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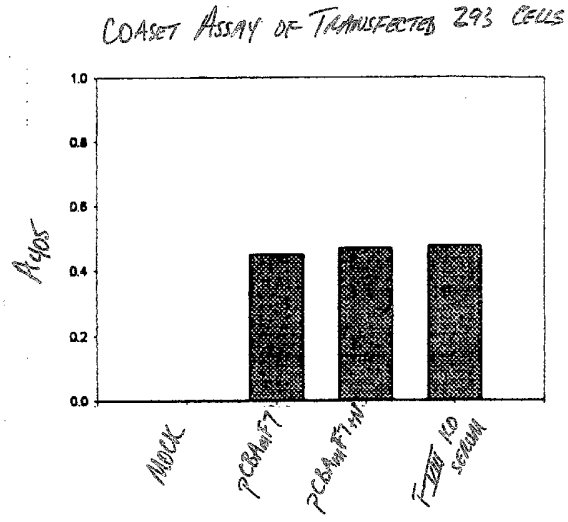
(54) Title: RAAV COMPOSITIONS AND METHODS FOR DELIVERY OF HUMAN FACTOR VII POLYPEPTIDES AND TREATMENT OF HEMOPHILIA A



(57) Abstract: Disclosed are improved recombinant adeno-associated viral (rAAV) vector compositions useful in the delivery of antihemophilic factor polypeptides to selected mammalian host cells. The disclosed rAAV vector compositions preferably comprise one or more polynucleotide sequences that express one or more mammalian Factor VII proteins, polypeptides, peptides, a operably linked to one or more promoter and/or enhancer sequences that are capable of expressing the encoded antihemophilic therapeutics in cells suitably transformed with the disclosed rAAV vector constructs, virions, and viral particles comprising the contructs of interest. These compositions, and methods for their use, including the manufacture of medicaments, have desirable therapeutic and/or prophylactic efficacy in the amelioration, treatment, and/or prevention of a variety of diseases, disorders, and dysfunctions in selected mammals, and in particular, humans diagnosed with Factor VII deficiency and/or hemophilia A.

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DESCRIPTION

rAAV COMPOSITIONS AND METHODS FOR DELIVERY OF HUMAN FACTOR VII POLYPEPTIDES AND TREATMENT OF HEMOPHILIA A

1. BACKGROUND OF THE INVENTION

The present application claims priority to United States Provisional Application Serial No. 60/392,725, filed June 28, 2002, the entire contents of which is specifically incorporated herein by reference.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and virology, and in particular, to methods for using recombinant adeno-associated virus (rAAV) compositions that express nucleic acid segments encoding therapeutic antihemophilic factor polypeptides useful in the treatment of complex human disorders, including for example, blood disorders such as hemophilia. In illustrative embodiments, methods are provided for preparing rAAV-based vector constructs that deliver one or more therapeutic antihemophilic factor compositions to cells, and in particular, human plasma protein Factor VII for the treatment of hemophilia or Factor VII deficiency.

1.2 DESCRIPTION OF RELATED ART

1.2.1 BLEEDING DISORDERS

Coagulopathic (bleeding and clotting) disorders encompass a wide range of medical problems that lead to poor blood clotting and continuous bleeding. These disorders can result from defects in the blood vessels or from abnormalities in the blood itself. The abnormalities may be in blood clotting factors or in platelets.

Coagulation, the process that controls bleeding, is a complex multi-component process that involves as many as twenty different plasma proteins, or blood clotting factors. Normally, a complex chemical process occurs using these clotting factors to form a substance called fibrin that stops bleeding. When certain coagulation factors are deficient or missing, the process doesn't occur normally.

1.2.2 HEMOPHILIA A

Hemophilia is a bleeding disorder caused by a deficiency in one of the blood clotting factors. Hemophilia A (or "classic" hemophilia) is a deficiency in clotting Factor VIII, and accounts for about 80 percent of all hemophilia cases.

Hemophilia A is a hereditary disorder in which the clotting ability of the blood is impaired and excessive bleeding results. Small wounds and punctures are usually not a problem. But uncontrolled internal bleeding can result in pain and swelling and permanent damage, especially to joints and muscles.

Severity of symptoms can vary, and severe forms become apparent early on. The incidence of hemophilia A is 1 out of 10,000 live male births. About 20,000 Americans have hemophilia. Women may have it, but the condition is very rare in females.

Hemophilia is caused by several different gene abnormalities. The severity of the symptoms of hemophilia A depends on how a particular gene abnormality affects the activity of factor VIII. When the activity is less than 1 percent of normal, episodes of severe bleeding occur and recur for no apparent reason.

The symptoms of hemophilia include bruising, spontaneous bleeding, bleeding into joints and associated pain and swelling, gastrointestinal tract and urinary tract hemorrhage, blood in the urine or stool, and prolonged bleeding from cuts, tooth extraction, *etc.*

1.2.3 FACTOR VII (PROCONVERTIN) DEFICIENCY

In addition to Factor VIII deficiency, some individuals suffer from a lack or deficiency in Factor VII. This factor is often referred to a "stable" factor or "proconvertin." Factor VII deficiency is an extremely rare disorder that can be inherited or acquired by persons without hemophilia who take Coumadin, a drug used to inhibit blood clotting. In this disorder, bleeding can vary from mild to severe within the same person over time. Bleeding doesn't always correspond with the severity of the deficiency shown in blood tests. A history of bleeding may occur in infancy or childhood. Gastrointestinal and central nervous system bleeding can also occur.

Factor VII deficiency occurs in approximately one in 500,000 males and females. Congenital Factor VII deficiency is distinguished from Acquired Factor VII Deficiency that may result from liver disease, vitamin K deficiency, or other malabsorption conditions.

Unlike hemophilia, Factor FVII deficiency is not sex-linked. It affects both males and females with equal frequency. It is also autosomal recessive, which means that if the clotting defect is inherited from a parent, the child will be a genetic carrier of the condition, but may or may not have symptoms. Those who have inherited a defective Factor VII gene from only one parent will usually have only moderate levels of the factor, but without symptoms.

The symptoms of Factor VII deficiency often include bleeding of mucous membranes, spontaneous nosebleeds, excessive bruising, prolonged menstrual bleeding, and bleeding into joints or muscles.

The diagnosis for Factor VII deficiency is usually made by testing for Factor VII in the blood, prolonged prothrombin time, or a normal partial thromboplastin time in combination with decreased Factor VII Assay.

Existing treatments for Factor VII deficiency rely on the administration of normal plasma or concentrates containing Factor VII. Severe bleeding is typically treated with fresh frozen plasma or PCCs (Prothrombin complex concentrates). However, because the life span of infused factor VII is very short (2 to 4 hours), patients require treatment every 2 to 6 hours for severe bleeding or surgery.

1.2.4 DEFICIENCIES IN THE PRIOR ART

Current treatment methods for factor VII deficiency and hemophilia A are costly, in high demand, and typically most often involve replacement therapy using plasma, plasma-derived or recombinant Factor VII or VIII. Such therapy is limited by the production of Factor VII or VIII and the short half-life of these Factors *in vitro*. In addition, patients treated *via* replacement therapy often build resistance to treatment due to an immune response to the recombinant factor. What is lacking in the prior art is treatment of Factor VII deficiency and hemophilia A by administration of therapeutically-effective amounts of human plasma protein Factor VII. What is also lacking in the prior art are methods for long-term therapy of patients who are either unresponsive or refractive to current therapy regimens.

2.0 SUMMARY OF THE INVENTION

The present invention overcomes these and other limitations inherent in the prior art by providing rAAV compositions that comprise a genetic construct that encodes one or more mammalian therapeutic polypeptides, for use in the amelioration, treatment and/prevention of a variety of bleeding disorders, such as for example, hemophilia and/or diseases or conditions that result from a lack, deficiency in, or absence of sufficient biologicall-active Factor VII peptide or polypeptides in one or more cells of the affected mammal. In illustrative embodiments, the invention discloses particular rAAV compositions useful in a variety of therapeutic and

diagnostic regimens and in the manufacture of medicaments for treating various mammalian bleeding disorders, including Factor VII deficiency and hemophilia A, in particular.

The invention provides compositions and methods for treating or ameliorating Factor VII polypeptide deficiencies in a mammal, and particularly Factor VII deficiency in a human, diagnosed with, at risk for developing, or presenting clinical symptoms of one or more bleeding disorders, such as Factor VII deficiency or hemophilia.

The invention provides methods for treating or reducing the severity or extent of Factor VII polypeptide deficiency in a human manifesting one or more of the disorders linked to a deficiency in Factor VII polypeptide in cells and tissues of a human in need thereof. In a general sense, the method involves administration of at least a first composition that comprises a rAAV-based genetic construct that encodes one or more Factor VII peptides, polypeptides, or proteins in a pharmaceutically-acceptable vehicle to the animal in an amount and for a period of time sufficient to prevent, treat, or ameliorate one or more symptoms of the Factor VII deficiency, defect, disorder, disease, or dysfunction in the animal suspected of suffering from one or more disorders linked to a deficiency in Factor VII.

The invention provides for superior advantages over the currently-existing products as it results in long-term expression of the Factor VII protein in the patient from a single, or few doses. Current products are extremely unstable and often in short supply as it is extremely difficult and costly to produce. Delivery of a gene therapy therapeuticum such as recombinant Factor VII *via* recombinant adeno-associated virus (rAAV)-mediated gene transfer overcomes the need for frequent and costly administration of exogenous Factor VII polypeptides, or administration of plasma, plasma concentrates, or plasma extracts containing the Factor VII polypeptide.

Because activated Factor VII acts through the extrinsic pathway of the clotting cascade and circumvents the necessity for functional Factor VIII, the present therapy method provides a

vital alternate form of treatment for hemophiliacs which are refractory to current replacement therapies. Additional benefits of the present therapy include the elimination of a need for repeated injections or exogenous Factor VII polypeptide. In fact, recipients of the current therapy would receive multiple benefits including, for example, the increased efficacy of treating the disease than with the currently available modalities, and an overall reduced health-care cost, as repeated injections of the purified protein are obviated by the gene therapy methods provided herein.

Many patients with hemophilia A are not able to control their disease with current replacement therapies and are left with few or no options. This invention provides a cost-effective option, with improved patient compliance, more convenient dosage and administration regimens, and less repeated injections. The recombinant AAV- Factor VII compositions disclosed herein also lower expenses due to far fewer dosings and increased stability and longevity of treatment. A single dose of recombinant AAV has been shown to produce therapeutic proteins over the course of weeks, months, and even years.

2.1 rAAV-Factor VII Vector Compositions

In a first embodiment, the invention provides an rAAV vector comprising a polypeptide that comprises at least a first nucleic acid segment that encodes a mammalian Factor VII peptide or polypeptide, and in particular, a biologically-active Factor VII (FVII) polypeptide, or biologically-active fragment thereof, operably linked to at least a first promoter capable of expressing the nucleic acid segment in a suitable host cell transformed with such a vector. In preferred embodiments, the nucleic acid segment encodes a mammalian, and in particular, a human Factor VII polypeptide, such as for example, one or more of the polypeptides as disclosed in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ

ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17.

In addition to therapeutic polynucleotides and polypeptides of human origin, the invention also encompasses treatment modalities involving the use of one or more other mammalian Factor VII genes or proteins, as may be desirable in the treatment of humans, or other mammals (such as for example, in veterinary medicine therapies), and as such, the rAAV vectors may comprise sequences of murine, porcine, feline, canine, bovine, ovine, equine, epine, caprine, or lupine origin. In an example presented herein as an illustrative embodiment of the practice of the invention, the rAAV-Factor VII constructs comprise at least a first genetic sequence that encodes a human Factor VII peptide, polypeptide, or protein, to provide therapeutic levels of the selected protein, *e.g.,* Factor VII, to the transfected cells.

Alternatively, the therapeutic constructs of the invention may encompass nucleic acid segments that encode modified hemophilia-inhibitory polypeptides obtained from any mammalian origin, and engineered by the hand of man to produce more desirable properties or characteristsics. For example, nucleic acids, peptides, and polypeptides of murine, primate, ovine, porcine, bovine, equine, epine, caprine, canine, feline, and/or lupine origin, may be used in their native or unmodified form, but also may be modified or site-specifically mutagenized, and/or otherwise genetically modified to be expressed in human cells such that their Factor VII biological activity is retained, increased, or prolonged.

Preferred rAAV vector backbones for the practice of the present invention include, but are not limited to, rAAV serotype 1 (rAAV1), rAAV serotype 2 (rAAV2), rAAV serotype 3 (rAAV3), rAAV serotype 4 (rAAV4) and rAAV serotype 5 (rAAV5) and rAAV serotype 6 (rAAV6).

The polynucleotides comprised in the vectors and viral particles of the present invention preferably comprise at least a first constitutive or inducible promoter operably linked to the nucleic acid segments disclosed herein. Such promoters may be homologous or heterologous promoters, and may be operatively positioned upstream of the nucleic acid segment encoding the therapeutic polypeptide of interest, such that the expression of the segment is under the control of the promoter. The construct may comprise a single promoter, or alternatively, two or more promoters may be used to facilitate expression of the therapeutic gene sequence. Exemplary promoters useful in the practice of the invention include, but are in no way limited to, those promoter sequences that are operable in mammalian, and in particular, human host cells, tissues, and organs, such as for example, a CMV promoter, a β-actin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter being particularly useful in the practice of the invention. In illustrative embodiments, a polynucleotide encoding a therapeutic polypeptide was placed under the control of the chicken β-actin (CBA) promoter and used to produce therapeutically effective levels of the encoded polypeptide when suitable host cells were transformed with the genetic construct.

The polynucleotides comprised in the vectors and viral particles of the present invention may also further optionally comprise one or more native, synthetic, homologous, heterologous, or hybrid enhancer or 5' regulatory elements, for example, a CMV enhancer, a synthetic enhancer, or a liver- or tissue-specific enhancer operably linked to the therapeutic polypeptide-encoding nucleic acid segments disclosed herein.

The polynucleotides and nucleic acid segments comprised within the vectors and viral particles of the present invention may also further optionally comprise one or more intron sequences.

The polynucleotides comprised in the vectors and viral particles of the present invention may also further optionally comprise one or more native, synthetic, homologous, heterologous, or hybrid post-transcriptional or 3' regulatory elements operably positioned relative to the therapeutic polypeptide-encoding nucleic acid segments disclosed herein to provide greater expression, stability, or translation of the encoded polypeptides. One such example is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), operably positioned downstream of the therapeutic gene of interest.

In illustrative embodiments, the invention concerns administration of one or more biologically-active neovascularization-inhibitory peptides or polypeptides that comprise an at least 20, at least 40, at least 60, at least 80, at least 100, at least 120, at least 140, at least 160, at least 180, at least 200, at least 220, at least 240, at least 260, at least 280, at least 300, or more contiguous amino acid sequence from one or more of the polypeptide sequences disclosed in Section 5.2 hereinbelow and particularly those polypeptides as recited in any one of SEQ ID NO:2, SEQ ID NO:4. SEQ ID NO:6. SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:17.

Likewise, in additional illustrative embodiments, the invention concerns administration of one or more biologically-active Factor VII polypeptides that are encoded by a nucleic acid segment that comprises at least 30, at least 60, at least 90, at least 120, at least 150, at least 180, at least 210, at least 240, at least 270, at least 300, at least 330, at least 360, at least 390, at least 420, at least 450, at least 480, at least 510, at least 540, at least 570, or at least 600, 700, 800, or 900, or more contiguous nucleic acid residues, up to and including substantially full-length, and full-length sequences from the DNA sequences disclosed in Section 5.2 hereinbelow and particularly those DNA sequences as recited in any one of SEQ ID NO:1, SEQ ID NO:3. SEQ ID NO:5. SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

Exemplary adeno-associated viral vector constructs and polynucleotides of the present invention include those that comprise, consist essentially of, or consist of at least a first nucleic acid segment that encodes a peptide or polypeptide that is at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical to the sequence of SEQ ID NO:2, SEQ ID NO:4. SEQ ID NO:6. SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:17, wherein the peptide or polypeptide has Factor VII activity when administered to, and expressed in, a suitable mammalian cell.

