(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 May 2014 (01.05.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/063753 A1

(51) International Patent Classification:
A61K 38/36 (2006.01) *C12N 15/86* (2006.01)
A61K 48/00 (2006.01)

(21) International Application Number:
PCT/EP2012/071297

(22) International Filing Date:
26 October 2012 (26.10.2012)

(25) Filing Language: English

(26) Publication Language: English

(71) Applicant: **VRIJE UNIVERSITEIT BRUSSEL** [BE/BE]; Pleinlaan 2, B-1050 Brussel (BE).

(72) Inventors: **VANDENDRIESSCHE, Thierry**; Tiensesteenweg 250, B-3360 Bierbeek (BE). **CHUAH, Marinee**; Tiensesteenweg 250, B-3360 Bierbeek (BE).

(74) Agents: **VANHALST, Koen Victor Rachel** et al.; De Clercq & Partners, E. Gevaertdreef 10a, B-9830 Sint-Martens-Latem (BE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/063753 A1

(54) **Title**: HYPER-ACTIVE FACTOR IX VECTORS FOR LIVER-DIRECTED GENE THERAPY OF HEMOPHILIA 'B' AND METHODS AND USE THEREOF

(57) **Abstract**: The present invention relates to vectors containing liver-specific regulatory sequences and a factor IX gene containing a hyper-activating mutation, methods employing these vectors and uses of these vectors. Expression cassettes and vectors containing these liver-specific regulatory elements and hyper-active factor IX genes are also disclosed. The present invention is particularly useful for applications using gene therapy.

HYPER-ACTIVE FACTOR IX VECTORS FOR LIVER-DIRECTED GENE THERAPY OF HEMOPHILIA 'B' AND METHODS AND USE THEREOF

FIELD OF THE INVENTION

The invention relates to expression vectors for gene therapy with improved liver-specific expression capabilities, particularly for use as a gene therapy means for restoring coagulation factor IX (FIX) in the treatment of hemophilia, more particularly in liver-directed gene therapy of hemophilia B.

BACKGROUND OF THE INVENTION

Hemophilia B is an X-linked, recessive bleeding disorder caused by deficiency of clotting factor IX (FIX). The clinical presentation for hemophilia B is characterized by episodes of spontaneous and prolonged bleeding. There are an estimated 1 in 20,000 individuals who suffer from hemophilia B. Currently, hemophilia B is treated with protein replacement therapy using either plasma-derived or recombinant FIX. Although FIX protein replacement markedly improved the life expectancy of patients suffering from hemophilia, they are still at risk for severe bleeding episodes and chronic joint damage, since prophylactic treatment is restricted by the short half-life, the limited availability and the high cost of purified FIX, which can approach 100.000$/patient/year. In addition, the use of plasma-derived factors obtained from contaminated blood sources increases the risk of viral transmission. Gene therapy offers the promise of a new method of treating hemophilia B, since the therapeutic window is relatively broad and levels slightly above 1% of normal physiologic levels are therapeutic. If successful, gene therapy could provide constant FIX synthesis which may lead to a cure for this disease. The different modalities for gene therapy of hemophilia have been extensively reviewed (Chuah et al., 2012a, 2012b, 2012c; VandenDriessche et al., 2012; High 2001, 2011; Matrai et al., 2010a, 2010b).

The liver is the main physiological site of FIX synthesis and hence hepatocytes are well suited target cells for hemophilia gene therapy. From this location, FIX protein can easily enter into the circulation. Moreover, the hepatic niche may favor the induction of immune tolerance towards the transgene product (Annoni et al., 2007; Follenzi et al., 2004; Brown et al., 2007; Herzog et al., 1999; Matrai et al., 2011; Matsui et al., 2009). Liver-directed gene therapy for hemophilia can be accomplished with different viral vectors including retroviral (Axelrod et al., 1990; Kay et al., 1992; VandenDriessche et al., 1999, Xu et al.,

2003, 2005), lentiviral (Ward et al., 2011, Brown et al., 2007, Matrai et al., 2011), adeno-associated viral (AAV) (Herzog et al., 1999) and adenoviral vectors (Brown et al., 2004)(Ehrhardt & Kay, 2002) In particular, AAV is a naturally occurring replication defective non-pathogenic virus with a single stranded DNA genome. AAV vectors have a favorable safety profile and are capable of achieving persistent transgene expression. Long-term expression is predominantly mediated by episomally retained AAV genomes. More than 90% of the stably transduced vector genomes are extra-chromosomal, mostly organized as high-molecular-weight concatemers. Therefore, the risk of insertional oncogenesis is minimal, especially in the context of hemophilia gene therapy where no selective expansion of transduced cells is expected to occur. Nevertheless, oncogenic events have been reported following AAV-based gene transfer (Donsante et al., 2007) but it has been difficult to reproduce these findings in other model systems (Li et al., 2011). The major limitation of AAV vectors is the limited packaging capacity of the vector particles (i.e. approximately 4.7 kb), constraining the size of the transgene expression cassette to obtain functional vectors (Jiang et al., 2006). Several immunologically distinct AAV serotypes have been isolated from human and non-human primates (Gao et al., 2002, Gao et al. 2004), although most vectors for hemophilia gene therapy were initially derived from the most prevalent AAV serotype 2. The first clinical success of AAV-based gene therapy for congenital blindness underscores the potential of this gene transfer technology (Bainbridge et al., 2008).

AAV-mediated hepatic gene transfer is an attractive alternative for gene therapy of hemophilia for both liver and muscle-directed gene therapy (Herzog et al., 1997, 1999, 2002; Arruda et al., 2010; Fields et al., 2001; Buchlis et al., 2012; Jiang et al., 2006; Kay et al., 2000). Preclinical studies with the AAV vectors in murine and canine models of hemophilia or non-human primates have demonstrated persistent therapeutic expression, leading to partial or complete correction of the bleeding phenotype in the hemophilic models (Snyder et al., 1997, 1999; Wang et al., 1999, 2000; Mount et al., 2002; Nathwani et al., 2002). Particularly, hepatic transduction conveniently induces immune tolerance to FIX that required induction of regulatory T cells (Tregs) (Mingozzi et al., 2003; Dobrzynski et al., 2006). Long-term correction of the hemophilia phenotype without inhibitor development was achieved in inhibitor-prone null mutation hemophilia B dogs treated with liver-directed AAV2-FIX gene therapy (Mount et al, 2002). In order to further reduce the vector dose, more potent FIX expression cassettes have been developed. This could be accomplished by using stronger promoter/enhancer elements, codon-optimized FIX or self-complementary, double-stranded AAV vectors (scAAV) that overcome one of the

limiting steps in AAV transduction (i.e. single-stranded to double-stranded AAV conversion) (McCarty, 2001, 2003; Nathwani et al, 2002, 2006, 2011; Wu et al., 2008). Alternative AAV serotypes could be used (e.g. AAV8) that result in increased transduction into hepatocytes, improve intra-nuclear vector import and reduce the risk of T cell activation (Gao et al., 2002; Vandenberghe et al., 2006). Liver-directed gene therapy for hemophilia B with AAV8 or AAV9 is more efficient than when lentiviral vectors are used, at least in mice, and resulted in less inflammation (VandenDriessche et al., 2007, 2002). Furthermore, recent studies indicate that mutations of the surface-exposed tyrosine residues allow the vector particles to evade phosphorylation and subsequent ubiquitination and, thus, prevent proteasome-mediated degradation, which resulted in a 10-fold increase in hepatic expression of FIX in mice (Zhong et al., 2008).

These liver-directed preclinical studies paved the way toward the use of AAV vectors for clinical gene therapy in patients suffering from severe hemophilia B. Hepatic delivery of AAV-FIX vectors resulted in transient therapeutic FIX levels (maximum 12% of normal levels) in subjects receiving AAV-FIX by hepatic artery catheterization (Kay et al., 2000). However, the transduced hepatocytes were able to present AAV capsid-derived antigens in association with MHC class I to T cells (Manno et al., 2006, Mingozzi et al., 2007). Although antigen presentation was modest, it was sufficient to flag the transduced hepatocytes for T cell-mediated destruction. Recently, gene therapy for hemophilia made an important step forward (Nathwani et al., 2011; Commentary by VandenDriessche & Chuah, 2012). Subjects suffering from severe hemophilia B (<1% FIX) were injected intravenously with self-complementary (sc) AAV8 vectors expressing codon-optimized FIX from a liver-specific promoter. This AAV8 serotype exhibits reduced cross-reactivity with pre-existing anti-AAV2 antibodies. Interestingly, its uptake by dendritic cells may be reduced compared to conventional AAV2 vectors, resulting in reduced T cell activation (Vandenberghe et al., 2006). In mice, AAV8 allows for a substantial increase in hepatic transduction compared to AAV2, though this advantage may be lost in higher species, like dog, rhesus monkeys and man. Subjects received escalating doses of the scAAV8-FIX vector, with two participants per dose. All of the treated subjects expressed FIX above the therapeutic 1% threshold for several months after vector administration, yielding sustained variable expression levels (i.e. 2 to 11% of normal levels). The main difference with the previous liver-directed AAV trial is that for the first time sustained therapeutic FIX levels could be achieved after gene therapy. Despite this progress, T-cell mediated clearance of AAV-transduced hepatocytes remains a concern consistent with elevated liver enzyme

levels in some of the patients. Transient immune suppression using a short course of glucocorticoids was used in an attempt to limit this vector-specific immune response.

Non-viral vectors typically rely on a plasmid-based gene delivery system, where only the naked DNA is delivered, potentially in conjunction with physicochemical methods that facilitate transfection. Consequently, the non-viral approach maybe less immunogenic and potentially safer than viral vectors, though innate immune response may still occur. The non-viral gene transfer method is simple, but the efficiency is generally low compared to most viral vector-mediated gene transfer approaches. Efficient in vivo gene delivery of non-viral vectors remains a bottleneck. Typically, for hepatic gene delivery, plasmids are administered by hydrodynamic injection. In this case, a hydrodynamic pressure is generated by rapid injection of a large volume of DNA solution into the circulation, in order to deliver the gene of interest in the liver (Miao et al., 2000). Efforts are being made to adapt hydrodynamic injection towards a clinically relevant modality by reducing the volume of injection along with maintaining localized hydrodynamic pressure for gene transfer. Alternative approaches based on targetable nanoparticles are being explored to achieve target specific delivery of FIX into hepatocytes. Expression could be prolonged by removing bacterial backbone sequences which interfere with long term expression (i.e. mini-circle DNA) Finally, to increase the stability of FIX expression after non-viral transfection, transposons could be used that result in stable genomic transgene integration. We and others have shown that transposons could be used to obtain stable clotting factor expression following in vivo gene therapy (Yant et al., 2000; Mates, Chuah et al., 2009, VandenDriessche et al., 2009; Kren et al.,2009; Ohlfest et al., 2004).

An exemplary state of the art vector for liver-specific expression of FIX is described in WO2009/130208 and is composed of a single-stranded AAV vector that contains the TTR/Serp regulatory sequences driving a factor cDNA. A FIX first intron was included in the vector and a poly-adenylation signal. Using said improved vector yielded about 25-30% stable circulating factor IX.

Nevertheless, in order to translate viral-vector based gene therapy for hemophilia to the clinic, the safety concerns associated with administering large vector doses to the liver and the need for manufacturing large amounts of clinical-grade vector must be addressed. Increasing the potency (efficacy per dose) of gene transfer vectors is crucial towards achieving these goals. It would allow using lower doses to obtain therapeutic benefit, thus reducing potential toxicities and immune activation associated with in vivo administration,

and easing manufacturing needs. One way to increase potency is to engineer the transgene sequence itself to maximize expression and biological activity per vector copy. We have shown that FIX transgenes optimized for codon usage and carrying an R338L amino acid substitution associated with clotting hyperactivity and thrombophilia (Simioni et al., 2009), increase the efficacy of gene therapy using lentiviral vector up to 15-fold in hemophilia B mice, without detectable adverse effects, substantially reducing the dose requirement for reaching therapeutic efficacy and thus facilitating future scale up and its clinical translation (Cantore et al., 2012).

SUMMARY OF THE INVENTION

It is an object of the present invention to increase the efficiency and safety of liver-directed gene therapy for hemophilia B. The above objective is accomplished by providing a vector comprising a nucleic acid expression cassette with specific regulatory elements that enhance liver-directed gene expression, while retaining tissue specificity, in conjunction with the use of a human FIX gene containing a hyper-activating mutation.

The resulting vector and nucleic acid expression cassette results in unexpectedly high expression levels of FIX in the liver, due to its unique combination of regulatory elements and the choice of vector type and transgene. The combined effect of these elements could not have been predicted. In WO2009/130208 for example, the given vector yielded about 25-30% stable circulating factor IX. In the current application the new vector obtained 500-600% of stable circulating factor IX levels. This represents a more than 20-fold increase in FIX levels due to the unique combination of elements of the nucleic acid expression cassette and vector of the present invention.

The invention therefore provides the following aspects:

Aspect 1. A vector containing a nucleic acid expression cassette comprising liver-specific regulatory sequences and a gene encoding for coagulation factor IX (FIX) containing a hyper-activating mutation and a transcriptional termination signal.

Aspect 2. The vector according to aspect 1, further comprising an intron.

Aspect 3. The vector according to claim 1 or 2 wherein said hyper-activating mutation in coagulation factor IX corresponds to an R338L amino acid substitution.

Aspect 4. The vector according to aspect 1, 2 or 3 wherein said gene encoding for coagulation factor IX is codon-optimized.

Aspect 5. The vector according to any one of aspects 1 to 4 wherein said liver-specific regulatory elements are derived from the transthyrethin promoter.

Aspect 6. The vector according to any one of aspects 1 to 5 wherein said liver-specific regulatory elements contain sequences from the serpin promoter.

Aspect 7. The vector according to any one of aspects 1 to 6 wherein said transcriptional termination signal is derived from the bovine growth hormone polyadenylation signal.

Aspect 8. The vector according to any one of aspects 1 to 7 wherein said intron is derived from the minute virus of mice (MVM).

Aspect 9. The vector according to any one of aspects 1 to 8 wherein said vector is a viral vector.

Aspect 10. The vector according to aspect 9, wherein said vector is derived from the adeno-associated virus (AAV).

Aspect 11. The vector according to aspects 9 or 10 wherein said vector is a self-complementary AAV.

Aspect 12. The vector according to any one of aspects 1 to 8 wherein said vector is a non-viral vector.

Aspect 13. The vector according to aspect 12 wherein said vector is a transposon-based vector.

Aspect 14. The vector according to aspects 12 or 13 wherein said vector is a PiggyBac-based vector.

Aspect 15. The use of the vector according to any one of aspects 1 to 14 for the manufacture of a medicament to treat hemophilia B.

Aspect 16. A method to obtain levels of factor IX in plasma equal to or higher than the therapeutic threshold concentration of 10mU/ml plasma in a subject, comprising the transduction or transfection of the vector according to any one of aspects 1 to 14 into a subject.

Aspect 17. The method according to aspect 16, wherein the transduction of the vector according to any one of aspects 1 to 11 into the subject is done at a dose lower than $2x10^{11}$ vg/kg.

Aspect 18. The method according to aspect 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 100 mU/ml in a subject, wherein the transduction of the vector according to any one of aspects 1 to 11 into the subject is done at a dose lower than or equal than $6x10^{11}$ vg/kg.

Aspect 19. The method according to aspect 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 50 mU/ml in a subject, wherein the transduction of the vector according to any one of aspects 1 to 11 into the subject is done at a dose lower than or equal than $6x10^{11}$ vg/kg.

20. The aspect according to claim 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 200 mU/ml in a subject, wherein the transduction of the vector according to any one of aspects 1 to 11 into the subject is done at a dose lower than or equal than $2x10^{12}$ vg/kg.

21. The aspect according to claim 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 150 mU/ml in a subject, wherein the transduction of the vector according to any one of aspects 1 to 11 into the subject is done at a dose lower than or equal than $2x10^{12}$ vg/kg.

22. The aspect according to any one of claims 16 to 21, wherein said transduction or transfection is by intravenous administration.

23. The aspect according to any one of claims 16 to 22, wherein said subject is a mammalian subject, preferably a human subject.

24. A method for treating hemophilia B in a mammalian subject, comprising performing the method according to any one of aspects 16 to 23.

25. A pharmaceutical composition comprising a vector according to any one of aspects 1 to 14 and a pharmaceutically acceptable carrier, optionally further comprising an active ingredient for treating hemophilia B.

26. The pharmaceutical composition according to aspect 25, for use in treating hemophilia B.

27. The use of a vector according to anyone of aspects 1 to 14 for the manufacturing of a medicament for treating hemophilia B.

28. The pharmaceutical composition according to aspect 25 or 26, or the use of a vector for treating hemophilia according to aspect 27, wherein said treatment results in levels of factor IX in plasma of the treated subject that are equal to or higher than the therapeutic

threshold concentration of 10 mU/ml plasma in a subject, preferably equal to or higher than the therapeutic concentration of 50 mU/ml plasma in a subject, more preferably equal to or higher than the therapeutic concentration of 100 mU/ml plasma in a subject, even more preferably equal to or higher than the therapeutic concentration of 150 mU/ml plasma in a subject and even more preferably equal to or higher than the therapeutic concentration of 200 mU/ml plasma in a subject.

