder being treated, and the stability of the gene or RNA product. One of skill in the art can readily determine a rAAV virion dose range to treat a patient having a particular disease or disorder based on the aforementioned factors, as well as other factors that are well known
15 in the art.

An effective amount of an rAAV is an amount sufficient to target infect an animal, target a desired tissue. In some embodiments, an effective amount of an rAAV is an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend primarily on factors such as the species, age, weight, health of the subject, and the
20 tissue to be targeted, and may thus vary among animal and tissue. For example, a effective amount of the rAAV is generally in the range of from about 1 ml to about 100 ml of solution containing from about 10^9 to 10^{16} genome copies. In some cases, a dosage between about 10^{11} to 10^{12} rAAV genome copies is appropriate. In certain preferred embodiments, 10^{12} rAAV genome copies is effective to target heart, liver, and pancreas tissues. In some cases,
25 stable transgenic animals are produced by multiple doses of an rAAV.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens.

30 Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound in each therapeutically-useful composition may be prepared in such a way that a suitable dosage will

be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

The rAAV constructs and compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a host.

Delivery vehicles such as liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, may be used for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered transgenes may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art. Recently, liposomes were developed with improved serum stability and circulation half-times (U.S. Pat. No. 5,741,516). Further, various methods of liposome and liposome like preparations as

potential drug carriers have been described (; U.S. Pat. Nos. 5,567,434; 5,552,157; 5,565,213; 5,738,868 and 5,795,587).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures. In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs, radiotherapeutic agents, viruses, transcription factors and allosteric effectors into a variety of cultured cell lines and animals. In addition, several successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 μ m. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 $\text{\AA}$, containing an aqueous solution in the core.

Alternatively, nanocapsule formulations of the rAAV may be used. Nanocapsules can generally entrap substances in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μ m) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use.

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the rAAV compositions to a host. Sonophoresis (e.g., ultrasound) has been used and described in U.S. Pat. No. 5,656,016 as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U.S. Pat. No. 5,779,708), microchip devices (U.S. Pat. No. 5,797,898), ophthalmic formulations (Bourlais et al., 1998), transdermal matrices (U.S. Pat. Nos. 5,770,219 and 5,783,208) and feedback-controlled delivery (U.S. Pat. No. 5,697,899).

Compositions and Kits

The multicistronic expression constructs described herein may, in some embodiments, be assembled into pharmaceutical or diagnostic or research kits to facilitate their use in therapeutic, diagnostic or research applications. A kit may include one or more containers housing the components of the invention and instructions for use. Specifically, such kits may include one or more agents described herein, along with instructions describing the intended

application and the proper use of these agents. In certain embodiments agents in a kit may be in a pharmaceutical formulation and dosage suitable for a particular application and for a method of administration of the agents. Kits for research purposes may contain the components in appropriate concentrations or quantities for running various experiments.

5 The kit may be designed to facilitate use of the methods described herein by researchers and can take many forms. Each of the compositions of the kit, where applicable, may be provided in liquid form (*e.g.*, in solution), or in solid form, (*e.g.*, a dry powder). In certain cases, some of the compositions may be constitutable or otherwise processable (*e.g.*, to an active form), for example, by the addition of a suitable solvent or other species (for
10 example, water or a cell culture medium), which may or may not be provided with the kit. As used herein, "instructions" can define a component of instruction and/or promotion, and typically involve written instructions on or associated with packaging of the invention. Instructions also can include any oral or electronic instructions provided in any manner such that a user will clearly recognize that the instructions are to be associated with the kit, for
15 example, audiovisual (*e.g.*, videotape, DVD, *etc.*), Internet, and/or web-based communications, *etc.* The written instructions may be in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which instructions can also reflect approval by the agency of manufacture, use or sale for animal administration.

20 The kit may contain any one or more of the components described herein in one or more containers. As an example, in one embodiment, the kit may include instructions for mixing one or more components of the kit and/or isolating and mixing a sample and applying to a subject. The kit may include a container housing agents described herein. The agents may be in the form of a liquid, gel or solid (powder). The agents may be prepared sterilely,
25 packaged in syringe and shipped refrigerated. Alternatively it may be housed in a vial or other container for storage. A second container may have other agents prepared sterilely. Alternatively the kit may include the active agents premixed and shipped in a syringe, vial, tube, or other container. The kit may have one or more or all of the components required to administer the agents to an animal, such as a syringe, topical application devices, or iv needle
30 tubing and bag, particularly in the case of the kits for producing specific somatic animal models.

Hairpin RNAs and RNAi

Some aspects of this invention provide methods for the use of expression constructs provided herein to effect RNA interference (RNAi) in a target cell. In some embodiments, the construct used to effect RNAi in the target cell is a multicistronic expression construct as
5 provided herein comprising a hairpin RNA expression cassette.

RNAi, a key cellular pathway utilizing double-stranded RNAs, such as shRNAs or microRNAs as provided herein, as sequence-specific regulators, can be harnessed for a wide spectrum of potential therapeutic and basic research applications. RNAi has been proposed as a cellular response to the presence of double-stranded RNA (dsRNA) in the cell. It is
10 believed that dsRNAs, for example, small hairpin RNAs (shRNAs) and microRNAs (miRNAs) are cleaved into ~20-base pair (bp) duplexes of small-interfering RNAs (siRNAs) by Dicer. It is further believed that siRNAs, either exogenously introduced into a cell or generated by Dicer from shRNA, miRNA, or other substrates, bind to the RNA-induced silencing complex ("RISC"), where they are unwound and the sense strand, also called the
15 "passenger strand" is discarded. The antisense strand of the siRNA, also referred to as the "guide strand", complexed with RISC then binds to a complementary target sequence, for example, a target sequence comprised in an mRNA, which is subsequently cleaved by Slicer, resulting in inactivation of the nucleic acid molecule comprising the target sequence. As a result, the expression of mRNAs containing the target sequence is reduced.

20 Target sequence recognition and binding by the siRNA guide strand is not completely stringent. While guide strands complementary to a given target sequence have been demonstrated to effect efficient target sequence cleavage and inhibition of expression, it is also known in the art that nucleic acid sequences containing sequences corresponding to the siRNA guide strand can also be efficiently targeted. In connection with RNAi, two nucleic
25 acid sequences are referred to as corresponding sequences, if they are either complementary, or if the degree of complementarity is high enough to allow recognition and binding of the target sequence by the guide strand under physiological conditions. Methods and algorithms to engineer efficient siRNA sequences complementary or corresponding to a given target sequence, or to determine whether a given siRNA sequence will effect inhibition of target
30 sequence expression, are well known to those in the art. Suitable algorithms are publicly available (e.g., from Whitehead Institute for Biomedical Research, Cambridge, MA, at jura.wi.mit.edu/bioc/siRNAext/). Methods to engineer and synthesize hairpin RNAs and nucleic acid sequences encoding such hairpin RNAs are also well known to those of skill in the art. The term "hairpin RNA" refers to a self-complementary, single-stranded RNA

comprising a first nucleic acid sequence and a second nucleic acid sequence complementary to the first nucleic acid sequence positioned in a manner that allows the two sequences to anneal. In some embodiments, the two complementary sequences are separated by a third nucleic acid sequence, the hairpin loop sequence, that is not self-complementary and remains single-stranded after the two complementary sequences have annealed to form the double-stranded “stem” region of the hairpin, thus allowing formation of a hairpin structure. In some embodiments, the first nucleic acid sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides long. In some embodiments, the first nucleic acid sequence is between about 50 to about 100 nucleotides long. Accordingly, a nucleic acid encoding a hairpin structure comprises a self-complementary sequence that may form a secondary structure by self-annealing of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or about 50 to about 100 consecutive nucleotides forming intramolecular base pairs. In some embodiments, the stem region of a hairpin RNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 base pair mismatches.

A hairpin RNA, accordingly, may be any RNA comprising a self-complementary nucleic acid sequence able to form a hairpin structure, for example, as described above. Non-limiting examples for hairpin RNAs are mRNAs comprising a hairpin structure, tRNAs, “small hairpin RNAs” also referred to as “short hairpin RNAs” (shRNAs) and “microRNAs”, also referred to as “miRNAs” or miRs. A nucleic acid molecule encoding a hairpin RNA is necessarily a nucleic acid comprising a self-complementary nucleic acid sequence (encoding the stem-loop-stem structure of the hairpin RNA). In single-stranded form, such encoding nucleic acids may form a hairpin structure by self annealing of the self-complementary nucleic acid sequence in analogy to the encoded hairpin RNA. Without wishing to be bound by theory, it is believed that a hairpin RNA expressed in a target cell is processed by the cell’s RNAi machinery, as described in more detail elsewhere herein and as known to those of skill in the art, to form a small interfering RNA (siRNA). As described elsewhere herein and as known to those of skill in the related arts, siRNAs can efficiently and specifically inhibit expression of their target gene. Nucleic acid sequences encoding hairpin RNAs and methods and algorithms for designing and generating hairpin RNAs targeted to a given gene are well known to those of skill in the art. As used herein, the term “target sequence” refers to a nucleotide sequence targeted by a nucleic acid molecule, for example a nucleic acid molecule provided herein, as part of the RNAi mechanism. For example, in some

embodiments, a target sequence is a sequence of an mRNA encoding a protein to which a given nucleic acid molecule, for example a nucleic acid molecule complexed with RISC would bind. In some embodiments, a target sequence is a sequence that is specific for a transcript to be targeted. For example, in some embodiments, a target sequence of a specific mRNA is a sequence unique to the specific mRNA.

In general, the guide strand of a given siRNA recognizes and binds to a target sequence that is complementary to the guide strand sequence. Stringent complementarity between guide strand and target sequence is, however, not required, as it is known in the art that a guide strand still efficiently recognizes and binds to a target sequence with single or multiple base pair mismatches. Accordingly, while stringent complementarity between guide strand and target sequence is preferred in some embodiments, a sufficient degree of complementarity between guide strand and target sequence is given, where the guide strand sequence and the target sequence are complementary for all but 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base pair mismatch. In some embodiments, a sufficient degree of complementarity between guide strand and target sequence is given, where the guide strand sequence and the target sequence share 100%, about 99%, about 98%, about 97%, about 96%, about 95%, about 94%, about 93%, about 92%, about 91%, about 90%, about 89%, about 88%, about 87%, about 86%, about 85%, about 84%, about 83%, about 82%, about 81%, or about 8%.

RNAi applications

Various methods and vectors for the expression of siRNAs in a variety of target cells are known in the art, including direct delivery of siRNA complexes or expression from a nucleic acid construct comprising a nucleic acid encoding an siRNA precursor, for example, a hairpin RNA, such as a shRNA or miRNA. In some embodiments, a nucleic acid construct for siRNA expression comprises an expression cassette including a self-complementary nucleic acid sequence encoding a transcript comprising a self-complementary region, for example, a hairpin RNA.

Methods for hairpin RNA and, thus, siRNA expression include, but are not limited to, transient expression methods, for example, methods involving transfection or transduction of non-integrating DNA or RNA constructs into a target cell, or constitutive expression methods, for example, methods involving transfection or transduction of nucleic acid constructs that integrate into the genome of the target cell or are maintained epichromosomally in the target cell.

The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention.

5

EXAMPLES

Materials and methods

Nucleic acid constructs

In order to test the positional effects in nucleic acid constructs harboring a plurality of expression cassettes and/or a plurality of self-complementary nucleic acid sequences, a series of non-viral and viral expression constructs was generated.

The expression cassettes used were

(1) a CMV/CB-intron-eGFP cassette, comprising a CMV enhancer (SEQ ID NO: 3), a CB promoter (SEQ ID NO: 4), an intron (SEQ ID NO: 5), a nucleic acid encoding enhanced green fluorescent protein (eGFP) (SEQ ID NO: 6), and a 3'UTR/polyadenylation signal/GRE element (SEQ ID NO: 7); and

(2) a U6siFluc cassette (SEQ ID NO: 10), comprising a U6 promoter (SEQ ID NO: 8) and a nucleic acid encoding an shRNA targeting a luciferase mRNA (siFluc) (SEQ ID NO: 9).

Some of the nucleic acid constructs were AAV constructs, further comprising a 5' Δ ITR (an inverted terminal repeat lacking a functional terminal resolution site, SEQ ID NO: 2) and a 3'ITR (a functional AAV ITR including a functional TRS, SEQ ID NO: 1). Both ITR and 5' Δ ITR sequences comprise self-complementary nucleic acid sequences.

In some of the constructs generated the expression cassettes (1) and (2) were in the same orientation, while in some other constructs they were in opposite, or inverted, orientation to each other. The constructs in which the expression cassettes were in opposite orientation, comprised an inverted U6siFluc cassette, U6Fluc^{inv} (SEQ ID NO: 11). The constructs generated, non-viral constructs A1 and A2, and AAV constructs A, B1, B2, C1, C2, D1, and D2, are disclosed in SEQ ID NOs: 12-20 and the respective vector maps are shown in Figures 1-9. For the AAV experiments, construct A, comprising only one expression cassette (the eGFP cassette) served as a control construct.

AAV Genome Copy (GC) number titration

The genome copy (GC) number of an AAV vector preparation is a measure of the number of AAV particles with full genome content in that preparation and can be determined by real time PCR, or Q-PCR. Typically, validated controls representing known GC benchmarks are assayed in parallel to a given AAV preparation and the actual GC number
5 can be determined from comparing the results obtained from the benchmark samples to the given AAV preparation.

AAV infectious titer determination (Q-PCR)

AAV titers can be determined by standard techniques, for example, the infectious
10 center assay (ICA). Alternatively, Q-PCR assays can be employed to determine AAV titers. Typically, such Q-PCR assays are based upon limiting dilution of the vector and an endpoint or 50% endpoint determination of viral DNA replication using real-time PCR. AAV vectors may be serially diluted and a cell line expressing AAV rep and cap may be co-infected with these dilutions plus wildtype Ad5 in parallel replicates. The presence of AAV rep and
15 adenovirus helper genes allows for the replication of AAV DNA. After a suitable incubation period, DNA is extracted and endpoint or 50% endpoint determination is performed. Validation samples may be included. A GC:infectivity (GC:I) ratio may be calculated based upon the results of both the GC copy number titration and Q-PCR assay results. The GC:I ratio is used as a measure of infectivity of the preparation with low GC:I ratios indicating
20 more infectious vector lots. The GC:I ratios between different lots of the same serotype are useful in assessing the relative potency of a particular preparation.

EXAMPLE 1

**Expression of eGFP and siRNA from expression constructs comprising an eGFP
25 and a hairpin expression cassette in different positional configurations.**

The AAV constructs A, B1, C1, and D1, comprising the U6siFluc cassette at three different positions (see Figure 9, upper panel) were assayed for eGFP expression levels by transfecting mammalian cells with plasmid DNA containing the respective construct. Construct A served as a control construct. Figure 9, lower panel, shows representative
30 fluorescence microscopy images of transfected cells. Surprisingly, no significant difference in eGFP fluorescence was observed in cells transfected with the different AAV constructs, indicating that the inclusion of the U6siFluc cassette upstream, downstream, or within the intron of the eGFP expression cassette did not affect overall eGFP expression. The finding that placement of the U6siFluc cassette in proximity to the CMV/CB enhancer/promoter

element does not affect eGFP expression is remarkable in that it is contrary to a large body of evidence suggesting that placement of two promoters close to each other can lead to promoter interference with significant inhibition of one or both promoters. Further, our results constitute the first demonstration that positioning a hairpin expression cassette within an intron of a second expression cassette does not affect the expression of the gene product encoded by the second expression cassette.

To compare siFluc expression levels among the AAV constructs mentioned above, they were further assayed for their ability to inhibit expression of their target luciferase mRNA. Cells expressing the target luciferase mRNA were transfected with plasmid DNA comprising the respective construct and luciferase activity was measured subsequent to transfection (Figure 9, middle panel). As a negative control, a plasmid RNAi vector, pRNA, comprising a mock hairpin RNA not targeting luciferase under the control of a U6 promoter was transfected to establish a “no knockdown” luciferase expression baseline. Luciferase activity after transfection with negative control pRNA-U6-mock was normalized to a relative value of 100. As a positive knockdown control, a pRNA vector comprising a nucleic acid encoding siFluc (a hairpin RNA targeting luciferase) under the control of a U6 promoter was transfected to establish a level of luciferase expression knockdown achieved with a well established hairpin expression vector. The positive control pRNA-U6-siFluc achieved knockdown of luciferase expression to 36% of the baseline level (Figure 9). As expected, transfection of the luciferase-expressing cells with construct A, an AAV construct not comprising a siFluc encoding nucleic acid sequence, did not result in appreciable knockdown of luciferase expression (96% luciferase activity). In contrast, transfection of each the AAV constructs B1, C1, and D1 resulted in luciferase expression knockdown to levels comparable to those observed after transfection of the positive control vector (43%-45%). Surprisingly, no appreciable differences in knockdown efficiency were observed between these constructs, suggesting that siRNA expression can efficiently be effected from hairpin RNA expression cassettes at either position.

Together, the eGFP and siFluc expression results indicate that a hairpin expression cassette can be positioned upstream, downstream, or within an intron of a second expression cassette, for example, a reporter expression cassette without affecting the expression level of the hairpin RNA encoded by the hairpin RNA expression cassette or the gene product encoded by the second expression cassette. Further, the results indicate that a hairpin RNA expression cassette can be positioned in close proximity of a promoter of a second expression cassette without affecting the expression levels of the encoded hairpin RNA or the gene

product encoded by the second expression cassette, for example, through promoter interference. Finally, a siRNA can efficiently be expressed from a hairpin RNA expression cassette positioned within an intron of a second expression cassette, without affecting the expression level of the gene product, for example, a reporter (e.g., eGFP), encoded by the second expression cassette.