Exemplary polynucleotides of the present invention also include those sequences that comprise, consist essentially of, or consist of at least a first nucleic acid segment that encodes a polypeptide that is at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical to the amino acid sequence of any one of SEQ ID NO:2, SEQ ID NO:4. SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, or SEQ ID NO:17, wherein the peptide or polypeptide encoded by the nucleic acid segment has Factor VII activity when administered to a mammalian eye.

Particularly preferred adeno-associated viral vector constructs and polynucleotides of the present invention include those that comprise, consist essentially of, or consist of at least a first nucleic acid segment that is at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about

98%, or at least about 99% identical to the sequence of any one of SEQ ID NO:1, SEQ ID NO:3. SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13, wherein the segment encodes a peptide or polypeptide that has Factor VII activity when administered to, and expressed in, a suitable mammalian cell.

Exemplary polynucleotides of the present invention also include those sequences that comprise, consist essentially of, or consist of at least a first nucleic acid segment that is at least about 75%, at least about 76%, at least about 77%, at least about 78%, at least about 79%, at least about 80%, at least about 81%, at least about 82%, at least about 83%, at least about 84%, at least about 85%, at least about 86%, at least about 87%, at least about 88%, at least about 89%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identical to the sequence of any one of SEQ ID NO:1, SEQ ID NO:3. SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13, wherein the peptide or polypeptide encoded by the nucleic acid segment has Factor VII activity when administered to a mammalian eye.

2.2 rAAV Viral Particles and Virions, and Host Cells Comprising Them

Other aspects of the invention concern rAAV particles and virions that comprise the vectors of the present invention, pluralities of such particles and virions, as well as pharmaceutical compositions and host cells that comprise one or more of the rAAV vectors disclosed herein, such as for example pharmaceutical formulations of the rAAV vectors or virions intended for administration to a mammal through suitable means, such as, by intramuscular, intravenous, or direct injection to selected cells, tissues, or organs of the mammal, for example, to the muscle tissue, the circulatory system, or directly to one or more organs of the selected mammal, such as for example, by direct administration to the liver, or to liver cells. Typically, such compositions will be formulated with pharmaceutically-

acceptable excipients, buffers, diluents, adjuvants, or carriers, as described hereinbelow, and may further comprise one or more liposomes, lipids, lipid complexes, microspheres, microparticles, nanospheres, or nanoparticle formulations to facilitate administration to the selected organs, tissues, and cells for which therapy is desired.

Further aspects of the invention include mammalian host cells, and pluralities thereof that comprise one or more of the adeno-associated viral vectors, virions, or viral particles as disclosed herein. Particularly preferred cells are human host cells, and in particular, human bone, blood, liver, pancreatic, kidney, muscle, heart, lung, epithelial, endothelial, or vascular cells.

2.3 THERAPEUTIC KITS AND PHARMACEUTICAL COMPOSITIONS

The compositions of the invention also will optionally further comprising at least a first pharmaceutical vehicle, and particularly those formulations that are acceptable for administration to a human through one or more conventional routes of administration, such as for example, oral, nasal, inhalation, trasndermal, intravenous, subcutaneous, or intramuscular administration. The compositions of the invention may also further comprise one or more liposomes, lipids, proteins, peptides, polypeptides, nucleic acids, polysaccharides, antibodies, antigens, antigen binding fragments, enzymes, lipid complexes, or at least a first detectable label, marker, or tag. The rAAV particles may be conjugated or otherwise associated with one or more surfaces of a micro- or nanoparticle, such as for example, the nanospheres and microspheres described herein.

Therapeutic kits for treating or ameliorating the symptoms of hemophilia, bleeding or clotting disorders, or other condition resulting from defect, deficiency, or dysfunction of the native Factor FII polypeptide in a mammal are also part of the present invention. Such kits typically comprise one or more of the disclosed AAV vector constructs, virion or virus

particles, host cells, or therapeutic AAV compositions described herein, and instructions for using the kit.

Another important aspect of the present invention concerns methods of use of the disclosed vectors, virions, compositions, and host cells described herein in the preparation of medicaments for treating or ameliorating the symptoms of a bleeding disorder, clotting disorder, hemophilia, or other conditions resulting from a defect, deficiency, or dysfunction of Factor VII polypeptide in a mammal. Such methods generally involve administration to a mammal, or human in need thereof, one or more of the disclosed vectors, virions, host cells, or compositions, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a defect, dysfunction, or deficiency in the affected mammal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms. Such symptoms may include, but are not limited to, increased bleeding time, increased clotting time, or hemophilia in affected animals.

Another aspect of the invention concerns compositions that comprise one or more of the disclosed adeno-associated viral vectors, virions, viral particles, and host cells as described herein. Pharmaceutical compositions comprising such are particularly contemplated to be useful in therapy, and particularly in the preparation of medicaments for treating Factor VII deficiency, dysfunction, or defect in affected mammals, and humans in particular.

The present invention also provides for a host cell that comprises the rAAV-Factor VII compositions disclosed herein. Preferably, such host cells are mammalian cells, with human host cells being particular preferred. For example, the host cell may be a human

pancreas, kidney, muscle, epithelial, endothelial, perivascular, liver, heart, lung, brain, blood, bone, or nerve cell.

2.4 THERAPEUTIC METHODS

The invention also provides methods for delivering therapeutically-effective amounts of a biologically-active Factor VII polypeptide to a mammal in need thereof. Such methods generally comprise at least the step of providing or administering to such a mammal, one or more of the AAV-Factor VII compositions disclosed herein. For example, the method may involve providing to such a mammal, one or more of the rAAV vectors, virions, viral particles, host cells, or pharmaceutical compositions as described herein. Preferably such providing or such administration will be in an amount and for a time effective to provide a therapeutically-effective amount of one or more of the Factor VII peptides or polypeptides disclosed herein to selected cells, tissues, or organs of the mammal, and in particular, therapeutically-effective levels to the cells, tissues, or organs of the mammal. Such methods may include systemic injection(s) of the therapeuticum, or may even involve direct or indirect administration, injection, or introduction of the therapeutic compositions to particular cells, tissues, or organs of the mammal, such as for example, by direct injection into muscle or liver tissues.

The invention also provides methods of treating, ameliorating the symptoms, and reducing the severity of Factor VII deficiency in an animal. These methods generally involve at least the step of providing to an animal in need thereof, one or more of the rAAV vector compositions disclosed herein in an amount and for a time effective to treat the Factor VII deficiency or other related dysfunction in the animal. As described above, such methods may involve systemic injection(s) of the therapeuticum, or may even involve

direct or indirect administration, injection, or introduction of the therapeutic compositions to particular cells, tissues, or organs of the animal.

In one embodiment, the invention provides a method for treating, preventing, or ameliorating the symptoms of a Factor VII protein, peptide, or polypeptide deficiency or dysfunction in a mammal. The method generally involves administering to a mammal in need thereof, one or more of the disclosed rAAV-Factor VII vector compositions disclosed herein, in an amount and for a time sufficient to treat, prevent, or ameliorate the symptoms of the Factor VII deficiency or the bleeding disorder or dysfunction, such as *e.g.*, hemophilia, in the mammal. In preferred embodiments, the mammal is a human that is has, is at risk for developing, or has been diagnosed with one or more diseases, disorders, or dysfunctions that result from the deficiency or lack of one or more Factor VII peptides, polypeptides, or proteins normally present in a healthy subject.

In such cases, the compositions of the invention may be administered to the patient in an amount and for a time sufficient to treat or prevent the symptoms of the Factor VII deficiency or dysfunction through a single dose, or by administration of a plurality of doses given over a relatively short, or even relatively long period of therapy. The patient may require only one or two administrations of the disclosed rAAV constructs to achieve relatively short-term, relatively medium-term, or even relatively long-term treatment. For example, one or two administrations of the disclosed compositions may provide sufficient therapeutic levels of the Factor VII composition for a period of several days, several weeks, or several months. Alternatively, three or four administrations of the disclosed compositions either over a relatively short, or relatively long administration period, may provide sufficient therapeutic levels of the Factor VII composition for a period of several weeks, several months, several years, or even tens of years, up to and including the natural lifetime of the treated mammal.

When relatively short-term therapy is warranted, the therapeutic effectiveness of a single administration or of multiple administrations of the disclosed compositions may persist for a period of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days or more, and even up to an including a period of about 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 days or more. When relatively medium-term therapy is warranted, the therapeutic effectiveness of a single administration or of multiple administrations of the disclosed compositions may persist for a period of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks or more, and even up to an including a period of about 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 weeks or more, such as for example, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, or about 30 weeks or more, and even up to an including a period of about 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 weeks or more. Likewise, when relatively long-term therapy is warranted, the therapeutic effectiveness of a single administration or of multiple administrations of the disclosed compositions may persist for a period of about 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 weeks or more, and even up to an including a period of about 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60, 70, 80, 90, 100, or even 200 or 300 weeks or more. As such, the inventors contemplate that particular therapeutic regimens involving one or more of the compositions disclosed herein will provide a biologically- effective amount of the Factor VII peptide, polypeptide, or protein, to the individual to which such compositions have been administered, for periods of at least about 1 month, at least about 2 months, at least about 3 months, at least about 4 months, at least about 5 months, at least about 6 months, at least about 7 months, at least about 8 months, at least about 9 months, at least about 10 months, at least about 11 months, at least about 12 months, and up to and including periods of therapy that persist in the treated individual for periods of at least about 1 year, at least about 2 years, at least about 3 years, at least about 4 years, year, at least about 6 years, at least about 7

years, at least about 8 years, at least about 9 years, or even at least about 10 or more years, up to and including the natural lifetime of the treated individual.

The rAAV-Factor VII compositions disclosed herein may be administered by any of the conventional drug delivery methods, such as for example, orally, intranasally, transdermally, intramuscularly, intravenously, subcutaneously, intrathecally, intraperitoneally, or by absorption, inhalation or direct injection into at least a first organ or at least a first tissue of the patient as may be required. Exemplary organs and tissues which may find particular benefit through administration of one or more of the compositions disclosed herein include, but are not limited to, the vascular or circulatory system, the pancreas, liver, heart, lung, brain, kidney, joint, bone, neural, and muscles.

In yet another embodiment, the invention provides kits for treating, preventing, or ameliorating the symptoms of a Factor VII protein, peptide, or polypeptide-related deficiency or disorder in a mammal, comprising (i) one or more rAAV-Factor VII composition disclosed herein; and (ii) instructions for using the kit in diagnostic, therapeutic, or prophylactic treatment regimens.

3. BRIEF DESCRIPTION OF THE DRAWING

The following drawing forms part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawing, in which like reference numerals identify like elements, and in which:

FIG. 1 shows that functional Factor VII can be produced *in vitro*. The chromogenic Coaset assay was used to determine the levels of functional Factor VII secreted. Factor VIII KO plasma was used as a positive control as these mice have been shown to produce normal levels of Factor VII.

4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

The practical feature of this invention is the ability to treat hemophilia patients that are resistant to current therapy. AAV-mediated gene delivery has demonstrated long-term correction of the diseased state with a minimal pathology. Optimally, a single delivery of AAV-Factor VII should result in disease correction for the lifetime of the individual.

4.1 ADENO-ASSOCIATED VIRUS

Adeno-associated virus (AAV) is a single-stranded DNA parvovirus with a 4.7 kb genome and a particle diameter of approximately 20 nm. The AAV genome is flanked by two identical inverted terminal repeat (ITR) sequences (Lusby *et al.*, 1980). These ITRs provide all the *cis*-acting sequence required for replication, packaging and integration (Samulski *et al.*, 1989). There are two large open reading frames (Srivastava *et al.*, 1983). The open reading frame in the right half of the genome (*cap*) encodes 3 overlapping coat proteins (VP1, VP2 and VP3). The open reading frame in the left half (*rep* gene) encodes 4 regulatory proteins with overlapping sequences which are known as *Rep* proteins (*Rep*78, *Rep*68, *Rep*52 and *Rep*40), because frame shift mutations at most locations within the open reading frame inhibit viral DNA

replication (Hermonat *et al.*, 1984). The *Rep* proteins are multi-functional DNA binding proteins. The functions of the *Rep* proteins in viral DNA replication include helicase activity and a site-specific, strand-specific endonuclease (nicking) activity (Ni *et al.*, 1994).

AAV infects a broad spectrum of vertebrates from birds to humans, although in nature specific types are species specific (Berns, 1996). In humans AAV can infect a large variety of cells derived from different tissues. The infection of AAV is ubiquitous within the population with about 90% of adults being seropositive (Cukor *et al.*, 1983). In spite of its omnipresence, AAV has never been associated with any human disease. In this sense, rAAV is the safest of the currently used gene therapy vectors.

Because of its propensity to establish latency and because it has not been implicated as a pathogen, AAV has been of considerable interest as a potential vector for human gene therapy (Flotte and Ferkol, 1997; Flotte and Carter, 1995). In general, rAAV vectors are produced by deleting the viral coding sequences and substituting the transgene of interest under control of a non-AAV promoter between the two AAV inverted terminal repeats (ITRs). When the *rep* and *cap* proteins are expressed *in trans* in Ad-infected cells, rAAV genomes can be efficiently packaged. Considerations in the development of AAV as a vector have included difficulties in attaining high vector titers and the limited insertional capacity (>5 kb). Although these issues can still be improved, recently developed packaging techniques for high titer and Ad-contamination free vectors, and strategies to overcome the packaging limitation, have dramatically impacted the applications of rAAV (Zolotukhin *et al.*, 1999; Duan *et al.*, 2000; Yan *et al.*, 2000). Unlike adenovirus vectors, rAAV vectors are remarkably nonimmunogenic with little host response (Jooss *et al.*, 1998; Song *et al.*, 1998). In addition to the above unique features, rAAV have mediated long-term transgene expression in a wide variety of tissues, including muscle (Song *et al.*, 1998; Kessler *et al.*, 1996; Xiao *et al.*, 1996; Clark *et al.*, 1997; Snyder *et al.*, 1997a), lung (Flotte *et al.*, 1993), liver (Snyder *et al.*, 1997b; Xiao *et al.*, 1998;

Song *et al.*, 2001a; Xu *et al.*, 2001), brain (Kaplitt *et al.*, 1994) and eye (Flannery *et al.*, 1997). Thus rAAV vectors appear to have significant advantages over other commonly used viral vectors.

Six serotypes of AAV have been cloned and sequenced. Of the six AAV serotypes, serotype 2 (AAV2) is the best-characterized and has been predominantly used in gene transfer studies. Membrane-associated heparan sulfate proteoglycan is the primary receptor for AAV type 2 (Summerford and Samulski, 1998). Human fibroblast growth factor receptor 1 and $\alpha_V\beta_5$ integrin are co-receptors for AAV2 (Qing *et al.*, 1999; Summerford *et al.*, 1999). Serotypes 1 and 6 share >99% amino acid homology in their capsid proteins. Sequence analysis supports a recombination event between seroType I and 2. Comparison of the serotype capsid amino acid sequences suggests that serotypes, 1, 2, and 3 share homology across the three capsids in accord with heparan sulfate binding (Summerford and Samulski, 1998). In contrast, AAV type 4 and 5 are the most divergent of the six AAV serotypes, exhibiting only 60% homology to AAV2 or to each other. AAV4 and AAV5 require different sialic acid-containing glycoproteins for binding and transduction of target cells. The different tropisms of AAV serotypes provide opportunities to optimize the transduction efficiency in different target cells. Data showed that of the serotypes, AAV1 mediated the highest transgene expression in skeletal muscle and murine islets (Chao *et al.*, 2000).