29. The pharmaceutical composition according to aspect 25 or 26, or the use of a vector for treating hemophilia according to aspect 27, wherein said treatment comprises the transduction of the vector according to any one of aspect 1 to 14 into the subject at a dose lower than or equal than $2x10^{12}$ vg/kg, preferably at a dose lower than or equal than $6x10^{11}$ vg/kg, more preferably at a dose lower than or equal than $2x10^{11}$vg/kg.

BRIEF DESCRIPTION OF THE FIGURES

The present invention is illustrated by the following figures which are to be considered for illustrative purposes only and in no way limit the invention to the embodiments disclosed therein:

Figure 1 A) shows a schematic diagram of the pdsAAVsc SerpEnh TTRmin MVM FIXcoptMT-bghpA construct with indication where the liver-specific Serpin regulatory element (SerpEnh) is inserted upstream of the transthyretin minimal promoter. Abbreviations used are: ITR: viral inverted terminal repeat; TTRmin: transthyretin minimal promoter; MVM: MVM intron (minute virus mouse); FIXcoptMT (or F9): codon-optimized FIX; bGHpA: polyadenylation signal of bovine growth hormone; **B)** shows the sequence of the pdsAAVsc SerpEnh TTRmin MVM FIXcoptMT-bghpA construct (SEQ ID No. 1) and **C)** shows the sequence of the pdsAAVsc SerpEnh TTRmin MVM FIXcoptPADUA-bghpA (SEQ ID No. 2).

Figure 2 shows FIX activity after intravenous injection of AAV9-pdsAAVsc SerpEnh TTRmin MVM FIXcoptMT-bghpA or AAV9 pdsAAVsc SerpEnh TTRmin MVM FIXcoptPADUA-bghpA in FIX-deficient hemophilia B mice. AAV vectors expressing either the human codon-optimized FIX cDNA were designated as AAV-co-hFIX or the human codon-optimized FIX-R338L cDNA as AAV-co-padua-hFIX . hFIX activity levels were determined using a chromogenic activity assay on citrated plasma. Mice were injected with different vectors dose of the cognate self-complementary AAV9 vectors (10^9 vg, $5x10^9$ vg, $2x10^{10}$ vg)

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes.

Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. The term “comprising” also encompasses the more specific embodiments defined as “consisting of” and “consisting essentially of”.

Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order.

It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Plainsview, New York (1989); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999), for definitions and terms of the art.

The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

The term “coagulation factor IX” has the meaning as known in the art. Synonyms of coagulation factor IX are “FIX” or “Christmas factor” or “F9” and can be used interchangeably. In particular, the term “coagulation factor IX” encompasses the human protein encoded by the mRNA sequence as defined in Genbank accession number NM_000133.

Preferably, said FIX is a mutated FIX, which is hyperactive or hyper-functional as compared to the wild type FIX. Modifying functional activity of human coagulation factor can be done by bioengineering e.g. by introduction of point mutations. By this approach a hyperactive R338A variant was reported, which showed a 3 fold increased clotting activity compared to the wild type human FIX in an *in vitro* activated partial thromboplastin time assay (APPT) (Chang et al., 1998) and a 2 to 6-fold higher specific activity in hemophilia B mice transduced with the mutant FIX gene (Schuettrumpf et al., 2005). Further exemplary FIX point-mutants or domain exchange mutants with even higher clotting activities have been described: FIX, with the EGF-1 domain replaced with the EGF-1 domain from FVII, alone or in combination with a R338A point mutation (Brunetti-Pierri et al., 2009), the V86A/E277A/R338A triple mutant (Lin et al., 2010), the Y259F, K265T, and/or Y345T single, double or triple mutants (Milanov, et al., 2012), and the G190V point mutant (Kao et al., 2010), all incorporated herein by reference. In a particularly preferred embodiment, the FIX mutant is the one described by Simioni et al., in 2009 and denominated as the "factor IX Padua" mutant, causing X-linked thrombophilia. Said mutant factor IX is hyperactive and carries an R338L amino acid substitution. In a preferred embodiment of the present invention, the FIX transgene used in expression vector encodes the human FIX protein, most preferably the FIX transgene encodes for the Padua mutant of the human FIX protein.

A "regulatory element" as used herein refers to transcriptional control elements, in particular non-coding cis-acting transcriptional control elements, capable of regulating and/or controlling transcription of a gene, in particular tissue-specific transcription of a gene. Regulatory elements comprise at least one transcription factor binding site (TFBS), more in particular at least one binding site for a tissue-specific transcription factor, most particularly at least one binding site for a liver-specific transcription factor. Typically, regulatory elements as used herein increase or enhance promoter-driven gene expression when compared to the transcription of the gene from the promoter alone, without the regulatory elements. Thus, regulatory elements particularly comprise enhancer sequences, although it is to be understood that the regulatory elements enhancing transcription are not limited to typical far upstream enhancer sequences, but may occur at any distance of the gene they regulate. Indeed, it is known in the art that sequences regulating transcription may be situated either upstream (e.g. in the promoter region) or downstream (e.g. in the 3'UTR) of the gene they regulate in vivo, and may be located in the immediate vicinity of the gene or further away. Of note, although regulatory elements as disclosed herein typically are naturally occurring sequences, combinations of (parts of)

such regulatory elements or several copies of a regulatory element, i.e. non-naturally occurring sequences, are themselves also envisaged as regulatory element. Regulatory elements as used herein may be part of a larger sequence involved in transcriptional control, e.g. part of a promoter sequence. However, regulatory elements alone are typically not sufficient to initiate transcription, but require a promoter to this end. 'Liver-specific expression', as used in the application, refers to the preferential or predominant expression of a (trans)gene (as RNA and/or polypeptide) in the liver as compared to other tissues. According to particular embodiments, at least 50% of the (trans)gene expression occurs within the liver. According to more particular embodiments, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100% of the (trans)gene expression occurs within the liver. According to a particular embodiment, liver-specific expression entails that there is no 'leakage' of expressed gene product to other organs, such as spleen, muscle, heart and/or lung. The same applies mutatis mutandis for hepatocyte-specific expression, which may be considered as a particular form of liver-specific expression. Throughout the application, where liver-specific is mentioned in the context of expression, hepatocyte-specific expression is also explicitly envisaged. Similarly, where tissue-specific expression is used in the application, cell-type specific expression of the cell type(s) predominantly making up the tissue is also envisaged.

The term “functional fragment” as used in the application refers to fragments of the sequences disclosed herein that retain the capability of regulating liver-specific expression, i.e. they still confer tissue specificity and they are capable of regulating expression of a (trans)gene in the same way (although possibly not to the same extent) as the sequence from which they are derived. Fragments comprise at least 10 contiguous nucleotides from the sequence from which they are derived. In further particular embodiments, fragments comprise at least 15, at least 20, at least 25, at least 30, at least 35 or at least 40 contiguous nucleotides from the sequence from which they are derived. As used herein, the term “nucleic acid expression cassette” refers to nucleic acid molecules that include one or more transcriptional control elements (such as, but not limited to promoters, enhancers and/or regulatory elements, polyadenylation sequences, and introns) that direct (trans)gene expression in one or more desired cell types, tissues or organs. Typically, they will also contain the FIX transgene as defined herein.

The term “operably linked” as used herein refers to the arrangement of various nucleic acid molecule elements relative to each such that the elements are functionally connected and are able to interact with each other. Such elements may include, without limitation, a

promoter, an enhancer and/or a regulatory element, a polyadenylation sequence, one or more introns and/or exons, and a coding sequence of a gene of interest to be expressed (i.e., the transgene). The nucleic acid sequence elements, when properly oriented or operably linked, act together to modulate the activity of one another, and ultimately may affect the level of expression of the transgene. By modulate is meant increasing, decreasing, or maintaining the level of activity of a particular element. The position of each element relative to other elements may be expressed in terms of the 5' terminus and the 3' terminus of each element, and the distance between any particular elements may be referenced by the number of intervening nucleotides, or base pairs, between the elements.

As used in the application, the term “promoter” refers to nucleic acid sequences that regulate, either directly or indirectly, the transcription of corresponding nucleic acid coding sequences to which they are operably linked (e.g. a transgene or endogenous gene). A promoter may function alone to regulate transcription or may act in concert with one or more other regulatory sequences (e.g. enhancers or silencers). In the context of the present application, a promoter is typically operably linked to regulatory elements to regulate transcription of a transgene.

When a regulatory element as described herein is operably linked to both a promoter and a transgene, the regulatory element can (1) confer a significant degree of liver specific expression in vivo (and/or in hepatocytes/ hepatic cell lines in vitro) of the transgene, and/or (2) can increase the level of expression of the transgene in the liver (and/or in hepatocytes/hepatocyte cell lines in vitro). A 'minimal promoter' as used herein is part of a full- size promoter still capable of driving expression, but lacking at least part of the sequence that contributes to regulating (e.g. tissue-specific) expression. This definition covers both promoters from which (tissue-specific) regulatory elements have been deleted- that are capable of driving expression of a gene but have lost their ability to express that gene in a tissue-specific fashion and promoters from which (tissue-specific) regulatory elements have been deleted that are capable of driving (possibly decreased) expression of a gene but have not necessarily lost their ability to express that gene in a tissue-specific fashion. Minimal promoters have been extensively documented in the art, a non-limiting list of minimal promoters is provided in the specification.

Typically, the nucleic acid expression cassette in the expression vector according to the invention comprises a plasmid origin, a promotor and or enhancer, a (trans)gene, a transcription terminator, and a selection gene. In a typical embodiment of the present

invention, said nucleic acid expression cassette in the expression vector comprises the following elements (cf. Figure 1):

- an plasmid origin such as the f1 origin,
- an Inverted Terminal Repeat sequence (ITR), sometimes mutated,
- an enhancer, preferably the Serpin enhancer,
- a promoter, preferably the mTTR promoter,
- an intron sequence, preferably the MVM intron,
- a (trans)gene, preferably the FIX encoding gene, or its Padua mutant form,
- a trasncription terminator, preferably a polyadenylation signal such as the bGHpA,
- an Inverted Terminal Repeat sequence (ITR),
- an selection gene (e.g. an antibiotic resistance gene such as an ampicilin resistance gene), and
- a plasmid origin such as the pBR322 origin.

The combination of said elements results in an unexpectedly high expression level of FIX and in particular of the Padua mutant thereof in the liver of subjects. Preferably, the vector is an adeno-associated virus-derived vector, in combination with the Padua-mutant FIX gene.

The term “transgene” or “(trans)gene” as used herein refers to particular nucleic acid sequences encoding a polypeptide or a portion of a polypeptide to be expressed in a cell into which the nucleic acid sequence is inserted. However, it is also possible that transgenes are expressed as RNA, typically to lower the amount of a particular polypeptide in a cell into which the nucleic acid sequence is inserted. These RNA molecules include but are not limited to molecules that exert their function through RNA interference (shRNA, RNAi), micro-RNA regulation (miR), catalytic RNA, antisense RNA, RNA aptamers, etc. How the nucleic acid sequence is introduced into a cell is not essential to the invention, it may for instance be through integration in the genome or as an episomal plasmid. Of note, expression of the transgene may be restricted to a subset of the cells into which the nucleic acid sequence is inserted. The term 'transgene' is meant to include (1) a nucleic acid sequence that is not naturally found in the cell (i.e., a heterologous nucleic acid sequence); (2) a nucleic acid sequence that is a mutant form of a nucleic acid sequence naturally found in the cell into which it has been introduced ; (3) a nucleic acid sequence that serves to add additional copies of the same (i.e., homologous)

or a similar nucleic acid sequence naturally occurring in the cell into which it has been introduced ; or (4) a silent naturally occurring or homologous nucleic acid sequence whose expression is induced in the cell into which it has been introduced. By 'mutant form' is meant a nucleic acid sequence that contains one or more nucleotides that are different from the wild-type or naturally occurring sequence, i.e., the mutant nucleic acid sequence contains one or more nucleotide substitutions, deletions, and/or insertions. In some cases, the transgene may also include a sequence encoding a leader peptide or signal sequence such that the transgene product will be secreted from the cell. The term 'vector' as used in the application refers to nucleic acid molecules, usually double- stranded DNA, which may have inserted into it another nucleic acid molecule (the insert nucleic acid molecule) such as, but not limited to, a cDNA molecule. The vector is used to transport the insert nucleic acid molecule into a suitable host cell. A vector may contain the necessary elements that permit transcribing the insert nucleic acid molecule, and, optionally, translating the transcript into a polypeptide. The insert nucleic acid molecule may be derived from the host cell, or may be derived from a different cell or organism. Once in the host cell, the vector can replicate independently of, or coincidental with, the host chromosomal DNA, and several copies of the vector and its inserted nucleic acid molecule may be generated.

The term “vector” may thus also be defined as a gene delivery vehicle that facilitates gene transfer into a target cell. This definition includes both non-viral and viral vectors. Non-viral vectors include but are not limited to cationic lipids, liposomes, nanoparticles, PEG, PEI, etc. Viral vectors are derived from viruses including but not limited to: retrovirus, lentivirus, adeno- associated virus, adenovirus, herpesvirus, hepatitis virus or the like. Typically, but not necessarily, viral vectors are replication-deficient as they have lost the ability to propagate in a given cell since viral genes essential for replication have been eliminated from the viral vector. However, some viral vectors can also be adapted to replicate specifically in a given cell, such as e.g. a cancer cell, and are typiclly used to trigger the (cancer) cell-specific (onco)lysis.

Preferred vectors are derived from adeno-associated virus, adenovirus, retroviruses and Antiviruses. Alternatively, gene delivery systems can be used to combine viral and non-viral components, such as nanoparticles or virosomes (Yamada et al., 2003). Retroviruses and Antiviruses are RNA viruses that have the ability to insert their genes into host cell chromosomes after infection. Retroviral and lentiviral vectors have been developed that lack the genes encoding viral proteins, but retain the ability to infect cells and insert their genes into the chromosomes of the target cell (Miller, 1990; Naldini et al., 1996, VandenDriessche et al., 1999). The difference between a lentiviral and a classical

Moloney-murine leukemia-virus (MLV) based retroviral vector is that lentiviral vectors can transduce both dividing and non-dividing cells whereas MLV-based retroviral vectors can only transduce dividing cells.

Adenoviral vectors are designed to be administered directly to a living subject. Unlike retroviral vectors, most of the adenoviral vector genomes do not integrate into the chromosome of the host cell. Instead, genes introduced into cells using adenoviral vectors are maintained in the nucleus as an extrachromosomal element (episome) that persists for an extended period of time. Adenoviral vectors will transduce dividing and nondividing cells in many different tissues in vivo including airway epithelial cells, endothelial cells, hepatocytes and various tumors (Trapnell, 1993; Chuah et al., 2003). Another viral vector is derived from the herpes simplex virus, a large, double-stranded DNA virus. Recombinant forms of the vaccinia virus, another dsDNA virus, can accommodate large inserts and are generated by homologous recombination.

Adeno-associated virus (AAV) is a small ssDNA virus which infects humans and some other primate species, not known to cause disease and consequently causing only a very mild immune response. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. These features make AAV a very attractive candidate for creating viral vectors for gene therapy, although the cloning capacity of the vector is relatively limited. In a preferred embodiment of the invention, the vector used is therefore derived from adeno associated virus.

In a further particular aspect, the nucleic acid regulatory elements, the nucleic acid expression cassettes and the vectors described herein can be used in gene therapy. Gene therapy protocols, intended to achieve therapeutic gene product expression in target cells, in vitro, but also particularly *in vivo*, have been extensively described in the art. These include, but are not limited to, intramuscular injection of plasmid DNA (naked or in liposomes), interstitial injection, instillation in airways, application to endothelium, intra-hepatic parenchyme, and intravenous or intra-arterial administration (e.g. intra-hepatic artery, intra-hepatic vein). Various devices have been developed for enhancing the availability of DNA to the target cell. A simple approach is to contact the target cell physically with catheters or implantable materials containing DNA. Another approach is to utilize needle-free, jet injection devices which project a column of liquid directly into the target tissue under high pressure. These delivery paradigms can also be used to deliver viral vectors. Another approach to targeted gene delivery is the use of molecular conjugates, which consist of protein or synthetic ligands to which a nucleic acid-or DNA-

binding agent has been attached for the specific targeting of nucleic acids to cells (Cristiano et al., 1993).

According to a particular embodiment, the use of the nucleic acid regulatory elements, nucleic acid expression cassettes or vectors as described herein is envisaged for gene therapy of liver cells. According to a further particular embodiment, the use of the regulatory elements, expression cassettes or vectors is for gene therapy *in vivo*. According to yet a further particular embodiment, the use is for a method of gene therapy to treat hemophilia, in particular to treat hemophilia B.

Gene transfer into mammalian hepatocytes has been performed using both *ex vivo* and *in vivo* procedures. The *ex vivo* approach requires harvesting of the liver cells, *in vitro* transduction with long-term expression vectors, and reintroduction of the transduced hepatocytes into the portal circulation (Kay et al., 1992; Chowdhury et al., 1991). *In vivo* targeting has been done by injecting DNA or viral vectors into the liver parenchyma, hepatic artery, or portal vein, as well as via transcriptional targeting (Kuriyama et al., 1991; Kistner et al., 1996). Recent methods also include intraportal delivery of naked DNA (Budker et al., 1996) and hydrodynamic tail vein transfection (Liu et al., 1999; Zhang et al., 1999).