EXAMPLE 2

Expression of eGFP and siFluc from constructs comprising an eGFP expression cassette and an siRNA expression cassette in different orientation configurations.

To investigate the effect of the orientation of the hairpin expression cassette relative to the second expression cassette on expression of siFluc and eGFP, constructs B2, C2, and D2, comprising the hairpin RNA expression cassette at the same position as constructs B1, C1, and D1, respectively, but in opposite orientation, were transfected into cells and eGFP expression was examined by fluorescent microscopy. Construct A served as a control construct. Figure 10, lower panel, shows representative fluorescence microscopy images of cells transfected with the respective constructs. Surprisingly, no significant difference in eGFP fluorescence was observed in cells transfected between the AAV constructs harboring the siFluc expression cassette in the same orientation as the eGFP cassette as compared to the constructs harboring the siFluc expression cassette in the same position, but in opposite orientation (B1 compared to B2, C1 to C2, and D1 to D2). These results indicate that the orientation of the U6siFluc cassette at either of the three positions did not affect overall eGFP expression. The finding that the orientation of the U6siFluc cassette does not affect eGFP expression is remarkable in that a large body of evidence suggests that placement of two expression cassettes in opposite orientation might lead to “collisions” of transcriptional machinery during the transcription process, especially, where the two promoters are oriented in a manner that transcription occurs in the direction of the other promoter. Further, our results constitute the first demonstration that positioning a hairpin expression cassette within an intron of a second expression cassette does not affect the expression of the gene product encoded by the second expression cassette, even if the hairpin expression cassette is in opposite orientation of the first expression cassette.

To compare siFluc expression levels among the AAV constructs mentioned above, they were further assayed for their ability to inhibit expression of their target luciferase mRNA. As described above, cells expressing the target luciferase mRNA were transfected with plasmid DNA comprising the respective construct and luciferase activity was measured

subsequent to transfection, using the same positive and negative pRNA controls as described above (Figure 10, middle panel). Surprisingly, transfection with the AAV constructs B2, C2, and D2 resulted in luciferase expression knockdown to 42%-46% of baseline levels. The knockdown levels observed with these constructs were comparable to those observed after
5 transfection with the initial AAV constructs harboring the hairpin RNA cassette in the original orientation. The fact that no appreciable differences in knockdown efficiency were observed in constructs harboring the hairpin RNA expression cassette at the same position, but in different orientations (B1 and B2, C1 and C2, D1 and D2, respectively), suggests that siRNA expression can efficiently be effected from hairpin RNA expression cassettes in either
10 orientation.

Together, the eGFP and siFluc expression results indicate that a hairpin expression cassette can be positioned upstream, downstream, or within an intron of a second expression cassette, for example, a reporter expression cassette, and in either the same or in the opposite direction of the second expression cassette, without affecting the expression level of the
15 hairpin RNA encoded by the hairpin RNA expression cassette or the gene product encoded by the second expression cassette. Further, the results indicate that a hairpin RNA expression cassette can be positioned in close proximity of a promoter of a second expression cassette, and in either the same or in the opposite orientation as the second expression cassette, without affecting the expression levels of the encoded hairpin RNA or the gene product encoded by
20 the second expression cassette, for example, through promoter interference. Finally, a siRNA can efficiently be expressed from a hairpin RNA expression cassette positioned within an intron of a second expression cassette, and either in the same or in the opposite orientation as the second expression cassette, without affecting the expression level of the gene product, for example, a reporter (e.g., eGFP), encoded by the second expression cassette.

25

EXAMPLE 3:

Effect of a hairpin expression cassette on yield and titer of AAV constructs.

In order to investigate the effect of a hairpin cassette on AAV packaging efficiency, the yield and titer of AAV construct A (no hairpin cassette) was compared to the yield and
30 titer of construct D1 (hairpin expression cassette cloned between eGFP expression cassette and 3'ITR in the same orientation as the eGFP cassette). Figure 11 shows a comparison of packaging efficiency between these two scAAV vectors (scAAV: AAV construct A; sh scAAV: AAV construct D2).

Both yield and titer were decreased in the sh scAAV vector as compared to non-hairpin scAAV vector, indicating that the presence of a hairpin RNA expression cassette in the configuration of AAV construct D1 can inhibit AAV packaging (Figure 11, upper panel). These findings were confirmed in different serotypes (AAV2 and AAV8, Figure 11, lower panel). While both titer and yield varied between serotypes, the inclusion of the hairpin expression cassette in the described configuration resulted in decreased titer and yield of the sh scAAV construct in both serotypes. These results indicate that the presence of a hairpin RNA cassette, a structure comprising a self-complementary nucleic acid sequence, positioned between the eGFP cassette and the 3'ITR in the same orientation as the eGFP cassette, can interfere with the packaging of the scAAV genome.

EXAMPLE 4:

Positional and orientation effects of a hairpin cassette on yield and titer of AAV constructs.

In order to investigate whether a hairpin RNA expression cassette could be introduced into an scAAV genome at a different position or in a different orientation than the configuration provided in AAV construct D1 without detrimental effect on AAV genome packaging efficiency, titers and yields of AAV constructs B1-D2 were determined (Figures 12-17; 5'ITR (3'-5'): B2; 5'ITR (5'-3'): B1; intron (5'-3'): C2; intron (3'-5'): C1; 3'ITR (3'-5'): D2; 5'ITR (5'-3'): D1; dsAAV shRNA: positive control scAAV vector).

A comparison of packaging efficiencies of constructs B1, B2, C1, C2, D1, and D2 with two different shRNA expression cassettes is shown in Figure 12. The results indicate that the different shRNA expression cassettes resulted in different packaging efficiencies. More importantly, an shRNA expression cassette positioned proximal to the 3'ITR had a detrimental effect on packaging regardless of shRNA identity. Constructs D1 and D2 were observed to achieve the lowest packaging efficiency with either shRNA, while B1 and B2 could be efficiently packaged with either shRNA expression cassette.

In order to elucidate the packaging efficiencies of the different AAV constructs in more detail, the genome copy number (GC) and the number of AAV particles (VP) was assessed for the 6 different AAV constructs B1-D2 with two different shRNA expression cassettes. Genome copy numbers were analyzed by qPCR for the 6 AAV constructs (Figures 13 and 14). Figure 13 shows the average yield obtained from a control dsAAV shRNA construct as a control. These Figures show that the titers of AAV constructs B1, B2, C1, and C2 were significantly higher than the titer of AAV construct D2. In Figure 14, the titer of

AAV construct D1 was significantly higher than the titer of D2, but did not reach the level of the titers of B1-C2. These data indicate that the positioning of the hairpin RNA cassette in close proximity to the functional 3'ITR is detrimental to efficient AAV genome packaging in both orientations, with the inverted orientation yielding significantly better titers, but still performing sub-par as compared to the B or C configurations. It should be noted, that the self-complementary nucleic acid comprised in the hairpin cassette is further removed from the 3'ITR in the D2 configuration than in the D1 configuration, which might explain the elevated titers obtained from the D2 construct configuration. One possible explanation of the observed phenomenon is that the self-complementary nucleic acid encoding the hairpin RNA interferes with the formation of the 3'ITR secondary structure required for correct genome nicking. This might lead to increased generation of AAV genome concatemers that cannot be packaged.

The results of the yield determination of the different AAV constructs was similar: AAV constructs B1-C2 achieved yields comparable to or higher than those obtained with the positive control vector, while AAV construct D1 yields were significantly diminished (Figure 14). Interestingly, AAV construct D2 yields were comparable to those of AAV constructs B1-C2, indicating that D2 achieves AAV preparations of similar yield, but of diminished infectivity as compared to constructs B1-C2.

Viral particle number was assessed for the 6 AAV constructs by SDS-PAGE and GC/VP ratios were calculated for each construct (Figure 15). While constructs B1, B2, and C1 showed similar GC/VP ratios, C1 showed a slight decrease in GC/VP ratio, D2 showed a further decrease, and D1 showed the lowest ratio for both shRNA expression cassettes assessed. Based on this observation, it was hypothesized that introduction of an shRNA expression cassette close to the 3'ITR of the AAV constructs might result in the formation of more empty particles during AAV production. This hypothesis was confirmed by electron microscopy of AAV virions produced from four different constructs as shown in Figure 16. Both constructs having the shRNA expression cassette between the 5'ITR and the EGFP coding sequence (upper two panels) showed normal morphology and quantity of virions. In contrast, both AAV constructs having shRNA expression cassette between the 3'ITR and the EGFP coding sequence (lower two panels) showed a remarkably reduced number of virions and abnormal virion morphology.

Figure 16 further shows a differential effect on packaging that is dependent on whether the shRNA expression cassette is positioned proximally or distally from the closest ITR. For example, an shRNA cassette positioned proximally to the 3'ITR resulted in the

lowest number of virions (lower right panel), while an shRNA cassette inserted distally to the 3' ITR resulted in a slightly higher number of virions.

Southern blot analysis of AAV replication was assessed for all AAV constructs at three different time points and the data obtained is shown in Figure 17.

5 Figure 18 shows the positional effects of shRNA expression cassettes in a single stranded AAV constructs on AAV production efficiency. The effects are similar to those observed in scAAV constructs in that an shRNA expression cassette cloned in inverse orientation close to the 3' ITR (Construct C in Figure 18) resulted in a detrimental effect on packaging efficiency, as assessed by fluorescent imaging. These data support the notion that
10 the results reported herein for positional effects of shRNA cassettes in scAAV vectors are applicable to ssAAV constructs as well.

Together, these results demonstrate that hairpin RNA expression cassettes can efficiently be expressed from scAAV vectors comprising a reporter expression cassette. In order to maximize packaging efficiency, yield, and infectivity of shRNA AAV preparations,
15 the hairpin RNA cassette may be positioned either between the Δ ITR and the reporter expression cassette, or within the intron, if present, of the reporter expression cassette. If positioned between the reporter expression cassette and a functional ITR, the hairpin cassette may either be inserted in an orientation in which the promoter of the hairpin RNA cassette is between the self-complementary nucleic acid sequence encoding the hairpin RNA and the
20 functional ITR, or, alternatively, at a minimum distance of at least 300 nucleotides from the ITR, with greater numbers between the self-complementary. The resulting multicistronic scAAV constructs can efficiently be packaged and expressed in target cells regardless of the orientation of the hairpin cassette.

25

30

SEQUENCES

The following sequences are exemplary sequences of nucleic acid constructs disclosed herein, or parts thereof. Exemplary nucleic constructs comprising some of the sequences provided below are shown in Figures 1-6.

5 5' ΔTRS ITR (5'ΔITR, AAV inverted terminal repeat with terminal resolution site deleted) (SEQ ID NO: 1):

ctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccggcgctcgggcgacctttggtcgcccggcctca
gtgagcgagcgagcgcgagagagggagtg

10 3' ITR (SEQ ID NO: 2):

aggaacccctagtgatggagttggccactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaagg
tcgcccgcgcccgggctttgcccgggcggcctcagtgagcgagcgagcgcgag

CMV enhancer (SEQ ID NO: 3):

15 tacggtaaattggcccgctggctgaccgcccacgaccccgccattgacgtcaataatgacgtatgtt
cccatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgccacttggca
gtacatcaagtgtatcatatgccaaagtacgccccctattgacgtcaatgacggtaaattggcccgctggcattat
gcccagtacatgaccttatgggactttcctacttggcagtacatctactcgaggccacgttctgctt

20

CB promoter (SEQ ID NO: 4):

tctccccatctccccccccctccccaccccccaattttgtatatttattttttaattattttgtgcagc
gatgggggcggggggggggggggggggggggcgcgcgccaggcggggcggggcggggcgaggggcggggcggggcga
25 ggcggagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttccttttatggcgaggcggcgggcgc
ggcgggccctataaaaagcgaagcgcgcgggcgggcgggagcgggatc

Intron (SEQ ID NO: 5):

gaactgaaaaaccagaaagttaactggtaagtttagtctttttgtcttttatttcaggtcccggatccg
30 gtggtggtgcaaataaagaactgctcctcagtggatggtgcctttacttctaggcctgtacggaagtgttactt
ctgctctaaaagctgcggaattgtaccc

eGFP encoding nucleic acid sequence (SEQ ID NO: 6):

atggtgagcaagggcgaggagctgttcaccgggggtggtgcccatcctggtcgagctggacggcgacgtaaacggc
35 cacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcacc
accggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgttccagccgctac
cccgaccacatgaagcagcagcacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttc

ttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacacccctggtgaaccgcatcgag
 ctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaac
 gtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggc
 agcgtgcagctcgccgaccactaccagcagaacacccccatcgggcgaaggccccgtgctgctgcccgacaaccac
 5 tacctgagcaccagtcggccctgagcaaagaccccaacgagaagcgcgatcacatgggtcctgctggagttcgtg
 accgcccgggatcactctcgccatggacgagctgtacaag

BGH 3'UTR/PolyA/GRE(partial) (SEQ ID NO: 7):

ctcgactgtgccttctagttgccagccatctgttggttgccccctccccgtgccttccttgaccctgga
 10 aggtgccactcccactgtcctttcctaataaaatgaggaaattgcacgcattgtctgagtaggtgtcattctat
 tctgggggggtgggggtggggcaggacagcaagggggaggattgggaagacaat

U6 promoter (SEQ ID NO: 8)

aattccccagtggaagacgcgcaggcaaaacgcaccacgtgacggagcgtgaccgcgcgccgagcgcg
 15 cgccaaggtcgggcaggaagagggcctatttcccatgattccttcatatttgcataacgatacaaggctgttag
 agagataattagaattaatttgactgtaaacacaaagatattagtagacaaaatacgtgacgtagaaagtaataatt
 tcttgggtagtttgagttttaaaattatgttttaa

20 Nucleic acid sequence encoding siFluc: SEQ ID NO: 9)

aatggactatcatatgcttaccgtaacttgaaagtatttcgatttcttgggtttatatatcttgtggaa
 aggacgcgggatccc**gcttacgctgagtacttcgattc**aagagatcgaagtactcagcgtaagttttttccaaa

Bold: first and second nucleic acid sequences encoding stem regions of hairpin RNA
 targeting luciferase mRNA; underlined: hairpin loop region.

25

U6siFluc (SEQ ID NO: 10):

aattccccagtggaagacgcgcaggcaaaacgcaccacgtgacggagcgtgaccgcgcgccgagcgcg
 cgccaaggtcgggcaggaagagggcctatttcccatgattccttcatatttgcataacgatacaaggctgttag
 agagataattagaattaatttgactgtaaacacaaagatattagtagacaaaatacgtgacgtagaaagtaataatt
 30 tcttgggtagtttgagttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtattt
 cgatttcttgggtttatatatcttgtggaaaggacgcgggatccc**gcttacgctgagtacttcgattc**aagagat
 cgaagtactcagcgttaagttttttccaaa

U6siFluc^{inv} (SEQ ID NO: 11):

gcttttggaaaaaacttacgctgagtacttcgatctcttgaatcgaagtactcagcgttaagcgggatcc
 cgcgctcctttccacaagatatataaaccacaagaaatcgaaataactttcaagttacggtaagcatatgatagtcca
 ttttaaaacataatttttaaaactgcaaactacccaagaaattattactttctacgtcacgtattttgtactaata
 tctttgtgtttacagtc aaattaattctaattatctcttaacagccttgtatcgatatatgcaaatatgaaggaa

tcatgggaaataggccctcttccctgcccagaccttggcgcgcgctcggcgcgcggtcacgctccgtcacgtggtgc
gttttgccctgcgcgtctttccactgggg

Non-viral constructs:

- 5 **Construct A1.** CMV/CB-intron-U6Fluc-intron-eGFP, U6Fluc cassette positioned within intron of eGFP cassette, both expression cassettes in same orientation (SEQ ID NO: 12, see Figure 1 for map)

tacggtaaattggcccgccctggctgaccgcccacgaccccgccattgacgtcaataatgacgtatgtt
cccatagtaacgccaatagggaactttccattgacgtcaatgggtggagtatTTTtacggtaaactgccacttggca
10 gtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaattggcccgccctggcattat
gccagtacatgaccttatgggaactttccctacttggcagtacatctactcgaggccacgttctgcttcaactctcc
ccatctccccccccctccccaccccccaattttgtattttattttattttttaatttttgtgcagcgatggggggcgg
ggggggggggggggggggggcgcgcgccaggcgggggcgggggcgagggggcgggggcgagggcgaggcgagaggt
gcgggcggcagccaatcagagcgggcgcgctccgaaagtTTTccttttatggcgagggcgggcgggcgggcgccctat
15 aaaaagcggaagcgcgcgggcgggcgggagcgggatcagccaccgcggtggcgggccctagagtcgatcgaggaactg
aaaaaccagaaagttaactggtaagtTTTtagtctttttgtctttttatttcagaattccccagtggaagacgcgcga
ggcaaaacgcaccacgtgacggagcgtgaccgcgcgccgagcgcgcgccaaggtcgggcaggaagagggcctatt
tcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattagaattaatttgactgtaaa
cacaagatattagtaaaaaatacgtgacgtagaaagtaataatttcttgggtagtttgacgtttttaaattatg
20 tttttaaattggactatcatatgcttaccgtaacttgaaagtatttcgatttcttgggtttatatatcttgtggaa
aggacgcgggatcccgcttacgctgagtaacttcgattcaagagatcgaagtactcagcgtaagtTTTTccaaag
tccccgatccggtggtggtgcaaatcaaagaactgctcctcagtggtatgtgcctttacttctaggcctgtacgg
aagtgttacttctgctctaaaagctgcggaattgtacccgcggccgatccaccggtcgccaccatggtgagcaag
ggcgaggagctgttcaccgggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagc
25 gtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccggcaagctg
cccgtagcctggccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatg
aagcagcacgacttcttcaagtccgccatgccgaaggctacgtccaggagcgccaccatcttcttcaaggacgac
ggcaactacaagacccgcgcccaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatc
gacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatg
30 gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctc
gccgaccactaccagcagaacacccccatcgggcgacggccccgtgctgctgcccgacaaccactacctgagcacc
cagtcgcgcctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgcccgcggg
atcactctcgcatggacgagctgtacaagtaagcgggccatcaagcttatcgataccgtcgactagagctcgtc
gatcagcctcgactgtgccttctagtTgcccagccatctgttggtttgccccctcccccgtagccttcttgaccctgg
35 aaggtgccactcccactgtcctttcctaataaaaatgaggaaattgcacgcattgtctgagtaggtgtcattcta
ttctgggggggtgggggtggggcaggacagcaagggggaggattgggaagacaat