The AAV life cycle is quite unusual (Berns and Linden, 1995). AAV binds to cells via a heparan sulfate proteoglycan receptor (Summerford and Samulski, 1998). Once attached, AAV entry is dependent upon the presence of a co-receptor, which may consist of either the fibroblast growth factor receptor (FGF-R) (Qing *et al.*, 1999) or the α_v-β_5 integrin molecule (Summerford *et al.*, 1999). Cells infected with AAV and a helper virus (or another adjunctive agent, such as UV irradiation or treatment with genotoxic agents) will undergo productive replication of AAV prior to cell lysis, which is induced by the helper rather than by AAV. Human cells infected

with AAV alone, however, will become persistently infected (Berns *et al.*, 1975). This latency pathway often results in colinear integration of AAV sequences within the host cell genome (Cheung *et al.*, 1980), often within a specific site on human chromosome 19, the AAVS1 site (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Kotin *et al.*, 1992; Samulski *et al.*, 1991; Samulski, 1993. While this site is not strictly homologous to AAV, it contains consensus elements required for binding and nicking by the AAV Rep protein, a non-structural protein that is also involved in productive replication and in transcriptional regulation of the virus (Weitzman *et al.*, 1994; Giraud *et al.*, 1994; Giraud *et al.*, 1995; Linden *et al.*, 1996). Once AAV is integrated, it will remain stable within infected cells for prolonged periods of time, up to 100 passages (Hoggan *et al.*, 1972). Episomal forms of the virus may also be present for extended periods in some circumstances (Afione *et al.*, 1996; Kearns *et al.*, 1996; Flotte *et al.*, 1994). If latently infected cells are subsequently infected with a helper virus, the genome will be excised and productive AAV replication and packaging will ensue (Senapathy *et al.*, 1984; Afione *et al.*, 1996).

The AAV genome consists of two 145-nucleotide inverted terminal repeat (ITR) sequences, each an identical palindrome at either terminus of the virus, flanking the two AAV genes, *rep* and *cap* (Tratschin *et al.*, 1984). The *rep* gene is transcribed from two promoters, the p5 promoter (at map position 5) and the p19 promoter (map position 19), which is embedded within the coding sequence of the longer Rep proteins. In each case, both the spliced and unspliced transcripts are processed and translated. This allows for the production of 4 Rep proteins, Rep78, Rep68, Rep52, and Rep40. Rep78 and Rep68 are multifunctional DNA binding proteins which possess helicase activity and site-specific, strand-specific nickase activity, both of which are required for terminal resolution of replicating AAV genomes (Im and Muzyczka, 1990). The long Rep proteins are also capable of binding to the chromosomal target sequence for AAV integration, the AAVS1 site, and these proteins are required for normal integration into this site. Finally, Rep78/68 are potent bi-functional transcription regulators that

generally activate transcription from AAV promoters during productive infection and repress their transcription during latent infection (Pereira and Muzyczka, 1997; Pereira *et al.*, 1997). The shorter Rep proteins, Rep52 and Rep40 act as modifier proteins for long Rep transcriptional activities, and are required for sequestration of single-stranded AAV genomes into capsids during productive infection.

The AAV *cap* gene is transcribed from the p40 promoter. The 5' end of the mRNA transcript from p40 contains an intron which can utilize either of two downstream splice acceptor sites. The longer of the two processed messages contains an ATG codon which is used in the translation of VP1, the longest of the three AAV capsid proteins. The shorter mRNA can initiate translation using either a non-canonical ACG start codon, which represents the start of VP2, or an ATG codon further downstream, which comprises the N-terminal Met of VP3 (Trempe and Carter, 1988). VP3 is the shortest and most abundant of the AAV capsid proteins, but all three are required for the production of infectious virions.

4.2 Recombinant AAV Vectors

Recombinant AAV (rAAV) vectors have been developed by replacement of the viral coding sequences with transgene of interest (Tratschin *et al.*, 1984; Hermonat and Muzyczka, 1984). The ITR sequences must be retained in rAAV since these serve as origins for viral DNA replication and contain the packaging signals. The maximum packaging capacity of rAAV is approximately 5 kb, including the ITRs, the transgene, its promoter, and polyadenylation signal (Flotte *et al.*, 1992; Dong *et al.*, 1996). The full exploitation of rAAV for gene transfer has been limited in the past primarily by the packaging and purification process. In particular, contamination of rAAV vector preparations with wild-type AAV has been found to alter the biological behavior of the vector, and limitations on the titers and infectivity of the vectors have limited their widespread use on the past. Recent advances in the packaging and purification

technology have resulted in a dramatic improvement in the expression levels that have been achievable *in vivo*. In particular, the use of adenoviral plasmids and of complementing *rep* gene expression constructs that express less of the longer Rep proteins (Rep68/78) has resulted in a substantial improvement in the efficiency of vector production on a per cell basis (Xiao *et al.*, 1998; Li *et al.*, 1997). The availability of packaging cell lines has also resulted in a substantial improvement in the scale-ability of the packaging process (Clark *et al.*, 1996; Flotte *et al.*, 1995; Gao *et al.*, 1998). Finally, the availability of several column chromatography methods, including heparin sulfate affinity column chromatography, has allowed for the elimination of CsCl banding, which in turn appears to have enhanced the infectivity of output particles (Zolotukhin *et al.*, 1999).

rAAV vectors are uniquely suitable for *in vivo* gene therapy for genetic and metabolic disorders, since they are non-toxic (Flotte *et al.*, 1993; Conrad *et al.*, 1996; Flotte and Carter, 1998), highly efficient when used at high titers, relatively non-immunogenic (Jooss *et al.*, 1998; Hernandez *et al.*, 1999; Beck *et al.*, 1999), and very stable in their pattern of expression. The utility of rAAV vectors for *in vitro* and *in vivo* gene transfer has now been well established. There appear to be important tissue specific differences in rAAV effects, however. rAAV vectors have been found to be particularly efficient for gene transfer into terminally differentiated cells such as neurons (Kaplitt *et al.*, 1994; McCown *et al.*, 1996; Peel *et al.*, 1997; Mandel *et al.*, 1997), myofibers (Xiao *et al.*, 1996; Kessler *et al.*, 1996; Clark *et al.*, 1997; Fisher *et al.*, 1997; Song *et al.*, 1998), and photoreceptor cells (Flannery *et al.*, 1997; Lewin *et al.*, 1998; Zolotukhin *et al.*, 1996; Rolling *et al.*, 1999). Gene transfer to the bronchial epithelium has also been demonstrated (Flotte *et al.*, 1993; Conrad *et al.*, 1996; Afione *et al.*, 1996; Flotte *et al.*, 1998; Halbert *et al.*, 1998), although the efficiency of transduction remains relatively low. Likewise, rAAV transduction of hepatocytes has also been studied, and has been found to be efficient enough to provide a potential therapeutic strategy for hemophilia B, by

providing persistent and therapeutic concentrations of human Factor IX in mice (Snyder *et al.*, 1997). However, in that study, *in situ* hybridization results indicated that only 5% of hepatocytes had been transduced (Miao *et al.*, 1998).

In the case of each of these two cell types, recent evidence has shown that the efficiency can be enhanced by altering the capsid to incorporate ligands for a receptor that is abundantly expressed on the cell surface and by optimizing the promoter usage (Wu *et al.*, 2000; Virella-Lowell *et al.*, 1999). Similar manipulations are also advantageous in pancreatic islet cells. Recent reports of severe dose-related clinical adverse events due to adenovirus, although not directly reflective of rAAV, underscore the necessity of minimizing the dose of vector whenever possible.

4.3 RAAV THERAPY FOR HUMAN DISEASES

During recent years, viral vector-based human gene therapy approaches have been developed as potentially effective, alternative treatment modalities for a variety of diseases. In particular, adeno-associated virus 2 (AAV), a defective parvovirus of human origin, has been demonstrated to be one such promising vector. AAV is an ideal vector for viral-based human gene therapy because it has not been associated with any known pathology and post-infection, the viral genome integrates into the human chromosome (Muzyczka, 1992). Recombinant adeno-associated virus (rAAV) vectors have important utility as vehicles for the *in vivo* delivery of polynucleotides to target host cells (Kessler *et al.*, 1996; Koeberl *et al.*, 1997; Kotin, 1994; Xiao *et al.*, 1996). rAAV vectors are useful vector for efficient and long-term gene transfer in a variety of mammalian tissues, *e.g.,* lung (Flotte *et al.*, 1993), muscle (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Clark *et al.*, 1997; Fisher *et al.*, 1997), brain (Kaplitt *et al.*, 1994; Klein *et al.*, 1998) retina (Flannery *et al.*, 1997; Lewin *et al.*, 1998), and liver (Snyder, 1997).

It has also been shown that rAAV can evade the immune response of the host by failing to transduce dendritic cells (Jooss *et al.*, 1998). Clinical trials have been initiated for several important mammalian diseases including hemophilia B, muscular dystrophy and cystic fibrosis (Flotte *et al.*, 1996; Wagner *et al.*, 1998; Flotte and Carter, 1995; Kay *et al.*, 2000). As with most gene therapy vectors, obstacles in the efficient use of rAAV vectors for a variety of disease models include sub-therapeutic levels of transduction and the ability to target the site(s) of gene transfer.

4.4 PROMOTERS AND ENHANCERS

Recombinant AAV vectors form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, to generate a biologically-active Factor VII polypeptide product from a transcribed gene.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a

promoter that is not normally associated with a biologically-active Factor VII gene in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the expression of the biologically-active Factor VII-encoding DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced DNA segment.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the biologically-active Factor

VII polypeptide-encoding nucleic acid segment in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter, such as a beta-actin, CMV, an HSV promoter, or even a human tissue-specific or otherwise inducible promoter. In certain aspects of the invention, the chicken beta-actin promoter has been demonstrated to be particularly desirable in some embodiments disclosed herein.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 1 and 2 below list several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of the present biologically-active therapeutic polypeptide-encoding nucleic acid segments comprised within the rAAV-Factor VII vectors and compositions of the present invention. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression, but merely to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchison and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990

PROMOTER/ENHANCER	REFERENCES
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Ornitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987a
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987

PROMOTER/ENHANCER	REFERENCES
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch *et al.*, 1990
$\alpha_{1\text{-Antitrypain}}$	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988

PROMOTER/ENHANCER	REFERENCES
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Choi *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989

PROMOTER/ENHANCER	REFERENCES
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a

ELEMENT	INDUCER	REFERENCES
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active Factor VII polypeptide or a ribozyme specific for such a biologically-active Factor VII polypeptide product, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous DNA segment. Engineered cells are thus cells having DNA segment introduced through the hand of man.

To express a biologically-active Factor VII encoding gene in accordance with the present invention one would prepare an rAAV expression vector that comprises a biologically-active Factor VII polypeptide-encoding nucleic acid segment under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of "recombinant expression" in this context. Particularly preferred recombinant vector constructs are those that comprise an rAAV vector. Such vectors are described in detail herein.

4.5 PHARMACEUTICAL COMPOSITIONS

In certain embodiments, the present invention concerns formulation of one or more of the rAAV compositions disclosed herein in pharmaceutically acceptable solutions for administration to a cell or an animal, either alone or in combination with one or more other modalities of therapy, and in particular, for therapy of the mammalian pancreas and tissues thereof, such as for example, islet cells.

It will also be understood that, if desired, nucleic acid segments, RNA, DNA or PNA compositions that express one or more of the biologically-active Factor VII therapeutic gene products as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, proteins or polypeptides or various pharmaceutically-active agents, including one or more systemic or direct administrations of therpeutic proteins, polypeptides, peptides, antisense compounds, ribozymes, or biologically active fragments, or variants thereof. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The

disclosed rAAV-Factor VII compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA, DNA, or PNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.,* oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In certain circumstances it will be desirable to deliver the AAV vector-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intraopancreatically, parenterally, intravenously, intramuscularly, intrathecally, or even orally, intraperitoneally, or by nasal inhalation, including those modalities as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds

as freebase or pharmacologically acceptable salts may be prepared in sterile water and may also suitably mixed with one or more surfactants, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.,* glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

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

aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

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

The AAV vector compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and

the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human, and in particular, when administered to human cells that express LDLR polypeptides. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

4.6 LIPOSOME-, NANOCAPSULE-, AND MICROPARTICLE-MEDIATED DELIVERY

In certain embodiments, the inventors contemplate the use of liposomes, nanocapsules, nanospheres, nanoparticles, nanofibers, microparticles, microspheres, microfibers, lipid particles, vesicles, and the like, for the introduction of the rAAV-Factor VII vectors of the present invention into suitable hosts, and in particular, humans. A microparticle is well understood in the art to be "a small unit of material with a phase boundary separating it from its

surroundings, such as a solid fragment or liquid droplet suspended in blood or water. The size of microparticles are typically on the order of 5 mm or less in diameter, and they may assume any shape, such as spherical, polygonal, fiber-like, or simply a fractured piece of a larger structure. Nanoparticles are understood to encompass even smaller particles, typically those having diameters in the 5 micron or less range. These particles can be made by a wide variety of known methods such as suspension, emulsion or dispersion polymerization. Ball-milling, or grinding (for instance of a larger piece cooled to low temperature if not brittle at room temperature) can be used to create smaller fragments. Aerosol spraying and solidification by cooling or photo cross-linking can also be employed to prepare microparticles or nanoparticles of preferred sizes and compositions. Ultrasonication, for example, may be used to disperse one liquid in another. Many microparticles can be obtained directly from commercial sources (for example, Bangs Laboratories) or simply made by precipitating iron oxides and using those particles without polymers, or co-precipitation them with other solids.

In additions, combinations of materials may also be used to prepare suitable microspheres. For example, polymers of degradable solids (polylactic acid, polyglycolic acid, and their copolymers) or hydrogels such as dextran or starch or alginate, or non-degradable hydrogels such as polyhydroxyethyl methacrylate (PHEMA), or even non-degradable solids like polystyrene or hydroxy apatite or plaster of paris may also be used, either alone, or in combination with one or more magnetic field-responsive compounds (*e.g.,* magnetite) to form mixed microparticles. Of course, even finely milled particles of magnetic metals (*e.g.,* iron metal) may also be used as particulate substrates, owing to their highly desirable magnetic properties.

Such nanoparticle- and microparticle-associated formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the rAAV-Factor VII vectors disclosed herein. Likewise, the inventors also contemplate the use of other delivery regimens to

improve introduction of the rAAV vectors into suitable animals. Such delivery regimens may include, for example, the use of nanocapsules, liposomes, lipid particles, and the like. The formation and use of liposomes for pharmaceutical administration is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically incorporated herein by reference in its entirety). Further, various methods of liposome and liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety). Exemplary microparticles and nanoparticles, and methods for their synthesis have been described in a variety of patents including, for example, U.S. Patents 6,383,470, 6,346,274, 6,238,294, 6,284,280, 6,331,310, 6,254,890, 6,177,088, 5,972,707, 6,2587,588, and 6,361,994, each specifically incorporated herein by reference in its entirety.

Magnetically responsive microparticles are useful in biological techniques requiring the separation of bound from free fractions. Magnetically responsive particles useful in immunoassays, for the separation of cells, as magnetic resonance imaging agents, etc. have been described in U.S. Pat. Nos. 3,215,572, 4,452,773, 4,795,698, 4,770,183, 4,695,392, 4,329,241, 4,230,685, 4,177,253, 5,069,216, 5,091,206, and 5,705,628, 5,597,530, all incorporated herein by reference.

The targeting of pharmaceutical substrates to particular portions of the body using magnetic field-responsive microparticles has also gained clinical importance in recent years. In particular, the use of magnetically responsive microparticles may be used to selectively target

one or more regions of the body by administration of the pharmaceutically-active ingredients bound to the particles, and then placing the body in a magnetic field, and using this field to enrich the population of magnetic microbeads in the focus of the magnetic field. U. S. Patent 6,178,871 (specifically incorporated herein by reference in its entirety) illustrates the use of external magnetic fields to target magnetic materials within an animal.

Such biologically-active magnetic particles may find use in a variety of preparative and diagnostic techniques, including those described herein. Among these is high gradient magnetic separation (HGMS) which uses a magnetic field to separate magnetic particles from suspension. In instances where these particles are attached to biological materials of interest (e.g., cells, drugs), the material of interest may thereby be separated from other materials not bound to the magnetic particles. Because of their magnetic properties, these materials also function as contrast agents for magnetic resonance imaging.

As used herein, the term "resuspendable coated particle" refers to a finely divided solid, which forms a colloidal suspension and may be separated from the suspension and subsequently resuspended. "Magnetic" encompasses material which may or may not be permanently magnetic, which also may be paramagnetic or superparamagnetic but which in all cases exhibits a response in a magnetic field, *i.e.,* is magnetically responsive. "Disrupted" particles are those which are too small to contain a complete magnetic domain or, alternatively, whose Brownian energy exceeds their magnetic moment. Generally, such particles are less than 0.03 mu. in size.