According to a further aspect, methods for expressing a protein in liver cells are provided, comprising the steps of introducing in liver cells the nucleic acid expression cassette (or a vector) as described herein and expressing the transgene protein product in the liver cells. These methods may be performed both in vitro and in vivo.

Methods of gene therapy for a subject in need thereof are also provided, comprising the steps of introducing in the liver of the subject a nucleic acid expression cassette containing a transgene encoding a therapeutic protein, and expressing a therapeutic amount of the therapeutic protein in the liver. According to a further embodiment, the method comprises the steps of introducing in the liver of the subject a vector comprising the nucleic acid expression cassette containing a transgene encoding a therapeutic protein, and expressing a therapeutic amount of the therapeutic protein in the liver. According to a very specific embodiment, the therapeutic protein encoded by the transgene in the nucleic acid expression cassette is factor IX, and the method is a method for treating hemophilia B. By expressing factor IX in the liver via gene therapy, hemophilia B can be treated (Snyder et al., 1999).

Except when noted differently, the terms "subject" or "patient" are used interchangeably and refer to animals, preferably vertebrates, more preferably mammals, and specifically

includes human patients and non-human mammals. "mammalian" subjects include, but are not limited to, humans, domestic animals, commercial animals, farm animals, zoo animals, sport animals, pet and experimental animals such as dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, cows; primates such as apes, monkeys, orang-utans, and chimpanzees; canids such as dogs and wolves; felids such as cats, lions, and tigers; equids such as horses, donkeys, and zebras; food animals such as cows, pigs, and sheep; ungulates such as deer and giraffes; rodents such as mice, rats, hamsters and guinea pigs; and so on. Accordingly, "subject" or "patient" as used herein means any mammalian patient or subject to which the compositions of the invention can be administered. Preferred patients or subjects are human subjects.

As used herein, the terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the development or spread of proliferative disease, e.g., cancer. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilised (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

As used herein, a phrase such as "a subject in need of treatment" includes subjects, such as mammalian subjects, that would benefit from treatment of a given condition, such as, hemophilia B. Such subjects will typically include, without limitation, those that have been diagnosed with the condition, those prone to have or develop the said condition and/or those in whom the condition is to be prevented.

The term "therapeutically effective amount" refers to an amount of a compound or pharmaceutical composition of the invention effective to treat a disease or disorder in a subject, i.e., to obtain a desired local or systemic effect and performance. In a particular embodiment, the term implies that levels of factor IX in plasma equal to or higher than the therapeutic threshold concentration of 10mU/ml (milli-units per milliliter) plasma, 50mU/ml plasma, 100mU/ml plasma, 150mU/ml or 200mU/ml plasma in a subject can be obtained by transduction or transfection of the vector according to any one the embodiments described herein into a subject. Due to the very high efficiency of the vector and nucleic acid expression cassette of the present invention, this high physiological level of factor IX in the subject can be obtained even by administering relatively low doses of vector. The term thus refers to the quantity of compound or pharmaceutical composition that elicits the

biological or medicinal response in a tissue, system, animal, or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the hemophilia being treated. In particular, these terms refer to the quantity of compound or pharmaceutical composition according to the invention which is necessary to prevent, cure, ameliorate, or at least minimize the clinical impairment, symptoms, or complications associated with hemophilia in either a single or multiple dose.

In particular, the transduction of the vector according to any one of the embodiments defined herein into the subject can be done at a dose lower than $2x10^{11}$ vg/kg (viral genomes per kilogram) to obtain a physiological factor IX level of 10mU/ml plasma or of 50mU/ml plasma in a subject.

Alternatively, if a level of factor IX of 100 mU/ml plasma needs to be reached in a subject, the transduction of the vector according to any one of the embodiments defined herein into the subject can be done at a dose lower than or equal to $6x10^{11}$ vg/kg.

Further, if a level of factor IX equal to 150 mU/ml plasma or higher needs to be reached, the transduction of the vector according to any one of the embodiments defined herein into the subject can be done at a dose lower than or equal than $2x10^{12}$ vg/kg. In a preferred embodiment, a level of factor IX of 200 mU/ml plasma or higher can be reached in a subject, when the transduction of the vector according to any one of the embodiments defined herein into the subject is done at a dose lower than or equal to $2x10^{12}$ vg/kg.

For hemophilia therapy, efficacy of the treatment can, for example, be measured by assessing the hemophilia-caused bleeding in the subject. In vitro tests such as, but not limited to the in vitro actived partial thromboplastin time assay (APPT), test factor IX chromogenic activity assays, blood clotting times, factor IX-specific ELISAs are also available. Any other tests for assessing the efficacy of the treatment known in the art can of course be used.

The compound or the pharmaceutical composition of the invention may be used alone or in combination with any of the know hemophilia therapies, such as the administration of recombinant or purified clotting factors. The compound or the pharmaceutical composition of the invention can thus be administered alone or in combination with one or more active compounds. The latter can be administered before, after or simultaneously with the administration of the said agent(s).

A further object of the invention are pharmaceutical preparations which comprise a therapeutically effective amount of the expression vector of the invention as defined herein, and a pharmaceutically acceptable carrier, i.e., one or more pharmaceutically acceptable carrier substances and/or additives, e.g., buffers, carriers, excipients, stabilisers, etc. The term "pharmaceutically acceptable" as used herein is consistent with the art and means compatible with the other ingredients of a pharmaceutical composition and not deleterious to the recipient thereof. The term "pharmaceutically acceptable salts" as used herein means an inorganic acid addition salt such as hydrochloride, sulfate, and phosphate, or an organic acid addition salt such as acetate, maleate, fumarate, tartrate, and citrate. Examples of pharmaceutically acceptable metal salts are alkali metal salts such as sodium salt and potassium salt, alkaline earth metal salts such as magnesium salt and calcium salt, aluminum salt, and zinc salt. Examples of pharmaceutically acceptable ammonium salts are ammonium salt and tetramethylammonium salt. Examples of pharmaceutically acceptable organic amine addition salts are salts with morpholine and piperidine. Examples of pharmaceutically acceptable amino acid addition salts are salts with lysine, glycine, and phenylalanine. The pharmaceutical composition according to the invention can be administered orally, for example in the form of pills, tablets, lacquered tablets, sugar-coated tablets, granules, hard and soft gelatin capsules, aqueous, alcoholic or oily solutions, syrups, emulsions or suspensions, or rectally, for example in the form of suppositories. Administration can also be carried out parenterally, for example subcutaneously, intramuscularly or intravenously in the form of solutions for injection or infusion. Other suitable administration forms are, for example, percutaneous or topical administration, for example in the form of ointments, tinctures, sprays or transdermal therapeutic systems, or the inhalative administration in the form of nasal sprays or aerosol mixtures, or, for example, microcapsules, implants or rods. The pharmaceutical composition can be prepared in a manner known per se to one of skill in the art. For this purpose, the expression vector according to the invention as defined herein, one or more solid or liquid pharmaceutically acceptable excipients and, if desired, in combination with other pharmaceutical active compounds, are brought into a suitable administration form or dosage form which can then be used as a pharmaceutical in human medicine or veterinary medicine.

According to another aspect, a pharmaceutical composition is provided comprising a nucleic acid expression cassette containing a transgene encoding a therapeutic protein, and a pharmaceutically acceptable carrier. According to another embodiment, the pharmaceutical composition comprises a vector containing the nucleic acid expression

cassette containing a transgene encoding a therapeutic protein, and a pharmaceutically acceptable carrier. According to further particular embodiments, the transgene encodes factor IX and the pharmaceutical composition is for treating hemophilia B.

The use of the nucleic acid expression cassette, its regulatory elements and the vector components as disclosed herein for the manufacture of these pharmaceutical compositions for use in treating hemophilia B is also envisaged.

It is to be understood that although particular embodiments, specific constructions and configurations, as well as materials, have been discussed herein for methods and applications according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention.

The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

EXAMPLES

Example 1: In vivo validation of liver-specific regulatory enhancer sequences expressing hyper-active FIX via AAV vector gene delivery.

Materials and methods

Vector construction

AAV-based vectors were constructed that express either the codon-optimized factor IX or the codon-optimized factor IX with the Padua R338L mutation from the TTRmin promoter operably linked to the serpin A regulatory sequence. The Serpin A regulatory sequence has been identified and described under patent application WO2009/130208.

An intron and poly-A sequence were also provided. The full sequence of the construct containing the codon-optimized factor IX is given in SEQ ID No.1 (Fig. 1B) and the construct containing the codon-optimized factor IX with the Padua R338L mutation in SEQ ID No.2 (Fig. 1C). The vectors were constructed by conventional cloning and DNA synthesis. A schematic overview of the AAV vector containing the codon-optimized huFIX is shown in Fig. 1A. The vector with the Padua R338L is identical except for the specific R338L mutation that results in FIX hyper-activity.

Cell lines and culture conditions

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine (Gln), 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Merelbeke, Belgium).

AAV vector production

As an example, the AAV serotype 9 viral vector was chosen to package the construct, known to be a promising vector for gene therapy (Vandendriessche et al. 2007). AAV vectors expressing human FIX were produced at high-titer by calcium phosphate transfection according to the manufacturer's instruction (Calcium phosphate transfection kit, Invitrogen) of 293 cells with AAV2-vector DNA (26 µg/10 cm dish), an adenoviral helper plasmid (52 µg/10 cm dish)and AAV helper plasmids expressing Rep2 and Cap9 (26 µg/10 cm dish) for production of AAV9 serotypes, as described in Gao et al. (2002), Mingozzi et al. (2003) and Gehrke (2003).

Two days post-transfection, cells were lysed by successive freeze-thaw cycles and sonication. Lysates were treated with benzonase (Merck) and deoxycholate (Sigma-Aldrich) and subsequently subjected to three successive rounds of cesium chloride density ultracentrifugation. The fractions containing the AAV particles were concentrated using an Amicon filter (Millipore) and washed with PBS 1mM MgCl2. Vector genome titers were determined by quantitative polymerase chain reaction (qPCR) using TaqMan® probes and primers specific for the polyadenylation signal (forward primer: 5'GCCTTCTAGTTGCCAGCCAT (SEQ ID No.3), probe: 5'TGTTTGCCCCTCCCCCGTGC (SEQ ID No.4), reverse primer : 5'GGCACCTTCCAGGGTCAAG (SEQ ID No.5)).

Animal studies

Animal procedures were approved by the animal Ethical Commission of the VUB. Animals were housed under Biosafety Level II conditions. Mice were injected with the AAV9 vectors as described in Vandendriessche et al. (2007). Briefly, 10^9 vg, $5x10^9$ vg, $2x10^{10}$ vg (vector genomes = vg) were injected (i.v.) into the tail vein of adult hemophilia B mice (3 mice/group). Blood was collected by retro-orbital bleeds under general anesthesia. Human FIX expression was determined in citrated mouse plasma using a chromogenic FIX

activity assay, according to the manufacturer (Hyphen Biomed, Neuville-sur-Oise, France) using serially diluted hFIX standards for calibration.

Results

AAV vectors expressing either the human codon-optimized FIX cDNA (designated as AAV-co-hFIX in Fig. 2) or the human codon-optimized FIX-R338L cDNA (designated as AAV-co-padua-hFIX in Fig. 2) from a chimeric liver-specific promoter (TTRmin/SerpENH) were injected into FIX-deficient hemophilic mice that suffered from hemophilia B. A dose-response was observed and the AAV vector expressing the codon-optimized FIX-R338L yielded significantly higher FIX activity than the codon-optimzed FIX control without the hyper-activating mutation. Remarkably, the AAV9-TTRminSerp-FIXIA vector reached therapeutic FIX levels at a relatively low dose (>50% of normal FIX levels at $1x10^9$ gc/mouse, >250% of normal FIX levels at $5x10^9$ gc/mouse and >700% of normal FIX levels at $2x10^{10}$ gc/mouse after 5 days), which underscores its potency. These levels typically increase more than 2-fold to stable levels in subsequent weeks, reaching respectively approximately >100%, >500% and >1400% FIX at doses of respectively, $1x10^9$ gc/mouse, $5x10^9$ gc/mouse and $2x10^{10}$ gc/mouse. These levels were still increasing in subsequent days following vector injection. Hence, this new vector produced unprecedented, high levels of human IX and can be used at much lower doses than described in the art to cure hemophilia B in a clinically relevant animal model.

References

ANNONI A, BROWN BD, CANTORE A, SERGI LS, NALDINI L, and RONCAROLO MG. (2009). In vivo delivery of a microRNA-regulated transgene induces antigen-specific regulatory T cells and promotes immunologic tolerance. Blood 114, 5152–5161

ARRUDA VR, STEDMAN HH, HAURIGOT V, and BUCHLIS G. (2010). Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B. Blood 115, 4678-88.

AXELROD JH, READ MS, BRINKHOUS KM, and VERMA IM. (1990). Phenotypic correction of factor IX deficiency in skin fibroblasts of hemophilic dogs. Proc Natl Acad Sci USA; 87, 5173-7.

BROWN BD, SHI CX, POWELL S HURLBUT D, GRAHAM FL, and LILLICRAP D. (2004). Helper-dependent adenoviral vectors mediate therapeutic factor VIII expression for

several months with minimal accompanying toxicity in a canine model of severe hemophilia A. Blood 103, 804-10.

BROWN BD, CANTORE A, ANNONI A, SERGI LS, LOMBARDO A, DELLA VALLE P, D'ANGELO A, and NALDINI L. (2007). A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. Blood 110, 4144-52.

Brunetti-Pierri N, Grove NC, Zuo Y, Edwards R, Palmer D, Cerullo V, Teruya J, Ng P.

Bioengineered factor IX molecules with increased catalytic activity improve the therapeutic index of gene therapy vectors for hemophilia B. Hum Gene Ther. 2009 May;20(5):479-85.

BUCHLIS G, PODSAKOFF GM, RADU A, HAWK SM, FLAKE AW, MINGOZZI F, and HIGH KA. (2012). Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. Blood 119, 3038-41.

BUDKER V, ZHANG G, KNECHTLE S, WOLFF JA. Naked DNA delivered intraportally expresses efficiently in hepatocytes. (1996) Gene Ther. Jul;3(7):593-8.

CANTORE A, NAIR N, DELLA VALLE P, DI MATTEO M, MÀTRAI J, SANVITO F, BROMBIN C, DI SERIO C, D'ANGELO A, CHUAH M, NALDINI L, VANDENDRIESSCHE T. Hyper-functional coagulation factor IX improves the efficacy of gene therapy in hemophilic mice. Blood. 2012. Oct 4.

CHANG, J., JIN, J., LOLLAR, P., BODE, W., BRANDSTETTER, H., HAMAGUCHI, N., STRAIGHT, D. L. &STAFFORD, D. W. (1998). Changing residue 338 in human factor IX from arginine to alanine causes an increase in catalytic activity. J Biol Chem 273(20): 12089-12094.

CHOWDHURY JR, GROSSMAN M, GUPTA S, CHOWDHURY NR, BAKER JR JR, WILSON JM. (1991) Long-term improvement of hypercholesterolemia after ex vivo gene therapy in LDLR-deficient rabbits. Science. Dec 20;254(5039):1802-5.

CHUAH MK, SCHIEDNER G, THORREZ L, BROWN B, JOHNSTON M, GILLIJNS V, HERTEL S, VAN ROOIJEN N, LILLICRAP D, COLLEN D, VANDENDRIESSCHE T, and KOCHANEK S. (2003). Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. Blood 101, 1734-43.

Chuah MK, Nair N, VandenDriessche T. Recent progress in gene therapy for hemophilia.

Hum Gene Ther. 2012a Jun;23(6):557-65.

Chuah MK, Nair N, VandenDriessche T. Recent progress in gene therapy for hemophilia. Hum Gene Ther. 2012b Jun;23(6):557-65.

Chuah MK, VandenDriessche T. Platelet-directed gene therapy overcomes inhibitory antibodies to factor VIII. J Thromb Haemost. 2012c Aug;10(8):1566-9

DONSANTE A, MILLER DG, LI Y, VOGLER C, BRUNT EM, RUSSELL DW, and SANDS MS. (2007). AAV vector integration sites in mouse hepatocellular carcinoma. Science 317, 477.

DOBRZYNSKI E, FITZGERALD JC, CAO O, MINGOZZI F, WANG L, and HERZOG RW (2006) Prevention of cytotoxic T lymphocyte responses to factor IX-expressing hepatocytes by gene transfer-induced regulatory T cells. Proc Natl Acad Sci USA 103, 4592 – 4597.

EHRHARDT A, and KAY MA. (2002). A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. Blood 99, 3923-30.

FIELDS PA, ARRUDA VR, ARMSTRONG E, KIRK CHU, MINGOZZI, F. HAGSTROM, J., HERZOG R, HIGH KA. (2001). Risk and prevention of anti-factor IX formation in AAV-mediated gene transfer in the context of a large deletion of F9. Mol. Ther. 4, 201–210.