Construct A2. CMV/CB-intron-U6Fluc^{inv}-intron-eGFP, U6Fluc cassette positioned within intron of eGFP cassette, expression cassettes in opposite orientation (SEQ ID NO: 13, see Figure 2 for map):

tacggtaaataggcccgccctgggtgacgcgccaacgaccccgccattgacgtcaataatgacgtatgtt
 5 cccatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgccacttggca
 gtacatcaagtgtatcatatgccagtagcggccctattgacgtcaatgacggtaaataggcccgccctggcattat
 gccagtagcatgaccttatgggactttccctacttggcagtagcatctactcgaggccacgttctgcttcaactctcc
 ccatctccccccccctccccaccccccaattttgtattttatttttttaattttttgtgcagcgatggggggcg
 gggggggggggggggggggcgcgccgagggcgggggcgggggcgagggggcgggggcgagggcgagaggt
 10 gcgggcgagccaatcagagcgggcgcgctccgaaagtttcttttatggcgagggcgggcgggcgggcgccctat
 aaaaagcgaagcgcgcgggcgggcgggagcgggatcagccaccgcggtggcgggccctagagtcgatcgaggaaactg
 aaaaaccagaaagttaactggtaagtttagtctttttgtctttttatttcaggcttttggaaaaaacttacgctga
 gtacttcgatctcttgaatcgaagtactcagcgtaagcgggatcccgcgctcctttccacaagatatataaaccga
 agaaatcgaaatactttcaagttacggtaagcatatgatagtcatttttaaaacataatttttaaaactgcaaact
 15 acccaagaaattattactttctacgtcacgtattttgtactaataatctttgtgtttacagtc aaattaattctaa
 ttatctcttaacagccttgtatcgtatatgcaaataatgaaggaatcatgggaaataggccctcttccctgccga
 ccttggcgcgcgctcgggcgcgcggtcacgctccgctcacgtgggtgcggttttgccctgcgctctttccactgggggt
 cccggatccgggtgggtgggtgcaaatacaagaactgctcctcagtggtggtgcttacttctaggcctgtacgga
 agtggtacttctgctctaaaagctgcggaattgtaccgcggccgatccaccggtcgccaccatgggtgagcaagg
 20 gcgaggagctgttcaccgggggtgggtgcccatcctgggtcgagctggacggcgacgtaaacggccacaagttcagcg
 tgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccggcaagctgc
 ccgtgccctggccaccctcgtgaccaccctgacctacggcggtgacgtgcttcagccgctaccccgaccacatga
 agcagcacgacttcttcaagtccgccaatgccgaaggctacgtccaggagcgacccatcttcttcaaggacgacg
 gcaactacaagaccccgcgccgaggtgaagttcgagggcgacaccctgggtgaaccgcatcgagctgaagggcatcg
 25 acttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatgg
 ccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcg
 ccgaccactaccagcagaacaccccccatcggcgacggccccgtgctgctgcccgaacaaccactacctgagcacc
 agtccgccttgagcaaaagaccccaacgagaagcgcgatcacatgggtcctgctggagttcgtgaccgcccggga
 tcaactctcggcattggacgagctgtacaagtaaagcgggccatcaagcttatcgataccgtcgactagagctcgctg
 30 atcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctcccccggtgccttcttgaccctgga
 aggtgccactcccactgtcctttcctaataaaaatgaggaaattgcatcgcatgtgtgagtaggtgtcattctat
 tctgggggggtgggggtggggcaggacagcaagggggaggattgggaagacaat

AAV constructs:

35 **Construct A.** AAV 5'ΔITR CMV/CB -intron-eGFP 3'ITR (SEQ ID NO: 14, see Figure 3 for map).

ctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggcgctcgggcgacctttgggtcgcccc
 gcctcagtgagcgagcgagcgcgagaggggagtgtagccatgctctaggaagatcaattcggtacaattcacg
 cgtcgacattgattattgactctgggtcggttacataacttacggtaaataggcccgccctggctgaccgccaacgac

cccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatggg
 tggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccagtacgccccctattgacg
 tcaatgacggtaaatggcccgctggcattatgccagtacatgaccttatgggactttcctacttggcagtaca
 tctactcgaggccacgttctgtcttcaactctccccatctccccccctccccaccccccaattttgtatttatttat
 5 tttttaattattttgtgcagcgatgggggccccggggggggggggggggggggggcgcgccaggcgggggcgggcggg
 gcgagggggcgggcgggcgaggcgagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttcctt
 ttatggcgaggcgggcgggcgggcgggccctataaaaagcgaagcgcgcgggcgggcgaggcgggatcagccaccg
 cgggtggcgggccctagagtcgatcgaggaactgaaaaaccagaaagttaactggtaagttagtctttttgtcttt
 tatttcaggtcccggatccggtggtggtgcaaatcaaagaactgctcctcagtggatggtgcctttacttctagg
 10 cctgtacggaagtgttacttctgtctctaaaagctgcggaattgtaccccgcgccgatccaccggtcgccaccatg
 gtgagcaagggcgaggagctgttcaccgggggtggtgcccacctcctggtcgagctggacggcgacgtaaacggccac
 aagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccacc
 ggcaagctgcccgtgccctggcccaccctcgtgaccacccctgacctacggcgtgcagtgttcagccgctacccc
 gaccacatgaagcagcacgacttcttcaagtccgcatgcccgaaggctacgtccaggagcgcaccatcttcttc
 15 aaggacgacggcaactacaagaccccgcgccgaggtgaagttcgagggcgacacccctggtgaaccgcatcgagctg
 aagggcacgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtc
 tatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagc
 gtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactac
 ctgagcaccagtcggccctgagcaaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgacc
 20 gccgcccgggatcactctcggcattggacgagctgtacaagtaaagcggccatcaagcttatcgataccgtcgacta
 gagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttggttgccccctcccccgctgccttct
 tgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcatgtctgagtaggt
 gtcatttctattctgggggggtgggggtggggcaggacagcaagggggaggattgggaagacaattaggtagataagt
 agcatggcggggttaatcattaactacaaggaacccctagtgatggagttggccactccctctctgcgcgctcgct
 25 cgctcactgaggccggggcgaccaaaggtcgcccgacgccccgggcttgccccggggcgccctcagtgagcgagcgag
 cgcgag

Construct B1. AAV 5' Δ ITR U6shFluc CMV/CB -intron-eGFP 3'ITR, U6 promoter
 between Δ ITR and shRNA encoding nucleic acid, expression cassettes in same orientation
 30 (SEQ ID NO: 15, see Figure 4 for map):

ctgcgcgctcgctcgctcactgaggccgccccgggcaaagccccgggcgctcgggcgacctttggtcgcccg
 gcctcagtgagcgagcgagcgcgagagagggagtgtagccatgctctaggaagatcaattcggtaacaattcaaa
 tccccagtggaagacgcgcaggcaaaacgcaccacgtgacggagcgtgaccgcgcgcccagcgcgcgccaagg
 tcgggcaggaagagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataa
 35 ttagaattaatttgactgtaaacacaaagatattagtagcaaaatacgtgacgtagaaagtaataatttcttgggt
 agtttgagtttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttct
 tgggtttatatatcttgtggaaaggacgcgggatcccgttacgctgagtagtctcgattcaagagatcgaagtac
 tcagcgtaagttttttccaaacgcgtcgacattgattattgactctggtcggttacataacttacggtaaatggcc
 cgccctggctgaccgcccacgacccccgccattgacgtcaataatgacgtatgttcccatagtaacgccaatagg
 40 gactttccattgacgtcaatgggtggagtagtttacggtaaaactgcccacttggcagtacatcaagtgtatcatat

gccaaagtacgccccctattgacgtcaatgacggtaaattggcccgctggcattatgcccagtacatgaccttatg
 ggacttttctacttggcagtacatctactcgaggccacgttctgcttcactctccccatctccccccctcccca
 cccccaattttgtattttattttatttttaattattttgtgcagcgatggggcgggggggggggggggggggcg
 cgcgccaggcgggcgggcgggcgaggggcgggcgggcgaggcgagaggtgcggcgccagccaatcagag
 5 cggcgcgctccgaaagtttcttttatggcgaggcgggcgggcgggcgggccctataaaaagcgaagcgcgcgggcg
 ggcgggagcgggatcagccaccgcggtggcgggcctagagtcgatcgaggaaactgaaaaaccagaaagttaactg
 gtaagttagtctttttgtctttttatttcaggtcccggatccggtggtggtgcaaatcaaagaactgctcctcag
 tggatggtgcctttacttctaggcctgtacggaagtgttacttctgctctaaaagctgcggaattgtacccgcgg
 ccgatccaccggtcgccaccatggtgagcaagggcgaggagctgttcaccgggggtggtgcccacctcctggtcgagc
 10 tggacggcgacgtaaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctga
 ccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcg
 tgcagtgttcagccgctaccccgaccacatgaagcagcagacttcttcaagtccgcatgcccgaaggctacg
 tccaggagcgccaccatcttcttcaaggacgaaggcaactacaagaccgcgcggaggtgaagttcgagggcgaca
 ccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagt
 15 acaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatcc
 gccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacaccccccatcggcgacggccccg
 tgctgctgcccgcacaaccactacctgagcaccagtcggccctgagcaaagaccccaacgagaagcgcgatcaca
 tgggtcctgctggagttcgtgaccgcccggggatcactctcggcattggacgagctgtacaagtaagcgggccatc
 aagcttatcgataccgtcgactagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgt
 20 ttgccccctccccgtgccttcccttgaccctggaaggtgccactcccactgtcctttcctaataaaaatgaggaaat
 tgcacgcattgtctgagtaggtgtcattctattctgggggggtgggggtggggcaggacagcaagggggaggattg
 ggaagacaattaggtagataagtagcatggcggggttaattattaactacaaggaaccctagtgatggagttggc
 cactccctctctgcgcgctcgctcgctcactgaggccggggcgaccaaaggtcgcccgacgcccgggctttgcccg
 ggcgccctcagtgagcgagcgagcgcgcgag

25

Construct B2. AAV 5'ΔITR U6siFluc^{inv} CMV/CB -intron-eGFP 3'ITR, U6

promoter flanks shRNA encoding nucleic acid sequence on the opposite side of the ΔITR, expression cassettes in opposite orientation (SEQ ID NO: 16, see Figure 5 for map):

ctgcgcgctcgctcgctcactgaggccgccccgggcaaagccccgggcgtcgggcgacctttggtcgccccg
 30 gcctcagtgagcgagcgagcgcgagagagggagtgtagccatgctctaggaagatcaattcggtacaattcagc
 ttttggaaaaaacttacgctgagtagtctcgtatctcttgaatcgaagtactcagcgtaagcgggatcccgcgctct
 ttccacaagatatataaacccaagaaatcgaaatactttcaagttacggtaagcatatgatagtcatttttaaaa
 cataatttttaaaactgcaaactaccaagaaattattactttctacgtcacgtattttgtactaatatctttgtg
 tttacagtcataattaatttctaattatctctctaacagccttgatcgatatgcaaatatgaaggaatcatggga
 35 aataggccctcttccctgcccgcaccttggcgcgcgctcggcgcgcggtcacgctccgtcacgtggtgcgttttgcc
 tgcgcgctcttccactggggcgcgctcgacattgattattgactctggtcggttacataacttacggtaaatggccc
 gcctgggtgaccgcccacgaccccgcccatcgacgtcaataatgacgtatggttcccatagtaacgccaataggg
 actttccattgacgtcaatgggtggagttttacggtaaaactgcccaacttggcagtacatcaagtgtatcatatg
 ccaagtacgccccctattgacgtcaatgacggtaaatggcccgctggcattatgccagtacatgaccttatgg
 40 gactttcctacttggcagtacatctactcgaggccacgttctgcttcactctccccatctccccccctccccac

ccccaattttgtattttatttattttttaattattttgtgcagcgcgatgggggcggggggggggggggggggggggcgcg
gcgccaggcgggggcggggcggggcgaggggcggggcgaggcggagaggtgcggcggcagccaatcagagc
ggcgcgctccgaaagtcttcttttatggcgaggcggcgggcgggcgggccctataaaaaagcgaagcgcgcggcg
gcgggagcgggatcagccaccgcggtggcgggccctagagtcgatcgaggaaactgaaaaaccagaaagttaactgg
5 taagtttagtctttttgtcttttatttcaggtcccggatccggtgggtggtgcaaatacaagaactgctcctcagt
ggatggtgcctttacttctagggcctgtacggaagtgttacttctgctctaaaagctgcggaattgtaccgcggc
cgatccaccggctgcgccaccatgggtgagcaagggcgaggagctgttcaccgggggtgggtgcccatcctggtcgagct
ggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgac
cctgaagttcatctgcaccaccggcaagctgcccggtgcctggccaccctcgtgaccaccctgacctacggcgt
10 gcagtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtcggccatgcccgaggctacgt
ccaggagcgcaccatcttcttcaaggacgacggcaactacaagaccgcgcggaggtgaagttcgagggcgacac
cctgggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagta
caactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccg
ccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacaccccccatcggcgacggccccgt
15 gctgctgcccgaacaaccactacctgagcacccagtcggccctgagcaaagaccccaacgagaagcgcgatcacat
ggctcctgctggagttcgtgaccgcgcggggatcactctcgcatggacgagctgtacaagtaaagcggccatca
agcttatcgataccgtcgactagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgtt
tgccctcccccggtgccttccttgaccctggaaggtgccactcccactgtccttctctaataaaatgaggaaatt
gcacgcattgtctgagtaggtgtcattctattctgggggggtgggggtggggcgaggacagcaagggggaggattgg
20 gaagacaattaggtagataagtagcatggcggggttaatcattaactacaaggaacccctagtgatggagttggcc
actccctctctgcgcgctcgctcgctcactgaggccggggcgaccaaaggtcgcccgacgcccgggctttgcccg
gcggcctcagtgagcgaagcgaagcgcgcga

Construct C1. AAV 5'ΔITR CMV/CB -intron-U6siFluc-intron-eGFP 3'ITR,
25 U6siFluc cassette positioned within intron of eGFP cassette, expression cassettes in same
orientation (SEQ ID NO: 17, see Figure 6 for map):

ctgcgcgcctcgctcgctcactgagggccgcccgggcaaagcccgggcgctcgggcgacctttgggtcgccccg
gcctcagtgagcgagcgagcgcgagagagggagtgtagccatgctctaggaagatcaattcggtacaattcacg
cgtcgacattgattattgactctgggtcgttacataacttacggtaaattggcccgctgggtgaccgccccaaacgac
30 cccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatggg
tgaggtattttacggtaaactgccacttggcagtacatcaagtgtatcatatgccaaagtacgccccctattgacg
tcaatgacggtaaattggcccgctggcattatgccagtacatgaccttatgggacttttctacttggcagtaca
tctactcgaggccacgttctgcttcactctccccatctccccccctccccaccccccaattttgtatttatttat
tttttaattattttgtgagcgatggggggcgggggggggggggggggggggcgcgcgccaggcgggggcgggggcg
35 gcgagggggcgggggcgggggcgaggcgagaggtgcgggcggcagccaatcagagcgggcgcgctccgaaagtttcctt
ttatggcgaggcgggcgggcgggcgggccctataaaaagcgaagcgcgcgggcgggcgggagcgggatcagccaccg
cggtggcgggccctagagtcgacgaggaactgaaaaaccagaaagttaactggtaagttagtctttttgtcttt
tatttcagaattccccagtggaagacgcgagggcaaaacgcaccacgtgacggagcgtgaccgcgcgccgagcg
cgcgccaaggctcgggcaggaagagggcctatttcccatgattccttcataatttgcatatacgatacaaggctgtt
40 agagagataattagaattaattttgactgtaaacacaaagatatagtacaaaatacgtgacgtagaaaagtaataa

tttcttgggtagtttgcagtttttaaattatgtttttaaattggactatcatatgcttaccgtaacttgaaagtat
 ttcgattttcttgggtttatatatcttgtggaaaggacgcgggatcccgcttacgctgagtacttcgattcaagag
 atcgaagtactcagcgttaagttttttccaaagtcccggtccgggtggtgcaaatacaagaactgctcctcag
 tggatgttgcttttacttctagggcctgtacggaagtgttacttctgctctaaaagctgcggaattgtaccgcgg
 5 ccgatccaccggtcgccaccatggtgagcaagggcgaggagctgttcaccgggggtggtgcccatcctggtcgagc
 tggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctga
 cctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccacctgacctacggcg
 tgcagtgttccagccgtaccccgaccacatgaagcagcagacttcttcaagtccgcatgccgaaggctacg
 tccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgcgaggtgaagttcgagggcgaca
 10 cctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagt
 acaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatcc
 gccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccg
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 tgggtcctgctggagttcgtgaccgcgcgcgggatcactctcgccatggacgagctgtacaagtaaagcggccatc
 15 aagcttatcgataccgtcgactagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgt
 ttgcccctccccgtgccttcttgacctggaaggtgccactcccactgtccttctctaataaaatgaggaaat
 tgcategcattgtctgagtaggtgtcattctattctgggggggtgggggtggggcaggacagcaagggggaggattg
 ggaagacaattaggtagataagtagcatggcgggttaatcattaactacaaggaacccctagtgatggagttggc
 cactccctctctgcgcgctcgctcgctcactgagggcggggcgaccaaaggtcgcccgacgcccgggctttgcccg
 20 ggcgccctcagtgagcgcgagcgcgcgcg

