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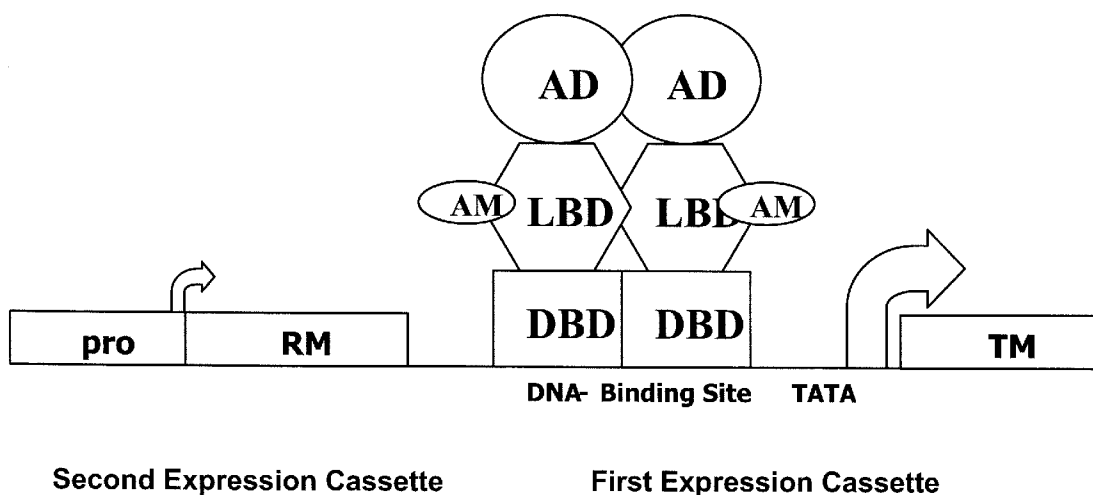
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(54) Title: GM-CSF GENE THERAPY FOR TREATMENT OF CROHN'S DISEASE USING AN IMPROVED REGULATED EXPRESSION SYSTEM



(57) Abstract: The present invention provides an improved, expression system for the regulated expression of an encoded protein or nucleic acid therapeutic molecule in the cells of a subject, for use in the treatment of disease. In particular, the present invention provides an improved, regulated gene expression system, and pharmaceutical compositions and uses thereof for treatment of disease.

GM-CSF GENE THERAPY FOR TREATMENT OF CROHN'S DISEASE USING AN IMPROVED REGULATED EXPRESSION SYSTEM

FIELD OF THE INVENTION

5 The present invention relates to an improved expression system for the regulated expression of an encoded protein or nucleic acid therapeutic molecule, for use in the treatment of disease. In particular, the present invention relates to an improved regulated gene expression system, and pharmaceutical compositions and uses thereof for treatment of disease.

BACKGROUND OF THE INVENTION

10 The delivery of nucleic acids encoding therapeutic molecules (TMs) for treatment of diseases is thought to provide enormous potential as a therapeutic modality over conventional treatment methods. In particular, the delivery of nucleic acids encoding a therapeutic protein, in gene therapy, has the potential to provide significant advantages over conventional
15 therapies requiring the administration of bolus protein. These potential advantages include, e.g., the long-term and regulated expression of a TM in the cells of a patient resulting in maximum therapeutic efficacy and minimum side effects and, also, the avoidance of toxic and infectious impurities, and systemic impurities.

 For example, the delivery of bolus protein for the treatment of disease is known to
20 result in adverse side effects including, e.g., those related to infectious and toxic impurities, systemic toxicity, injection-site necrosis, influenza-like symptoms, chills, fever, fatigue, anorexia, and weight loss. In some cases these events are dose limiting and may lead to cessation of treatment altogether. Further, it is known that continuous exposure to some protein therapeutics may result in tolerance over time. Thus, there is a need for a regulated
25 expression system that can provide a sustained or long-term, therapeutically efficacious level of a TM, with the additional feature of a means to rapidly reduce or modulate the level of TM within a dynamic therapeutic window. More particularly, there is a need for a regulated expression system which has the capability to be turned off should the concentration of TM reach a level that is potentially toxic. Moreover, the ability to titrate the level of TM would
30 allow dosing to be adjusted where there is a potential for an increase in tolerance to the TM over time.