FOLLENZI A, BATTAGLIA M, LOMBARDO A, ANNONI A, RONCAROLO MG, and NALDINI L. (2004). Targeting lentiviral vector expression to hepatocytes limits transgene-specific immune response and establishes long-term expression of human antihemophilic factor IX in mice. Blood 103, 3700-9.

GAO GP, ALVIRA MR, WANG L, JOHNSTON J, WILSON JM. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci USA 99, 11854 -9.

GAO G, VANDENBERGH LH, ALVIRA MR LU Y, CALCEDO R, ZHOU X, and WILSON JM. (2004). Clades of Adeno- associated viruses are widely disseminated in human tissues. J. Viro l78, 6381 – 6388.

HERZOG RW, YANG EY, COUTO LB, HAGSTROM JN, ELWELL D, FIELDS PA, BURTON M, BELLINGER DA, READ MS, BRINKHOUS KM, PODSAKOFF GM, NICHOLS TC, KURTZMAN GJ, and HIGH KA. (1999). Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. Nat Med. 5, 56–63.

HERZOG RW, MOUNT JD, ARRUDA VR, HIGH KA, and LOTHROP CD Jr. (2001). Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. Mol Ther. 4, 192-200.

HERZOG RW, HAGSTROM JN, KUNG SH, TAI SJ, WILSON JM, FISHER KJ, and HIGH KA. (1997) Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proc Natl Acad Sci USA. 94, 5804 – 5809.

HERZOG RW, FIELDS PA, ARRUDA VR, BRUBAKER JO, ARMSTRONG E, MCCLINTOCK D, BELLINGER DA, COUTO LB, NICHOLS TC, HIGH KA (2002) Influence of vector dose on factor IX-specific T and B cell responses in muscle- directed gene therapy. Hum Gene Ther 13, 1281–1291.

HIGH KA. (2001). Gene Transfer as an approach to treating Hemophilia. Circ Res. 88, 137-144.

HIGH KA. (2011) Gene therapy for hemophilia: a long and winding road. J Thromb Haemost. 9 Suppl. 1: 2-11.

BAINBRIDGE J, SMITH AJ, BARKER S, et al. (2008) Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis. N Engl J Med. 358, 2231-2239.

JIANG H, LILLICRAP D, and PATARROYO-WHITE S. (2006). Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. Blood. 108, 107-15.

KAO, C. Y., LIN, C. N., YU, I. S., TAO, M. H., WU, H. L., SHI, G. Y., YANG, Y. L., KAO, J. T. &LIN, S. W. (2010). FIX-Triple, a gain-of-function factor IX variant, improves haemostasis in mouse models without increased risk of thrombosis. Thromb Haemost 104(2): 355-365.

KAY MA, BALEY P, ROTHENBERG S, LELAND F, FLEMING L, PONDER KP, LIU T, FINEGOLD M, DARLINGTON G, POKORNY W, WOO SLC. (1992) Expression of human alpha 1 -antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc Natl Acad Sci U S A. Jan 1 ;89(1):89-93.

KAY MA, MANNO CS, RAGNI MV, COUTO LB, MCCLELLAND A, GLADER B, CHEW AJ, TAI SJ, HERZOG RW, ARRUDA V, JOHNSON F, SCALLAN C, SKARSGARD E, FLAKE AW, and HIGH KA. (2000). Evidence for gene transfer and expression of factor IX in hemophilia B patients treated with an AAV vector. Nat Genet. 24, 257- 61.

KISTNER A, GOSSEN M, ZIMMERMANN F, JERECIC J, ULLMER C, LYBBERT H, BUJARD H. (1996) Doxycycline- mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc Natl Acad Sci U S A. Oct 1;93(20): 10933-8.

KREN BT, UNGER GM, SJEKLOCHA L, TROSSEN AA, KORMAN V, DIETHELEM-OKITA BM, REDING MT, and STEER CJ. (2009). Nanocapsule-delivered Sleeping Beauty mediates therapeutic Factor VIII expression in liver sinusoidal endothelial cells of hemophilia A mice. J Clin Invest. 19, 2086-99.

KURIYAMA S, YOSHIKAWA M, ISHIZAKA S, TSUJII T, LKENAKA K, KAGAWA T, MORITA N, MIKOSHIBA K. (1991) A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector. Cell Struct Funct. Dec;16(6):503-10.

LI H, MALANI N, HAMILTON SR, SCHLACHTERMAN A, BUSSADORI G, EDMONSON SE, SHAH R, ARRUDA VR, MINGOZZI F, WRIGHT JF, BUSHMAN FD, and HIGH KA. (2011). Assessing the potential for AAV vector genotoxicity in a murine model. Blood. 117, 3311-9.

LIN, C. N., KAO, C. Y., MIAO, C. H., HAMAGUCHI, N., WU, H. L., SHI, G. Y., LIU, Y. L., HIGH, K. A. &LIN, S. W. (2010). Generation of a novel factor IX with augmented clotting activities in vitro and in vivo. J Thromb Haemost 8(8): 1773-1783.

LIU F, SONG Y, LIU D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. Jul;6(7):1258-66.

MANNO CS, PIERCE GF, and ARRUDA VR. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med. 12, 342-7.

MÁTÉS L, CHUAH MK, BELAY E, JERCHOW B, MANOJ N, ACOSTA-SANCHEZ A, GRZELA DP, SCHMITT A, BECKER K, MATRAI J, MA L, SAMARA-KUKO E, GYSEMANS C, PRYPUTNIEWICZ D, MISKEY C, FLETCHER B, VANDENDRIESSCHE T,, IVICS Z, and IZSVAK Z. (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet. 41, 753-61.

MÁTRAI J, CHUAH MK, and VANDENDRIESSCHE T. (2010a). Pre clinical and clinical progress in hemophilia gene therapy. Curr Opin Hematol. 17, 387-92.

MÁTRAI J, CHUAH MK, and VANDENDRIESSCHE T. (2010b). Recent advances in lentiviral vector development and applications. Mol Ther. 18, 477-90.

MÁTRAI J, CANTORE A, BARTHOLOMAE CC, ANNONI A, WANG W, ACOSTA-SANCHEZ A, SAMARA-KUKO E, DE WAELE L, MA L, GENOVESE P, DAMO M, ARENS A, GOUDY K, NICHOLS TC, VON KALLE C, L CHUAH MK, RONCAROLO MG, SCHMIDT M, VANDENDRIESSCHE T, and NALDINI L. (2011). Hepatocyte-targeted expression by integrase-defective lentiviral vectors induces antigen-specific tolerance in mice with low genotoxic risk. Hepatology 53, 1696-707.

MATSUI H, SHIBATA M, BROWN B, LABELLE A, HEGADRON C, ANDREWS C, CHUAH M, VANDENDRIESSCHE T, MIAO CH, HOUGH C, and LILLICRAP D. (2009). A murine model for induction of long-term immunologic tolerance to factor VIII does not require persistent detectable levels of plasma factor VIII and involves contributions from Foxp3+ T regulatory cells. Blood. 114, 677-85.

MATSUI H, HEGADORN C, OZELO M, BURNETT E, TUTTLE A, LABELLE A, McCARY PB Jr., NALDINI L, BROWN B, HOUGH C, and LILLICRAP D. (2011). A microRNA-regulated and GP64-pseudotyped lentiviral vector mediates stable expression of FVIII in a murine model of Hemophilia A. Mol Ther. 19, 723-30.

McCARTY DM, MONAHAN PE, and SAMULSKI RJ. (2001). Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. Gene Ther. 8, 1248-54.

McCARTY DM, FU H, MONAHAN PE, TOULSON CE, NAIK P, and SAMULSKI RJ. (2003). Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. Gene Ther. 10, 2112-8.

MIAO CH, OHASHI K, PATIJN GA, MEUSE L, YE X, THOMPSON AR, and KAY MA .(2000). Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro. Mol Ther. 1, 522-32.

MILANOV, ET AL., 2012 Engineered factor IX variants bypass FVIII and correct hemophilia A phenotype in mice Blood 119:602-611.

MILLER AD. (1990) Retrovirus packaging cells. Hum Gene Ther. Spring;1 (1):5-14.

MINGOZZI F, LIU YL, DOBRZYNSKI E, KAUFHOLD A, LIU JH, WANG Y, ARRUDA VR, HIGH KA, and HERZOG RW. (2003). Induction of immune tolerance to coagulation factor IX antigen by in vivo hepatic gene transfer. J Clin Invest. 111, 1347-56.

MINGOZZI F, MAUS MV, HUI DJ, SABATINO DE, MURPHY SL, RASKO JE, RAGINI MV, MANNO CS, SOMMER J, JIANG H, PIERCE GF, ERTL HC, and HIGH KA. (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. Nat Med. 13, 419-22.

MOUNT JD, HERZOG RW, TILLSON DM, GOODMAN SA, ROBINSON N, MCCLELAND ML, BELLINGER D, NICHOLS TC, ARRUDA VR, LOTHROP CD JR, and HIGH KA. (2002). Sustained phenotypic correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. Blood 99, 2670-6.

NALDINI L, BLOMER U, GALLAY P, ORY D, MULLIGAN R, GAGE FH, VERMA IM, TRONO D. (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. Apr 12;272(5259):263-7.

NATHWANI AC, DAVIDOFF AM, HANAWA H, YUNYU HU, HOFFER FA, NIKANOROV A, SLAUGHTER C, NG CYC, ZHOU J, LOZIER J, MANDRELL TD, VANIN EF, and NIENHUIS AW. (2002). Sustained high- level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. Blood 100, 1662–1669.

NATHWANI AC, GRAY JT, NG CY, ZHOU J, SPENCE Y, WADDINGTON SN, TUDDENHAM EG, KEMBALL COOK G, McINTOSH J, BOON-SPIJKER M, MERTENS K, DAVIDOFF AM. (2006).Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. Blood 107, 2653-61.

NATHWANI AC, TUDDENHAM EG, RANGARAJAN S, ROSALES C, MCINTOSH J, LINCH DC, CHOWDARY P, RIDDELL A, PIE AJ, HARRINGTON C, O'BEIRNE J, SMITH K, PASI J, GLADER B, RUSTAGI P, NG CY, KAY MA, ZHOU J, SPENCE Y, MORTON CL, ALLAY J, COLEMAN J, SLEEP S, CUNNINGHAM JM, SRIVASTAVA D, BASNER-TSCHAKARJAN E, MINGOZZI F, HIGH KA, GRAY JT, REISS UM, NIENHUIS AW, and DAVIDOFF AM. (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med. 365, 2357-2365.

OHLFEST JR, FRANDSEN JL, FRITZ S, LOBITZ PD, PERKINSON SG, CLARK KJ, NELSESTUEN G, KEY NS, MCLVOR RS, HACKETT PB, and LARGAESPADA DA. (2004). Phenotypic correction and long-term expression of factor VIII in hemophilic mice by immunotolerization and nonviral gene transfer using the Sleeping Beauty transposon system. Blood 105, 2691-8.

SCHUETTRUMPF, J., HERZOG, R. W., SCHLACHTERMAN, A., KAUFHOLD, A., STAFFORD, D. W. &ARRUDA, V. R. (2005). Factor IX variants improve gene therapy efficacy for hemophilia B. Blood 105(6): 2316-2323.

SIMIONI, P., TORMENE, D., TOGNIN, G., GAVASSO, S., BULATO, C., IACOBELLI, N. P., FINN, J. D., SPIEZIA, L., RADU, C. &ARRUDA, V. R. (2009). X-linked thrombophilia with a mutant factor IX (factor IX Padua). N Engl J Med 361(17): 1671-1675.

SNYDER RO, MIAO CH, PATIJN GA, SPRATT SK, DANOS O, NAGY D, GOWN AM, WINTHER B, MEUSE L, COHEN LK, THOMPSON AR, and KAY MA. (1997). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet. 16, 270 – 276.

SNYDER RO, MIAO C, MEUSE L, TUBB J, DONAHUE BA, HUI-FENG LIN, STAFFORD DW, PATEL S, THOMPSON AR, NICHOLS T, READ MS , BELLINGER DA, BRINKHOUS KM, and KAY MA. (1999). Correction of hemophilia B in canine and murine models using recombinant adeno- associated viral vectors. Nat Med. 5, 64–70.

TRAPNELL BC. (1993) Adenoviral vectors for gene transfer. Adv. Drug Del. Rev. 12: 185-199.

VANDENBERGHE LH, WANG L, SOMANATHAN S, ZHI Y, FIGUEREDO J, CALCEDO R, SANMIGUEL J, DESAI RA, CHEN CS, JOHNSTON J, GRANT RL, GAO G, and WILSON JM. (2006). Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. Nat Med. 12, 967-71.

VANDENDRIESSCHE T, VANSLEMBROUCK V, GOOVAERTS I, ZWINNEN H, VANDERHAEGHEN ML, COLLEN D, and CHUAH MK. (1999). Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor VIII-deficient mice. Proc Natl Acad Sci USA. 96, 10379-84.

VANDENDRIESSCHE T, THORREZ L, NALDINI L, FOLLENZI A, MOONS L, ZWI BERNEMAN, COLLEN D, and CHUAH MK. (2002). Lentiviral vectors containing the human immunodeficiency virus type-1 central polypurine tract can efficiently transduce nondividing hepatocytes and antigen-presenting cells in vivo. Blood 100, 813-22.

VANDENDRIESSCHE T, THORREZ L, ACOSTA-SANCHEZ A, PETRUS I, WANG L, MA L, DE WAELE L, IWASAKI Y, GILLIJNS V, WILSON JM, COLLEN D, and CHUAH MK. (2007). Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for hemophilia B gene therapy. J Thromb Haemost. 5, 16-24.

VANDENDRIESSCHE T, IVICS Z, IZSVÁK Z, and CHUAH MK. (2009). Emerging potential of transposons for gene therapy and generation of induced pluripotent stem cells. Blood 114, 1461-8.

VANDENDRIESSCHE T, and CHUAH MK. (2012). Clinical progress in gene therapy: sustained partial correction of the bleeding disorder in patients suffering from severe hemophilia B. Hum Gene Ther. 23, 4-6.

WANG L, TAKABE K, BIDLINGMAIER SM, ILL CR, and VERMA IM. (1999). Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. Proc Natl Acad Sci USA 96, 3906–3910.

WANG L, NICHOLS TC, READ MS, BELLINGER DA, and VERMA IM. (2000). Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. Mol Ther. 1, 154–158.

WANG L, CAO O, SWALM B, DOBRZYNSKI E, MINGOZZI F, and HERZOG RW (2005) Major role of local immune responses in antibody formation to factor IX in AAV gene transfer. Gene Ther 12, 1453-464.

WARD NJ, BUCKLEY SM, WADDINGTON SN, VANDENDRIESSCHE T, CHUAH MK, NATHWANI AC, McLNTOSH J, TUDDENHAM EG, KINNON C, THRASHER AJ, and McVEY JH (2010) Codon optimization of human factor VIII cDNAs leads to high-level expression. Blood 117, 798-807.

WU Z, SUN J, ZHANG T, YIN C, YIN F, VAN DYKE T, SAMULSKI RJ, and MONAHAN PE. (2008). Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. Mol Ther. 16, 280-9.

XU L, GAO C, and SANDS MS. (2003). Neonatal or hepatocyte growth factor-potentiated adult gene therapy with a retroviral vector results in therapeutic levels of canine factor IX for hemophilia B. Blood 101, 3924 – 3932.

XU L, NICHOLS TC, SARKAR R, Mc CORQUODALE S, BELLINGER DA, PONDER KP. (2005). Absence of a desmopressin response after therapeutic expression of factor VIII in hemophilia A dogs with liver-directed neonatal gene therapy. Proc Natl Acad Sci USA 102, 6080–6085.

YAMADA T, IWASAKI Y, TADA H, IWABUKI H, CHUAH MK, VANDENDRIESSCHE T, FUKUDA H, KONDO A, UEDA M, SENO M, TANIZAWA K, KURODA S. (2003) Nanoparticles for the delivery of genes and drugs to human hepatocytes. Nat Biotechnol. Aug;21 (8):885-90.

YANT SR, MEUSE L, CHIU W, IVICS Z, IZSVAK Z, and KAY MA. (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. Nat Genet. 25, 35-41.

ZHANG G, BUDKER V, WOLFF JA. (1999) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. Jul 1 ;10(10):1735-7.

ZHONG L, LI B, MAH CS, GOVINDASAMY L, AGBANDJE-MCKENNA, COOPER M, HERZOG RW, ZOLOTUKHIN I, WARRINGTON JR. KH, WEIGEL-VAN AKEN K, HOBBS JA, ZOLOTUKHIN S, MUZYCZKA N, and SRIVASTAVA A (2008). Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. Proc Natl Acad Sci USA 105, 7827-32.

CLAIMS:

1. A vector containing a nucleic acid expression cassette comprising liver-specific regulatory sequences and a gene encoding for coagulation factor IX (FIX) containing a hyper-activating mutation and a transcriptional termination signal.

2. The vector according to claim 1, further comprising an intron.

3. The vector according to claim 1 or 2 wherein said hyper-activating mutation in coagulation factor IX corresponds to an R338L amino acid substitution.