Construct C2. AAV 5'ΔITR CMV/CB -intron-U6siFluc^{inv}-intron-eGFP 3'ITR,
 U6siFluc cassette positioned within intron of eGFP cassette, expression cassettes in opposite
 orientation (SEQ ID NO: 18, see Figure 7 for map):

25 ctgcgcgctcgctcgctcactgagggcccgccgggcaaagcccgggcgctcgggcgacctttgggtcgcccg
 gcctcagtgagcgcgagcgcgcgagagggagtgtagccatgctctaggaagatcaattcggtacaattcacg
 cgtcgacattgattattgactctgggtcggtacataacttacggtaaatggcccgcctggctgaccgcccacgcac
 cccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatggg
 tggagtatttacggtaaaactgcccacttggcagtagcatcaagtgtatcatatgccaagtacgccccctattgacg
 30 tcaatgacggtaaatggcccgcctggcattatgccagtagcatgaccttatgggactttcctacttggcagtaca
 tctactcgaggccacgttctgcttcactctccccatctccccccccctccccaccccccaattttgtattttat
 tttttaattattttgtgcagcgatgggggccccggggggggggggggggggggcgcgcgccaggcggggcggggccccg
 ggcgggggccccggggcgaggcgggagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttcctt
 ttatggcgaggcggcgggcgggcgggccctataaaaagcgaagcgcgcggcgggcgggagcgggatcagccaccg
 35 cgggtggcgggccctagagtcgatcgaggaaactgaaaaaccagaaagttaactggtaagtttagtcttttgtcttt
 tatttcaggcttttggaaaaaacttacgctgagtacttcgatctcttgaatcgaagtactcagcgttaagcgggat
 cccgcgtcctttccacaagatatataaacccaagaaatcgaaatactttcaagttacggtaagcatatgatagtc
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 tatctttgtgtttacagtc aaattaatttctaattatctcttaacagccttgtagtgcgtaataatgaagg
 40 aatcatgggaaataggccctcttccctgcccgccttggcgcgcgctcgggcgcgcggtcacgctccgtcacgtggt

gcgttttgctgcgctctttccactgggggtcccggatccggtggtggtgcaaatacaagaactgctcctcagt
 ggatgttgcttttacttctaggcctgtacggaagtgttacttctgctctaaaagctgcggaattgtaccgcggc
 cgatccaccggtcgccaccatggtgagcaagggcgaggagctgttcacccgggtggtgcccatcctggtcgagct
 ggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgac
 5 cctgaagttcatctgcaccaccggcaagctgcccgtgccttggcccaccctcgtgaccaccctgacctacggcgt
 gcagtgttccagccgtaccccgaccacatgaagcagcagcacttcttcaagtccgccatgccgaaggctacgt
 ccaggagcgacccatcttcttcaaggacgacggcaactacaagaccgcgcccaggtgaagttcgagggcgacac
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 10 ccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgt
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 gaagacaattaggtagataagtagcatggcgggttaatcattaactacaaggaacccttagtgatggagttggcc
 actccctctctgcgcgtcgctcgctcactgaggccggggcgaccaaaggtcgcccgacgcccgggctttgcccg
 gcgccctcagtgagcgagcgagcgcgag

20 **Construct D1.** AAV 5'ΔITR CMV/CB -intron-eGFP U6siFluc 3'ITR, U6 promoter
 flanking shRNA encoding sequence on opposite site of functional ITR, expression cassettes
 in same orientation (SEQ ID NO: 19, see Figure 8 for map):

ctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggctcgggcgacctttggtcgcccg
 gcctcagtgagcgagcgagcgcgagagagggagtgtagccatgctctaggaagatcaattcggtacaattcacg
 25 cgtcgacattgattattgactctggtcggttacataacttacggtaaatggcccgctggctgaccgcccacgac
 ccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatggg
 tggagtatttacggtaaaactgccacttggcagtagcatcaagtgtatcatatgccaaagtacgccccctattgacg
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 tctactcgaggccacgttctgcttccactctccccatctccccccccctcccccccccaattttgtatttatttat
 30 tttttaattattttgtgcagcgatgggggccccggggggggggggggggggggcgcgccaggcgggggcgggcggg
 gcgagggggcgggcgggcgaggcgagaggtgcggcggcagccaatcagagcggcgcgctccgaaagtttcctt
 ttatggcgaggcgggcgggcgggcgccctataaaaagcgaagcgcgcgggcgggcgaggcgggatcagccaccg
 cggtggcgccctagagtcgatcgaggaaactgaaaaaccagaaagttaactggtaagtttagtctttttgtcttt
 tatttcagggtcccgatccggtggtggtgcaaatacaagaactgctcctcagtggatggtgcctttacttctagg
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 gtgagcaagggcgaggagctgttcacccgggtggtgcccatcctggtcgagctggacggcgacgtaaacggccac
 aagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccacc
 ggcaagctgcccgtgccttggcccaccctcgtgaccaccctgacctacggcgtgcagtgttccagccgtacccc
 gaccacatgaagcagcagcacttcttcaagtccgccatgccgaaggctacgtccaggagcgacccatcttcttc
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aagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgctc
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 gtgcagctcgccgaccactaccagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactac
 ctgagcaccagtcgcccctgagcaaagacccccaacgagaagcgcgatcacatggtcctgctggagttcgtgacc
 5 gcccgcgggatcactctcggcattggaagagctgtacaagtaaagcggccatcaagcttatcgataccgtcgacta
 gagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgtttgcccctccccgtgccttctt
 tgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcatgtctgagtaggt
 gtcattctattctggggggtgggggtggggcaggacagcaagggggaggattgggaagacaaaattccccagtggg
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 10 agggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataattagaattaatt
 tgactgtaaacacaaagatattagtaaaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttt
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 tcttgtggaaaggacgcgggatcccg**cttactgagtagtacttcgattcaagagatcgaagtactcagcgtaagtt**
 ttttccaaattaggtagataagtagcatggcgggttaatcattaactacaaggaacccctagtgatggagttggc
 15 cactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaaggtcgcccgacgcccgggctttgcccg
ggcggcctcagtgagcgagcgagcgcgag

Bold: hairpin RNA encoding sequence; underlined: 3'ITR. Distance between hairpin RNA encoding sequence and 3'ITR: 52 nucleotides.

20 **Construct D2.** AAV 5'ΔITR CMV/CB -intron-eGFP U6siFluc^{inv} 3'ITR, U6promoter between shRNA encoding nucleic acid sequence and ITR, expression cassettes in opposite orientation (SEQ ID NO: 20, see Figure 9 for map):

ctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggcgctcgggcgacctttgggtcgcccg
 gcctcagtgagcgagcgagcgcgagagagggagtgtagccatgctctaggaagatcaattcggtacaattcacg
 25 cgtcgacattgattattgactctggtcgttacataacttacggtaaattggcccgctggctgaccgcccacgac
 ccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatggg
 tggagtatTTTtacggtaaactgcccacttggcagtagcatcaagtgtatcatatgccaagtacgccccctattgacg
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 30 tttttaattatTTTgtgcagcgatgggggcggggggggggggggggggggggcgcgcgccaggcggggcggggcggg
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 35 cctgtacggaagtgttacttctgctctaaaagctgcggaattgtaccccgggcgatccaccggtcgccaccatg
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 5 gccgcccgggatcactctcggcattggacgagctgtacaagtaaagcggccatcaagcttatcgataccgtcgacta
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 tgaccctggaaggtgccactcccactgtcctttcctaataaaaatgaggaaattgcatcgcatgtctgagtaggt
 gtcattctattctgggggggtgggggtggggcaggacagcaagggggaggattgggaagacaagcttttgaaaaaa
cttacgctgagtacttcgatctcttgaatcgaagtactcagcgtaagcgggatcccgcgctcctttccacaagata
 10 tataaaccacaagaaatcgaaatactttcaagttacggtaagcatatgatagtcatttttaaacataatttttaa
 actgcaaactaccaagaattattactttctacgtcacgtattttgtactaatatctttgtgtttacagtcaaa
 ttaattctaattatctcttaacagccttgatcgatatgcaaatatgaaggaatcatgggaaataggccctct
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 cactgggggttaggtagataagtagcatggcgggttaatcattaactacaaggaacccctagtgatggagttggcc
 15 actccctctctgcgcgctcgctcgctcactgaggccggcgaccaaaggtcgcccgacgcccgggctttgcccgg
gcggcctcagtgagcgagcgagcgcgag

Bold: hairpin RNA encoding sequence; underlined: 3'ITR. Distance between hairpin RNA encoding sequence and 3'ITR: 377 nucleotides.

20 SCOPE AND EQUIVALENTS

While several embodiments of the present invention have been described and
 illustrated herein, those of ordinary skill in the art will readily envision a variety of other
 means and/or structures for performing the functions and/or obtaining the results and/or one
 or more of the advantages described herein, and each of such variations and/or modifications
 25 is deemed to be within the scope of the present invention. More generally, those skilled in the
 art will readily appreciate that all methods, reagents, and configurations described herein are
 meant to be exemplary and that the actual methods, reagents, and configurations will depend
 upon the specific application or applications for which the teachings of the present invention
 is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than
 30 routine experimentation, many equivalents to the specific embodiments of the invention
 described herein. It is, therefore, to be understood that the embodiments described herein are
 presented by way of example only and that, within the scope of the appended claims and
 equivalents thereto, the invention may be practiced otherwise than as specifically described
 and claimed. The present invention is directed to each individual feature, system, article,
 35 material, reagent, kit, and/or method described herein. In addition, any combination of two or
 more such features, systems, articles, materials, kits, and/or methods, if such features,

systems, articles, materials, reagents, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

5 All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents, and/or ordinary meanings of the defined terms.

The indefinite articles “a” and “an”, as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

10 The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified unless clearly indicated to the contrary. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one
15 embodiment, to A without B (optionally including elements other than B); in another embodiment, to B without A (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in
20 a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein
25 shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of”, when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in
30 reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified

within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently, “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

10 It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one act, the order of the acts of the method is not necessarily limited to the order in which the acts of the method are recited.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 64371-1254 Seq 09-OCT-13 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

<110> University of Massachusetts
Gao, Guangping
Xie, Jun

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<211> 2101

<212> DNA

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<220>

<223> synthetic polynucleotide

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<212> DNA

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<223> synthetic polynucleotide

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CLAIMS:

1. A recombinant AAV (rAAV) construct, comprising a recombinant AAV (rAAV) vector comprising inverted terminal repeats (ITRs),
 - a first expression cassette, comprising a nucleic acid encoding a gene product
 5 under the control of a first promoter positioned between the ITRs of the vector, and an intron, wherein the intron is positioned between the transcriptional start site of the first expression cassette and the nucleic acid encoding the gene product, and
 - a second expression cassette, comprising a self-complementary nucleic acid sequence under the control of a second promoter,
 - 10 wherein the second expression cassette is positioned within the intron of the first expression cassette in proximity with the first promoter.
2. The rAAV construct of claim 1, wherein the gene product is a reporter.
3. The rAAV construct of claim 2, wherein the reporter is a protein.
4. The rAAV construct of claim 3, wherein the protein is a fluorescent protein, an
 15 enzyme catalyzing a reaction yielding a detectable product, or a surface antigen.
5. The rAAV construct of claim 4, wherein the enzyme is a luciferase, a beta-glucuronidase, a chloramphenicol acetyltransferase, an aminoglycoside phosphotransferase, an aminocyclitol phosphotransferase, or a Puromycin N-acetyl-transferase.
6. The rAAV construct of any one of claims 1-5, wherein the self-complementary
 20 sequence encodes a hairpin RNA.
7. The rAAV construct of claim 6, wherein the hairpin RNA is a small hairpin RNA or a microRNA.

8. The rAAV construct of any one of claims 1-7, wherein the first promoter is an RNA polymerase II promoter.

9. The rAAV construct of any one of claims 1-8, wherein the second promoter is an RNA polymerase III promoter.

5 10. The rAAV construct of any one of claims 1-9, wherein the second promoter is a U6 or an H1 promoter.

11. The rAAV construct of any one of claims 1-10, wherein the first and the second expression cassette are in the same orientation.

12. The rAAV construct of any one of claims 1-10, wherein the first and the
10 second expression cassette are in opposite orientations.

13. The rAAV construct of any one of claims 1-12, wherein the rAAV vector is a self-complementary rAAV vector.

14. The rAAV construct of any one of claims 1-13, wherein the nucleic acid construct is integrated into the genome of a cell expressing a target gene.

15 15. The rAAV construct of any one of claims 1-14, wherein the nucleic acid construct comprises a nucleic acid encoding a hairpin RNA comprising a sequence complementary or corresponding to a sequence of an RNA transcribed from a target gene.

16. The rAAV construct of claim 15, wherein the target gene is a oncogene, a tumor suppressor gene, a gene involved in neovascularization of tissue, a viral gene, or a gene
20 encoding a receptor mediating uptake of viral particles.

17. A composition comprising the rAAV construct of any one of claims 1-16 and a pharmaceutically acceptable salt.

18. Use of the recombinant AAV construct of any one of claims 1 to 16 for inhibiting expression of a target gene in a cell.

19. The use of claim 18, wherein the recombinant AAV construct comprises a self-complementary nucleic acid sequence, and the self-complementary nucleic acid sequence
5 comprises a sequence corresponding to a sequence of an RNA encoded by the target gene.

20. The use of claim 18 or 19, wherein the recombinant AAV construct comprises a nucleic acid sequence encoding a reporter, and wherein the use further comprises detecting expression of the reporter in the cell.

21. The use of any one of claims 18-20, further comprising
10 determining a change in the phenotype of the cell.

22. The use of claim 21, wherein the change in the phenotype is a change in proliferation rate, change in cell size, change in cell viability, change in cell sensitivity to a drug, change in modulation of a cellular pathway in response to drug treatment, or a change in a level of expression of a gene of interest.

15 23. The use of any one of claims 18-22, wherein the cell is a cell in a subject.

24. The use of claim 23, wherein the recombinant AAV construct is for administration to the subject via an intravenous, intraperitoneal, intraocular, intramuscular, intraarticular, intracranial, intranasal, or endobronchial route.

25. The use of claim 23 or 24, wherein the subject has been diagnosed with a
20 disease and inhibition of the target gene is known to prevent or alleviate a symptom and/or progression of the disease.

26. A kit, comprising

a container; and

the rAAV construct of any one of claims 1-16 housed in the container.

27. A cell, comprising

the rAAV construct of any one of claims 1 to 16.