 Of particular interest and need is the delivery of a gene encoding a therapeutic protein that can be expressed in target patient cells, to remedy a condition resulting in or caused by a disease, or to stop or slow the progression of a disease. For example, the
35 etiologies of many disease states are the result of the expression of one or more defective gene products or the defective expression of one or more gene products, e.g., the expression of a mutated protein, or the over or under expression of a protein, respectively. Thus, conventional treatment methods include the administration of recombinant proteins to correct such defective protein expression or expression of a defective protein. However, the

administration of protein therapeutics to a patient is known to result in the generation of antibodies against the protein and its rejection by the patient immune system as foreign.

Known methods of treatment for multiple sclerosis (MS) include the administration of an IFN- β protein therapeutic. MS is a chronic inflammatory autoimmune disorder of the central nervous system that affects approximately 400,000 patients in North America and approximately one million people worldwide. MS is a disease that affects more women than men, with onset typically between 20 and 40 years of age. Further, the disease is progressive, and in the early stages is characterized by a relapsing and remitting phase that is characterized by "attacks" or "relapses" of neurological dysfunction that are sub-acute over hours to days followed by periods of improvement that may last months (B.M. Keegan et al. (2002) *Annu. Rev. Med.* 53: 258-302; J. Noseworthy (2000) 343: 938-52). The symptoms include, for example, disruption of coordinated movement of the eyes, limbs, and axial muscles leading to paralysis. The course of the disease may evolve over several years with neurological symptoms that worsen until the patient becomes severely disabled. The symptoms and signs of MS can reflect demyelination of neuronal axons in the brain resulting in impaired conductance of neural impulses along the axon. Moreover, the pathology of MS can manifest itself as acute focal inflammatory demyelination and axonal loss that eventually results in, e.g., chronic multifocal sclerotic plaques from which the disease gets its name (A. Compston and A. Coles (2002) *Lancet* 359: 1221-31; L. Steinman (1996) *Cell* 85: 299-302).

Thus far, there is no cure for MS and virtually all of the approved treatments target the inflammatory component of the disease. Recombinant interferon beta (IFN- β), first introduced in 1993 by Schering AG, represented a breakthrough in the treatment of MS by demonstrating a clear benefit in decreasing the number of relapses in MS patients (overall by 30-37% annually), slowing the progression and reducing the disability associated with the disease. These effects are manifested by a significant reduction in the number of demyelinating lesions in the brain of treated MS patients as determined by magnetic resonance imaging (MRI).

There are currently three IFN- β products approved for the relapsing–remitting form of MS: 1) Betaseron® or Betaferon® (Schering); 2) Avonex® (Biogen); and 3) Rebif® (Serono). Additionally, Betaseron® has been approved for secondary progressive MS in EU, Canada, and Europe. These approved IFN- β products are purified recombinant protein preparations. In the case of Betaseron®/Betaferon® (IFN- β 1b) the recombinant protein may be purified from a bacterial cell culture (e.g., *E. coli*) that expresses the protein. In the case of Avonex® and Rebif® (IFN- β 1a), the recombinant protein is purified from a mammalian cell culture that expresses the protein. These IFN- β products for MS can be administered by subcutaneous (s.c.) or intramuscular (i.m.) injection of a bolus protein solution at a frequency ranging from once a week to every other day.

Further, the Type I interferons (e.g., IFN- β) have been approved for several indications in addition to MS, including several cancer and viral disease indications. However, it is known that such IFN protein therapeutics can cause dose-dependent side

effects, e.g., flu-like symptoms, nausea, and leukopenia in patients (E.U. Walther (1999) Neurology 53: 1622-27). These side effects can result in an intolerance to further IFN therapy. Also, it is known that some patients receiving subcutaneous (s.c.) or intramuscular (i.m.) injections of IFN protein experience local injection site reactions that can become necrotic, which can result in the discontinuation of the IFN therapy (A. Bayas and R. Gold (2003) J. Neurol. 250(4): IV3-IV8). Further it is known that some MS patients undergoing IFN- β therapy for MS generate neutralizing antibodies that may limit the therapeutic benefit of the drug over time (S.M. Malucchi (2004) Neurology 62: 2031-37). Lastly, pharmacokinetic studies have shown that IFNs have a short half-life in the circulation, with levels becoming undetectable within a few hours following bolus delivery of the recombinant protein to a patient (R. Wils Clin. Pharmacokinet. 19: 390-99; P. Salmon et al. J. Interferon Cytokine Res. 16: 759-64; P.-A. Buchwalder et al. (2000) J. Interferon Cytokine Res. 20: 57-66).

Ulcerative colitis (UC) and Crohn's disease (CD), also known as inflammatory bowel disease (IBD), are chronic disorders with a prevalence estimated at 1 to 2 per 2000 people. IBD, including CD, is a serious, life-long chronic inflammation throughout the gastrointestinal (GI) tract with alternating periods of active disease and disease remission. Current treatment of CD relies primarily on suppression of the immune response. Many patients cannot tolerate this type of treatment or show no improvement, strongly indicating that improved drugs are needed.