4. The vector according to claim 1, 2 or 3 wherein said gene encoding for coagulation factor IX is codon-optimized.

5. The vector according to any one of claims 1 to 4 wherein said liver-specific regulatory elements are derived from the transthyrethin promoter.

6. The vector according to any one of claims 1 to 5 wherein said liver-specific regulatory elements contain sequences from the serpin promoter.

7. The vector according to any one of claims 1 to 6 wherein said transcriptional termination signal is derived from the bovine growth hormone polyadenylation signal.

8. The vector according to any one of claims 1 to 7 wherein said intron is derived from the minute virus of mice (MVM).

9. The vector according to any one of claims 1 to 8 wherein said vector is a viral vector.

10. The vector according to claim 9, wherein said vector is derived from the adeno-associated virus (AAV).

11. The vector according to claims 9 or 10 wherein said vector is a self-complementary AAV.

12. The vector according to any one of claims 1 to 8 wherein said vector is a non-viral vector.

13. The vector according to claim 12 wherein said vector is a transposon-based vector.

14. The vector according to claims 12 or 13 wherein said vector is a PiggyBac-based vector.

15. The use of the vector according to any one of claims 1 to 14 for the manufacture of a medicament to treat hemophilia B.

16. A method to obtain levels of factor IX in plasma equal to or higher than the therapeutic threshold concentration of 10 mU/ml plasma in a subject, comprising the transduction or transfection of the vector according to any one of claims 1 to 14 into a subject.

17. The method according to claim 16, wherein the transduction of the vector according to any one of claims 1 to 11 into the subject is done at a dose lower than $2x10^{11}$ vg/kg.

18. The method according to claim 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 100 mU/ml in a subject, wherein the transduction of the vector according to any one of claims 1 to 11 into the subject is done at a dose lower than or equal than $6x10^{11}$ vg/kg.

19. The method according to claim 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 50 mU/ml in a subject, wherein the transduction of the vector according to any one of claims 1 to 11 into the subject is done at a dose lower than or equal than $6x10^{11}$ vg/kg.

20. The method according to claim 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 200 mU/ml in a subject, wherein the transduction of the vector according to any one of claims 1 to 11 into the subject is done at a dose lower than or equal than $2x10^{12}$ vg/kg.

21. The method according to claim 16, used to obtain levels of factor IX in plasma equal to or higher than the therapeutic concentration of 150 mU/ml in a subject, wherein the transduction of the vector according to any one of claims 1 to 11 into the subject is done at a dose lower than or equal than $2x10^{12}$ vg/kg.

22. The method according to any one of claims 16 to 21, wherein said transduction or transfection is by intravenous administration.

23. The method according to any one of claims 16 to 22, wherein said subject is a mammalian subject, preferably a human subject.

24. A method for treating hemophilia B in a mammalian subject, comprising performing the method according to any one of claims 16 to 23.

25. A pharmaceutical composition comprising a vector according to any one of claims 1 to 14 and a pharmaceutically acceptable carrier, optionally further comprising an active ingredient for treating hemophilia B.

26. The pharmaceutical composition according to claim 25, for use in treating hemophilia B.

27. The use of a vector according to anyone of claims 1 to 14 for the manufacturing of a medicament for treating hemophilia B.

28. The pharmaceutical composition according to claim 25 or 26, or the use of a vector for treating hemophilia according to claim 27, wherein said treatment results in levels of factor IX in plasma of the treated subject that are equal to or higher than the therapeutic threshold concentration of 10 mU/ml plasma in a subject, preferably equal to or higher than the therapeutic concentration of 50 mU/ml plasma in a subject, more preferably equal to or higher than the therapeutic concentration of 100 mU/ml plasma in a subject, even more preferably equal to or higher than the therapeutic concentration of 150 mU/ml plasma in a subject and even more preferably equal to or higher than the therapeutic concentration of 200 mU/ml plasma in a subject.

29. The pharmaceutical composition according to claim 25 or 26, or the use of a vector for treating hemophilia according to claim 27, wherein said treatment comprises the transduction of the vector according to any one of claims 1 to 14 into the subject at a dose lower than or equal than $2x10^{12}$ vg/kg, preferably at a dose lower than or equal than $6x10^{11}$ vg/kg, more preferably at a dose lower than or equal than $2x10^{11}$vg/kg.

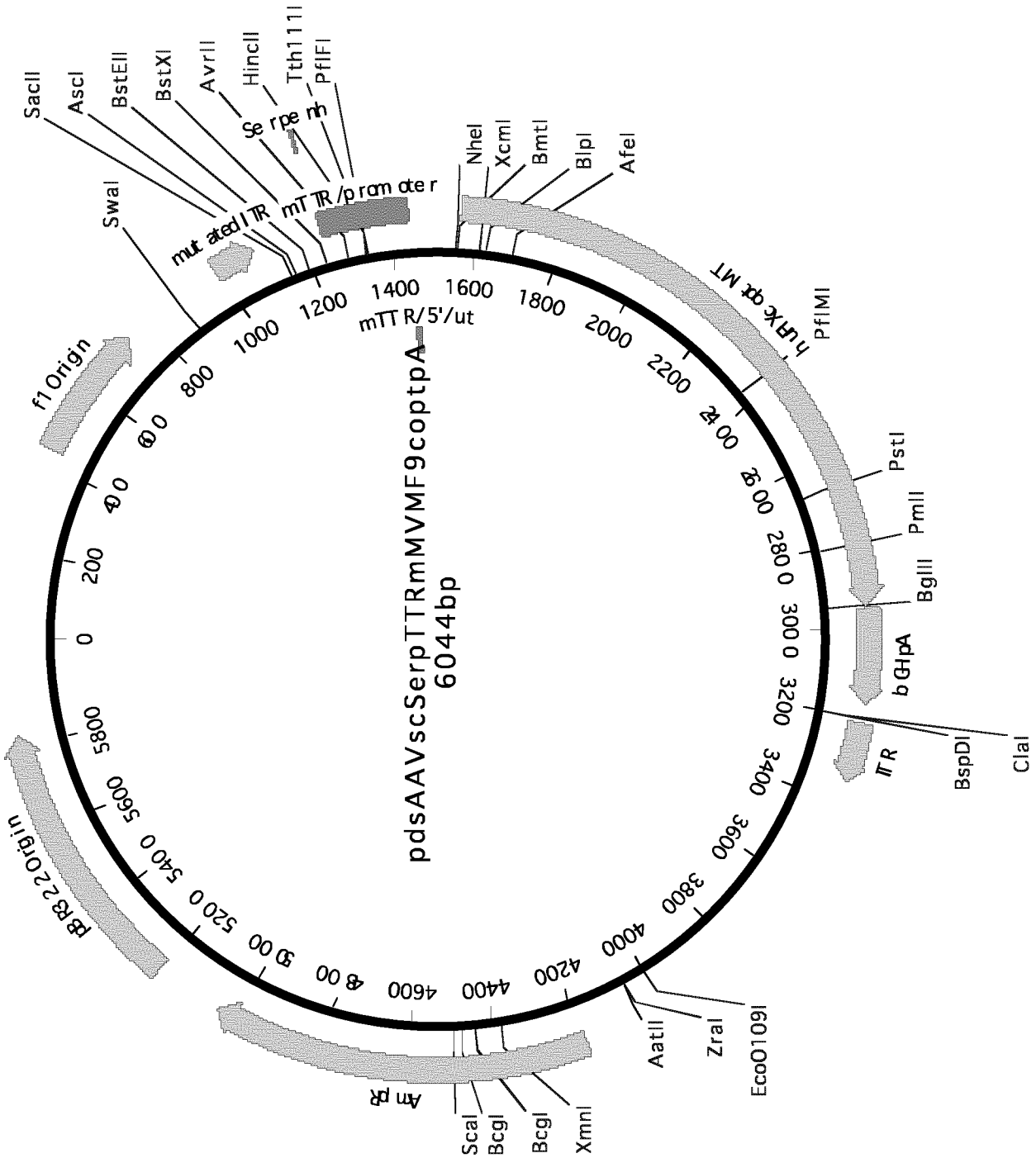

pdsAAVscSerpTTRmMVMF9coptpA
6044bp
mTTR/5'/ut
mTTR/promoter
mutatedITR
SacII
AscI
BstEII
BstXI
AvrII
HincII
Tth111I
PflFI
SwaI
NheI
XcmI
BmtI
BlpI
AfeI
hAAT/KozMT
PflMI
PstI
PmlI
BglII
bGHpA
ITR
BspDI
ClaI
f1Origin
pBR322Origin
AmpR
ScaI
BcgI
BcgI
XmnI
AatII
ZraI
EcoO109I