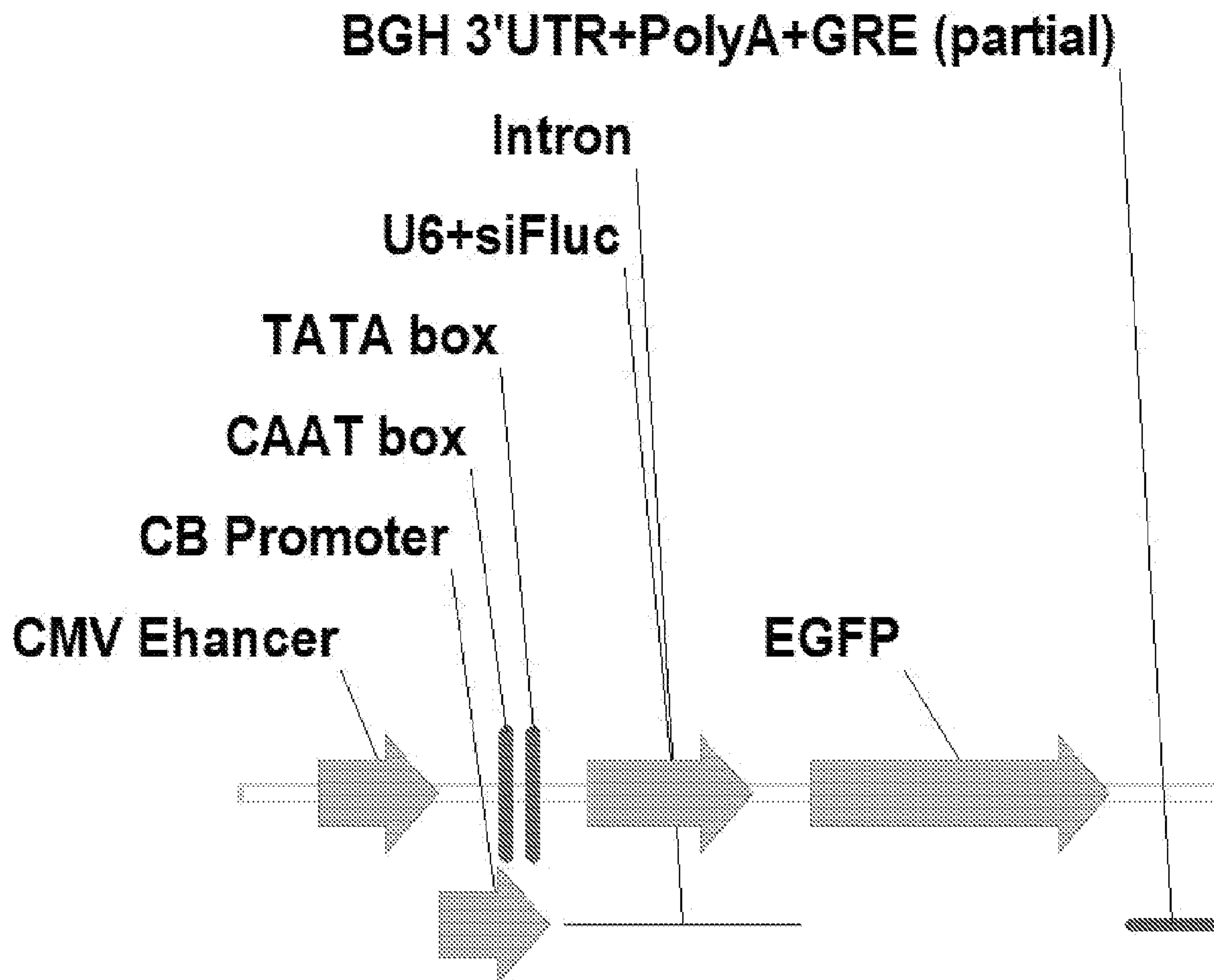


FIG. 1

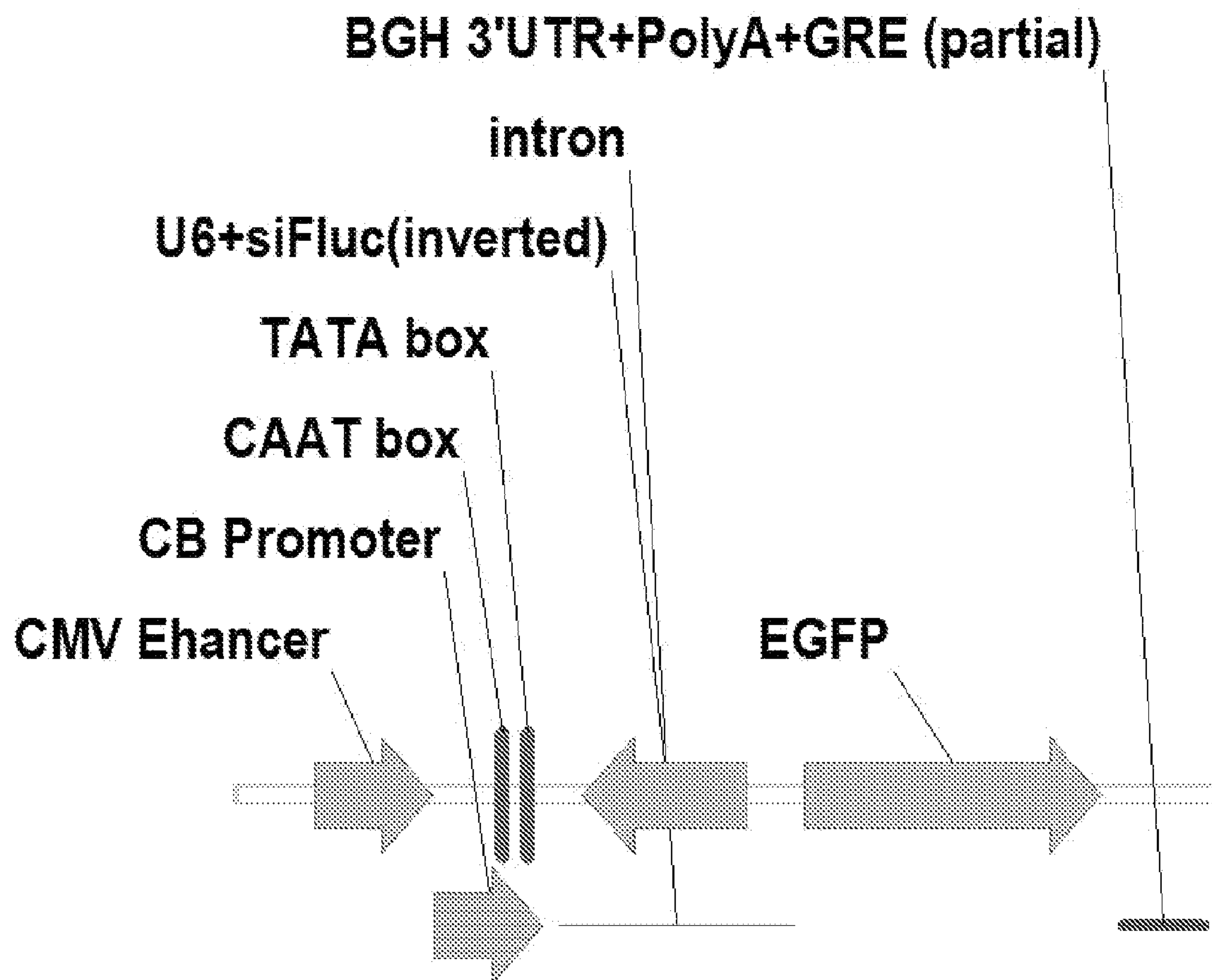


FIG. 2

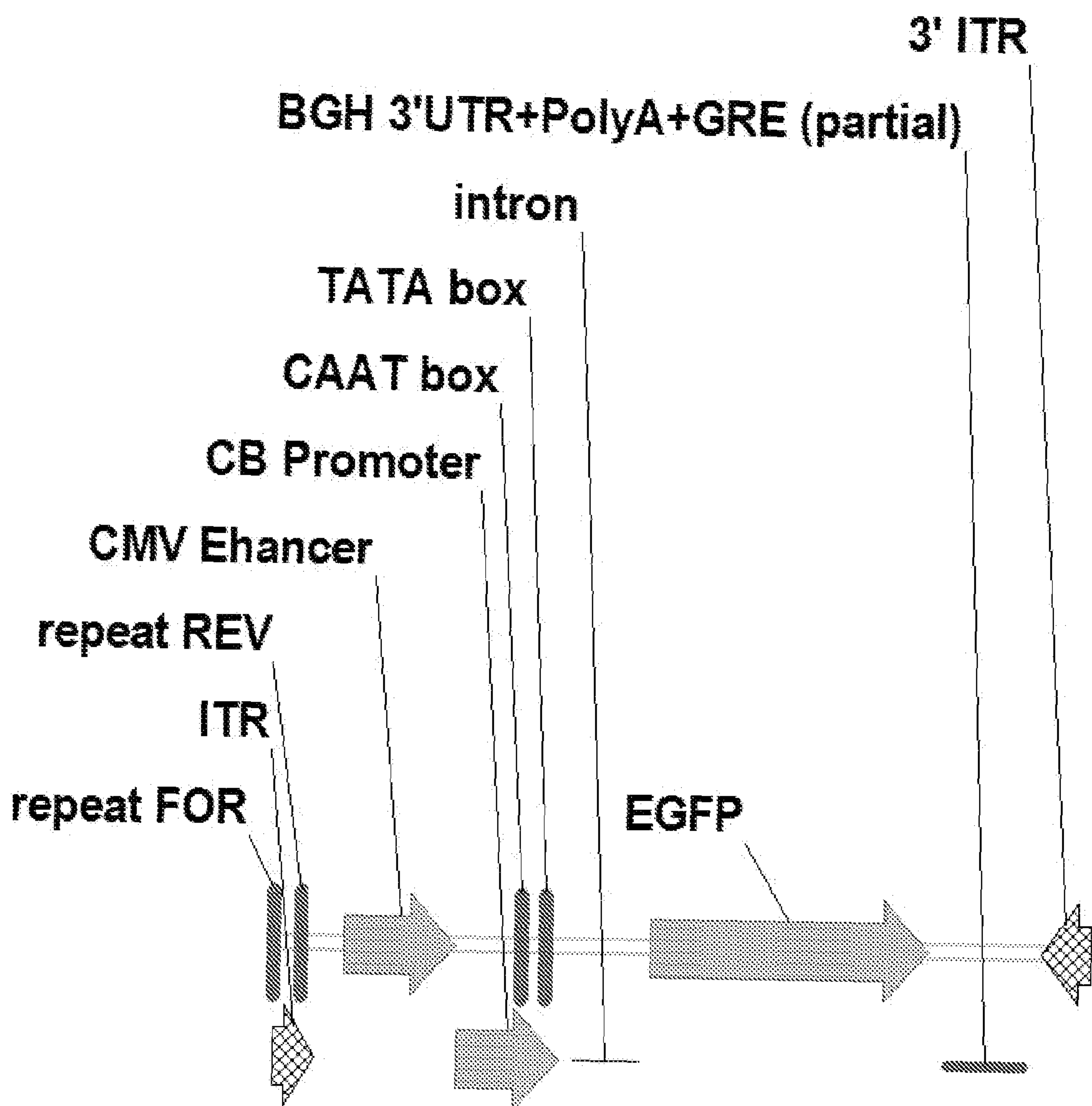


FIG. 3

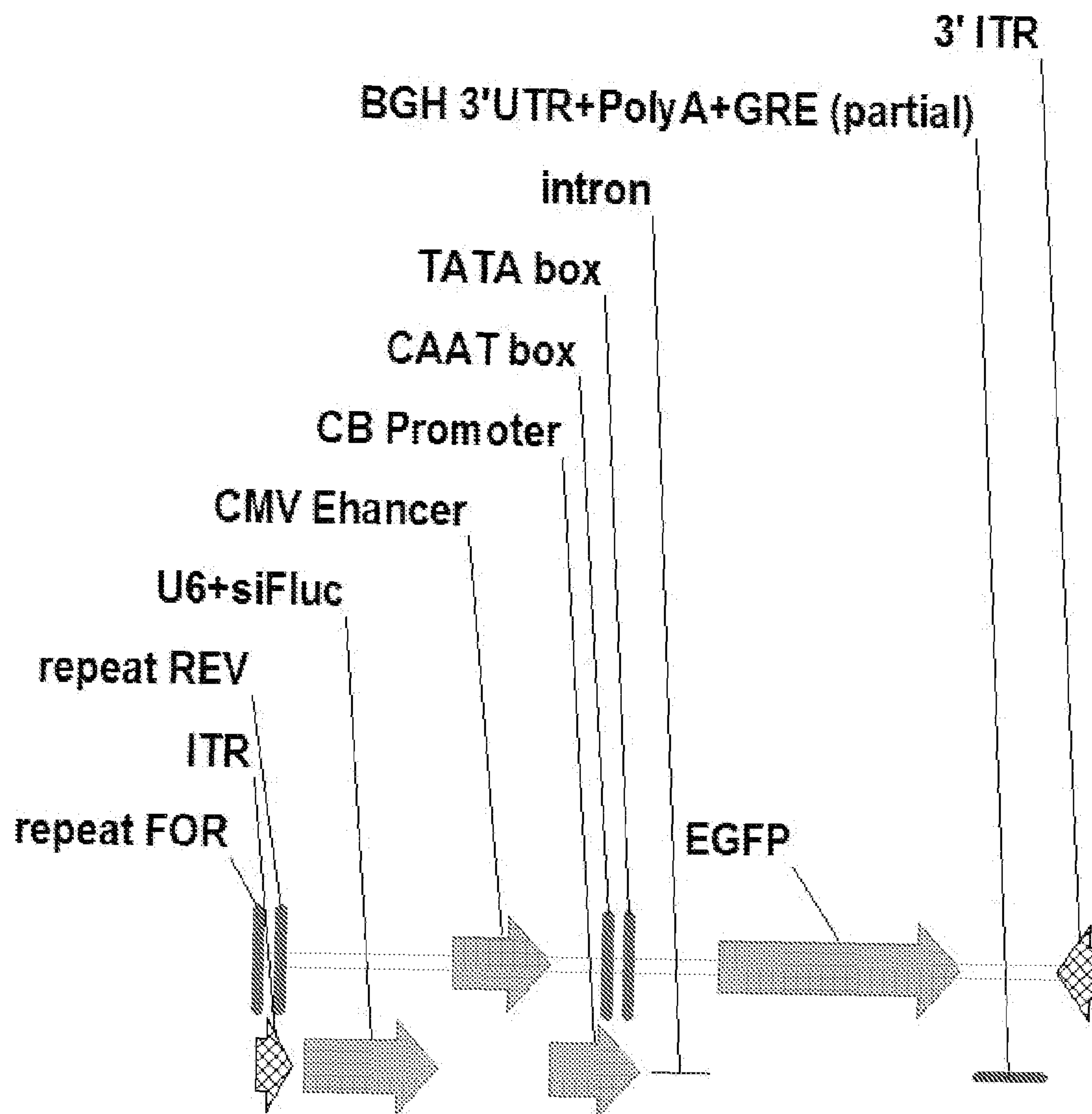


FIG. 4

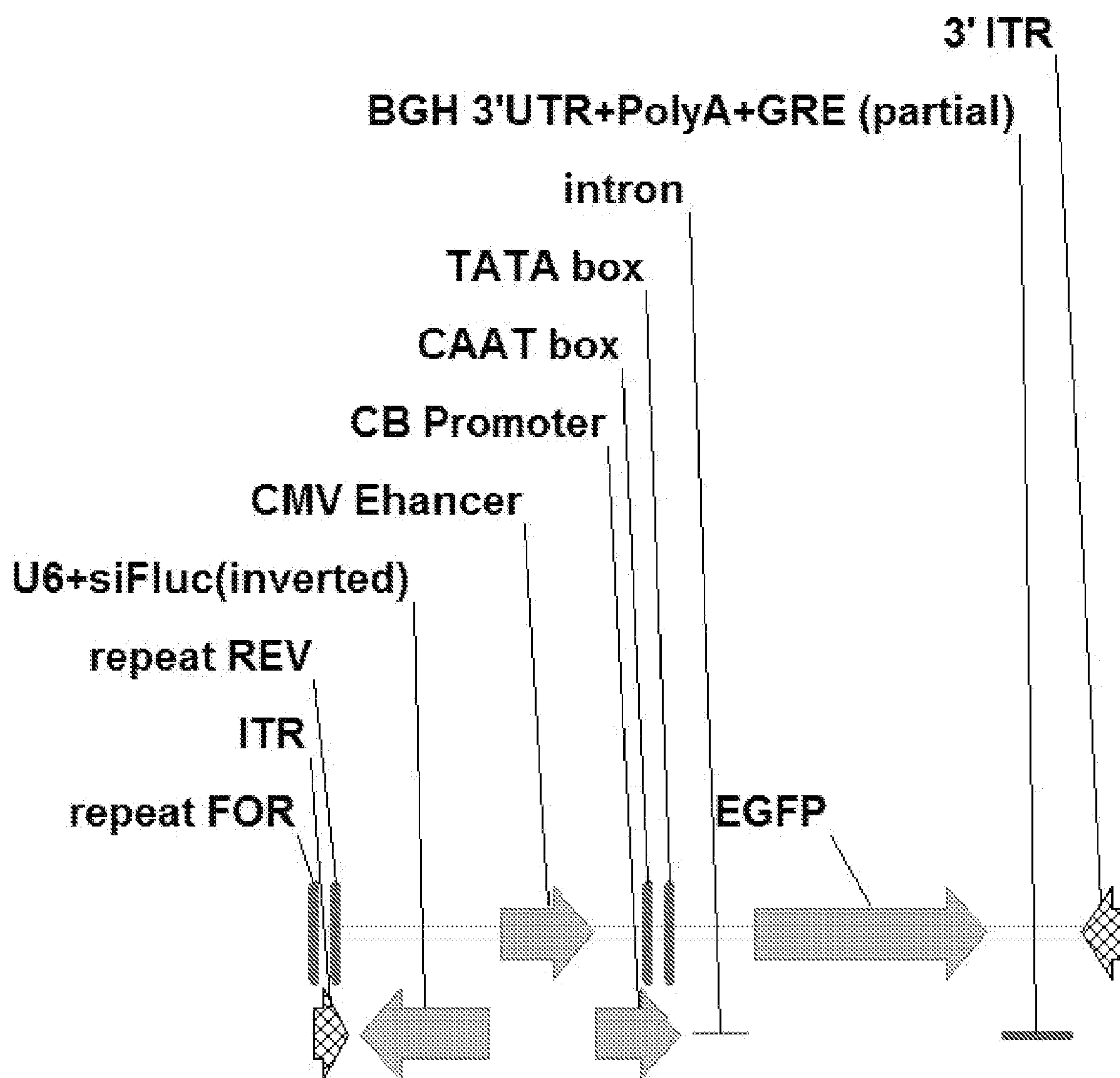


FIG. 5