Liposomes have been used effectively to introduce genes, drugs (Heath and Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta and Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller and Baltimore, 1984), transcription factors and allosteric effectors (Nicolau and Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been

completed (Lopez-Berestein *et al.,* 1985a; 1985b; Coune, 1988; Sculier *et al.,* 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori and Fukatsu, 1992).

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

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1988), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often

is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Targeting is generally not a limitation in terms of the present invention. However, should specific targeting be desired, methods are available for this to be accomplished. Antibodies may be used to bind to the liposome surface and to direct the antibody and its drug contents to specific antigenic receptors located on a particular cell-type surface. Carbohydrate determinants (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous injection of liposomal preparations would be used, but other routes of administration are also conceivable.

Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the AAV vector-based polynucleotide compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-

Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be are easily made, as described (Couvreur *et al.*, 1980; Couvreur, 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

4.7 Additional Modes of Delivery

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the disclosed rAAV vector based polynucleotide compositions to a target cell or animal. Sonophoresis (*i.e.*, ultrasound) has been used and described in U. S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U. S. Patent 5,779,708), microchip devices (U. S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U. S. Patent 5,770,219 and U. S. Patent 5,783,208) and feedback-controlled delivery (U. S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

4.8 Therapeutic and Diagnostic Kits

The invention also encompasses one or more compositions together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular rAAV-polynucleotide delivery formulations, and in the preparation of therapeutic agents for administration to a mammal, and

in particularly, to a human, for one or more of the deficiencies, dysfunctions, or abnormalities described herein. In particular, such kits may comprise one or more of the disclosed microsphere-conjugated rAAV compositions in combination with instructions for using the viral vector in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include murines, bovines, equines, ovines, epines, porcines, canines, felines, and other animals of veterinary significance which may benefit from the therapeutic and/or prophylactic methods disclosed herein. The composition may include partially or significantly purified rAAV compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

Therapeutic kits may also be prepared that comprise at least one of the compositions disclosed herein and instructions for using the composition as a therapeutic agent. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed rAAV composition(s) may be placed, and preferably suitably aliquoted. Where a second biologically-active therapeutic polypeptide composition is also provided, the kit may also contain a second distinct container means into which this second composition may be placed. Alternatively, the plurality of biologically-active therapeutic polypeptide compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container means. The kits of the present invention will also typically include a

means for containing the vial(s) in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.9 METHODS OF NUCLEIC ACID DELIVERY AND DNA TRANSFECTION

In some embodiments, it may be desirable to use other methods for the transfer of expression constructs into target mammalian cells. Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use, as discussed below. Likewise, in some applications, it may be desirable to transfer a naked DNA expression construct into cells using methods such as particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them. Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force. The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In certain embodiments, it is contemplated that one or more polynucleotide compositions disclosed herein will be used to transfect an appropriate host cell. Technology for introduction of nucleic acids into cells is well known to those of skill in the art. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984; Suzuki et al., 1998; Vanbever *et al.*, 1998), direct microinjection (Capecchi, 1980; Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Takakura, 1998) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990; Klein *et al.*, 1992), and receptor-mediated

transfection (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

4.10 COMPLEMENTARITY AND HOMOLOGY OF POLYNUCLEOTIDE SEQUENCES

The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an nucleotide sequence, is substantially complementary to all or a portion of the selected target sequence, and thus will specifically bind to a portion of the selected target sequence, such as for example, in a hybridization methodology. As such, typically the sequences will be highly complementary to the selected target sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the complementary portion of the sequence (*e.g.*, sequences that are about 90% homologous, about 91% homologous, about 92% homologous, about 93% homologous, about 94% homologous, about 95% homologous, about 96% homologous, about 97% homologous, about 98% homologous, or about 99% homologous, *etc.*). In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary (*e.g.*, 100% homologous or 100% complementary) to the selected sequence to which the nucleotide sequence specifically binds, and therefore have zero mismatches along the complementary stretch. For example, in the case of oligonucleotides that bind to a specific cellular mRNA (*e.g.*, antisense molecules), highly complementary sequences will typically bind quite specifically to the target sequence region of the candidate mRNA sequence and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product. Such methodologies are particularly preferred for therapies in which it is desirable to reduce, alter, or eliminate expression of one or more polypeptides in a selected cell through the introduction of one or more rAAV-vectored antisense oligonucleotides.

In such cases, substantially complementary oligonucleotide sequences will typically be greater than about 80 percent complementary (or '% exact-match') to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to all or a portion of the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

4.11 PEPTIDE NUCLEIC ACID COMPOSITIONS

In certain embodiments, the inventors contemplate the use of peptide nucleic acids (PNAs) in the practice of the methods of the invention. PNA is a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone (Good and Nielsen, 1997). PNAs may be utilized in a number of methods that traditionally have used RNA or DNA. Often PNA sequences perform better in techniques than the corresponding RNA or DNA sequences and have utilities that are not inherent to RNA or DNA. An excellent review of PNA including methods of making, characteristics of, and methods of using, is provided by Corey (1997) and is incorporated herein by reference. As such, in certain embodiments, one may prepare PNA sequences that are complementary to one or more portions of the particular selected mRNA sequence, and such PNA compositions may be used to regulate, alter, decrease, or reduce the translation of the specific mRNA, and thereby alter the level of polypeptide encoded by the targeted mRNA in a host cell to which such PNA compositions have been administered.

PNAs have 2-aminoethyl-glycine linkages replacing the normal phosphodiester backbone of DNA (Nielsen *et al.*, 1993; Hanvey *et al.*, 1992; Hyrup and Nielsen, 1996; Nielsen, 1995). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules; secondly, PNAs are achiral, which avoids the need to develop a stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc (Dueholm *et al.*, 1992) or Fmoc (Bonham *et al.*, 1995) protocols for solid-phase peptide synthesis, although other methods, including a modified Merrifield method, have been used (Christensen *et al.*, 1995).

PNA monomers or ready-made oligomers are commercially available from PerSeptive Biosystems (Framingham, MA, USA). PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols (Norton *et al.*, 1995).

4.12 NUCLEIC ACID AMPLIFICATION AND SITE-SPECIFIC MUTAGENESIS

In certain embodiments, it may be desirable to prepared modified nucleotide compositions, such as, for example, in the generation of the nucleic acid segments that encode either parts of the AAV vector itself, or the promoter, or even the therapeutic gene delivered by such rAAV vectors. Various means exist in the art, and are routinely employed by the artisan to generate modified nucleotide compositions.

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent polypeptides, through specific mutagenesis of the underlying polynucleotides that encode them. The technique, well-known to those of skill in the art, further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed polynucleotide sequences to alter the activity or effectiveness of such viral vector constructs in a transformed host cell. Likewise in certain embodiments, the inventors contemplate the mutagenesis of the viral genome itself to facilitate improved infectivity, replication, stability, activity, or viral titers, as well as efficiency of transfection both *in vitro* and/or *in vivo*.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector which includes within its sequence a DNA sequence which encodes the desired polypeptide(s). An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected polynucleotide segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of polypeptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding a desired polypeptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation that result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent

process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent 4,237,224, specifically incorporated herein by reference in its entirety. Nucleic acids, used as a template for amplification methods, may be isolated from cells according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to the ribozymes or conserved flanking regions are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer," as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of

the product *via* chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even *via* a system using electrical or thermal impulse signals (Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best-known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U. S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and each incorporated herein by reference in entirety.

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641, filed December 21, 1990, incorporated herein by reference in its entirety. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in Eur. Pat. Appl. No. 320308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair

will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U. S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase (QβR), described in Intl. Pat. Appl. Publ. No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA), described in U. S. Patent Nos. 5,455,166, 5,648,211, 5,712,124 and 5,744,311, each incorporated herein by reference, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products

that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in Great Britain Patent 2202328, and in Intl. Pat. Appl. Publ. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact, available to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer that has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double-stranded DNA, and then transcribed once

again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, Eur. Pat. Appl. No. 329822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not

produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, 1990 incorporated by reference).

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols (Sambrook *et al.*, 1989). Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U. S. Patent 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

4.13 BIOLOGICAL FUNCTIONAL EQUIVALENTS

Modification and changes may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that still possesses desirable characteristics. As mentioned above, it is often desirable to introduce one or more mutations into a specific polynucleotide and/or encoded polypeptide sequence. In certain circumstances, the resulting encoded polypeptide sequence is altered by this mutation, or in other cases, the sequence of the polypeptide is unchanged by one or more mutations in the encoding polynucleotide.

When it is desirable to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, second-generation molecule, the amino acid changes may be

achieved by changing one or more of the codons of the encoding DNA sequence, according to Table 3.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the polynucleotide sequences disclosed herein, without appreciable loss of their biological utility or activity.

TABLE 3

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a

protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity

value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take several of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.14 EXEMPLARY DEFINITIONS

In accordance with the present invention, polynucleotides, nucleic acid segments, nucleic acid sequences, and the like, include, but are not limited to, DNAs (including and not limited to genomic or extragenomic DNAs), genes, peptide nucleic acids (PNAs) RNAs (including, but not limited to, rRNAs, mRNAs and tRNAs), nucleosides, and suitable nucleic acid segments either obtained from native sources, chemically synthesized, modified, or otherwise prepared in whole or in part by the hand of man.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and compositions similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and compositions are described herein. For purposes of the present invention, the following terms are defined below:

A, an: In accordance with long standing patent law convention, the words "a" and "an" when used in this application, including the claims, denotes "one or more".

Expression: The combination of intracellular processes, including transcription and translation undergone by a polynucleotide such as a structural gene to synthesize the encoded peptide or polypeptide.

Promoter: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Regulatory Element: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Structural gene: A gene or sequence region that is expressed to produce an encoded peptide or polypeptide.

Transformation: A process of introducing an exogenous polynucleotide sequence (*e.g.*, a vector, a recombinant DNA or RNA molecule) into a host cell or protoplast in which that exogenous nucleic acid segment is incorporated into at least a first chromosome or is capable of autonomous replication within the transformed host cell. Transfection, electroporation, and naked nucleic acid uptake all represent examples of techniques used to transform a host cell with one or more polynucleotides.

Transformed cell: A host cell whose nucleic acid complement has been altered by the introduction of one or more exogenous polynucleotides into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell, or from the progeny or offspring of any generation of such a transformed host cell.

Vector: A nucleic acid molecule (typically comprised of DNA) capable of replication in a host cell and/or to which another nucleic acid segment can be operatively linked so as to

bring about replication of the attached segment. A plasmid, cosmid, or a virus is an exemplary vector.

The terms "substantially corresponds to", "substantially homologous", or "substantial identity" as used herein denotes a characteristic of a nucleic acid or an amino acid sequence, wherein a selected nucleic acid or amino acid sequence has at least about 70 or about 75 percent sequence identity as compared to a selected reference nucleic acid or amino acid sequence. More typically, the selected sequence and the reference sequence will have at least about 76, 77, 78, 79, 80, 81, 82, 83, 84 or even 85 percent sequence identity, and more preferably at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 percent sequence identity. More preferably still, highly homologous sequences often share greater than at least about 96, 97, 98, or 99 percent sequence identity between the selected sequence and the reference sequence to which it was compared. The percentage of sequence identity may be calculated over the entire length of the sequences to be compared, or may be calculated by excluding small deletions or additions which total less than about 25 percent or so of the chosen reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, in the case of sequence homology of two or more polynucleotide sequences, the reference sequence will typically comprise at least about 18-25 nucleotides, more typically at least about 26 to 35 nucleotides, and even more typically at least about 40, 50, 60, 70, 80, 90, or even 100 or so nucleotides. Desirably, which highly homologous fragments are desired, the extent of percent identity between the two sequences will be at least about 80%, preferably at least about 85%, and more preferably about 90% or 95% or higher, as readily determined by one or more of the sequence comparison algorithms well-known to those of skill in the art, such as e.g., the FASTA program analysis described by Pearson and Lipman (1988).

The term "naturally occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by the hand of man in a laboratory is naturally-occurring. As used herein, laboratory strains of rodents that may have been selectively bred according to classical genetics are considered naturally occurring animals.

As used herein, a "heterologous" is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter which does not naturally occur adjacent to the referenced structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

"Transcriptional regulatory element" refers to a polynucleotide sequence that activates transcription alone or in combination with one or more other nucleic acid sequences. A transcriptional regulatory element can, for example, comprise one or more promoters, one or more response elements, one or more negative regulatory elements, and/or one or more enhancers.

As used herein, a "transcription factor recognition site" and a "transcription factor binding site" refer to a polynucleotide sequence(s) or sequence motif(s) which are identified as being sites for the sequence-specific interaction of one or more transcription factors, frequently taking the form of direct protein-DNA binding. Typically, transcription factor binding sites can be identified by DNA footprinting, gel mobility shift assays, and the like, and/or can be predicted on the basis of known consensus sequence motifs, or by other methods known to those of skill in the art.

As used herein, the term "operably linked" refers to a linkage of two or more polynucleotides or two or more nucleic acid sequences in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

"Transcriptional unit" refers to a polynucleotide sequence that comprises at least a first structural gene operably linked to at least a first *cis*-acting promoter sequence and optionally linked operably to one or more other *cis*-acting nucleic acid sequences necessary for efficient transcription of the structural gene sequences, and at least a first distal regulatory element as may be required for the appropriate tissue-specific and developmental transcription of the structural gene sequence operably positioned under the control of the promoter and/or enhancer elements, as well as any additional *cis* sequences that are necessary for efficient transcription and translation (*e.g.,* polyadenylation site(s), mRNA stability controlling sequence(s), *etc.*

The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an oligonucleotide sequence, is substantially complementary to all or a portion of the selected sequence, and thus will specifically bind to a portion of an mRNA encoding the selected sequence. As such, typically the sequences will be highly complementary to the mRNA "target" sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches

throughout the complementary portion of the sequence. In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary to the sequence to which the oligonucleotide specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region of the mRNA and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product.

Substantially complementary oligonucleotide sequences will be greater than about 80 percent complementary (or '% exact-match') to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to all or a portion of the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*,

nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

As used herein, the terms "gene transfer" and "gene delivery" refer to methods or systems for reliably inserting a particular polynucleotide sequence (*e.g.,* a nucleic acid segment DNA) into targeted cells. In particularly preferred embodiments, the nucleotide sequence comprises at least a portion of biologically active Factor VII.

As used herein, the terms "vector," and "gene transfer vector" refer to any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc., which is capable of replication when associated with the proper control sequences and/or which can transfer nucleic acid sequences between cells. Thus, the term includes cloning and expression vectors, as well as viral vectors.

As used herein, the terms "host" and "expression host" refer to organisms and/or cells which harbor an exogenous DNA sequence (*e.g., via* transfection), an expression vector or vehicle, as well as organisms and/or cells that are suitable for use in expressing a recombinant gene or protein. It is not intended that the present invention be limited to any particular type of cell or organism. Indeed, it is contemplated that any suitable organism and/or cell will find use in the present invention as a host.

As used herein, the terms "viral replicons" and "viral origins of replication" refer to viral DNA sequences that allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. In some embodiments, vectors which contain either the SV40 or polyoma virus origin of replication replicate to high copy number,

while vectors which contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at low copy number may be utilized in other embodiments.

As used herein, the term "wild type" ("wt") refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.,* altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "heterologous" as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a "heterologous" region of a nucleic acid construct or a vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a nucleic acid construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Similarly, a cell transfected with a construct which is not normally present in the cell would be considered heterologous for purposes of this invention. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

As used herein, "coding sequence" or a sequence which "encodes" a particular antigen, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated

(in the case of mRNA) into a polypeptide in vitro or in vivo, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

The term DNA "control sequences" refers collectively to regulatory elements such as promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate recipient cell.

"Operably linked" or "operatively linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" or "operatively linked" to the coding sequence.

As used herein, the term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily

associated in its natural source. Isolated nucleic acid is such present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs which encode a multitude of proteins. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (i.e., the oligonucleotide or polynucleotide may single-stranded), but may contain both the sense and anti-sense strands (i.e., the oligonucleotide or polynucleotide may be double-stranded).