Figure 1

Figure 1B: SEQ ID 1

```
         10         20         30         40         50
CAGCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
GTCGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGTTG

         60         70         80         90        100
AGTTGCGCAGCCTGAATGGCGAATGGAATTCCAGACGATTGAGCGTCAAA
TCAACGCGTCGGACTTACCGCTTACCTTAAGGTCTGCTAACTCGCAGTTT

        110        120        130        140        150
ATGTAGGTATTTCCATGAGCGTTTTTCCTGTTGCAATGGCTGGCGGTAAT
TACATCCATAAAGGTACTCGCAAAAAGGACAACGTTACCGACCGCCATTA

        160        170        180        190        200
ATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCA
TAACAAGACCTATAATGGTCGTTCCGGCTATCAAACTCAAGAAGATGAGT

        210        220        230        240        250
GGCAAGTGATGTTATTACTAATCAAAGAAGTATTGCGACAACGGTTAATT
CCGTTCACTACAATAATGATTAGTTTCTTCATAACGCTGTTGCCAATTAA

        260        270        280        290        300
TGCGTGATGGACAGACTCTTTTACTCGGTGGCCTCACTGATTATAAAAAC
ACGCACTACCTGTCTGAGAAAATGAGCCACCGGAGTGACTAATATTTTTG

        310        320        330        340        350
ACTTCTCAGGATTCTGGCGTACCGTTCCTGTCTAAAATCCCTTTAATCGG
TGAAGAGTCCTAAGACCGCATGGCAAGGACAGATTTTAGGGAAATTAGCC

        360        370        380        390        400
CCTCCTGTTTAGCTCCCGCTCTGATTCTAACGAGGAAAGCACGTTATACG
GGAGGACAAATCGAGGGCGAGACTAAGATTGCTCCTTTCGTGCAATATGC

        410        420        430        440        450
TGCTCGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGC
ACGAGCAGTTTCGTTGGTATCATGCGCGGGACATCGCCGCGTAATTCGCG
                                              _________>

        460        470        480        490        500
GGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCC
CCGCCCACACCACCAATGCGCGTCGCACTGGCGATGTGAACGGTCGCGGG
_____________________F1 ORIGIN_____________________>

        510        520        530        540        550
TAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCC
ATCGCGGGCGAGGAAAGCGAAAGAAGGGAAGGAAAGAGCGGTGCAAGCGG
_____________________F1 ORIGIN_____________________>

        560        570        580        590        600
GGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATT
CCGAAAGGGGCAGTTCGAGATTTAGCCCCCGAGGGAAATCCCAAGGCTAA
_____________________F1 ORIGIN_____________________>

        610        620        630        640        650
```

```
TAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTT
ATCACGAAATGCCGTGGAGCTGGGGTTTTTTGAACTAATCCCACTACCAA
_____________________F1 ORIGIN_____________________>

       660       670       680       690       700
CACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTG
GTGCATCACCCGGTAGCGGGACTATCTGCCAAAAAGCGGGAAACTGCAAC
_____________________F1 ORIGIN_____________________>

       710       720       730       740       750
GAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACT
CTCAGGTGCAAGAAATTATCACCTGAGAACAAGGTTTGACCTTGTTGTGA
_____________________F1 ORIGIN_____________________>

       760       770       780       790       800
CAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTT
GTTGGGATAGAGCCAGATAAGAAAACTAAATATTCCCTAAAACGGCTAAA

       810       820       830       840       850
CGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAAT
GCCGGATAACCAATTTTTTACTCGACTAAATTGTTTTTAAATTGCGCTTA

       860       870       880       890       900
TTTAACAAAATATTAACGTTTACAATTTAAATATTTGCTTATACAATCTT
AAATTGTTTTATAATTGCAAATGTTAAATTTATAAACGAATATGTTAGAA

       910       920       930       940       950
CCTGTTTTTGGGGCTTTTCTGATTATCAACCGGGGTACATATGATTGACA
GGACAAAAACCCCGAAAAGACTAATAGTTGGCCCCATGTATACTAACTGT

       960       970       980       990      1000
TGCTAGTTTTACGATTACCGTTCATCGCCTGCACTGCGCGCTCGCTCGCT
ACGATCAAAATGCTAATGGCAAGTAGCGGACGTGACGCGCGAGCGAGCGA
                                  ___MUTATEDITR____>

      1010      1020      1030      1040      1050
CACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCC
GTGACTCCGGCGGGCCCGTTTCGGGCCCGCAGCCCGCTGGAAACCAGCGG
_____________________MUTATEDITR_____________________>

      1060      1070      1080      1090      1100
CGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGAATTCACGCGT
GCCGGAGTCACTCGCTCGCTCGCGCGTCTCTCCCTCACCTTAAGTGCGCA
_________________MUTATEDITR_________________>

      1110      1120      1130      1140      1150
GGTACGATCTGAATTCGGTACAATTCACGCGTGGTACGGCCGCGGTACCG
CCATGCTAGACTTAAGCCATGTTAAGTGCGCACCATGCCGGCGCCATGGC

      1160      1170      1180      1190      1200
GCGCGCCGGGGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTA
CGCGCGGCCCCCTCCGACGACCACTTATAATTGGTTCCAGTGGGGTCAAT

                            >Serp_enh   >mTTR/promoter
                                  |     |
```

```
      1210      1220      1230     |1240 |    1250
TCGGAGGAGCAAACAGGGGCTAAGTCCACACGCGTGGTACCGTCTGTCTG
AGCCTCCTCGTTTGTCCCCGATTCAGGTGTGCGCACCATGGCAGACAGAC

      1260      1270      1280      1290      1300
CACATTTCGTAGAGCGAGTGTTCCGATACTCTAATCTCCCTAGGCAAGGT
GTGTAAAGCATCTCGCTCACAAGGCTATGAGATTAGAGGGATCCGTTCCA

      1310      1320      1330      1340      1350
TCATATTTGTGTAGGTTACTTATTCTCCTTTTGTTGACTAAGTCAATAAT
AGTATAAACACATCCAATGAATAAGAGGAAAACAACTGATTCAGTTATTA

      1360      1370      1380      1390      1400
CAGAATCAGCAGGTTTGGAGTCAGCTTGGCAGGGATCAGCAGCCTGGGTT
GTCTTAGTCGTCCAAACCTCAGTCGAACCGTCCCTAGTCGTCGGACCCAA

                                          >mTTR/5'/ut
                                                |
      1410      1420      1430      1440    |  1450
GGAAGGAGGGGGTATAAAAGCCCCTTCACCAGGAGAAGCCGTCACACAGA
CCTTCCTCCCCCATATTTTCGGGGAAGTGGTCCTCTTCGGCAGTGTGTCT

            >MVMint
               |
      1460    | 1470      1480      1490      1500
TCCACAAGCTCCTGAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGG
AGGTGTTCGAGGACTTCTCCATTCCCAAATTCCCTACCAACCAACCACCC

      1510      1520      1530      1540      1550
GTATTAATGTTTAATTACCTGGAGCACCTGCCTGAAATCACTTTTTTTCA
CATAATTACAAATTAATGGACCTCGTGGACGGACTTTAGTGAAAAAAAGT

      1560      1570      1580      1590      1600
GGTTGGCTAGCATGCAGCGCGTGAACATGATCATGGCCGAGAGCCCCGGC
CCAACCGATCGTACGTCGCGCACTTGTACTAGTACCGGCTCTCGGGGCCG
            _____________HUFIXCOPTMT_______________>

      1610      1620      1630      1640      1650
CTGATCACCATCTGCCTGCTGGGCTACCTGCTGAGCGCCGAGTGCACCGT
GACTAGTGGTAGACGGACGACCCGATGGACGACTCGCGGCTCACGTGGCA
_____________________HUFIXCOPTMT_____________________>

      1660      1670      1680      1690      1700
GTTCCTGGACCACGAGAACGCCAACAAGATCCTGAACCGCCCCAAGCGCT
CAAGGACCTGGTGCTCTTGCGGTTGTTCTAGGACTTGGCGGGGTTCGCGA
_____________________HUFIXCOPTMT_____________________>

      1710      1720      1730      1740      1750
ACAACAGCGGCAAGCTGGAGGAGTTCGTGCAGGGCAACCTGGAGCGCGAG
TGTTGTCGCCGTTCGACCTCCTCAAGCACGTCCCGTTGGACCTCGCGCTC
_____________________HUFIXCOPTMT_____________________>

      1760      1770      1780      1790      1800
TGCATGGAGGAGAAGTGCAGCTTCGAGGAGGCCCGCGAGGTGTTCGAGAA
ACGTACCTCCTCTTCACGTCGAAGCTCCTCCGGGCGCTCCACAAGCTCTT
```

```
_____________________HUFIXCOPTMT_____________________>

      1810      1820      1830      1840      1850
CACCGAGCGCACCACCGAGTTCTGGAAGCAGTACGTGGACGGCGACCAGT
GTGGCTCGCGTGGTGGCTCAAGACCTTCGTCATGCACCTGCCGCTGGTCA
_____________________HUFIXCOPTMT_____________________>

      1860      1870      1880      1890      1900
GCGAGAGCAACCCCTGCCTGAACGGCGGCAGCTGCAAGGACGACATCAAC
CGCTCTCGTTGGGGACGGACTTGCCGCCGTCGACGTTCCTGCTGTAGTTG
_____________________HUFIXCOPTMT_____________________>

      1910      1920      1930      1940      1950
AGCTACGAGTGCTGGTGCCCCTTCGGCTTCGAGGGCAAGAACTGCGAGCT
TCGATGCTCACGACCACGGGGAAGCCGAAGCTCCCGTTCTTGACGCTCGA
_____________________HUFIXCOPTMT_____________________>

      1960      1970      1980      1990      2000
GGACGTGACCTGCAACATCAAGAACGGCCGCTGCGAGCAGTTCTGCAAGA
CCTGCACTGGACGTTGTAGTTCTTGCCGGCGACGCTCGTCAAGACGTTCT
_____________________HUFIXCOPTMT_____________________>

      2010      2020      2030      2040      2050
ACAGCGCCGACAACAAGGTGGTGTGCAGCTGCACCGAGGGCTACCGCCTG
TGTCGCGGCTGTTGTTCCACCACACGTCGACGTGGCTCCCGATGGCGGAC
_____________________HUFIXCOPTMT_____________________>

      2060      2070      2080      2090      2100
GCCGAGAACCAGAAGAGCTGCGAGCCCGCCGTGCCCTTCCCCTGCGGCCG
CGGCTCTTGGTCTTCTCGACGCTCGGGCGGCACGGGAAGGGGACGCCGGC
_____________________HUFIXCOPTMT_____________________>

      2110      2120      2130      2140      2150
CGTGAGCGTGAGCCAGACCAGCAAGCTGACCCGCGCCGAGGCCGTGTTCC
GCACTCGCACTCGGTCTGGTCGTTCGACTGGGCGCGGCTCCGGCACAAGG
_____________________HUFIXCOPTMT_____________________>

      2160      2170      2180      2190      2200
CCGACGTGGACTACGTGAACAGCACCGAGGCCGAGACCATCCTGGACAAC
GGCTGCACCTGATGCACTTGTCGTGGCTCCGGCTCTGGTAGGACCTGTTG
_____________________HUFIXCOPTMT_____________________>

      2210      2220      2230      2240      2250
ATCACCCAGAGCACCCAGAGCTTCAACGACTTCACCCGCGTGGTGGGCGG
TAGTGGGTCTCGTGGGTCTCGAAGTTGCTGAAGTGGGCGCACCACCCGCC
_____________________HUFIXCOPTMT_____________________>

      2260      2270      2280      2290      2300
CGAGGACGCCAAGCCCGGCCAGTTCCCCTGGCAGGTGGTGCTGAACGGCA
GCTCCTGCGGTTCGGGCCGGTCAAGGGGACCGTCCACCACGACTTGCCGT
_____________________HUFIXCOPTMT_____________________>

      2310      2320      2330      2340      2350
AGGTGGACGCCTTCTGCGGCGGCAGCATCGTGAACGAGAAGTGGATCGTG
TCCACCTGCGGAAGACGCCGCCGTCGTAGCACTTGCTCTTCACCTAGCAC
```

```
_____________________HUFIXCOPTMT_____________________>

      2360       2370       2380       2390       2400
ACCGCCGCCCACTGCGTGGAGACCGGCGTGAAGATCACCGTGGTGGCCGG
TGGCGGCGGGTGACGCACCTCTGGCCGCACTTCTAGTGGCACCACCGGCC
_____________________HUFIXCOPTMT_____________________>

      2410       2420       2430       2440       2450
CGAGCACAACATCGAGGAGACCGAGCACACCGAGCAGAAGCGCAACGTGA
GCTCGTGTTGTAGCTCCTCTGGCTCGTGTGGCTCGTCTTCGCGTTGCACT
_____________________HUFIXCOPTMT_____________________>

      2460       2470       2480       2490       2500
TCCGCATCATCCCCCACCACAACTACAACGCCGCCATCAACAAGTACAAC
AGGCGTAGTAGGGGGTGGTGTTGATGTTGCGGCGGTAGTTGTTCATGTTG
_____________________HUFIXCOPTMT_____________________>

      2510       2520       2530       2540       2550
CACGACATCGCCCTGCTGGAGCTGGACGAGCCCCTGGTGCTGAACAGCTA
GTGCTGTAGCGGGACGACCTCGACCTGCTCGGGGACCACGACTTGTCGAT
_____________________HUFIXCOPTMT_____________________>

      2560       2570       2580       2590       2600
CGTGACCCCCATCTGCATCGCCGACAAGGAGTACACCAACATCTTCCTGA
GCACTGGGGGTAGACGTAGCGGCTGTTCCTCATGTGGTTGTAGAAGGACT
_____________________HUFIXCOPTMT_____________________>

      2610       2620       2630       2640       2650
AGTTCGGCAGCGGCTACGTGAGCGGCTGGGGCCGCGTGTTCCACAAGGGC
TCAAGCCGTCGCCGATGCACTCGCCGACCCCGGCGCACAAGGTGTTCCCG
_____________________HUFIXCOPTMT_____________________>

      2660       2670       2680       2690       2700
CGCAGCGCCCTGGTGCTGCAGTACCTGCGCGTGCCCCTGGTGGACCGCGC
GCGTCGCGGGACCACGACGTCATGGACGCGCACGGGGACCACCTGGCGCG
_____________________HUFIXCOPTMT_____________________>

      2710       2720       2730       2740       2750
CACCTGCCTGCGCAGCACCAAGTTCACCATCTACAACAACATGTTCTGCG
GTGGACGGACGCGTCGTGGTTCAAGTGGTAGATGTTGTTGTACAAGACGC
_____________________HUFIXCOPTMT_____________________>

      2760       2770       2780       2790       2800
CCGGCTTCCACGAGGGCGGCCGCGACAGCTGCCAGGGCGACAGCGGCGGC
GGCCGAAGGTGCTCCCGCCGGCGCTGTCGACGGTCCCGCTGTCGCCGCCG
_____________________HUFIXCOPTMT_____________________>

      2810       2820       2830       2840       2850
CCCCACGTGACCGAGGTGGAGGGCACCAGCTTCCTGACCGGCATCATCAG
GGGGTGCACTGGCTCCACCTCCCGTGGTCGAAGGACTGGCCGTAGTAGTC
_____________________HUFIXCOPTMT_____________________>

      2860       2870       2880       2890       2900
CTGGGGCGAGGAGTGCGCCATGAAGGGCAAGTACGGCATCTACACCAAGG
GACCCCGCTCCTCACGCGGTACTTCCCGTTCATGCCGTAGATGTGGTTCC
```

```
_____________________HUFIXCOPTMT____________________>

      2910       2920       2930       2940       2950
TGAGCCGCTACGTGAACTGGATCAAGGAGAAGACCAAGCTGACCTGGAGA
ACTCGGCGATGCACTTGACCTAGTTCCTCTTCTGGTTCGACTGGACCTCT
____________________HUFIXCOPTMT____________________>

      2960       2970       2980       2990       3000
TCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGC
AGACTAGTCGGAGCTGACACGGAAGATCAACGGTCGGTAGACAACAAACG
   ____________________BGHPA ____________________>

      3010       3020       3030       3040       3050
CCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCT
GGGAGGGGGCACGGAAGGAACTGGGACCTTCCACGGTGAGGGTGACAGGA
____________________BGHPA ____________________>

      3060       3070       3080       3090       3100
TTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATT
AAGGATTATTTTACTCCTTTAACGTAGCGTAACAGACTCATCCACAGTAA
____________________BGHPA ____________________>

      3110       3120       3130       3140       3150
CTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA
GATAAGACCCCCCACCCCACCCCGTCCTGTCGTTCCCCCTCCTAACCCTT
____________________BGHPA ____________________>

      3160       3170       3180       3190       3200
GACAATAGCAGGCATGCTGGGGATCTGATAGCAGGCATGCTGGGGAGAGA
CTGTTATCGTCCGTACGACCCCTAGACTATCGTCCGTACGACCCCTCTCT
_________BGHPA _________>

      3210       3220       3230       3240       3250
TCGATCTAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGC
AGCTAGATCCTTGGGGATCACTACCTCAACCGGTGAGGGAGAGACGCGCG
       ____________________ITR____________________>

      3260       3270       3280       3290       3300
TCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCT
AGCGAGCGAGTGACTCCGGCGGGCCCGTTTCGGGCCCGCAGCCCGCTGGA
____________________ITR____________________>

      3310       3320       3330       3340       3350
TTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCC
AACCAGCGGGCCGGAGTCACTCGCTCGCTCGCGCGTCTCTCCCTCACCGG
____________________ITR____________________>

      3360       3370       3380       3390       3400
AACCCCCCCCCCCCCCCCCCTGCATGCAGGCGATTCTCTTGTTTGCTCCA
TTGGGGGGGGGGGGGGGGGGACGTACGTCCGCTAAGAGAACAAACGAGGT
__>

      3410       3420       3430       3440       3450
GACTCTCAGGCAATGACCTGATAGCCTTTGTAGAGACCTCTCAAAAATAG
CTGAGAGTCCGTTACTGGACTATCGGAAACATCTCTGGAGAGTTTTTATC
```

```
      3460       3470       3480       3490       3500
CTACCCTCTCCGGCATGAATTTATCAGCTAGAACGGTTGAATATCATATT
GATGGGAGAGGCCGTACTTAAATAGTCGATCTTGCCAACTTATAGTATAA

      3510       3520       3530       3540       3550
GATGGTGATTTGACTGTCTCCGGCCTTTCTCACCCGTTTGAATCTTTACC
CTACCACTAAACTGACAGAGGCCGGAAAGAGTGGGCAAACTTAGAAATGG

      3560       3570       3580       3590       3600
TACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCTAAAAATT
ATGTGTAATGAGTCCGTAACGTAAATTTTATATACTCCCAAGATTTTTAA

      3610       3620       3630       3640       3650
TTTATCCTTGCGTTGAAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGT
AAATAGGAACGCAACTTTATTTCCGAAGAGGGCGTTTTCATAATGTCCCA

      3660       3670       3680       3690       3700
CATAATGTTTTTGGTACAACCGATTTAGCTTTATGCTCTGAGGCTTTATT
GTATTACAAAAACCATGTTGGCTAAATCGAAATACGAGACTCCGAAATAA

      3710       3720       3730       3740       3750
GCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTTG
CGAATTAAAACGATTAAGAAACGGAACGGACATACTAAATAACCTACAAC

      3760       3770       3780       3790       3800
GAATTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACA
CTTAAGGACTACGCCATAAAAGAGGAATGCGTAGACACGCCATAAAGTGT

      3810       3820       3830       3840       3850
CCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAA
GGCGTATACCACGTGAGAGTCATGTTAGACGAGACTACGGCGTATCAATT

      3860       3870       3880       3890       3900
GCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGT
CGGTCGGGGCTGTGGGCGGTTGTGGGCGACTGCGCGGGACTGCCCGAACA

      3910       3920       3930       3940       3950
CTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTG
GACGAGGGCCGTAGGCGAATGTCTGTTCGACACTGGCAGAGGCCCTCGAC

      3960       3970       3980       3990       4000
CATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGG
GTACACAGTCTCCAAAAGTGGCAGTAGTGGCTTTGCGCGCTCTGCTTTCC

      4010       4020       4030       4040       4050
GCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTT
CGGAGCACTATGCGGATAAAAATATCCAATTACAGTACTATTATTACCAA

      4060       4070       4080       4090       4100
TCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTAT
AGAATCTGCAGTCCACCGTGAAAAGCCCCTTTACACGCGCCTTGGGGATA

      4110       4120       4130       4140       4150
TTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT
```

```
AACAAATAAAAAGATTTATGTAAGTTTATACATAGGCGAGTACTCTGTTA

      4160       4170       4180       4190       4200
AACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATT
TTGGGACTATTTACGAAGTTATTATAACTTTTTCCTTCTCATACTCATAA
                                                 _________>

      4210       4220       4230       4240       4250
CAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCC
GTTGTAAAGGCACAGCGGGAATAAGGGAAAAAACGCCGTAAAACGGAAGG
___________________AMPR_____________________________>

      4260       4270       4280       4290       4300
TGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATC
ACAAAAACGAGTGGGTCTTTGCGACCACTTTCATTTTCTACGACTTCTAG
___________________AMPR_____________________________>

      4310       4320       4330       4340       4350
AGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAG
TCAACCCACGTGCTCACCCAATGTAGCTTGACCTAGAGTTGTCGCCATTC
___________________AMPR_____________________________>

      4360       4370       4380       4390       4400
ATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTT
TAGGAACTCTCAAAAGCGGGGCTTCTTGCAAAAGGTTACTACTCGTGAAA
___________________AMPR_____________________________>

      4410       4420       4430       4440       4450
TAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAG
ATTTCAAGACGATACACCGCGCCATAATAGGGCATAACTGCGGCCCGTTC
___________________AMPR_____________________________>

      4460       4470       4480       4490       4500
AGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTAC
TCGTTGAGCCAGCGGCGTATGTGATAAGAGTCTTACTGAACCAACTCATG
___________________AMPR_____________________________>

      4510       4520       4530       4540       4550
TCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATT
AGTGGTCAGTGTCTTTTCGTAGAATGCCTACCGTACTGTCATTCTCTTAA
___________________AMPR_____________________________>

      4560       4570       4580       4590       4600
ATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTC
TACGTCACGACGGTATTGGTACTCACTATTGTGACGCCGGTTGAATGAAG
___________________AMPR_____________________________>

      4610       4620       4630       4640       4650
TGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATG
ACTGTTGCTAGCCTCCTGGCTTCCTCGATTGGCGAAAAAACGTGTTGTAC
___________________AMPR_____________________________>

      4660       4670       4680       4690       4700
GGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGC
CCCCTAGTACATTGAGCGGAACTAGCAACCCTTGGCCTCGACTTACTTCG
```

```
_____________________AMPR_____________________>

      4710      4720      4730      4740      4750
CATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAA
GTATGGTTTGCTGCTCGCACTGTGGTGCTACGGACATCGTTACCGTTGTT
_____________________AMPR_____________________>

      4760      4770      4780      4790      4800
CGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAA
GCAACGCGTTTGATAATTGACCGCTTGATGAATGAGATCGAAGGGCCGTT
_____________________AMPR_____________________>

      4810      4820      4830      4840      4850
CAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCG
GTTAATTATCTGACCTACCTCCGCCTATTTCAACGTCCTGGTGAAGACGC
_____________________AMPR_____________________>

      4860      4870      4880      4890      4900
CTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTG
GAGCCGGGAAGGCCGACCGACCAAATAACGACTATTTAGACCTCGGCCAC
_____________________AMPR_____________________>

      4910      4920      4930      4940      4950
AGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCC
TCGCACCCAGAGCGCCATAGTAACGTCGTGACCCCGGTCTACCATTCGGG
_____________________AMPR_____________________>

      4960      4970      4980      4990      5000
TCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGA
AGGGCATAGCATCAATAGATGTGCTGCCCCTCAGTCCGTTGATACCTACT
_____________________AMPR_____________________>

      5010      5020      5030      5040      5050
ACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGT
TGCTTTATCTGTCTAGCGACTCTATCCACGGAGTGACTAATTCGTAACCA
_____________________AMPR_____________________>

      5060      5070      5080      5090      5100
AACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTT
TTGACAGTCTGGTTCAAATGAGTATATATGAAATCTAACTAAATTTTGAA
__>

      5110      5120      5130      5140      5150
CATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCAT
GTAAAAATTAAATTTTCCTAGATCCACTTCTAGGAAAAACTATTAGAGTA

      5160      5170      5180      5190      5200
GACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCG
CTGGTTTTAGGGAATTGCACTCAAAAGCAAGGTGACTCGCAGTCTGGGGC

      5210      5220      5230      5240      5250
TAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATC
ATCTTTTCTAGTTTCCTAGAAGAACTCTAGGAAAAAAAGACGCGCATTAG
      _________________PBR322 ORIGIN_________________>
```

```
      5260       5270       5280       5290       5300
TGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCC
ACGACGAACGTTTGTTTTTTTGGTGGCGATGGTCGCCACCAAACAAACGG
_____________________PBR322 ORIGIN_____________________>

      5310       5320       5330       5340       5350
GGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAG
CCTAGTTCTCGATGGTTGAGAAAAAGGCTTCCATTGACCGAAGTCGTCTC
_____________________PBR322 ORIGIN_____________________>

      5360       5370       5380       5390       5400
CGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCAC
GCGTCTATGGTTTATGACAGGAAGATCACATCGGCATCAATCCGGTGGTG
_____________________PBR322 ORIGIN_____________________>

      5410       5420       5430       5440       5450
TTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTT
AAGTTCTTGAGACATCGTGGCGGATGTATGGAGCGAGACGATTAGGACAA
_____________________PBR322 ORIGIN_____________________>

      5460       5470       5480       5490       5500
ACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACT
TGGTCACCGACGACGGTCACCGCTATTCAGCACAGAATGGCCCAACCTGA
_____________________PBR322 ORIGIN_____________________>

      5510       5520       5530       5540       5550
CAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGT
GTTCTGCTATCAATGGCCTATTCCGCGTCGCCAGCCCGACTTGCCCCCCA
_____________________PBR322 ORIGIN_____________________>

      5560       5570       5580       5590       5600
TCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATA
AGCACGTGTGTCGGGTCGAACCTCGCTTGCTGGATGTGGCTTGACTCTAT
_____________________PBR322 ORIGIN_____________________>

      5610       5620       5630       5640       5650
CCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGG
GGATGTCGCACTCGATACTCTTTCGCGGTGCGAAGGGCTTCCCTCTTTCC
_____________________PBR322 ORIGIN_____________________>

      5660       5670       5680       5690       5700
CGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGG
GCCTGTCCATAGGCCATTCGCCGTCCCAGCCTTGTCCTCTCGCGTGCTCC
_____________________PBR322 ORIGIN_____________________>

      5710       5720       5730       5740       5750
GAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCG
CTCGAAGGTCCCCCTTTGCGGACCATAGAAATATCAGGACAGCCCAAAGC
_____________________PBR322 ORIGIN_____________________>

      5760       5770       5780       5790       5800
CCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGA
GGTGGAGACTGAACTCGCAGCTAAAAACACTACGAGCAGTCCCCCCGCCT
_____________________PBR322 ORIGIN_____________________>
```

```
      5810      5820      5830      5840      5850
GCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTT
CGGATACCTTTTTGCGGTCGTTGCGCCGGAAAAATGCCAAGGACCGGAAA
______PBR322 ORIGIN_______>

      5860      5870      5880      5890      5900
TGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGT
ACGACCGGAAAACGAGTGTACAAGAAAGGACGCAATAGGGGACTAAGACA

      5910      5920      5930      5940      5950
GGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCC
CCTATTGGCATAATGGCGGAAACTCACTCGACTATGGCGAGCGGCGTCGG

      5960      5970      5980      5990      6000
GAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCA
CTTGCTGGCTCGCGTCGCTCAGTCACTCGCTCCTTCGCCTTCTCGCGGGT

      6010      6020      6030      6040
ATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG
TATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTAC
```