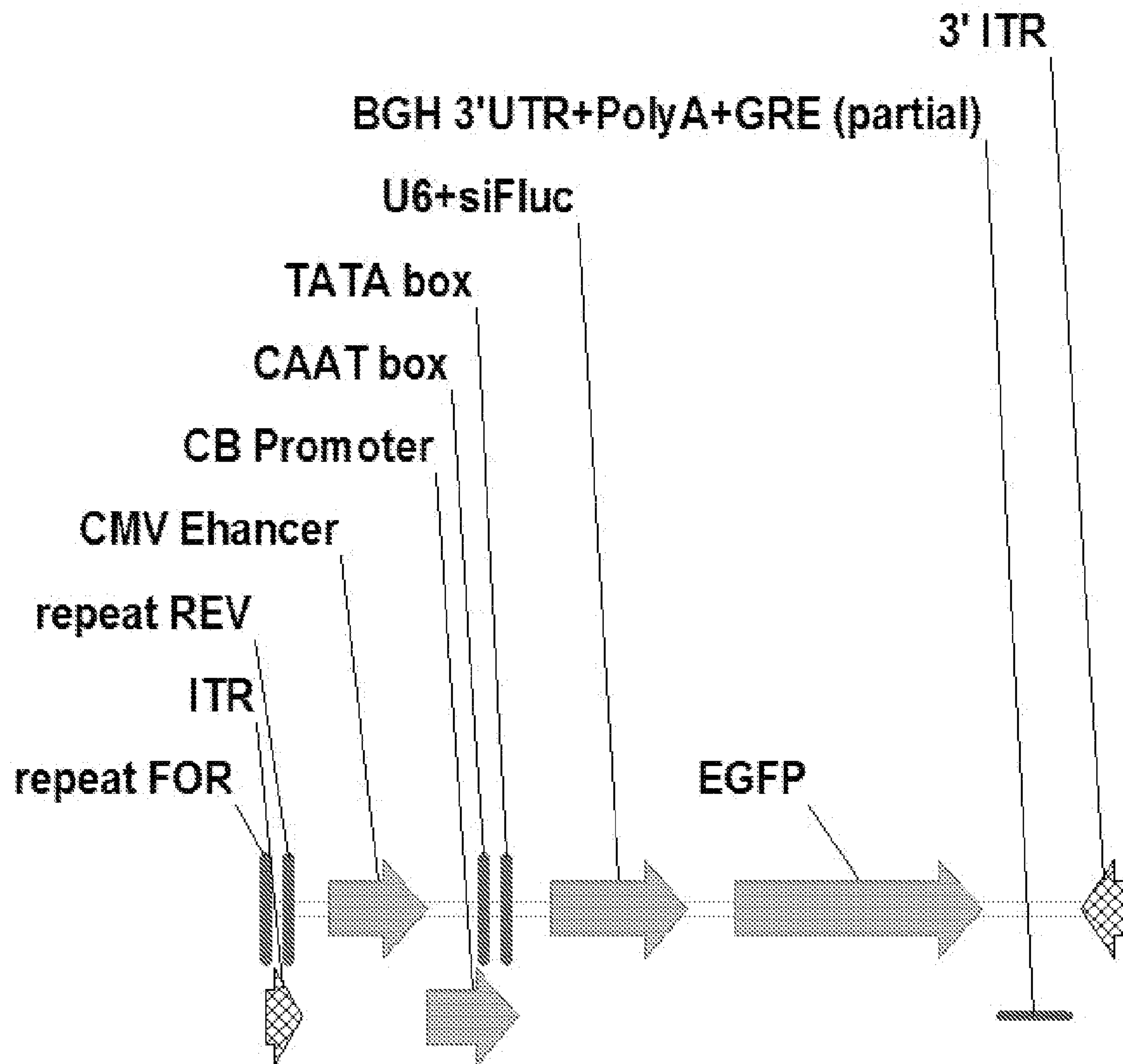


FIG. 6

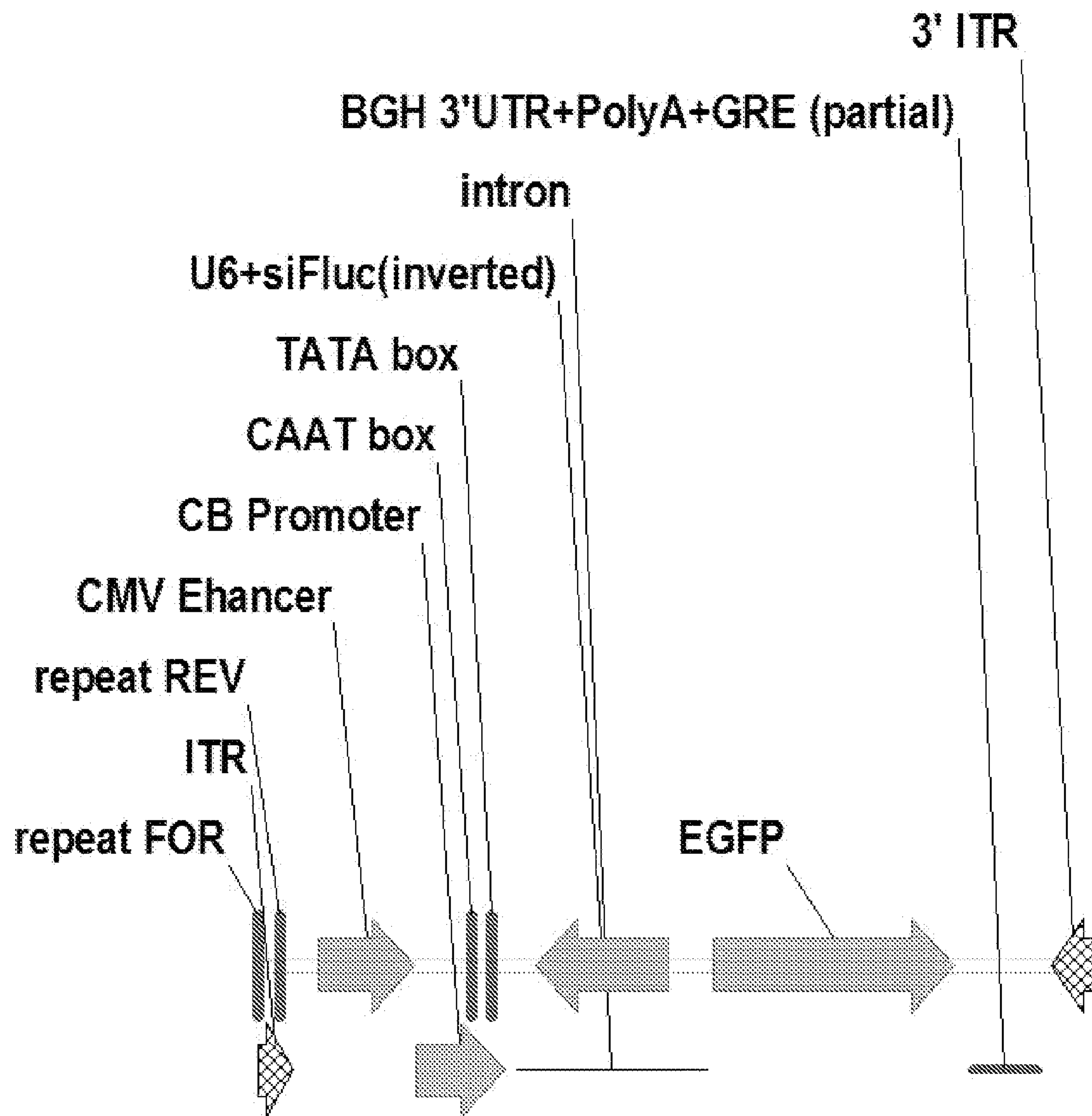


FIG. 7

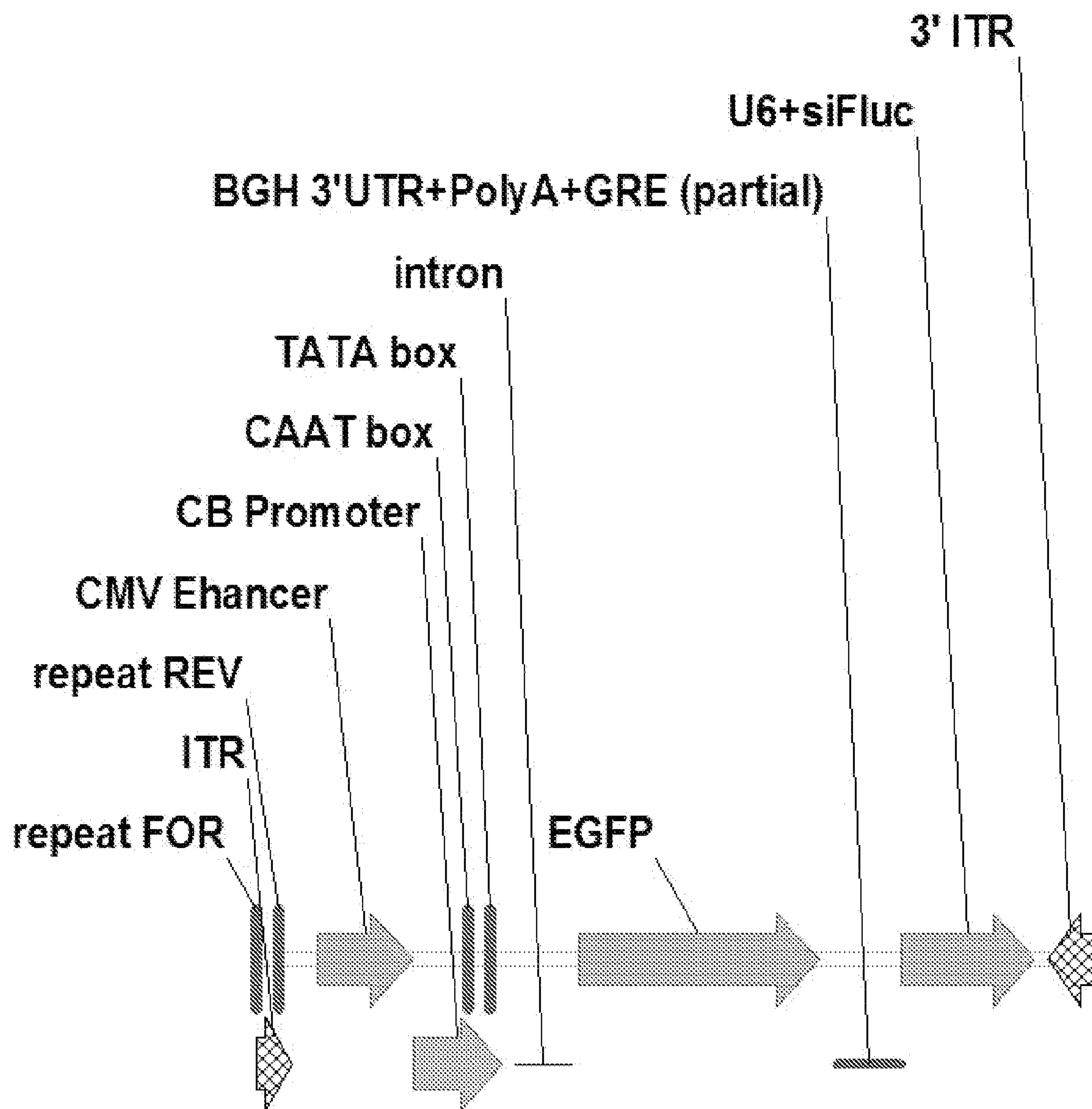


FIG. 8

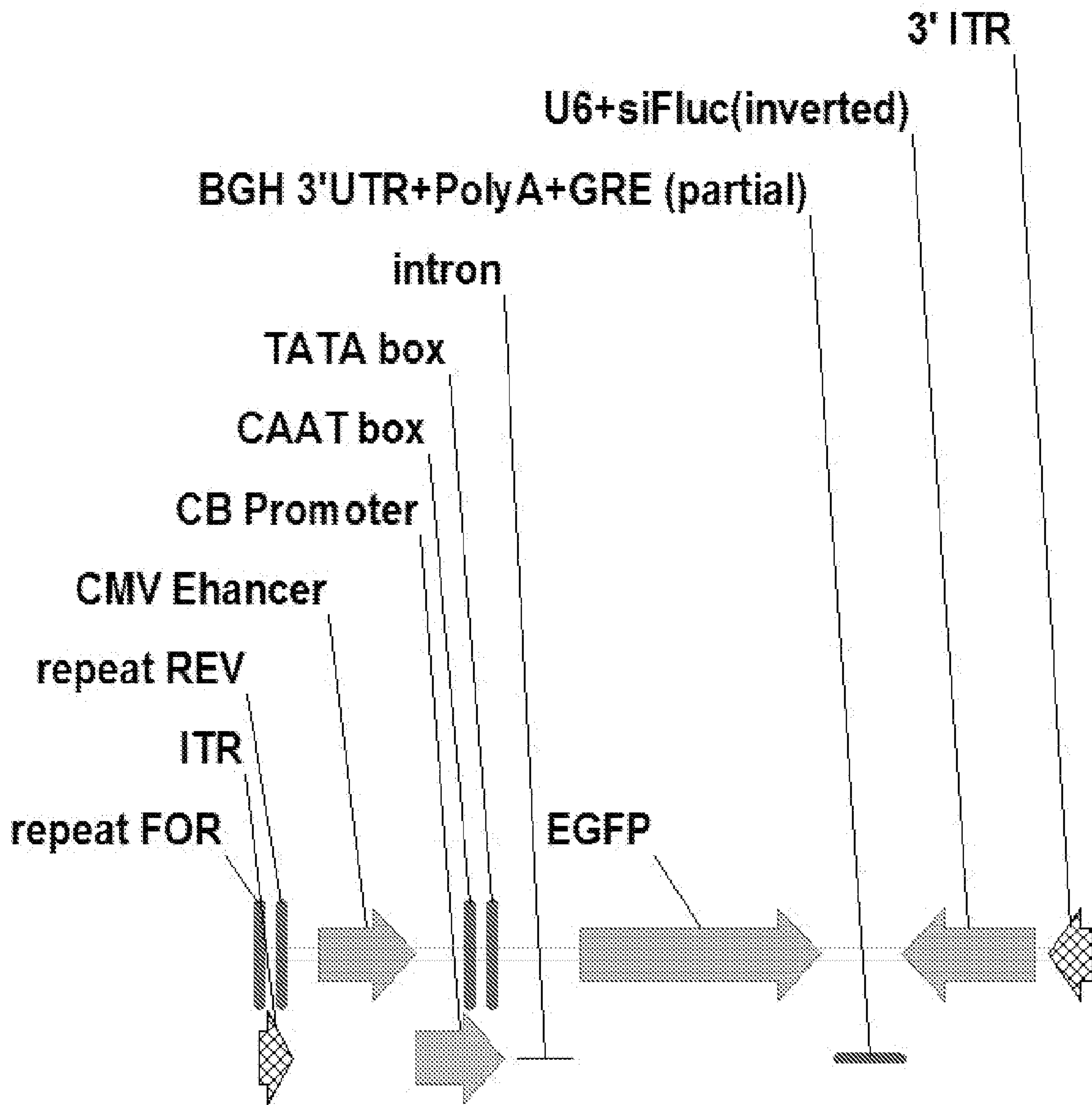


FIG. 9

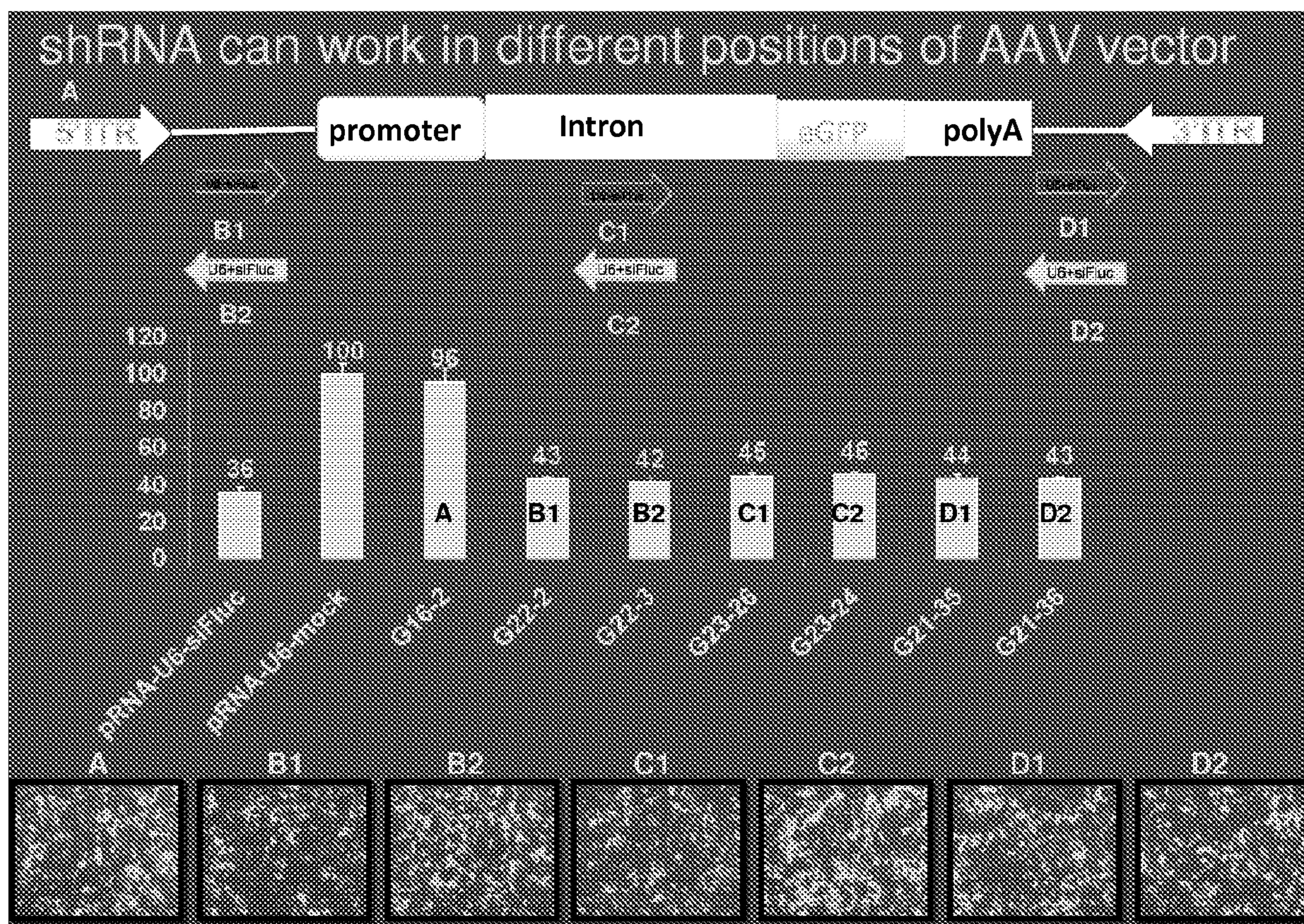


FIG. 10

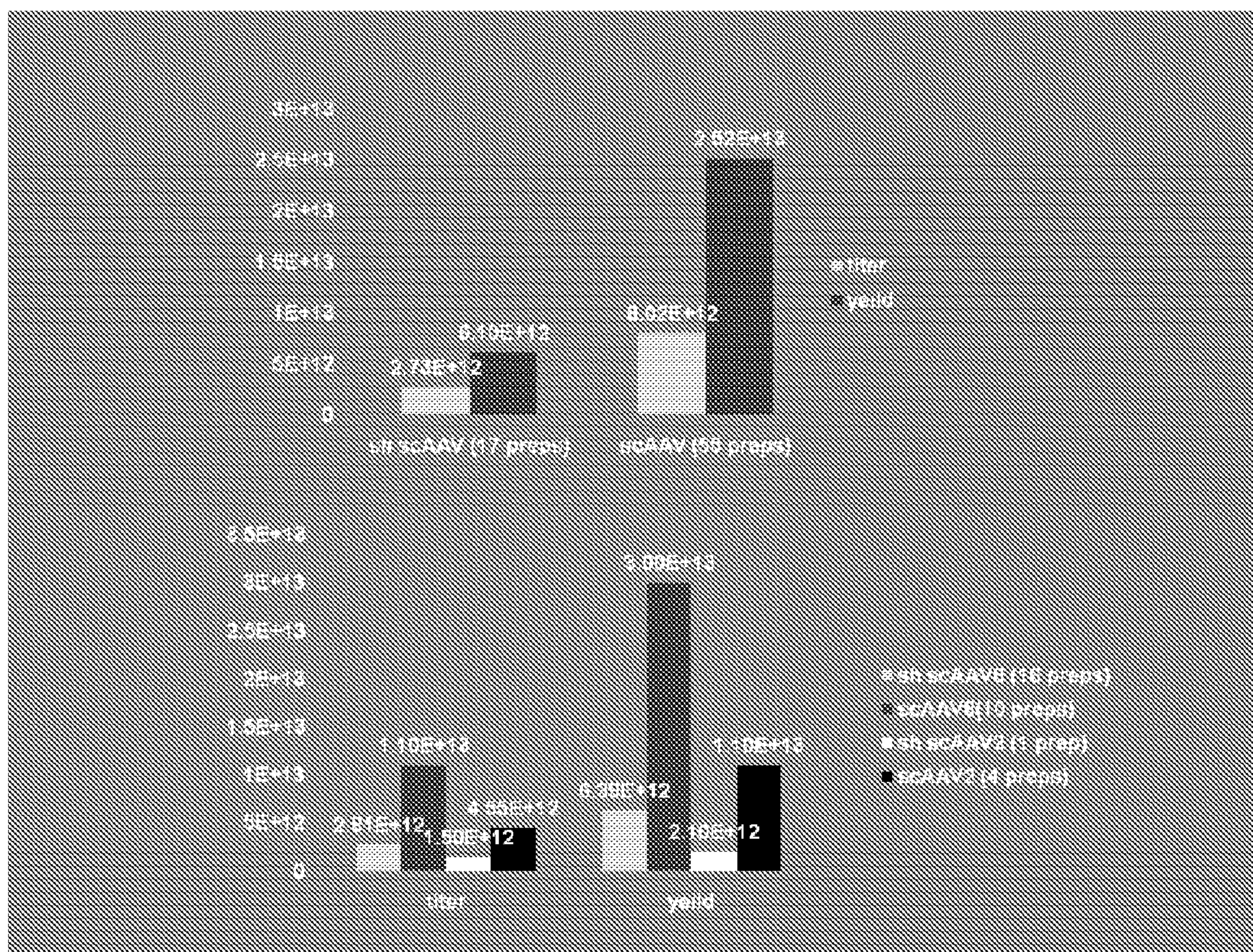