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, antibodies may be purified by removal of contaminating non-immunoglobulin proteins; they may also purified by the removal of immunoglobulin that does not bind the antigen of interest (*e.g.,* at least a portion of Factor VII). The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind the antigen of interest (*e.g.,* at least a portion of Factor VII) results in an increase in the percent of desired antigen-reactive immunoglobulins in the sample. In another example, recombinant polypeptides of Factor VII are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

A "composition comprising a given polynucleotide sequence" as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise an aqueous solution.

As used herein, the term "at risk" is used in references to individuals who are at risk for experiencing hemorrhagic episodes. In particularly preferred embodiments, the individuals are hemophiliacs with mild, moderate, or severe hemophilia.

As used herein, the term "subject" refers to any animal (*i.e.,* vertebrates and invertebrates), while the term "vertebrate subject" refers to any member of the subphylum Chordata. It is intended that the term encompass any member of this subphylum, including, but not limited to humans and other primates, rodents (*e.g.,* mice, rats, and guinea pigs), lagomorphs (*e.g.,* pikas, hares, rabbits), bovines (*e.g.,* cattle), ovines (*e.g.,* sheep), caprines (*e.g.,* goats), porcines (*e.g.,* swine), equines (*e.g.,* horses), canines (*e.g.,* dogs, wolves), felines (*e.g.,* lions, tigers, cheetahs, domestic cats), domestic fowl (*e.g.,* chickens, turkeys, ducks, geese, other gallinaceous birds, *etc.*), as well as feral or wild animals, including, but not limited to, such animals as ungulates, including members of the Artiodactyla (*e.g.,* antelope, deer, *etc.*) and the Perissodactyla (*e.g.,* rhinoceros, tapir, horse, zebra, *etc.*), *etc.* It is not intended that the term be limited to a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are encompassed by the term.

As defined herein, a "therapeutically effective amount" or "therapeutic effective dose" is an amount or dose of AAV vectors or AAV virions capable of producing sufficient amounts of Factor VII to decrease the time it takes for a subject's blood to clot.

5. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate

that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – RAAV COMPOSITIONS EXPRESSING FACTOR VII POLYPEPTIDES

Replacement therapy using plasma-derived concentrates or recombinant factor VIII administered after bleeding episodes is the current mode of therapy for hemophilia A. Although prophylaxis with protein has been shown to significantly reduce spontaneous bleeds, treatment efficacy is limited by the short half-life of FVIII *in vivo*, high production costs, repeated intravenous administrations, and development of host antibodies to the therapeutic protein. Gene therapy approaches may circumvent these problems, and as little as 2% of normal FVIII activity can result in therapeutic effects (Connelly *et al.*, 1996; Kay and High, 1999).

Adeno-associated virus 2 (AAV)-based vectors have emerged at the forefront of gene therapy. Previously, AAV has been used effectively for the treatment of factor IX (FIX) deficiency in both murine and canine models (Koeberl *et al.*, 1997; Snyder *et al.*, 1997; Chao *et al.*, 1999; Herzog *et al.*, 1999; Hagstrom *et al.*, 2000), leading to current phase I clinical trials.

The present example describes the use of a recombinant AAV-based vector system for the treatment of a clinically relevant murine model of hemophilia A. This study demonstrates expression of Factor VII polypeptide from rAAV vectors expressing the FVII gene, administered either intravenously or intramuscularly.

5.1.2 METHODS FOR PREPARING RAAV VECTORS

rAAV is most often produced by co-transfection of rAAV vector plasmid and wt AAV helper plasmid into Ad-infected 293 cells (Hermonat and Muzyczka, 1984). Recent

improvements in AAV helper design (Li *et al.*, 1997) as well as construction of non-infectious mini-Ad plasmid helper (Grimm *et al.*, 1998; Xiao *et al.*, 1998; Salvetti, 1998) have eliminated the need for Ad infection, and made it possible to increase the yield of rAAV up to 10^5 particles per transfected cell in a crude lysate. Scalable methods of rAAV production that do not rely on DNA transfection have also been developed (Chiorini *et al.*, 1995; Inoue and Russell, 1998; Clark *et al.*, 1995). These methods, which generally involve the construction of producer cell lines and helper virus infection, are suitable for high-volume production.

The conventional protocol for downstream purification of rAAV involves the stepwise precipitation of rAAV using ammonium sulfate, followed by two or preferably, three rounds of CsCl density gradient centrifugation. Each round of CsCl centrifugation involves fractionation of the gradient and probing fractions for rAAV by dot-blot hybridization or by PCR™ analysis.

The AAV vectors and rAAV virions of the present invention can be produced using standard methodology known to those of skill in the art. Such methods typically involve one or more steps such as: (a) introducing an AAV vector into an appropriate mammalian host cell; (b) introducing an AAV helper construct into the host cell, where the helper construct includes AAV coding regions capable of being expressed in the host cell to complement AAV helper functions missing from the AAV vector; (c) introducing one or more helper viruses and/or accessory function vectors into the host cell, wherein the helper virus and/or accessory function vectors provide accessory functions capable of supporting efficient recombinant AAV ("rAAV") virion production in the host cell; and (d) culturing the host cell to produce rAAV virions. The AAV vector, AAV helper construct and the helper virus or accessory function vector(s) can be introduced into the host cell either simultaneously or serially, using standard transfection techniques.

5.1.3 INCORPORATION OF rAAV VECTORS INTO CELLS

In various embodiments of the invention, DNA may be delivered to a suitable mammalian host cell as an expression vector construct. Preferred gene therapy vectors of the present invention are generally viral vectors. Adeno-associated virus (AAV) is particularly attractive for gene transfer because it does not induce any pathogenic response and can integrate into the host cellular chromosome (Kotin *et al.*, 1990). The AAV terminal repeats (TRs) are the only essential *cis*-components for the chromosomal integration (Muzyczka and McLaughin, 1988). These TRs are reported to have promoter activity (Flotte *et al.*, 1993). They may promote efficient gene transfer from the cytoplasm to the nucleus or increase the stability of plasmid DNA and enable longer-lasting gene expression. Studies using recombinant plasmid DNAs containing AAV TRs have attracted considerable interest. AAV-based plasmids have been shown to drive higher and longer transgene expression than the identical plasmids lacking the TRs of AAV in most cell types (Shafron *et al.*, 1998).

AAV (Ridgeway, 1988; Hermonat and Muzyczka, 1984) is a parovirus, discovered as a contamination of adenoviral stocks. It is a ubiquitous virus (antibodies are present in 85% of the U. S. human population) that has not been linked to any disease. It is also classified as a dependovirus, because its replication is dependent on the presence of a helper virus, such as adenovirus.

There are several factors that prompted researchers to study the possibility of using rAAV as an expression vector. One is that the requirements for delivering a gene to integrate into the host chromosome are surprisingly few. It is necessary to have the 145-bp ITRs, which are only 6% of the AAV genome. This leaves room in the vector to assemble a 4.5-kb DNA insertion. AAV is also a good choice of delivery vehicles due to its safety. There is a relatively complicated rescue mechanism: not only wild type adenovirus but also AAV genes are required to mobilize rAAV. Likewise, AAV is not pathogenic and not associated with any disease. The

removal of viral coding sequences minimizes immune reactions to viral gene expression, and therefore, rAAV does not evoke an inflammatory response. AAV therefore, represents an ideal candidate for delivery of the present anti-hemophilia constructs.

Retroviruses have promise as gene delivery vectors due to their ability to integrate their genes into the host genome, transferring a large amount of foreign genetic material, infecting a broad spectrum of species and cell types and of being packaged in special cell-lines.

Of course, in using viral delivery systems, one may desire to purify the virion sufficiently to render it essentially free of undesirable contaminants, such as defective interfering viral particles or endotoxins and other pyrogens such that it will not cause any untoward reactions in the cell, animal or individual receiving the vector construct. One preferred means of purifying the vector involves the use of buoyant density gradients, such as cesium chloride gradient centrifugation, heparin affinity chromatography (Clark *et al.*, 1999), or non-ionic iodixinol gradients followed by heparin affinity chromatography (Zolotukhin *et al.*, 1999).

The titer of AAV in a given sample may be determined using any one of the methods routinely accepted in the AAV arts. For example, the inventors routinely use the methods of QC-PCR™ or infectious center assay, as described in detail in the Examples and by Zolotukhin *et al.* (1999), to determine the titer of a viral stock.

Likewise, the infectivity of a given AAV sample may be determined using any one of the methods routinely accepted in the AAV arts. For example, the inventors routinely use the methods of Hermonat and Muzyczka (1984) or Clark *et al.* (1999) to determine the infectivity of a given AAV stock.

5.1.4 MATERIALS AND METHODS

5.1.4.1 CELLS, PLASMIDS, AND VIRUSES

Adenovirus-transformed human embryonic kidney cells (293 cells), the human hepatocellular carcinoma cell line Hep G2, the human cervical carcinoma cell line HeLa, and the mouse myoblast cell line C2C12 may be obtained from the American Type Culture Collection (ATCC, Manassas, VA). Monolayer cells may be maintained at 37°C, 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The albumin promoter may be obtained from K. Zaret (Foxchase, Philadelphia, PA). A truncated hybrid cytomegalovirus immediate-early enhancer chicken *b*-actin (CBA) promoter may be generated by using *Bsp*120I to delete 700 bp from the 39 end of the promoter, according to standard cloning methods. rAAV plasmids pCMVp-*lacZ*, UF5 (pCMVp-GFP), and UF11 (pCBAp-GFP) have been described previously (Kessler *et al.*, 1996; Klein *et al.*, 1998). rAAV plasmids containing the appropriate DNA sequence encoding the desired mammalian Factor VII polypeptide may be generated by standard cloning methods. The splice donor/acceptor (SD and SA, respectively) elements in pCBA-HC-SD and pSALC may be cloned from the intron region of the pCI cloning vector (Stratagene, La Jolla, CA). rAAV vectors may be generated, purified, and titered at the University of Florida (Gainesville, FL) Vector Core Laboratory as previously described (Zolotukhin *et al.*, 1999).

5.1.4.2 IN VITRO TRANSFECTION AND TRANSDUCTION

In vitro transfections may be performed by the calcium phosphate–DNA coprecipitation method as described by Sambrook *et al.* (1989). For *in vitro* infections, cells may be infected with the Factor VII vectors at the indicated multiplicities of infection (MOIs) and coinfected with wild-type adenovirus type 5 at an MOI of 1. Forty-eight hours

posttransfection or infection, conditioned medium may be assayed for the presence of functional FVII by the Coatest kit assay (Chromogenix, Milan, Italy).

For the cotransduction studies, HeLa or 293 cells may be infected with the indicated vectors at the indicated MOIs. In addition, HeLa cells, may also be coinfected with wild-type adenovirus type 5 at an MOI of 1. HeLa cells (48 hr postinfection) and 293 cells (72 hr postinfection) may be subjected to fluorescence-activated cell sorting (FACS) analysis at a suitable facility, such as the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) Flow Cytometry Core (FACScan; BD Immunocytometry Systems, San Jose, CA). *lacZ*-positive cells may be identified with an ImaGene Red C12RG *lacZ* gene expression kit, as per the kit protocol (Molecular Probes,

Eugene, OR).

5.1.4.3 ANIMALS

A suitable model of hemophilia A Factor VII deficiency, such as for example a mouse FVII knockout [KO] mouse may be utilized in the practice of the present invention. One-day-old FVII KO mice may be administered a suitable concentration of infectious particles (IP) (for example about 2.4×10^8) via the superficial temporal vein as described by Sands and Barker (1999). Seven-month-old FVII KO mice may also be administered a suitable concentration of IP via intramuscular injection into the gastrocnemius of the hind leg. Beginning, for example, about 4 weeks postinjection, plasma may then be collected from tail vein bleeds and functional mFVII activity determined by a suitable Factor VII assay, such as the Coatest assay. Plasma samples are obtained by tail bleeding of anesthetized animals followed by the immediate addition of sodium citrate to a final concentration of about 0.38% (wt./vol.). Samples are centrifuged at 2000 3 *g* for 10 min at 25°C. The plasma fraction is collected and frozen immediately. Samples are thawed quickly

at 37°C immediately before testing. Serial dilutions of pooled normal C57BL/6 mouse plasma diluted in pooled FVII KO mouse plasma is then used to derive the standard curve. Inhibitor formation is detected by Bethesda assay as described previously, with the following modifications (Kasper *et al.*, 1975). Treated mouse plasma is mixed with an equivalent volume of pooled normal C57BL/6 mouse plasma and incubated at 37°C for 2 hr. The residual mFVII activity of each sample is plotted on the established Bethesda Inhibitor Assay standard curve to determine the anti-mFVII inhibitor titer.

5.1.4.4 IMMUNOHISTOCHEMISTRY

Tissue samples are fixed in 10% buffered formalin, embedded, and sectioned (4-*m*m thickness). Sections are immunostained with a suitable anti-mFVII antibody, the mouse-on-mouse (M.O.M.) immunostaining kit (Vector Laboratories, Burlingame, CA), and 3,39-diaminobenzidine (DAB), and then counterstained with Gill's hematoxylin (Vector Laboratories). Photographs are taken with a light microscope (Zeiss, Thornwood, NY), camera (Olympus America, Melville, NY) and MagnaFire digital recording system (Pro Image Digital, Teddington, Middlesex, UK). Relative transduction efficiency is determined as described by Nakai *et al.* (2002). Briefly, the number of positively stained cells and total number of cells are counted. Ten fields of approximately 500 nuclei per field are counted for each section.

5.1.4.5 WESTERN BLOT AND SEMIQUANTITATIVE WESTERN BLOT

Plasma samples are subjected to cryoprecipitation as described (Bi *et al.*, 1996). Cryoprecipitate samples were analyzed by the Laemmli gel method, using precast 8–16% Tris–glycine sodium dodecyl sulfate (SDS)–polyacrylamide minigels (Invitrogen, Carlsbad, CA). Western transfer is performed with a Novex Western blot module (Invitrogen) and

Hybond ECL nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ). Blots are hybridized with a suitable anti-FVII antibody (Bi *et al.*, 1996; Sarkar *et al.*, 2000) (diluted 1:50) and horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:1000) (Amersham Pharmacia Biotech). Hybridization is detected with the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). For semiquantitative Western blot, equivalent amounts of treated animal plasma or standard samples containing 10, 25, 50, 75, and 100% normal C57BL/6 plasma is subjected to cryoprecipitation and analyzed by the Laemmli gel method, using precast 8% Tris–glycine SDS–polyacrylamide minigels (Invitrogen). Western transfer and hybridization visualization is performed as described above. Autoradiographs are scanned with an Astra 6450 scanner (UMAX Technologies, Dallas, TX) and optical density is determined with Scion Image Release Beta 4.0.2 software (Scion, Frederick, MD). The standard curve is determined by plotting optical density versus percent normal FVII.

5.1.2 RESULTS

FIG. 1 shows that functional Factor VII can be produced *in vitro*. The chromogenic Coaset assay was used to determine the levels of functional Factor VII secreted. Factor VIII KO plasma was used as a positive control as these mice have been shown to produce normal levels of Factor VII.