Figure 1C: SEQ ID 2

```
        10         20         30         40         50
CAGCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
GTCGTCGACCGCATTATCGCTTCTCCGGGCGTGGCTAGCGGGAAGGGTTG

        60         70         80         90        100
AGTTGCGCAGCCTGAATGGCGAATGGAATTCCAGACGATTGAGCGTCAAA
TCAACGCGTCGGACTTACCGCTTACCTTAAGGTCTGCTAACTCGCAGTTT

       110        120        130        140        150
ATGTAGGTATTTCCATGAGCGTTTTTCCTGTTGCAATGGCTGGCGGTAAT
TACATCCATAAAGGTACTCGCAAAAAGGACAACGTTACCGACCGCCATTA

       160        170        180        190        200
ATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCA
TAACAAGACCTATAATGGTCGTTCCGGCTATCAAACTCAAGAAGATGAGT

       210        220        230        240        250
GGCAAGTGATGTTATTACTAATCAAAGAAGTATTGCGACAACGGTTAATT
CCGTTCACTACAATAATGATTAGTTTCTTCATAACGCTGTTGCCAATTAA

       260        270        280        290        300
TGCGTGATGGACAGACTCTTTTACTCGGTGGCCTCACTGATTATAAAAAC
ACGCACTACCTGTCTGAGAAAATGAGCCACCGGAGTGACTAATATTTTTG

       310        320        330        340        350
ACTTCTCAGGATTCTGGCGTACCGTTCCTGTCTAAAATCCCTTTAATCGG
TGAAGAGTCCTAAGACCGCATGGCAAGGACAGATTTTAGGGAAATTAGCC

       360        370        380        390        400
CCTCCTGTTTAGCTCCCGCTCTGATTCTAACGAGGAAAGCACGTTATACG
GGAGGACAAATCGAGGGCGAGACTAAGATTGCTCCTTTCGTGCAATATGC

       410        420        430        440        450
TGCTCGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGC
ACGAGCAGTTTCGTTGGTATCATGCGCGGGACATCGCCGCGTAATTCGCG
                                              _________>

       460        470        480        490        500
GGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCC
CCGCCCACACCACCAATGCGCGTCGCACTGGCGATGTGAACGGTCGCGGG
_____________________F1 ORIGIN_____________________>

       510        520        530        540        550
TAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCC
ATCGCGGGCGAGGAAAGCGAAAGAAGGGAAGGAAAGAGCGGTGCAAGCGG
_____________________F1 ORIGIN_____________________>

       560        570        580        590        600
GGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATT
CCGAAAGGGGCAGTTCGAGATTTAGCCCCCGAGGGAAATCCCAAGGCTAA
_____________________F1 ORIGIN_____________________>
```

```
       610        620        630        640        650
TAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTT
ATCACGAAATGCCGTGGAGCTGGGGTTTTTTGAACTAATCCCACTACCAA
_____________________F1 ORIGIN____________________>

       660        670        680        690        700
CACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTG
GTGCATCACCCGGTAGCGGGACTATCTGCCAAAAAGCGGGAAACTGCAAC
_____________________F1 ORIGIN____________________>

       710        720        730        740        750
GAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACT
CTCAGGTGCAAGAAATTATCACCTGAGAACAAGGTTTGACCTTGTTGTGA
___________________F1 ORIGIN___________________>

       760        770        780        790        800
CAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTT
GTTGGGATAGAGCCAGATAAGAAAACTAAATATTCCCTAAAACGGCTAAA

       810        820        830        840        850
CGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAAT
GCCGGATAACCAATTTTTTACTCGACTAAATTGTTTTTAAATTGCGCTTA

       860        870        880        890        900
TTTAACAAAATATTAACGTTTACAATTTAAATATTTGCTTATACAATCTT
AAATTGTTTTATAATTGCAAATGTTAAATTTATAAACGAATATGTTAGAA

       910        920        930        940        950
CCTGTTTTTGGGGCTTTTCTGATTATCAACCGGGGTACATATGATTGACA
GGACAAAAACCCCGAAAAGACTAATAGTTGGCCCCATGTATACTAACTGT

       960        970        980        990       1000
TGCTAGTTTTACGATTACCGTTCATCGCCTGCACTGCGCGCTCGCTCGCT
ACGATCAAAATGCTAATGGCAAGTAGCGGACGTGACGCGCGAGCGAGCGA
                                  ___MUTATEDITR____>

      1010       1020       1030       1040       1050
CACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCC
GTGACTCCGGCGGGCCCGTTTCGGGCCCGCAGCCCGCTGGAAACCAGCGG
____________________MUTATEDITR___________________>

      1060       1070       1080       1090       1100
CGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGAATTCACGCGT
GCCGGAGTCACTCGCTCGCTCGCGCGTCTCTCCCTCACCTTAAGTGCGCA
_________________MUTATEDITR_________________>

      1110       1120       1130       1140       1150
GGTACGATCTGAATTCGGTACAATTCACGCGTGGTACGGCCGCGGTACCG
CCATGCTAGACTTAAGCCATGTTAAGTGCGCACCATGCCGGCGCCATGGC

      1160       1170       1180       1190       1200
GCGCGCCGGGGGGAGGCTGCTGGTGAATATTAACCAAGGTCACCCCAGTTA
CGCGCGGCCCCCTCCGACGACCACTTATAATTGGTTCCAGTGGGGTCAAT

                              >Serp_enh    >mTTR/promoter
```

```
                                        |     |
      1210      1220      1230      |1240 |    1250
TCGGAGGAGCAAACAGGGGCTAAGTCCACACGCGTGGTACCGTCTGTCTG
AGCCTCCTCGTTTGTCCCCGATTCAGGTGTGCGCACCATGGCAGACAGAC

      1260      1270      1280      1290      1300
CACATTTCGTAGAGCGAGTGTTCCGATACTCTAATCTCCCTAGGCAAGGT
GTGTAAAGCATCTCGCTCACAAGGCTATGAGATTAGAGGGATCCGTTCCA

      1310      1320      1330      1340      1350
TCATATTTGTGTAGGTTACTTATTCTCCTTTTGTTGACTAAGTCAATAAT
AGTATAAACACATCCAATGAATAAGAGGAAAACAACTGATTCAGTTATTA

      1360      1370      1380      1390      1400
CAGAATCAGCAGGTTTGGAGTCAGCTTGGCAGGGATCAGCAGCCTGGGTT
GTCTTAGTCGTCCAAACCTCAGTCGAACCGTCCCTAGTCGTCGGACCCAA

                                          >mTTR/5'/ut
                                                |
      1410      1420      1430      1440    |  1450
GGAAGGAGGGGGTATAAAAGCCCCTTCACCAGGAGAAGCCGTCACACAGA
CCTTCCTCCCCCATATTTTCGGGGAAGTGGTCCTCTTCGGCAGTGTGTCT

            >MVMint
               |
      1460    | 1470      1480      1490      1500
TCCACAAGCTCCTGAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGG
AGGTGTTCGAGGACTTCTCCATTCCCAAATTCCCTACCAACCAACCACCC

      1510      1520      1530      1540      1550
GTATTAATGTTTAATTACCTGGAGCACCTGCCTGAAATCACTTTTTTTCA
CATAATTACAAATTAATGGACCTCGTGGACGGACTTTAGTGAAAAAAAGT

      1560      1570      1580      1590      1600
GGTTGGCTAGCATGCAGCGCGTGAACATGATCATGGCCGAGAGCCCCGGC
CCAACCGATCGTACGTCGCGCACTTGTACTAGTACCGGCTCTCGGGGCCG
            _____________HUFIXCOPTMT_____________>

      1610      1620      1630      1640      1650
CTGATCACCATCTGCCTGCTGGGCTACCTGCTGAGCGCCGAGTGCACCGT
GACTAGTGGTAGACGGACGACCCGATGGACGACTCGCGGCTCACGTGGCA
_____________________HUFIXCOPTMT_____________________>

      1660      1670      1680      1690      1700
GTTCCTGGACCACGAGAACGCCAACAAGATCCTGAACCGCCCCAAGCGCT
CAAGGACCTGGTGCTCTTGCGGTTGTTCTAGGACTTGGCGGGGTTCGCGA
_____________________HUFIXCOPTMT_____________________>

      1710      1720      1730      1740      1750
ACAACAGCGGCAAGCTGGAGGAGTTCGTGCAGGGCAACCTGGAGCGCGAG
TGTTGTCGCCGTTCGACCTCCTCAAGCACGTCCCGTTGGACCTCGCGCTC
_____________________HUFIXCOPTMT_____________________>

      1760      1770      1780      1790      1800
TGCATGGAGGAGAAGTGCAGCTTCGAGGAGGCCCGCGAGGTGTTCGAGAA
```

```
ACGTACCTCCTCTTCACGTCGAAGCTCCTCCGGGCGCTCCACAAGCTCTT
_____________________HUFIXCOPTMT_____________________>

      1810      1820      1830      1840      1850
CACCGAGCGCACCACCGAGTTCTGGAAGCAGTACGTGGACGGCGACCAGT
GTGGCTCGCGTGGTGGCTCAAGACCTTCGTCATGCACCTGCCGCTGGTCA
_____________________HUFIXCOPTMT_____________________>

      1860      1870      1880      1890      1900
GCGAGAGCAACCCCTGCCTGAACGGCGGCAGCTGCAAGGACGACATCAAC
CGCTCTCGTTGGGGACGGACTTGCCGCCGTCGACGTTCCTGCTGTAGTTG
_____________________HUFIXCOPTMT_____________________>

      1910      1920      1930      1940      1950
AGCTACGAGTGCTGGTGCCCCTTCGGCTTCGAGGGCAAGAACTGCGAGCT
TCGATGCTCACGACCACGGGGAAGCCGAAGCTCCCGTTCTTGACGCTCGA
_____________________HUFIXCOPTMT_____________________>

      1960      1970      1980      1990      2000
GGACGTGACCTGCAACATCAAGAACGGCCGCTGCGAGCAGTTCTGCAAGA
CCTGCACTGGACGTTGTAGTTCTTGCCGGCGACGCTCGTCAAGACGTTCT
_____________________HUFIXCOPTMT_____________________>

      2010      2020      2030      2040      2050
ACAGCGCCGACAACAAGGTGGTGTGCAGCTGCACCGAGGGCTACCGCCTG
TGTCGCGGCTGTTGTTCCACCACACGTCGACGTGGCTCCCGATGGCGGAC
_____________________HUFIXCOPTMT_____________________>

      2060      2070      2080      2090      2100
GCCGAGAACCAGAAGAGCTGCGAGCCCGCCGTGCCCTTCCCCTGCGGCCG
CGGCTCTTGGTCTTCTCGACGCTCGGGCGGCACGGGAAGGGGACGCCGGC
_____________________HUFIXCOPTMT_____________________>

      2110      2120      2130      2140      2150
CGTGAGCGTGAGCCAGACCAGCAAGCTGACCCGCGCCGAGGCCGTGTTCC
GCACTCGCACTCGGTCTGGTCGTTCGACTGGGCGCGGCTCCGGCACAAGG
_____________________HUFIXCOPTMT_____________________>

      2160      2170      2180      2190      2200
CCGACGTGGACTACGTGAACAGCACCGAGGCCGAGACCATCCTGGACAAC
GGCTGCACCTGATGCACTTGTCGTGGCTCCGGCTCTGGTAGGACCTGTTG
_____________________HUFIXCOPTMT_____________________>

      2210      2220      2230      2240      2250
ATCACCCAGAGCACCCAGAGCTTCAACGACTTCACCCGCGTGGTGGGCGG
TAGTGGGTCTCGTGGGTCTCGAAGTTGCTGAAGTGGGCGCACCACCCGCC
_____________________HUFIXCOPTMT_____________________>

      2260      2270      2280      2290      2300
CGAGGACGCCAAGCCCGGCCAGTTCCCCTGGCAGGTGGTGCTGAACGGCA
GCTCCTGCGGTTCGGGCCGGTCAAGGGGACCGTCCACCACGACTTGCCGT
_____________________HUFIXCOPTMT_____________________>

      2310      2320      2330      2340      2350
AGGTGGACGCCTTCTGCGGCGGCAGCATCGTGAACGAGAAGTGGATCGTG
```

```
TCCACCTGCGGAAGACGCCGCCGTCGTAGCACTTGCTCTTCACCTAGCAC
_____________________HUFIXCOPTMT_____________________>

      2360      2370      2380      2390      2400
ACCGCCGCCCACTGCGTGGAGACCGGCGTGAAGATCACCGTGGTGGCCGG
TGGCGGCGGGTGACGCACCTCTGGCCGCACTTCTAGTGGCACCACCGGCC
_____________________HUFIXCOPTMT_____________________>

      2410      2420      2430      2440      2450
CGAGCACAACATCGAGGAGACCGAGCACACCGAGCAGAAGCGCAACGTGA
GCTCGTGTTGTAGCTCCTCTGGCTCGTGTGGCTCGTCTTCGCGTTGCACT
_____________________HUFIXCOPTMT_____________________>

      2460      2470      2480      2490      2500
TCCGCATCATCCCCCACCACAACTACAACGCCGCCATCAACAAGTACAAC
AGGCGTAGTAGGGGGTGGTGTTGATGTTGCGGCGGTAGTTGTTCATGTTG
_____________________HUFIXCOPTMT_____________________>

      2510      2520      2530      2540      2550
CACGACATCGCCCTGCTGGAGCTGGACGAGCCCCTGGTGCTGAACAGCTA
GTGCTGTAGCGGGACGACCTCGACCTGCTCGGGGACCACGACTTGTCGAT
_____________________HUFIXCOPTMT_____________________>

      2560      2570      2580      2590      2600
CGTGACCCCCATCTGCATCGCCGACAAGGAGTACACCAACATCTTCCTGA
GCACTGGGGGTAGACGTAGCGGCTGTTCCTCATGTGGTTGTAGAAGGACT
_____________________HUFIXCOPTMT_____________________>

      2610      2620      2630      2640      2650
AGTTCGGCAGCGGCTACGTGAGCGGCTGGGGCCGCGTGTTCCACAAGGGC
TCAAGCCGTCGCCGATGCACTCGCCGACCCCGGCGCACAAGGTGTTCCCG
_____________________HUFIXCOPTMT_____________________>

      2660      2670      2680      2690      2700
CGCAGCGCCCTGGTGCTGCAGTACCTGCGCGTGCCCCTGGTGGACCGCGC
GCGTCGCGGGACCACGACGTCATGGACGCGCACGGGGACCACCTGGCGCG
_____________________HUFIXCOPTMT_____________________>

      2710      2720      2730      2740      2750
CACCTGCCTGCTGAGCACCAAGTTCACCATCTACAACAACATGTTCTGCG
GTGGACGGACGACGTCGTGGTTCAAGTGGTAGATGTTGTTGTACAAGACGC
_____________________HUFIXCOPTMT_____________________>

      2760      2770      2780      2790      2800
CCGGCTTCCACGAGGGCGGCCGCGACAGCTGCCAGGGCGACAGCGGCGGC
GGCCGAAGGTGCTCCCGCCGGCGCTGTCGACGGTCCCGCTGTCGCCGCCG
_____________________HUFIXCOPTMT_____________________>

      2810      2820      2830      2840      2850
CCCCACGTGACCGAGGTGGAGGGCACCAGCTTCCTGACCGGCATCATCAG
GGGGTGCACTGGCTCCACCTCCCGTGGTCGAAGGACTGGCCGTAGTAGTC
_____________________HUFIXCOPTMT_____________________>

      2860      2870      2880      2890      2900
CTGGGGCGAGGAGTGCGCCATGAAGGGCAAGTACGGCATCTACACCAAGG
```

```
GACCCCGCTCCTCACGCGGTACTTCCCGTTCATGCCGTAGATGTGGTTCC
_____________________HUFIXCOPTMT_____________________>

      2910       2920       2930       2940       2950
TGAGCCGCTACGTGAACTGGATCAAGGAGAAGACCAAGCTGACCTGGAGA
ACTCGGCGATGCACTTGACCTAGTTCCTCTTCTGGTTCGACTGGACCTCT
___________________HUFIXCOPTMT___________________>

      2960       2970       2980       2990       3000
TCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGC
AGACTAGTCGGAGCTGACACGGAAGATCAACGGTCGGTAGACAACAAACG
   _____________________BGHPA _____________________>

      3010       3020       3030       3040       3050
CCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCT
GGGAGGGGGCACGGAAGGAACTGGGACCTTCCACGGTGAGGGTGACAGGA
_____________________BGHPA _____________________>

      3060       3070       3080       3090       3100
TTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATT
AAGGATTATTTTACTCCTTTAACGTAGCGTAACAGACTCATCCACAGTAA
_____________________BGHPA _____________________>

      3110       3120       3130       3140       3150
CTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA
GATAAGACCCCCCACCCCACCCCGTCCTGTCGTTCCCCCTCCTAACCCTT
_____________________BGHPA _____________________>

      3160       3170       3180       3190       3200