FIG. 11

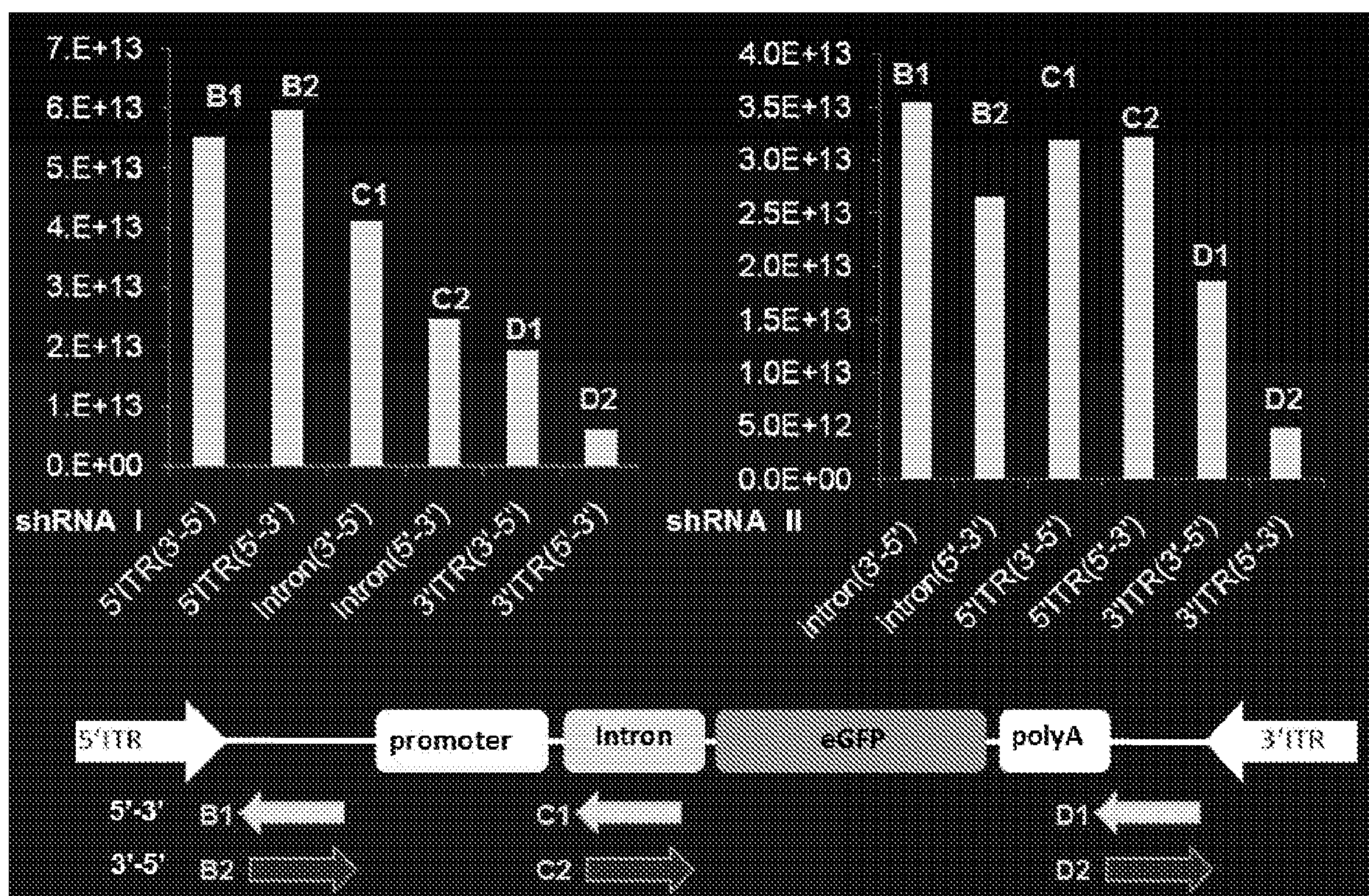


FIG. 12

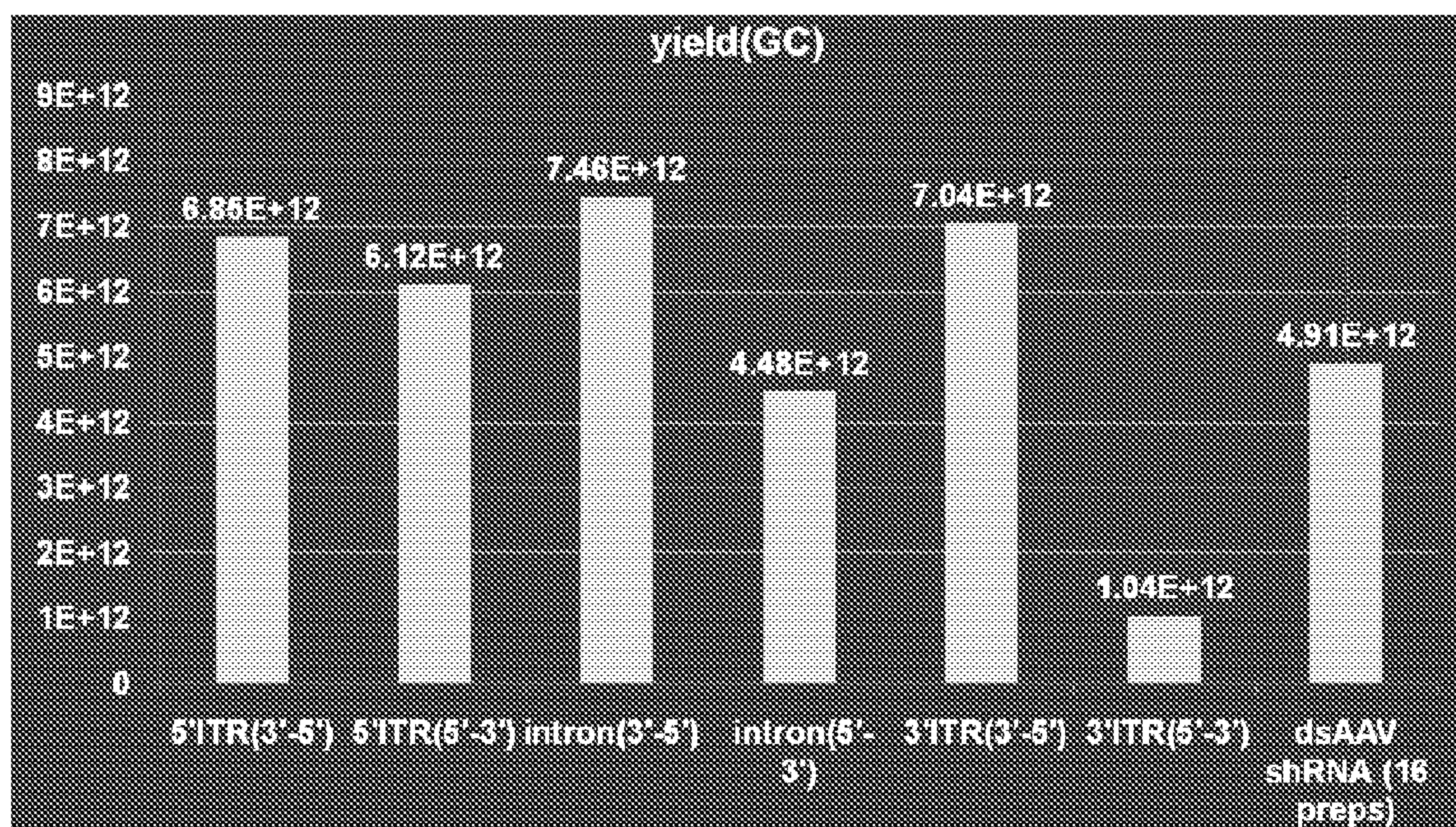


FIG. 13

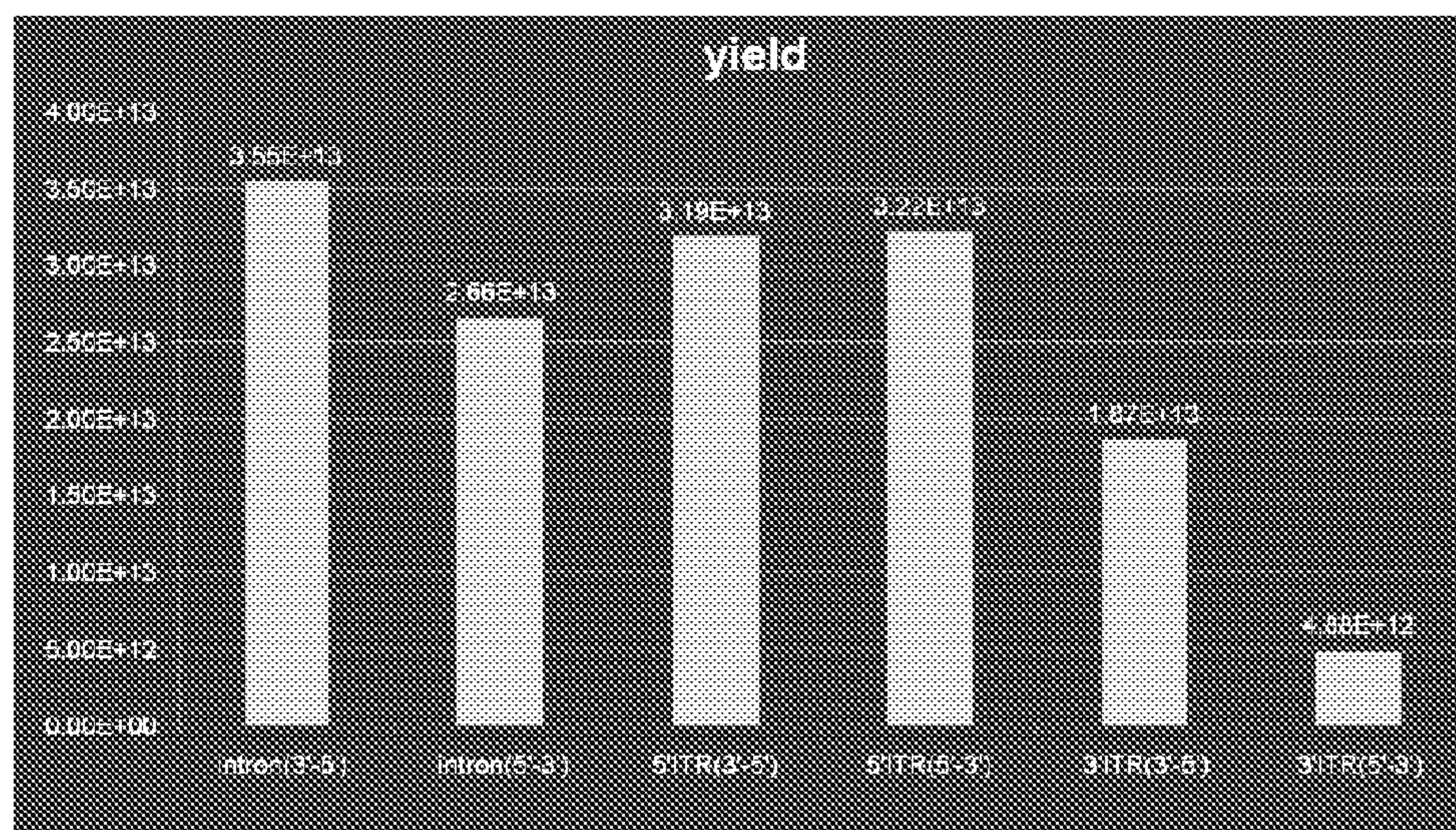


FIG. 14

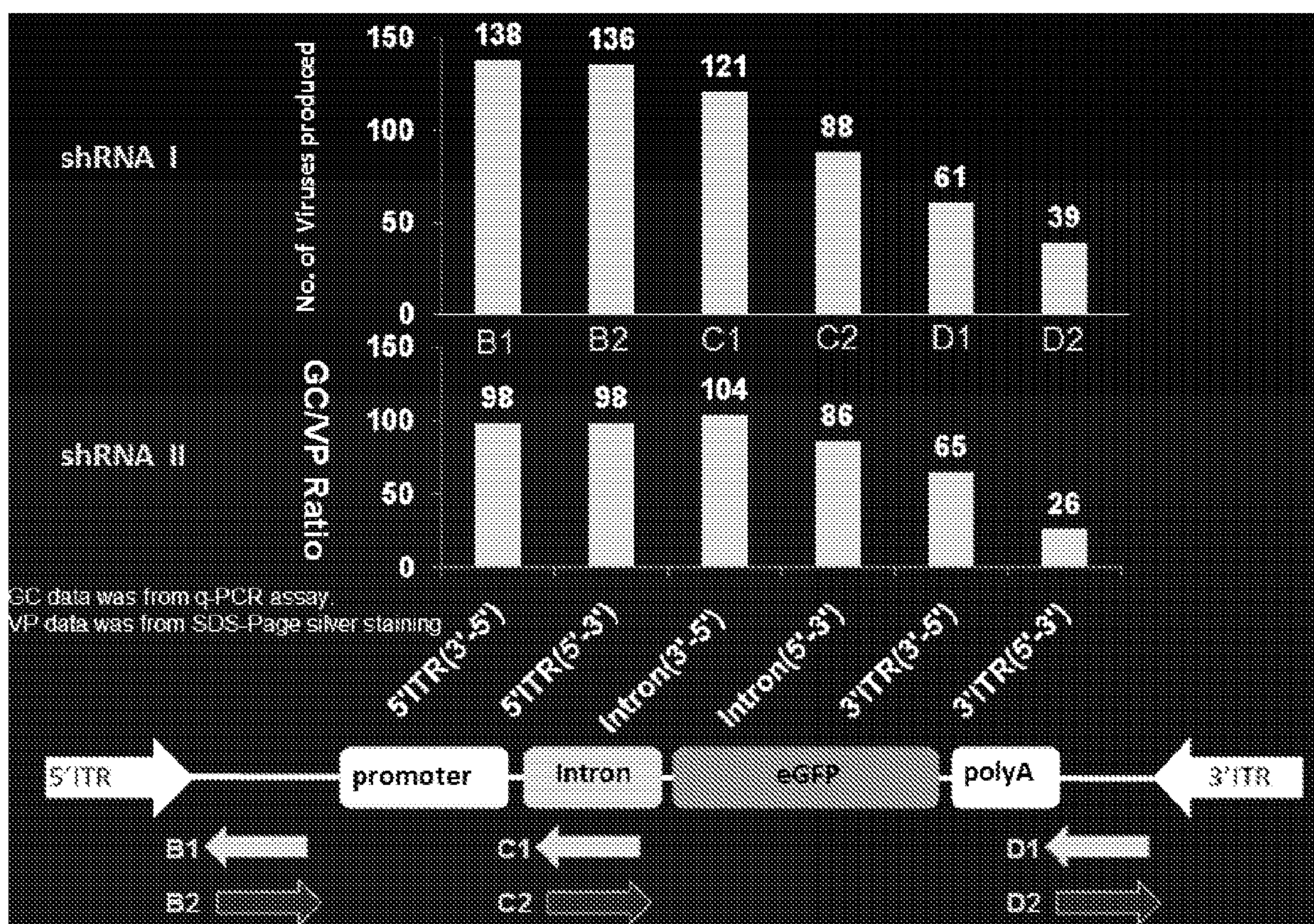


FIG. 15

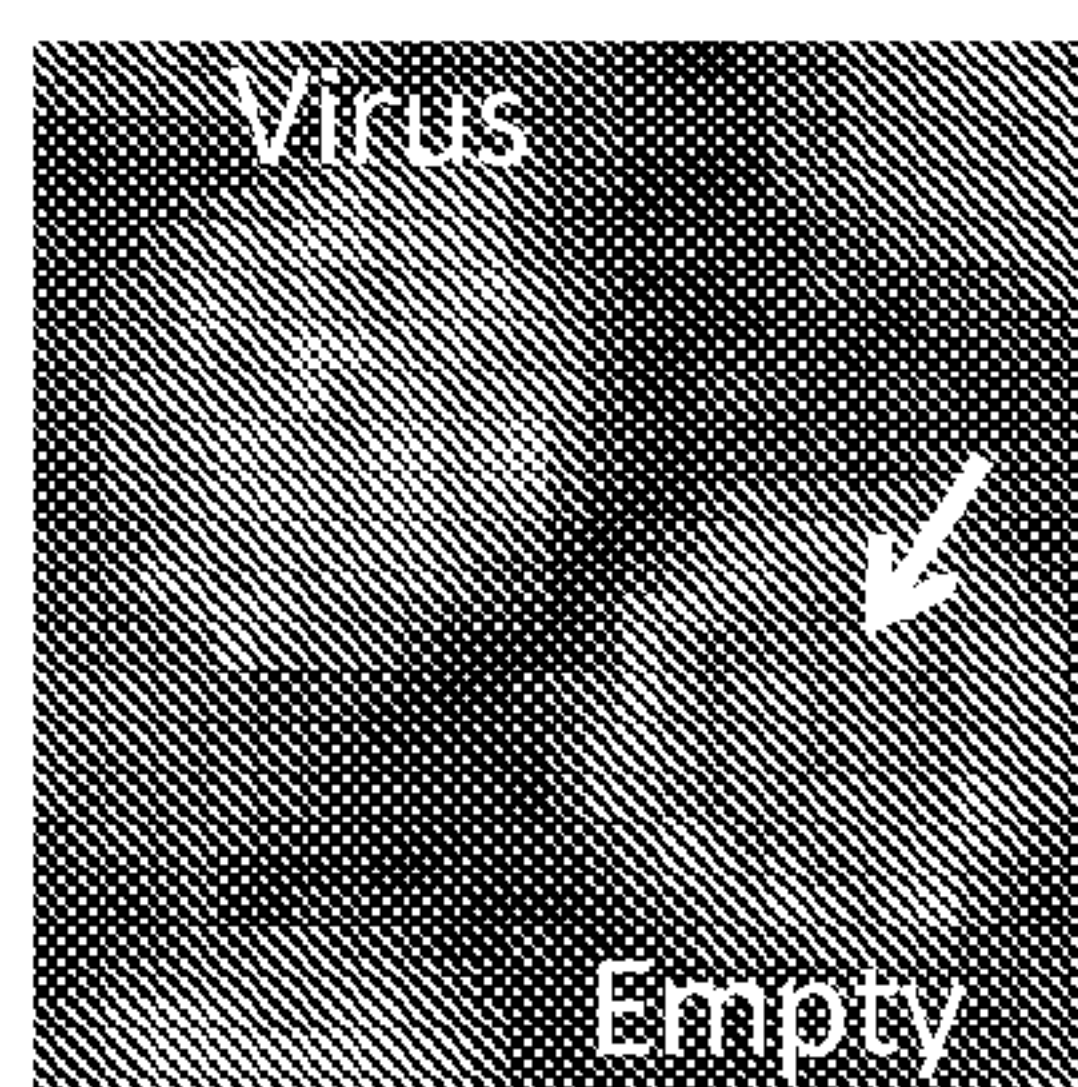