5.2 EXAMPLE 2 – ILLUSTRATIVE THERAPEUTIC POLYPEPTIDE SEQUENCES USEFUL IN THE PRACTICE OF THE PRESENT INVENTION AND DNA SEQUENCES ENCODING SUCH AMINO ACID SEQUENCES

5.2.1 DNA SEQUENCE ENCODING HUMAN FACTOR VII FROM GENBANK AF466933 (SEQ ID NO:1)

TCAACAGGCAGGGGCAGCACTGCAGAGATTTCATCATGGTCTCCCAGGCCCTCAGGCTC

CTCTGCCTTCTGCTTGGGCTTCAGGGCTGCCTGGCTGCAGGCGGGGTCGCTAAGGCCTC

AGGAGGAGAAACACGGGACATGCCGTGGAAGCCGGGGCCTCACAGAGTCTTCGTAACCC

AGGAGGAAGCCCACGGCGTCCTGCACCGGCGCCGGCGCGCCAACGCGTTCCTGGAGGAG

CTGCGGCCGGGCTCCCTGGAGAGGGAGTGCAAGGAGGAGCAGTGCTCCTTCGAGGAGGC

CCGGGAGATCTTCAAGGACGCGGAGAGGACGAAGCTGTTCTGGATTTCTTACAGTGATG

GGGACCAGTGTGCCTCAAGTCCATGCCAGAATGGGGGCTCCTGCAAGGACCAGCTCCAG

TCCTATATCTGCTTCTGCCTCCCTGCCTTCGAGGGCCGGAACTGTGAGACGCACAAGGA

TGACCAGCTGATCTGTGTGAACGAGAACGGCGGCTGTGAGCAGTACTGCAGTGACCACA

CGGGCACCAAGCGCTCCTGTCGGTGCCACGAGGGGTACTCTCTGCTGGCAGACGGGGTG

TCCTGCACACCCACAGTTGAATATCCATGTGGAAAAATACCTATTCTAGAAAAAAGAAA

TGCCAGCAAACCCCAAGGCCGAATTGTGGGGGGCAAGGTGTGCCCCAAAGGGGAGTGTC

CATGGCAGGTCCTGTTGTTGGTGAATGGAGCTCAGTTGTGTGGGGGGACCCTGATCAAC

ACCATCTGGGTGGTCTCCGCGGCCCACTGTTTCGACAAAATCAAGAACTGGAGGAACCT

GATCGCGGTGCTGGGCGAGCACGACCTCAGCGAGCACGACGGGGATGAGCAGAGCCGGC

GGGTGGCGCAGGTCATCATCCCCAGCACGTACGTCCCGGGCACCACCAACCACGACATC

GCGCTGCTCCGCCTGCACCAGCCCGTGGTCCTCACTGACCATGTGGTGCCCCTCTGCCT

GCCCGAACGGACGTTCTCTGAGAGGACGCTGGCCTTCGTGCGCTTCTCATTGGTCAGCG

GCTGGGGCCAGCTGCTGGACCGTGGCGCCACGGCCCTGGAGCTCATGGTGCTCAACGTG

CCCCGGCTGATGACCCAGGACTGCCTGCAGCAGTCACGGAAGGTGGGAGACTCCCCAAA

TATCACGGAGTACATGTTCTGTGCCGGCTACTCGGATGGCAGCAAGGACTCCTGCAAGG

GGGACAGTGGAGGCCCACATGCCACCCACTACCGGGGCACGTGGTACCTGACGGGCATC
GTCAGCTGGGGCCAGGGCTGCGCAACCGTGGGCCACTTTGGGGTGTACACCAGGGTCTC
CCAGTACATCGAGTGGCTGCAAAAGCTCATGCGCTCAGAGCCACGCCCAGGAGTCCTCC
TGCGAGCCCCATTTCCCTAGCCCA

5.2.2 Amino Acid Sequence of Human Factor VII From GenBank AF466933 (SEQ ID NO:2)

MVSQALRLLCLLLGLQGCLAAGGVAKASGGETRDMPWKPGPHRVFVTQEEAHGVLHRRR
RANAFLEELRPGSLERECKEEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNG
GSCKDQLQSYICFCLPAFEGRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEG
YSLLADGVSCTPTVEYPCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQ
LCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYV
PGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATA
LELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYR
GTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFP
(From Rieder *et al.*, unpublished)

5.2.3 DNA Sequence Encoding Human Factor VII Variant 1 from GenBank NM_000131 (SEQ ID NO:3)

ATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTGCTTGGGCTTCAGGGCTGCCTGGCTGC
AGGCGGGGTCGCTAAGGCCTCAGGAGGAGAAACACGGGACATGCCGTGGAAGCCGGGGCCTC
ACAGAGTCTTCGTAACCCAGGAGGAAGCCCACGGCGTCCTGCACCGGCGCCGGCGCGCCAAC
GCGTTCCTGGAGGAGCTGCGGCCGGGCTCCCTGGAGAGGGAGTGCAAGGAGGAGCAGTGCTC
CTTCGAGGAGGCCCGGGAGATCTTCAAGGACGCGGAGAGGACGAAGCTGTTCTGGATTTCTT
ACAGTGATGGGGACCAGTGTGCCTCAAGTCCATGCCAGAATGGGGGCTCCTGCAAGGACCAG
CTCCAGTCCTATATCTGCTTCTGCCTCCCTGCCTTCGAGGGCCGGAACTGTGAGACGCACAA

GGATGACCAGCTGATCTGTGTGAACGAGAACGGCGGCTGTGAGCAGTACTGCAGTGACCACA

CGGGCACCAAGCGCTCCTGTCGGTGCCACGAGGGGTACTCTCTGCTGGCAGACGGGGTGTCC

TGCACACCCACAGTTGAATATCCATGTGGAAAAATACCTATTCTAGAAAAAAGAAATGCCAG

CAAACCCCAAGGCCGAATTGTGGGGGGCAAGGTGTGCCCCAAAGGGGAGTGTCCATGGCAGG

TCCTGTTGTTGGTGAATGGAGCTCAGTTGTGTGGGGGGACCCTGATCAACACCATCTGGGTG

GTCTCCGCGGCCCACTGTTTCGACAAAATCAAGAACTGGAGGAACCTGATCGCGGTGCTGGG

CGAGCACGACCTCAGCGAGCACGACGGGGATGAGCAGAGCCGGCGGGTGGCGCAGGTCATCA

TCCCCAGCACGTACGTCCCGGGCACCACCAACCACGACATCGCGCTGCTCCGCCTGCACCAG

CCCGTGGTCCTCACTGACCATGTGGTGCCCCTCTGCCTGCCCGAACGGACGTTCTCTGAGAG

GACGCTGGCCTTCGTGCGCTTCTCATTGGTCAGCGGCTGGGGCCAGCTGCTGGACCGTGGCG

CCACGGCCCTGGAGCTCATGGTGCTCAACGTGCCCCGGCTGATGACCCAGGACTGCCTGCAG

CAGTCACGGAAGGTGGGAGACTCCCCAAATATCACGGAGTACATGTTCTGTGCCGGCTACTC

GGATGGCAGCAAGGACTCCTGCAAGGGGGACAGTGGAGGCCCACATGCCACCCACTACCGGG

GCACGTGGTACCTGACGGGCATCGTCAGCTGGGGCCAGGGCTGCGCAACCGTGGGCCACTTT

GGGGTGTACACCAGGGTCTCCCAGTACATCGAGTGGCTGCAAAAGCTCATGCGCTCAGAGCC

ACGCCCAGGAGTCCTCCTGCGAGCCCCATTTCCCTAG

5.2.4 Amino Acid Sequence of Human Factor VII Variant 1 from GenBank NM_000131 (SEQ ID NO:4)

MVSQALRLLCLLLGLQGCLAAGGVAKASGGETRDMPWKPGPHRVFVTQEEAHGVLHRRR

RANAFLEELRPGSLERECKEEQCSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNG

GSCKDQLQSYICFCLPAFEGRNCETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEG

YSLLADGVSCTPTVEYPCGKIPILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQ

LCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYV

PGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATA

LELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYR

GTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMRSEPRPGVLLRAPFP

(from Toso *et al., Biochem. J.*, 369(Pt 3):563-571, 2003).

5.2.5 DNA SEQUENCE ENCODING HUMAN FACTOR VII VARIANT 2 FROM GENBANK NM_019616 (SEQ ID NO:5)

ATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTGCTTGGGCTTCAGGGCTGCCTGGC

TGCAGTCTTCGTAACCCAGGAGGAAGCCCACGGCGTCCTGCACCGGCGCCGGCGCGCCA

ACGCGTTCCTGGAGGAGCTGCGGCCGGGCTCCCTGGAGAGGGAGTGCAAGGAGGAGCAG

TGCTCCTTCGAGGAGGCCCGGGAGATCTTCAAGGACGCGGAGAGGACGAAGCTGTTCTG

GATTTCTTACAGTGATGGGGACCAGTGTGCCTCAAGTCCATGCCAGAATGGGGGCTCCT

GCAAGGACCAGCTCCAGTCCTATATCTGCTTCTGCCTCCCTGCCTTCGAGGGCCGGAAC

TGTGAGACGCACAAGGATGACCAGCTGATCTGTGTGAACGAGAACGGCGGCTGTGAGCA

GTACTGCAGTGACCACACGGGCACCAAGCGCTCCTGTCGGTGCCACGAGGGGTACTCTC

TGCTGGCAGACGGGGTGTCCTGCACACCCACAGTTGAATATCCATGTGGAAAAATACCT

ATTCTAGAAAAAAGAAATGCCAGCAAACCCCAAGGCCGAATTGTGGGGGGCAAGGTGTG

CCCCAAAGGGGAGTGTCCATGGCAGGTCCTGTTGTTGGTGAATGGAGCTCAGTTGTGTG

GGGGGACCCTGATCAACACCATCTGGGTGGTCTCCGCGGCCCACTGTTTCGACAAAATC

AAGAACTGGAGGAACCTGATCGCGGTGCTGGGCGAGCACGACCTCAGCGAGCACGACGG

GGATGAGCAGAGCCGGCGGGTGGCGCAGGTCATCATCCCCAGCACGTACGTCCCGGGCA

CCACCAACCACGACATCGCGCTGCTCCGCCTGCACCAGCCCGTGGTCCTCACTGACCAT

GTGGTGCCCCTCTGCCTGCCCGAACGGACGTTCTCTGAGAGGACGCTGGCCTTCGTGCG

CTTCTCATTGGTCAGCGGCTGGGGCCAGCTGCTGGACCGTGGCGCCACGGCCCTGGAGC

TCATGGTGCTCAACGTGCCCCGGCTGATGACCCAGGACTGCCTGCAGCAGTCACGGAAG

GTGGGAGACTCCCCAAATATCACGGAGTACATGTTCTGTGCCGGCTACTCGGATGGCAG

CAAGGACTCCTGCAAGGGGGACAGTGGAGGCCCACATGCCACCCACTACCGGGGCACGT

GGTACCTGACGGGCATCGTCAGCTGGGGCCAGGGCTGCGCAACCGTGGGCCACTTTGGG

GTGTACACCAGGGTCTCCCAGTACATCGAGTGGCTGCAAAAGCTCATGCGCTCAGAGCC

ACGCCCAGGAGTCCTCCTGCGAGCCCCATTTCCCTAG

5.2.6 AMINO ACID SEQUENCE OF HUMAN FACTOR VII VARIANT 2 FROM GENBANK NM_019616 (SEQ ID NO:6)

MVSQALRLLCLLLGLQGCLAAVFVTQEEAHGVLHRRRRANAFLEELRPGSLERECKEEQ

CSFEEAREIFKDAERTKLFWISYSDGDQCASSPCQNGGSCKDQLQSYICFCLPAFEGRN

CETHKDDQLICVNENGGCEQYCSDHTGTKRSCRCHEGYSLLADGVSCTPTVEYPCGKIP

ILEKRNASKPQGRIVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKI

KNWRNLIAVLGEHDLSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDH

VVPLCLPERTFSERTLAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRK

VGDSPNITEYMFCAGYSDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFG

VYTRVSQYIEWLQKLMRSEPRPGVLLRAPFP

(from Toso *et al., Biochem. J.*, 369(Pt 3):563-571, 2003).