GACAATAGCAGGCATGCTGGGGATCTGATAGCAGGCATGCTGGGGAGAGA
CTGTTATCGTCCGTACGACCCCTAGACTATCGTCCGTACGACCCCTCTCT
_________BGHPA __________>

      3210       3220       3230       3240       3250
TCGATCTAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGC
AGCTAGATCCTTGGGGATCACTACCTCAACCGGTGAGGGAGAGACGCGCG
       _____________________ITR_____________________>

      3260       3270       3280       3290       3300
TCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCT
AGCGAGCGAGTGACTCCGGCGGGCCCGTTTCGGGCCCGCAGCCCGCTGGA
_____________________ITR_____________________>

      3310       3320       3330       3340       3350
TTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCC
AACCAGCGGGCCGGAGTCACTCGCTCGCTCGCGCGTCTCTCCCTCACCGG
_____________________ITR_____________________>

      3360       3370       3380       3390       3400
AACCCCCCCCCCCCCCCCCCTGCATGCAGGCGATTCTCTTGTTTGCTCCA
TTGGGGGGGGGGGGGGGGGGACGTACGTCCGCTAAGAGAACAAACGAGGT
__>

      3410       3420       3430       3440       3450
GACTCTCAGGCAATGACCTGATAGCCTTTGTAGAGACCTCTCAAAAATAG
```

```
CTGAGAGTCCGTTACTGGACTATCGGAAACATCTCTGGAGAGTTTTTATC

      3460       3470       3480       3490       3500
CTACCCTCTCCGGCATGAATTTATCAGCTAGAACGGTTGAATATCATATT
GATGGGAGAGGCCGTACTTAAATAGTCGATCTTGCCAACTTATAGTATAA

      3510       3520       3530       3540       3550
GATGGTGATTTGACTGTCTCCGGCCTTTCTCACCCGTTTGAATCTTTACC
CTACCACTAAACTGACAGAGGCCGGAAAGAGTGGGCAAACTTAGAAATGG

      3560       3570       3580       3590       3600
TACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCTAAAAATT
ATGTGTAATGAGTCCGTAACGTAAATTTTATATACTCCCAAGATTTTTAA

      3610       3620       3630       3640       3650
TTTATCCTTGCGTTGAAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGT
AAATAGGAACGCAACTTTATTTCCGAAGAGGGCGTTTTCATAATGTCCCA

      3660       3670       3680       3690       3700
CATAATGTTTTTGGTACAACCGATTTAGCTTTATGCTCTGAGGCTTTATT
GTATTACAAAAACCATGTTGGCTAAATCGAAATACGAGACTCCGAAATAA

      3710       3720       3730       3740       3750
GCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTTG
CGAATTAAAACGATTAAGAAACGGAACGGACATACTAAATAACCTACAAC

      3760       3770       3780       3790       3800
GAATTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACA
CTTAAGGACTACGCCATAAAAGAGGAATGCGTAGACACGCCATAAAGTGT

      3810       3820       3830       3840       3850
CCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAA
GGCGTATACCACGTGAGAGTCATGTTAGACGAGACTACGGCGTATCAATT

      3860       3870       3880       3890       3900
GCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGT
CGGTCGGGGCTGTGGGCGGTTGTGGGCGACTGCGCGGGACTGCCCGAACA

      3910       3920       3930       3940       3950
CTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTG
GACGAGGGCCGTAGGCGAATGTCTGTTCGACACTGGCAGAGGCCCTCGAC

      3960       3970       3980       3990       4000
CATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGG
GTACACAGTCTCCAAAAGTGGCAGTAGTGGCTTTGCGCGCTCTGCTTTCC

      4010       4020       4030       4040       4050
GCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTT
CGGAGCACTATGCGGATAAAAATATCCAATTACAGTACTATTATTACCAA

      4060       4070       4080       4090       4100
TCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTAT
AGAATCTGCAGTCCACCGTGAAAAGCCCCTTTACACGCGCCTTGGGGATA

      4110       4120       4130       4140       4150
```

```
TTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT
AACAAATAAAAAGATTTATGTAAGTTTATACATAGGCGAGTACTCTGTTA

      4160       4170       4180       4190       4200
AACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATT
TTGGGACTATTTACGAAGTTATTATAACTTTTTCCTTCTCATACTCATAA
                                                 _________>

      4210       4220       4230       4240       4250
CAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCC
GTTGTAAAGGCACAGCGGGAATAAGGGAAAAAACGCCGTAAAACGGAAGG
___________________AMPR____________________________>

      4260       4270       4280       4290       4300
TGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATC
ACAAAAACGAGTGGGTCTTTGCGACCACTTTCATTTTCTACGACTTCTAG
___________________AMPR____________________________>

      4310       4320       4330       4340       4350
AGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAG
TCAACCCACGTGCTCACCCAATGTAGCTTGACCTAGAGTTGTCGCCATTC
___________________AMPR____________________________>

      4360       4370       4380       4390       4400
ATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTT
TAGGAACTCTCAAAAGCGGGGCTTCTTGCAAAAGGTTACTACTCGTGAAA
___________________AMPR____________________________>

      4410       4420       4430       4440       4450
TAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAG
ATTTCAAGACGATACACCGCGCCATAATAGGGCATAACTGCGGCCCGTTC
___________________AMPR____________________________>

      4460       4470       4480       4490       4500
AGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTAC
TCGTTGAGCCAGCGGCGTATGTGATAAGAGTCTTACTGAACCAACTCATG
___________________AMPR____________________________>

      4510       4520       4530       4540       4550
TCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATT
AGTGGTCAGTGTCTTTTCGTAGAATGCCTACCGTACTGTCATTCTCTTAA
___________________AMPR____________________________>

      4560       4570       4580       4590       4600
ATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTC
TACGTCACGACGGTATTGGTACTCACTATTGTGACGCCGGTTGAATGAAG
___________________AMPR____________________________>

      4610       4620       4630       4640       4650
TGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATG
ACTGTTGCTAGCCTCCTGGCTTCCTCGATTGGCGAAAAAACGTGTTGTAC
___________________AMPR____________________________>

      4660       4670       4680       4690       4700
GGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGC
```

```
CCCCTAGTACATTGAGCGGAACTAGCAACCCTTGGCCTCGACTTACTTCG
_____________________AMPR_____________________>

      4710      4720      4730      4740      4750
CATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAA
GTATGGTTTGCTGCTCGCACTGTGGTGCTACGGACATCGTTACCGTTGTT
_____________________AMPR_____________________>

      4760      4770      4780      4790      4800
CGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAA
GCAACGCGTTTGATAATTGACCGCTTGATGAATGAGATCGAAGGGCCGTT
_____________________AMPR_____________________>

      4810      4820      4830      4840      4850
CAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCG
GTTAATTATCTGACCTACCTCCGCCTATTTCAACGTCCTGGTGAAGACGC
_____________________AMPR_____________________>

      4860      4870      4880      4890      4900
CTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTG
GAGCCGGGAAGGCCGACCGACCAAATAACGACTATTTAGACCTCGGCCAC
_____________________AMPR_____________________>

      4910      4920      4930      4940      4950
AGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCC
TCGCACCCAGAGCGCCATAGTAACGTCGTGACCCCGGTCTACCATTCGGG
_____________________AMPR_____________________>

      4960      4970      4980      4990      5000
TCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGA
AGGGCATAGCATCAATAGATGTGCTGCCCCTCAGTCCGTTGATACCTACT
_____________________AMPR_____________________>

      5010      5020      5030      5040      5050
ACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGT
TGCTTTATCTGTCTAGCGACTCTATCCACGGAGTGACTAATTCGTAACCA
_____________________AMPR_____________________>

      5060      5070      5080      5090      5100
AACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTT
TTGACAGTCTGGTTCAAATGAGTATATATGAAATCTAACTAAATTTTGAA
__>

      5110      5120      5130      5140      5150
CATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCAT
GTAAAAATTAAATTTTCCTAGATCCACTTCTAGGAAAAACTATTAGAGTA

      5160      5170      5180      5190      5200
GACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCG
CTGGTTTTAGGGAATTGCACTCAAAAGCAAGGTGACTCGCAGTCTGGGGC

      5210      5220      5230      5240      5250
TAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATC
ATCTTTTCTAGTTTCCTAGAAGAACTCTAGGAAAAAAAGACGCGCATTAG
      _________________PBR322 ORIGIN_________________>
```

```
      5260       5270       5280       5290       5300
TGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCC
ACGACGAACGTTTGTTTTTTTGGTGGCGATGGTCGCCACCAAACAAACGG
____________________PBR322 ORIGIN____________________>

      5310       5320       5330       5340       5350
GGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAG
CCTAGTTCTCGATGGTTGAGAAAAAGGCTTCCATTGACCGAAGTCGTCTC
____________________PBR322 ORIGIN____________________>

      5360       5370       5380       5390       5400
CGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCAC
GCGTCTATGGTTTATGACAGGAAGATCACATCGGCATCAATCCGGTGGTG
____________________PBR322 ORIGIN____________________>

      5410       5420       5430       5440       5450
TTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTT
AAGTTCTTGAGACATCGTGGCGGATGTATGGAGCGAGACGATTAGGACAA
____________________PBR322 ORIGIN____________________>

      5460       5470       5480       5490       5500
ACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACT
TGGTCACCGACGACGGTCACCGCTATTCAGCACAGAATGGCCCAACCTGA
____________________PBR322 ORIGIN____________________>

      5510       5520       5530       5540       5550
CAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGT
GTTCTGCTATCAATGGCCTATTCCGCGTCGCCAGCCCGACTTGCCCCCCA
____________________PBR322 ORIGIN____________________>

      5560       5570       5580       5590       5600
TCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATA
AGCACGTGTGTCGGGTCGAACCTCGCTTGCTGGATGTGGCTTGACTCTAT
____________________PBR322 ORIGIN____________________>

      5610       5620       5630       5640       5650
CCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGG
GGATGTCGCACTCGATACTCTTTCGCGGTGCGAAGGGCTTCCCTCTTTCC
____________________PBR322 ORIGIN____________________>

      5660       5670       5680       5690       5700
CGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGG
GCCTGTCCATAGGCCATTCGCCGTCCCAGCCTTGTCCTCTCGCGTGCTCC
____________________PBR322 ORIGIN____________________>

      5710       5720       5730       5740       5750
GAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCG
CTCGAAGGTCCCCCTTTGCGGACCATAGAAATATCAGGACAGCCCAAAGC
____________________PBR322 ORIGIN____________________>

      5760       5770       5780       5790       5800
CCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGA
GGTGGAGACTGAACTCGCAGCTAAAAACACTACGAGCAGTCCCCCCGCCT
____________________PBR322 ORIGIN____________________>
```

```
      5810      5820      5830      5840      5850
GCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTT
CGGATACCTTTTTGCGGTCGTTGCGCCGGAAAAATGCCAAGGACCGGAAA
______PBR322 ORIGIN_______>

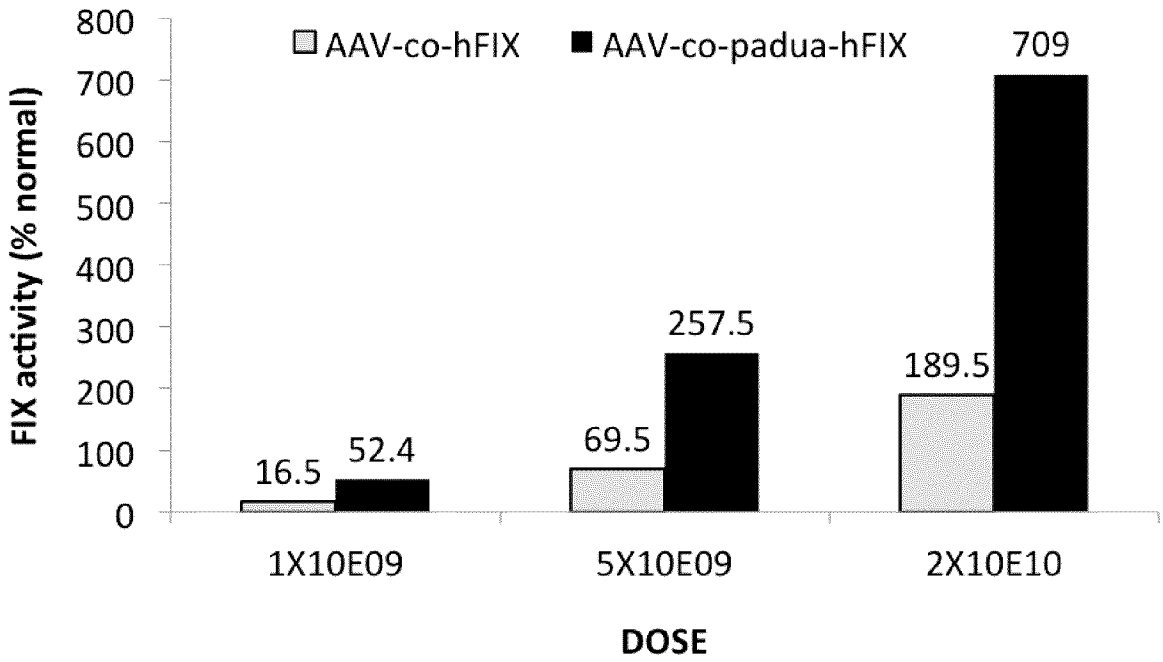
      5860      5870      5880      5890      5900
TGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGT
ACGACCGGAAAACGAGTGTACAAGAAAGGACGCAATAGGGGACTAAGACA

      5910      5920      5930      5940      5950
GGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCC
CCTATTGGCATAATGGCGGAAACTCACTCGACTATGGCGAGCGGCGTCGG

      5960      5970      5980      5990      6000
GAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCA
CTTGCTGGCTCGCGTCGCTCAGTCACTCGCTCCTTCGCCTTCTCGCGGGT

      6010      6020      6030      6040
ATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATG
TATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTAC
```

Figure 2


800
700
600
500
400
300
200
100
0
FIX activity (% normal)
AAV-co-hFIX
AAV-co-padua-hFIX
16.5
52.4
69.5
257.5
189.5
709
1X10E09
5X10E09
2X10E10
DOSE

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/071297

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/36 A61K48/00 C12N15/86
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. CANTORE ET AL: "Hyperfunctional coagulation factor IX improves the efficacy of gene therapy in hemophilic mice", BLOOD, vol. 120, no. 23, 4 October 2012 (2012-10-04), pages 4517-4520, XP55050690, ISSN: 0006-4971, DOI: 10.1182/blood-2012-05-432591 cited in the application the whole document ----- -/--	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2013	04/02/2013

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/071297

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHUETTRUMPF JOERG ET AL: "Factor IX variants improve gene therapy efficacy for hemophilia B", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 105, no. 6, 15 March 2005 (2005-03-15), pages 2316-2323, XP002555907, ISSN: 0006-4971, DOI: 10.1182/BLOOD-2004-08-2990 [retrieved on 2004-11-18] cited in the application the whole document -----	1-29