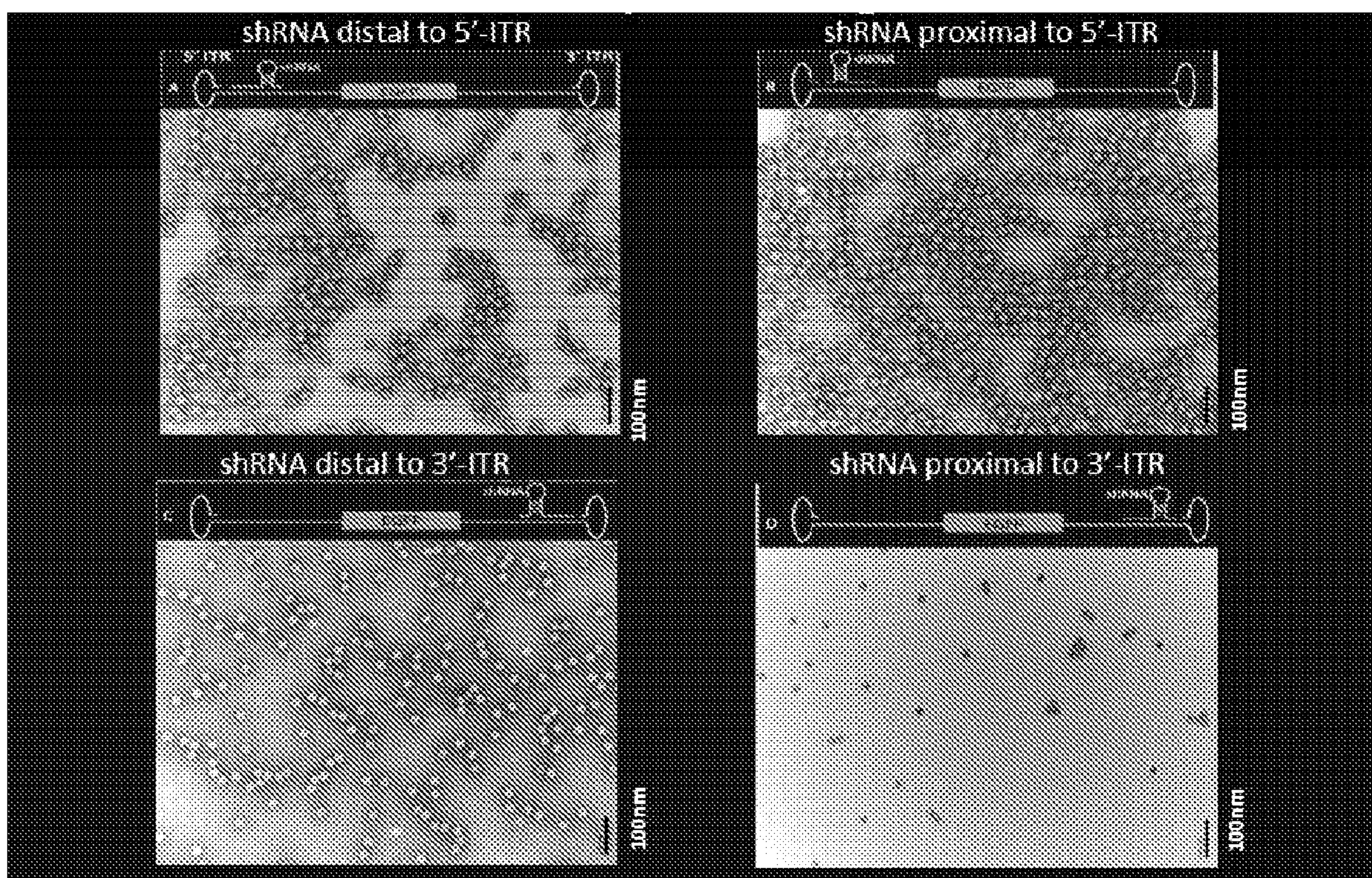


FIG. 16

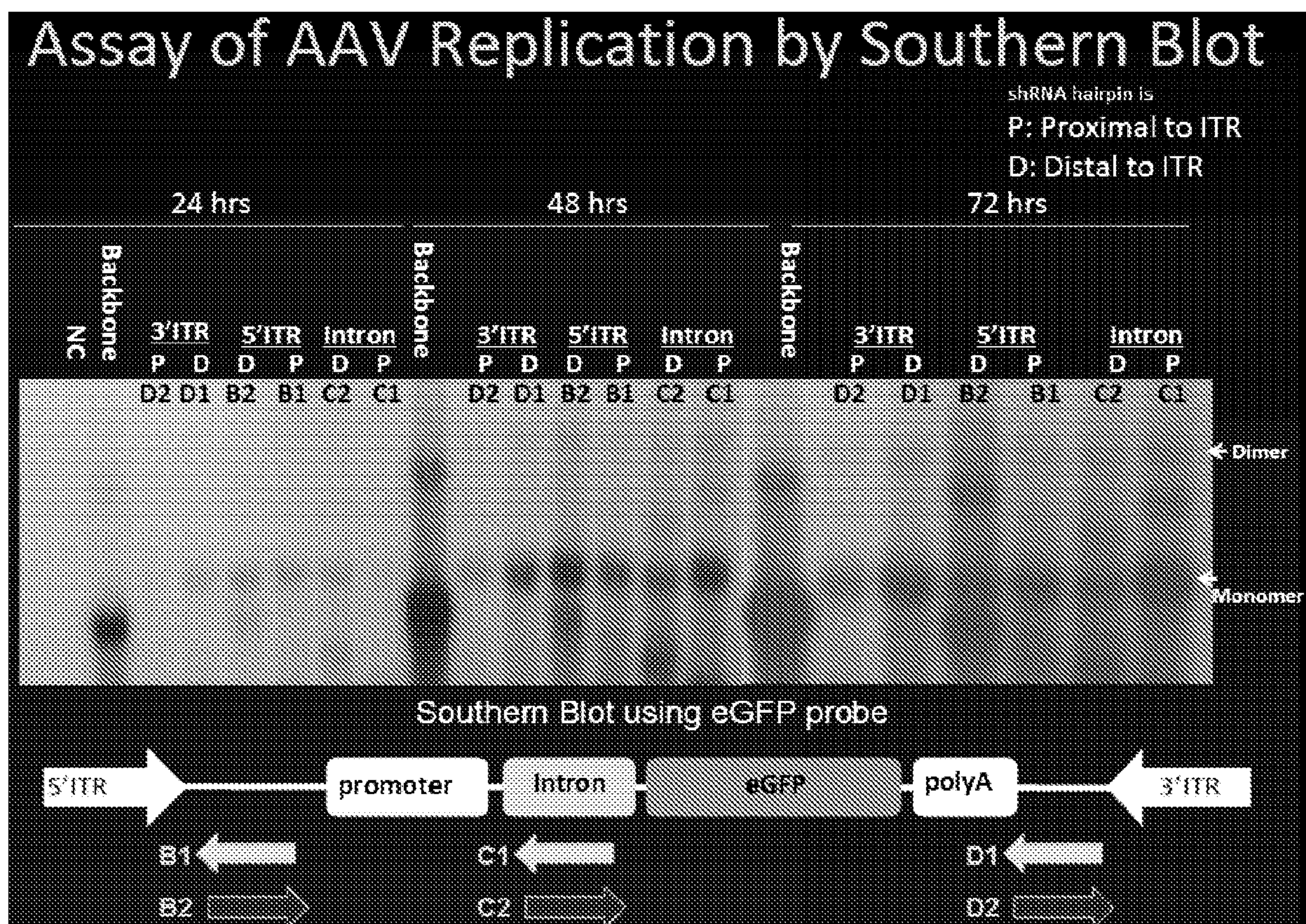


FIG. 17

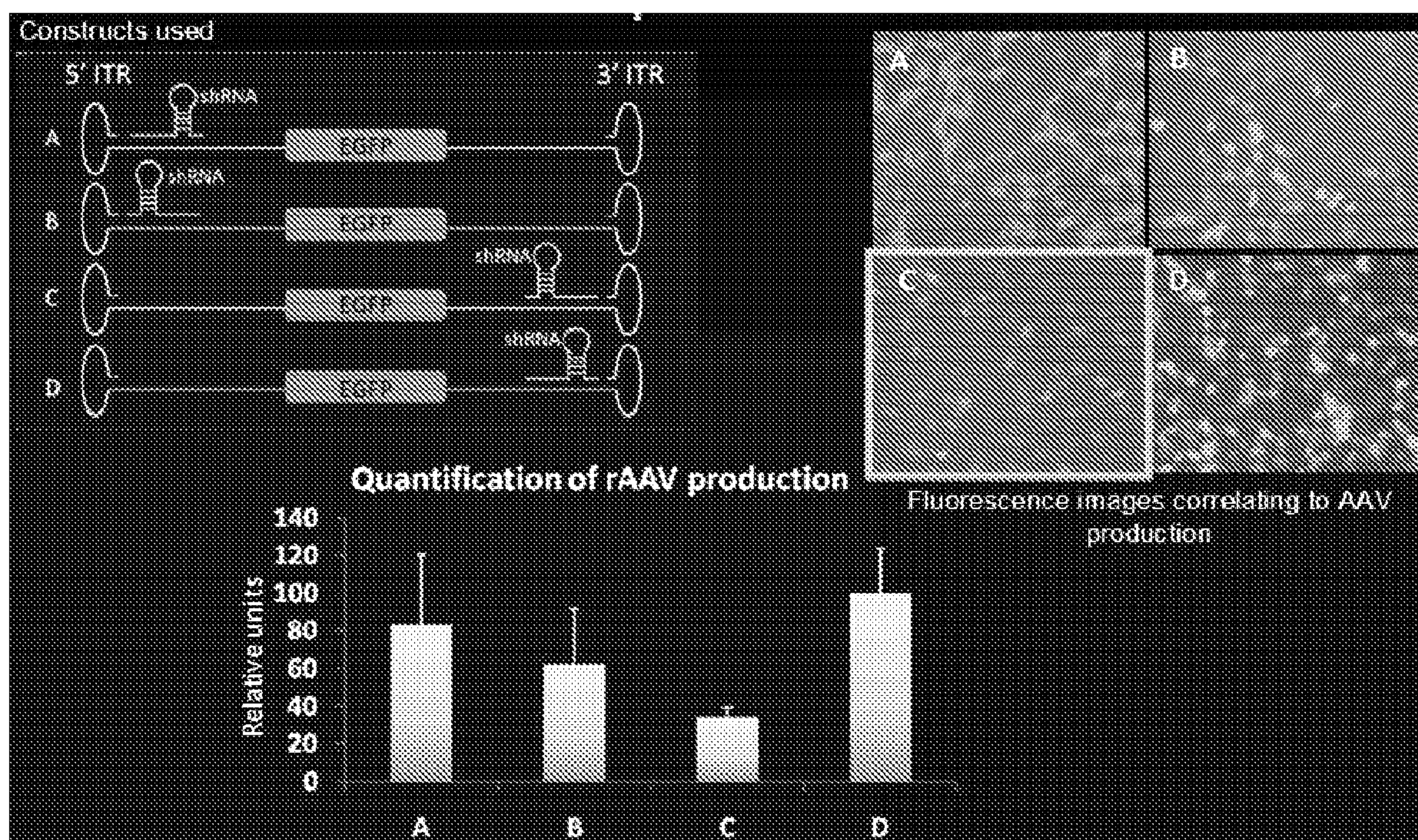


FIG. 18

BGH 3'UTR+PolyA+GRE (partial)

Intron

U6+siFluc

TATA box

CAAT box

CB Promoter

CMV Enhancer

EGFP