5.2.7 DNA SEQUENCE ENCODING RAT FACTOR VII FROM GENBANK NM_152846 (SEQ ID NO:7)

ATGGTTCCACAGACTCACGGACTGCTGCTTCTCTACTTTCTGCTCCAGCTCCAGGGACCCCT

AGGGGCTGTGGTTTTCATAACCCAGGAGGAAGCACACGGTGTCCTACACAGGCAAAGGCGTG

CCAACTCACTCCTAGAGGAGCTTTGGTCTAGCTCCTTGGAGAGGGAGTGCAATGAAGAGCGG

TGCTCCTTTGAGGAGGCCCGAGAGATCTTCAAGAGCCCTGAGAGAACCAAGCAGTTCTGGAC

TATTTACAGCGATGGCGACCAGTGTGCCTCGAATCCATGTCAGAACGGGGGTACCTGCCAGG

ATCACCTCAAGTCTTATGTCTGCTTCTGCCCCCTAGACTTTGAGGGCCGGAACTGTGAGAAA

AACAAGAATGAGCAGCTGATCTGTGCAAATGAAAATGGTGACTGTGACCAGTACTGCAGGGA

CCACGTAGGGACCAAGCGTACCTGTAGCTGTCACGAGGACTACGTGCTGCAGCCAGATGAGG

TGTCCTGCAAACCAAAAGTTGAGTACCCATGCGGGAGAATACCTGTTGTAGAAAAAAGAAAC

TTCAGCAGACCCCAAGGCCGGATTGTGGGAGGCTATGTGTGCCCCAAAGGGGAGTGCCCATG

GCAGGCTGTGCTGAAATTCAATGAGGCATTGCTGTGTGGGGCCGTCCTGCTGGACACCAGAT

GGATAGTAACTGCAGCCCACTGCTTCGATAAATTCGGGAAATTGGTAAACATCACAGTGGTG

TTGGGTGAACACGACTTCAGTGAGAAGGAGGGGACTGAGCAAGTACGGCTGGTGGAACAGGT

CATCATGCCCAACAAGTACACCCGCGGCAGGACTGACCATGACATCGCCCTGGTCCGCCTTC

ACCGGCCTGTAACCTTCACTGACTACGTGGTACCTCTGTGTCTGCCTGAACGGGCCTTCTCC

GAGAACACCCTAGCCAGCATCCGCTTCTCGAGGGTCAGCGGCTGGGGCCAGCTACTGGACCG

TGGTGCCACAGCTCTGGAGCTCATGGTCATCGAGGTGCCCCGGCTGATGACCCAGGACTGCC

TGGAGCATGCCAAACACAGTGCTAACACCCCCAGAATCACGGAGAACATGTTCTGCGCCGGC

TACATGGACGGCACCAAGGACGCCTGCAAGGGTGACAGTGGAGGCCCACACGCCACCCACTA

CCATGGCACTTGGTATCTGACAGGTGTGGTCAGCTGGGGGGAGGGCTGTGCAGCTATCGGCC

ACATCGGGGTGTACACCAGGGTCTCCCAGTACATAGACTGGCTGGTCAAATACATGGACTCC

AAGCTCCGGGTTGGGATTTCTCGAGTCTCCCTACTGTAG

5.2.8 Amino Acid Sequence of Rat Factor VII from GenBank NM_152846 (SEQ ID NO:8)

MVPQTHGLLLLYFLLQLQGPLGAVVFITQEEAHGVLHRQRRANSLLEELWSSSLERECNEER

CSFEEAREIFKSPERTKQFWTIYSDGDQCASNPCQNGGTCQDHLKSYVCFCPLDFEGRNCEK

NKNEQLICANENGDCDQYCRDHVGTKRTCSCHEDYVLQPDEVSCKPKVEYPCGRIPVVEKRN

FSRPQGRIVGGYVCPKGECPWQAVLKFNEALLCGAVLLDTRWIVTAAHCFDKFGKLVNITVV

LGEHDFSEKEGTEQVRLVEQVIMPNKYTRGRTDHDIALVRLHRPVTFTDYVVPLCLPERAFS

ENTLASIRFSRVSGWGQLLDRGATALELMVIEVPRLMTQDCLEHAKHSANTPRITENMFCAG

YMDGTKDACKGDSGGPHATHYHGTWYLTGVVSWGEGCAAIGHIGVYTRVSQYIDWLVKYMDS

KLRVGISRVSLL

5.2.9 DNA SEQUENCE ENCODING DANIO FACTOR VII FROM GENBANK NM_131819 (SEQ ID NO:9)

ATGAGTCTGCTGCTTGTGTTTTCTCTGCTCTGGAGTCTCCATTACTGCCATTCAGCAGCAGT
GTTCGTGCACAGAGATGAAGCTCACGAGGTGTTGATCAGGAGCAAAAGAGCCAACTCAGGCT
GGTTTGAGGAGCTGAAGACGGGGAATCTGGAGCGCGAGTGTCTGGAGGAGAAATGCTCGTAT
GAGGAGGCGCGCGAGGTGTTCGAGCACACAGAGGCCACGAATGAGTTCTGGAAGATCTACGA
TGTTAAAGATCACTGCGCATCCAGTCCATGTGAGCATGACGGGCTCTGCACCACACAGAACG
CGGACTCCTACATGTGTTTGTGTGCGCCGGGCTTCAGCGGACGCCACTGTGAGCAATCGATT
GGAGACGTTCTCGACTCCTGTCTGCATGATAACGGCGGCTGCGAACACTTCTGCACGGAGCA
GGACGGACGGAGAAACTGCTCCTGCGCAGACGGGTATTACCTAGATAACAGCGGGCAGAAGT
GCCGGAGTCACGAGGTGTTTCCATGTGGGAAGGTTCCTCTCCTGCAGGCTGGAAAAGCTGCG
GATCATCAGGTGGATCTCAGATCTCGTATCGTTGGAGGATCTGAATGTCCTAAAGGTCACTG
TCCGTGGCAGGTGCTGCTGAAGTACGGTGAGAAGGGTTTCTGTGGAGGTGTGATCTACAAGC
CCACCTGGATCCTCACAGCTGCTCACTGCTTGGAAAAGCTCAAGGTCAAGTTCCTCAGGATA
GTGGCAGGTGAGCATGATCTGGAGGTGGACGAGGGCACGGAGCAGCTCATCCAGGTGGATCA
GATGTTCACACACCCTGCGTACGTGTCTGAGACAGCGGACAGTGACATCGCCCTGCTGCGTC
TGCGCACCCCCATCGTCTACAGTGTGTATGCGGTGCCGGTGTGTTTGCCGCTGCGGGAGATG
GCGGAGCGCGAGCTGTGGGCGGTCAGCAAACACACGGTGAGCGGCTGGGGCAAACGCAGCGA
GGACGGGCCGACCTCTCGCCTGCTGCGCCGGCTGCTGGTGCCGCGCATCCGCACGCAGGAGT
GTGTGCAGGTCAGCAACCTCACGCTCACCAGCAACATGTTCTGCGCCGGATACATCGAGGGC
CGGCAGGACTCCTGTAAGGGTGACAGCGGCGGCCCGCTGGTGACCCGGTACCGAGACACCGC
CTTCCTACTGGGCATCGTGAGCTGGGGGAAAGGCTGCGCTCGCCCGGGCTCCTACGGCATCT
ACACACGCGTGTCCAACTACCTGCAGTGGATCCGACAAACAACCAACACCACGATACACTGA
TGAAGACATGACCCGGGTGCATTGCTCATCAAGATTGCTACTCTTAGGTGAACAATTAACAA
ATATTAACTATTATAGTTAATGTTTGTAAAAAATAGCAAAATTATATTGAAAATAAAAAATA

TTTATATTAATTATGAAGTGACGGCGATTACTTTAATTATCCAAGACGGTGTTATAGCCCAA

AATACCCAATAGTTGAGCATCAGCTGCTTTCCTGACATCCTGTACATATTAGACTCGGATCT

GATATTTTGCACAGGTTATATTGCATTTTTAGCAGGTATTTAATGATTTTGCTCTGATTAAT

CAGGAGATGTGCAGCTCATTATCTCCATATTATTAATGCTCAACTGTAGTAAACACTCG

5.2.10 AMINO ACID SEQUENCE OF DANIO FACTOR VII FROM GENBANK NM_131819 (SEQ ID NO:10)

MSLLLVFSLLWSLHYCHSAAVFVHRDEAHEVLIRSKRANSGWFEELKTGNLERECLEEKCSY

EEAREVFEHTEATNEFWKIYDVKDHCASSPCEHDGLCTTQNADSYMCLCAPGFSGRHCEQSI

GDVLDSCLHDNGGCEHFCTEQDGRRNCSCADGYYLDNSGQKCRSHEVFPCGKVPLLQAGKAA

DHQVDLRSRIVGGSECPKGHCPWQVLLKYGEKGFCGGVIYKPTWILTAAHCLEKLKVKFLRI

VAGEHDLEVDEGTEQLIQVDQMFTHPAYVSETADSDIALLRLRTPIVYSVYAVPVCLPLREM

AERELWAVSKHTVSGWGKRSEDGPTSRLLRRLLVPRIRTQECVQVSNLTLTSNMFCAGYIEG

RQDSCKGDSGGPLVTRYRDTAFLLGIVSWGKGCARPGSYGIYTRVSNYLQWIRQTTNTTIH

(From Sheehan *et al., Proc. Natl. Acad. Sci. USA*, 98(15):8768-8773, 2001.)

5.2.11 DNA SEQUENCE ENCODING MOUSE FACTOR VII FROM GENBANK NM_010172 (SEQ ID NO:11)

ATGGTTCCACAGGCGCATGGGCTGCTGCTTCTCTGCTTTCTGCTCCAGCTCCAGGGACCTCT

AGGGACTGCAGTTTTCATAACCCAGGAGGAAGCACATGGTGTCCTACACAGGCAAAGGCGTG

CCAACTCACTCCTGGAGGAGCTTTGGCCCGGCTCTCTGGAGAGAGAGTGCAATGAGGAACAG

TGCTCCTTTGAGGAGGCCCGGGAGATCTTCAAGAGCCCTGAGAGGACCAAGCAGTTCTGGAT

TGTTTACAGTGATGGGGACCAGTGTGCCTCGAATCCATGTCAGAACGTAGGTACCTGCCAGG

ATCATCTCAAGTCTTACGTCTGCTTCTGCCTCCTAGACTTTGAGGGTCGGAACTGTGAGAAA

AGCAAGAATGAGCAGCTGATCTGTGCAAATGAAAATGGTGACTGTGACCAGTACTGCAGGGA

CCATGTAGGGACCAAGCGTACCTGTAGCTGTCATGAGGACTACACGCTACAGCCAGATGAGG

TGTCCTGCAAACCAAAAGTTGAGTACCCGTGTGGGAGAATACCTGTTGTAGAAAAAAGAAAC

TCCAGCAGCCGCCAAGGCCGCATTGTGGGAGGCAACGTGTGCCCCAAAGGGGAGTGTCCATG

GCAGGCTGTGCTGAAAATCAATGGGTTATTGCTGTGTGGGGCCGTCCTGCTGGACGCCAGAT

GGATAGTGACCGCAGCCCACTGCTTCGATAATATCCGCTACTGGGGAAACATCACAGTGGTG

ATGGGTGAACATGACTTCAGTGAGAAGGATGGGGATGAGCAAGTACGACGGGTGACACAGGT

CATCATGCCCGACAAGTACATCCGCGGCAAGATCAACCACGACATTGCCCTGCTCCGCCTTC

ACCGGCCTGTGACCTTCACTGACTACGTGGTGCCCCTGTGTCTGCCTGAAAAGTCCTTCTCC

GAGAACACCCTAGCCAGAATCCGCTTCTCAAGGGTCAGTGGCTGGGGCCAGCTACTGGACCG

TGGTGCCACAGCCCTGGAACTCATGTCCATCGAGGTGCCCCGGCTGATGACCCAGGACTGTC

TGGAGCACGCCAAGCACAGCTCTAACACCCCCAAAATCACAGAGAACATGTTCTGCGCTGGC

TACATGGATGGTACCAAGGACGCCTGCAAGGGTGACAGCGGTGGCCCACATGCCACGCACTA

CCATGGCACATGGTATCTGACAGGTGTGGTCAGCTGGGGGGAGGGCTGTGCAGCTATTGGTC

ACATTGGGGTGTACACCAGGGTCTCCCAGTACATAGACTGGCTGGTCAGACACATGGACTCC

AAGCTCCAGGTTGGGGTTTTCCGACTCCCACTACTGTAG

5.2.12 AMINO ACID SEQUENCE OF MOUSE FACTOR VII FROM GENBANK NM_010172 (SEQ ID NO:12)

MVPQAHGLLLLCFLLQLQGPLGTAVFITQEEAHGVLHRQRRANSLLEELWPGSLERECNEEQ

CSFEEAREIFKSPERTKQFWIVYSDGDQCASNPCQNVGTCQDHLKSYVCFCLLDFEGRNCEK

SKNEQLICANENGDCDQYCRDHVGTKRTCSCHEDYTLQPDEVSCKPKVEYPCGRIPVVEKRN

SSSRQGRIVGGNVCPKGECPWQAVLKINGLLLCGAVLLDARWIVTAAHCFDNIRYWGNITVV

MGEHDFSEKDGDEQVRRVTQVIMPDKYIRGKINHDIALLRLHRPVTFTDYVVPLCLPEKSFS

ENTLARIRFSRVSGWGQLLDRGATALELMSIEVPRLMTQDCLEHAKHSSNTPKITENMFCAG

YMDGTKDACKGDSGGPHATHYHGTWYLTGVVSWGEGCAAIGHIGVYTRVSQYIDWLVRHMDS

KLQVGVFRLPLL

(From Aasrum and Prydz, *Biochemistry Mosc.*, 67(1):25-32, 2002.)

5.2.13 DNA SEQUENCE ENCODING CHICKEN FACTOR VII FROM GENBANK AF465268 (SEQ ID NO:13)

ATGGTTTCCAGGCAGTGCGTGGCTTTGCTGCTCTGCTTCCCGCTGCTGGTTCCTCCTTCTCT
GGAAGCAGTCTTTTTAAAGCAGGAAGAGGCAAACAGCATTTTTCAAAGGCACAGAAGAGCCA
ATAGCTTCTTTGAAGAGATAAAGCTGGGGCCACTAGAGCGAGAATGCATAGAAGAAAAGTGT
TCATTTGAGGAAGCAAGAGAGATCTACCGTGATGATGAGAGGACAAAAGAGTTCTGGCACAT
CTATTCTGACCCCAACCAGTGTGACTCCAGCCCCTGTCAGAACGGAGGGAGCTGCGATGACC
AGTTTCAGGATTATGTCTGCCGCTGTCCTCCGGAGTACGAGGGCAAAAGCTGTGAAACAGCT
GTGGCCGAGAACCTGAAGTGCATTTACGACAACGGCGGCTGTGAGCAGTACTGTGCTGACGA
GCAGTCTGAAAAACGAGTGTGCTTCTGTGCAGAGGGCTACGCTTTAGCGAGTGATGGAGTGT
CCTGCATTCCCCAAGTGAAATACCCTTGTGGAACGATACCAGTGCTGGCAAGAAAGAATACA
ACTGCTCAGGGGAGAATAGTAGGTGGTGTCACCTGTCCTCCGGGTGAATGTCCATGGCAAGC
CCTTATAATACAGGATCAGAAAGGGAAATGTGGGGGTAGTCTGCTCTCACCAGAGTGGGTGG
TGACTGCAGCTCATTGCCTGGACTACGCTCATTCCAAACAGCTCCGGGTGAGGCTGGGTGAA
TACTCAGTAAAAGTTGCTGAGAAAACTGAGCAAGAAAGTGGAGTTAGCAAGATCATCAGGCA
CGAAGAATACACCATTGGACAAGTCAATCATGACATTGCCCTCCTGAAGCTGGAAACACCCG
TGAATCTCACCGATTTCGTTGTGCCAATATGTTTGCCTGAAAAACGGTTTGCAGTGTACGAG
CTGTCCTCCATTAAGTTCTCAATGGTGAGCGGATGGGGACGGCTACTAGATGGAGGGGCTAC
TTCTACTTTTCTGATGCGAGTTCATTTGCCCCGTGTAAAGACACAAGAATGTGAAAAGCAGG
CTAATTTGAACATCACCGAGAATATGTTCTGTGCAGGAGACCTGACCGGTAAAAAAGACTCC
TGCAAGGGAGACAGTGGTGGACCTCACGCTACAAAGTACAAGAACACCTGGTTTCTGACTGG
GATTGTCAGCTGGGGAAAGGGTTGTGCTGTTGAAGGCAGCTACGGGGTGTACACAAGGGTAT
CCAGATACATCAACTGGTTG

5.2.14 AMINO ACID SEQUENCE OF CHICKEN FACTOR VII

FROM GENBANK AF465268 (SEQ ID NO:14)

MVSRQCVALLLCFPLLVPPSLEAVFLKQEEANSIFQRHRRANSFFEEIKLGPLERECIEEKC
SFEEAREIYRDDERTKEFWHIYSDPNQCDSSPCQNGGSCDDQFQDYVCRCPPEYEGKSCETA
VAENLKCIYDNGGCEQYCADEQSEKRVCFCAEGYALASDGVSCIPQVKYPCGTIPVLARKNT
TAQGRIVGGVTCPPGECPWQALIIQDQKGKCGGSLLSPEWVVTAAHCLDYAHSKQLRVRLGE
YSVKVAEKTEQESGVSKIIRHEEYTIGQVNHDIALLKLETPVNLTDFVVPICLPEKRFAVYE
LSSIKFSMVSGWGRLLDGGATSTFLMRVHLPRVKTQECEKQANLNITENMFCAGDLTGKKDS
CKGDSGGPHATKYKNTWFLTGIVSWGKGCAVEGSYGVYTRVSRYINWLKRHME
Davidson *et al.*, Unpublished)

5.2.15 AMINO ACID SEQUENCE OF RABBIT FACTOR VII

FROM SWISSPROT I43962 (SEQ ID NO:15)

MAPQARGLGLCSLLALQASLAAVFITQEEAHSVLRRQRRANSFLEELRPGSLERECKEELCS
FEEAREVFQSTERTKQFWITYNDGDQCASNPCQNGGSCEDQIQSYICFCLADFEGRNCEKNK
NDQLICMYENGGCEQYCSDHVGSQRSCRCHEGYTLLPNGVSCTPTVDYPCGKVPALEKRGAS
NPQGRIVGGKVCPKGECPWQAALMNGSTLLCGGSLLDTHWVVSAAHCFDKLSSLRNLTIVLG
EHDLSEHEGDEQVRHVAQLIMPDKYVPGKTDHDIALLRLLQPAALTNNVVPLCLPERNFSES
TLATIRFSRVSGWGQLLYRGALARELMAIDVPRLMTQDCVEQSEHNPGSPEVTGNMFCAGYL
DGSKDACKGDSGGPHATSYHGTYLTGVVSWGEGCARVGHVGVYTRVSRDTEWLSRLMRSKLH
HGIQRHPFP

5.2.16 AMINO ACID SEQUENCE OF MOUSE MUTATED FACTOR VII FROM SWISSPROT AAG00449 (SEQ ID NO:16)

MVPQAHGLLLLCFLLQLQGPLGTAVFITQEEAHGVLHRQRRANSLLEELWPGSLERECNEEQ
CSFEEAREIFKSPERTKQFWIVYSDGDQCASNPCQNVGTCQDHLKSYVCFCLLDFEGRNCEK
SKNEQLICANENGDCDQYCRDHVGTKRTCSCHEDYTLQPDEVSCKPKVEYPCGRIPVVEKRN
SSSRQGRIVGGNVCPKGECPWQAVLKINGLLLCGAVLLDARWIVTAAHCFDNIRYWGNITVV
MGEHDFSEKDGDEQVRRVTQVIMPDKYIRGKINHDIALLRLHRPVTFTDYVVPLCLPEKSFS
ENTLARIRFSRVSGWGQLLDRGATALELMSIEVPRLMTQDCLEHAKHSSNTPKITENMFCAG
YMDGTKDACAGDSGGPHATHYHGTWYLTGVVSWGEGCAAIGHIGVYTRVSQYIDWLVRHMDS
KLQVGVFRLPLLGSAEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCV
VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

5.2.17 AMINO ACID SEQUENCE OF BOVINE FACTOR VII FROM SWISSPROT P22457 (SEQ ID NO:17)

ANGFLEELLPGSLERECREELCSFEEAHEIFRNEERTRQFWVSYNDGDQCASSPCQNGGSCE
DQLRSYICFCPDGFEGRNCETDKQSQLICANDNGGCEQYCGADPGAGRFCWCHEGYALQADG
VSCAPTVEYPCGKIPVLEKRNGSKPQGRIVGGHVCPKGECPWQAMLKLNGALLCGGTLVGPA
WVVSAAHCFERLRSRGNLTAVLGEHDLSRVEGPEQERRVAQIIVPKQYVPGQTDHDVALLQL
AQPVALGDHVAPLCLPDPDFADQTLAFVRFSAVSGWGQLLERGVTARKLMVVLVPRLLTQDC
LQQSRQRPGGPVVTDNMFCAGYSDGSKDACKGDSGGPHATRFRGTWFLTGVVSWGEGCAAAG
HFGIYTRVSRYTAWLRQLMGHPPSRQGFFQVPLLP

6. REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference in whole or in part:

U. S. Patent 3,215,572

U. S. Patent 4,177,253

U. S. Patent 4,230,685

U. S. Patent 4,237,224

U. S. Patent 4,329,241

U. S. Patent 4,452,773

U. S. Patent 4,554,101

U. S. Patent 4,683,195

U. S. Patent 4,683,202

U. S. Patent 4,695,392

U. S. Patent 4,770,183

U. S. Patent 4,795,698

U. S. Patent 4,800,159

U. S. Patent 4,883,750

U. S. Patent 5,069,216

U. S. Patent 5,091,206

U. S. Patent 5,145,684

U. S. Patent 5,279,721

U. S. Patent 5,399,363

U. S. Patent 5,455,166

U. S. Patent 5,466,468

U. S. Patent 5,543,158

U. S. Patent 5,552,157

U. S. Patent 5,565,213

U. S. Patent 5,567,434

U. S. Patent 5,597,530

U. S. Patent 5,641,515

U. S. Patent 5,648,211

U. S. Patent 5,656,016

U. S. Patent 5,697,899

U. S. Patent 5,705,628

U. S. Patent 5,712,124

U. S. Patent 5,738,868

U. S. Patent 5,741,516

U. S. Patent 5,744,311

U. S. Patent 5,770,219

U. S. Patent 5,783,208

U. S. Patent 5,797,898

U. S. Patent 5,795,587

U. S. Patent 5,972,707

U. S. Patent 6,177,088

U. S. Patent 6,178,871

U. S. Patent 6,238,294

U. S. Patent 6,254,890

U. S. Patent 6,2587,588

U. S. Patent 6,284,280

U. S. Patent 6,331,310

U. S. Patent 6,346,274

U. S. Patent 6,361,994

U. S. Patent 6,383,470

European Patent Application 320308

European Patent Application 329822

Great Britain Patent 2202328

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the

compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

WHAT IS CLAIMED:

1. A recombinant adeno-associated viral vector comprising at least a first nucleic acid segment encoding a biologically-active Factor VII peptide, polypeptide or protein operably linked to at least a first promoter capable of expressing said segment in a mammalian host cell transformed with said vector.

2. The recombinant adeno-associated viral vector of claim 1, wherein said nucleic acid segment encodes a mammalian biologically-active Factor VII peptide, polypeptide or protein.

3. The recombinant adeno-associated viral vector of claim 2, wherein said nucleic acid segment encodes a biologically-active Factor VII peptide, polypeptide or protein that comprises a first contiguous sequence region of at least 60 amino acids from any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

4. The recombinant adeno-associated viral vector of claim 3, wherein said nucleic acid segment encodes a biologically-active Factor VII peptide, polypeptide or protein that comprises at least a first contiguous sequence region of at least 80 amino acids from any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID

NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

5. The recombinant adeno-associated viral vector of claim 4, wherein said nucleic acid segment encodes a biologically-active Factor VII peptide, polypeptide or protein that comprises at least a first contiguous sequence region of at least 100 amino acids from any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

6. The recombinant adeno-associated viral vector of claim 5, wherein said nucleic acid segment encodes a biologically-active Factor VII peptide, polypeptide or protein that comprises at least a first contiguous sequence region of at least 120 amino acids from any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

7. The recombinant adeno-associated viral vector of claim 6, wherein said nucleic acid segment encodes a biologically-active Factor VII peptide, polypeptide or protein that comprises the sequence of any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

8. The recombinant adeno-associated viral vector of claim 6, wherein said nucleic acid segment encodes a biologically-active Factor VII peptide, polypeptide or protein that comprises the sequence of any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

9. The recombinant adeno-associated viral vector of claim 1, wherein said nucleic acid segment encodes a biologically-active human Factor VII peptide, polypeptide or protein.

10. The recombinant adeno-associated viral vector of claim 9, wherein said nucleic acid segment encodes a biologically-active human Factor VII peptide, polypeptide or protein that comprises the sequence of SEQ ID NO:2.

11. The recombinant adeno-associated viral vector of claim 3, wherein said nucleic acid segment comprises at least a first contiguous sequence region of at least 180 nucleotides from any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

12. The recombinant adeno-associated viral vector of claim 11, wherein said nucleic acid segment comprises at least a first contiguous sequence region of at least 210 nucleotides from any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

13. The recombinant adeno-associated viral vector of claim 12, wherein said nucleic acid segment comprises at least a first contiguous sequence region of at least 240 nucleotides from any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

14. The recombinant adeno-associated viral vector of claim 13, wherein said nucleic acid segment comprises at least a first contiguous sequence region of at least 270 nucleotides from any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

15. The recombinant adeno-associated viral vector of claim 14, wherein said nucleic acid segment comprises at least a first contiguous sequence region of at least 300 nucleotides from any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

16. The recombinant adeno-associated viral vector of claim 15, wherein said nucleic acid segment comprises the nucleotide sequence of any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, or SEQ ID NO:13.

17. The recombinant adeno-associated viral vector of claim 16, wherein said nucleic acid segment comprises the nucleotide sequence of SEQ ID NO:1.

18. The recombinant adeno-associated viral vector of claim 1, wherein said promoter is a heterologous promoter.

19. The recombinant adeno-associated viral vector of claim 18, wherein said promoter is selected from the group consisting of a CMV promoter, a β-actin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

20. The recombinant adeno-associated viral vector of claim 19, wherein said promoter is a chicken β-actin promoter.

21. The recombinant adeno-associated viral vector of claim 1, wherein said vector further comprises at least a first enhancer.

22. The recombinant adeno-associated viral vector of claim 21, wherein said vector further comprises a CMV enhancer, a synthetic enhancer, a muscle-specific enhancer, a liver-specific enhancer, or a tissue-specific enhancer.

23. The recombinant adeno-associated viral vector of claim 1, wherein said vector further comprises at least a first intron sequence.

24. The recombinant adeno-associated viral vector of claim 1, wherein said polynucleotide further comprises a 3′ regulatory element operably linked to said nucleic acid segment.

25. The recombinant adeno-associated viral vector of claim 24, wherein said 3′ regulatory element comprises a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).

26. The recombinant adeno-associated viral vector of claim 1, wherein said nucleic acid segment is obtained from a human, primate, murine, porcine, bovine, ovine, canine, feline, equine, epine, caprine, avian, or lupine source.

27. The recombinant adeno-associated viral vector of claim 1, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 85% identical to the amino acid sequence of any of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

28. The recombinant adeno-associated viral vector of claim 27, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 90% identical to the amino acid sequence of any of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

29. The recombinant adeno-associated viral vector of claim 28, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 95% identical to the amino acid sequence of any of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

30. The recombinant adeno-associated viral vector of claim 29, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 98% identical to the amino acid sequence of any of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:15 or SEQ ID NO:17.

31. The recombinant adeno-associated viral vector of claim 1, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 85% identical to the amino acid sequence of SEQ ID NO:2.

32. The recombinant adeno-associated viral vector of claim 31, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 88% identical to the amino acid sequence of SEQ ID NO:2.

33. The recombinant adeno-associated viral vector of claim 32, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 91% identical to the amino acid sequence of SEQ ID NO:2.

34. The recombinant adeno-associated viral vector of claim 33, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 94% identical to the amino acid sequence of SEQ ID NO:2.

35. The recombinant adeno-associated viral vector of claim 34, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 97% identical to the amino acid sequence of SEQ ID NO:2.

36. The recombinant adeno-associated viral vector of claim 1, comprised within an adeno-associated viral particle.

37. The recombinant adeno-associated viral vector of claim 36, wherein said viral particle is an adeno-associated viral serotype 1 (AAV1), serotype 2 (AAV2), serotype 3 (AAV3), serotype 4 (AAV4), serotype 5 (AAV5), or serotype 6 (AAV6) viral particle.

32. The recombinant adeno-associated viral vector of claim 31, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 88% identical to the amino acid sequence of SEQ ID NO:2.

33. The recombinant adeno-associated viral vector of claim 32, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 91% identical to the amino acid sequence of SEQ ID NO:2.

34. The recombinant adeno-associated viral vector of claim 33, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 94% identical to the amino acid sequence of SEQ ID NO:2.

35. The recombinant adeno-associated viral vector of claim 34, wherein said nucleic acid segment encodes a biologically-active mammalian Factor VII polypeptide that is at least 97% identical to the amino acid sequence of SEQ ID NO:2.

36. The recombinant adeno-associated viral vector of claim 1, comprised within an adeno-associated viral particle.

37. The recombinant adeno-associated viral vector of claim 36, wherein said viral particle is an adeno-associated viral serotype 1 (AAV1), serotype 2 (AAV2), serotype 3 (AAV3), serotype 4 (AAV4), serotype 5 (AAV5), or serotype 6 (AAV6) viral particle.

38. The recombinant adeno-associated viral vector of claim 1, comprised within an isolated mammalian host cell.

39. A recombinant adeno-associated virion comprising the recombinant adeno-associated viral vector of claim 1.

40. A plurality of recombinant adeno-associated viral particles comprising the recombinant adeno-associated viral vector of claim 1.

41. A host cell comprising: (a) the recombinant adeno-associated viral vector of claim 1; (b) the virion of claim 39; or (c) the plurality of viral particles of claim 40.

42. The host cell of claim 41, wherein said host cell is a bone marrow, liver, kidney, spleen, endothelial, epithelial, heart, lung, pancreatic, cancer, tumor, bone, or blood cell.

43. The host cell of claim 42, wherein said host cell is a human cell.

44. The host cell of claim 43, wherein said host cell is a human liver cell.

45. A composition comprising: (a) the recombinant adeno-associated viral vector of claim 1; (b) the virion of claim 39; (c) the plurality of viral particles of claim 40, or the host cell of claim 41.

46. The composition of claim 45, further comprising a pharmaceutical excipient.

47. The composition of claim 45, further comprising a microparticle, nanoparticle, microsphere, nanosphere, liposome, lipid, or lipid complex.

48. The composition of claim 45, for use in therapy.

49. The composition of claim 48, for use in the therapy of hemophilia or Factor VII deficiency.

50. The composition of claim 49, for use in the therapy of human hemophilia or human Factor VII deficiency.

51. A kit for treating or ameliorating the symptoms of Factor VII deficiency in a mammal comprising (1) the recombinant adeno-associated viral vector of claim 1; the virion of

claim 39; the plurality of viral particles of claim 40; the host cell of claim 41; or the composition of claim 45; and (2) instructions for using said kit.

52. Use of the recombinant adeno-associated viral vector of claim 1, the virion of claim 39, the plurality of viral particles of claim 40, the host cell of claim 41, or the composition of claim 45, in the manufacture of a medicament for treating hemophilia, Factor VII deficiency, or a bleeding disorder in a mammal.

53. The use according to claim 52, wherein said vector, said virion, said particle, said host cell, or said composition is provided to said mammal by injection, infection, or direct administration to a cell, tissue, or organ of said mammal.

54. The use according to claim 53, wherein said mammal is human.

55. The use according to claim 54, wherein said mammal is a human that has, is suspected of having, or at risk for developing hemophilia A.

56. A method for providing an animal a biologically-active Factor VII peptide or polypeptide, said method comprising administering to said mammal: (a) the recombinant adeno-associated viral vector of claim 1; (b) the virion of claim 39; (c) the plurality of viral particles of claim 40; (c) the host cell of claim 41; or (d) the composition of claim 45, in an amount and for a time sufficient to provide said mammal with an effective amount of said biologically-active Factor VII peptide or polypeptide.

57. The method of claim 56, wherein said mammal has, is at risk for developing, or is diagnosed with hemophilia.

58. A method for treating or ameliorating the symptoms of a Factor VII polypeptide defect, deficiency or dysfunction in a mammal, said method comprising administering to said mammal: (a) the recombinant adeno-associated viral vector of claim 1; (b) the virion of claim 39; (c) the plurality of viral particles of claim 40; (c) the host cell of claim 41; or (d) the composition of claim 45, in an amount and for a time sufficient to treat or ameliorate the symptoms of said defect, deficiency or dysfunction in said mammal.

59. The method of claim 58, wherein said mammal has, is at risk for developing, or is diagnosed with hemophilia, a clotting deficiency, or a bleeding disorder.

60. A method for treating or ameliorating the symptoms of hemophilia in a mammal, said method comprising administering to said mammal: (a) the recombinant adeno-associated viral vector of claim 1; (b) the virion of claim 39; (c) the plurality of viral particles of claim 40; (c) the host cell of claim 41; or (d) the composition of claim 45, in an amount and for a time sufficient to treat or ameliorate the symptoms of hemophilia in said mammal.

61. The method of claim 60, wherein said mammal has, is at risk for developing, or is diagnosed with hemophilia A.

62. The method of claim 60, wherein said composition is administered to said human intramuscularly, intravenously, or by injection to at least one cell, tissue, or organ of said human.

63. The method of claim 60, wherein said vector, said virion, said particle, said cell, or said composition is provided to said mammal systemically, or by direct or indirect administration to a cell, tissue, or organ of said mammal.

64. The method of claim 63, wherein said vector, said virion, said particle, said cell, or said composition is provided to mammal by direct injection into the muscle tissue, or the liver of said mammal.

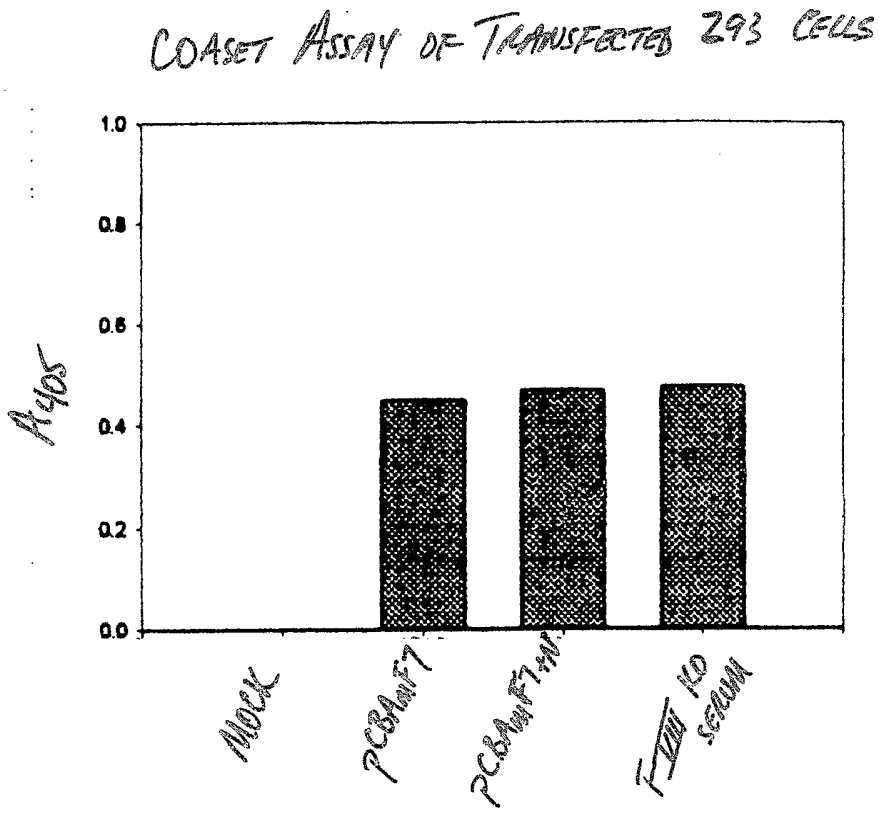
COASET ASSAY OF TRANSFECTED 293 CELLS
A405
1.0
0.8
0.6
0.4
0.2
0.0
MOCK
PCBAM F7
PCBAM F7 HP
F7 NO SERUM

FIG. 1