Current treatment paradigms being studied include the use of Sagramostim, which is the generic name for Leukine®, containing the protein granulocyte-macrophage colony-stimulating factor (GM-CSF) as the active ingredient. This approach strengthens the first line innate immune system by using GM-CSF to contain microbes, that in case of CD leads to a compensatory and persistent over-activation of the specific immune system with a chronic inflammation.

Leukine®, which is a GM-CSF protein expressed in yeast that differs from human GM-CSF by a leucine to arginine substitution at position 23, has now been tested in clinical phase I, II and III for treatment of CD by daily injections. Positive results from the phase II study and mixed results from phase III indicate potential for GM-CSF for this indication. The results suggest a treatment benefit, indicating that the new treatment paradigm, which is to strengthen the immune barrier of the GI tract instead of suppressing the immune system, may decrease disease severity and improve quality of life in patients with CD. Using GM-CSF protein might hold some intrinsic problems inherent in protein therapeutics, including the source of the recombinant protein, injection schedule, half life time and bioavailability. There is, therefore, a need for alternative candidates to overcome these obstacles.

Thus, there is a need for gene-based delivery of therapeutic proteins for the treatment of disease that provides regulated, long-term expression of the protein, resulting in therapeutic efficacy while minimizing dose-limiting toxic side effects. Such a regulated expression system could avoid many of the major limiting factors associated with current protein therapeutics. However, most known nucleic acid delivery systems are not suitable for

clinical use and do not afford regulated or long-term expression in cells. Only a few known nucleic acid delivery systems are reported to have an ability to regulate transgene expression under laboratory conditions, but the suitability and workability of these delivery systems for clinical use are not known (see e.g., M. Gossen and H. Bujard Science 268: 1766-69; D. No et al. (1996) Proc. Natl. Acad. Sci. USA 93: 3346-51; J.F. Amara et al. (1997) Proc. Natl. Acad. Sci. USA 94: 10618-23; Y. Wang (1994) Proc. Natl. Acad. Sci. USA 91: 8180-84; J.L. Nordstrom (2002) 13: 453-58).

SUMMARY OF THE INVENTION

The present invention provides an improved expression system for the regulated expression of an encoded protein or nucleic acid therapeutic molecule (TM) for use in the treatment of disease, wherein therapeutic efficacy of the TM can be maximized and side effects minimized. In particular, the present invention provides an improved regulated gene expression system, and pharmaceutical compositions and methods thereof for treatment of disease. The encoded TM can be a nucleic acid or protein that provides a therapeutic benefit to a subject having, or susceptible to, a disease. For example, such therapeutic benefit or activity includes, but is not limited to, the amelioration, modulation, diminution, stabilization, or prevention of a disease or a symptom of a disease.

In one aspect, the present invention provides an improved regulated expression system comprising at least a first expression cassette having a nucleic acid sequence encoding a TM, such that, when delivered to cells of a subject, the encoded TM is expressed, and the expression and/or activity of the TM is regulated in the presence of a regulator molecule (RM). Examples of such regulation include, but are not limited to, the induction, repression, increase, or decrease of TM expression and/or activity in the presence of an RM.

In one aspect of the present invention, the expression and/or activity of the TM is regulated in a dose-responsive or dose-dependent manner, e.g., according to the amount of a RM present in the cells of the subject or administered to the subject. In other aspects, the expression and/or activity of the TM is regulated in a dose-responsive or dose-dependent manner, e.g., according to the amount of an activator molecule (AM) or inactivator molecule (IM) present in the cells of the subject or administered to the subject.

In another aspect of the present invention, the expression and/or activity of the TM is orientation-dependent. For example, in one aspect, the expression and/or activity of the TM in cells is modulated with respect to the 5' to 3' orientation of the expression cassette encoding the TM, or with respect to the 5' to 3' orientation of the transcription or translation of the encoded TM. Consequently, TM expression and/or activity can be modulated by selection of a particular orientation of the expression cassette encoding the TM or the orientation of transcription or translation of the TM.

In another aspect, the regulated expression system of the present invention further comprises a second expression cassette encoding an RM, such that, when delivered to cells of a subject, the encoded RM is expressed and the presence thereof regulates the expression

and/or activity of the TM. In a preferred aspect, a first expression cassette encoding a TM and a second expression cassette encoding an RM of the present invention are present in a single vector. In another preferred aspect, the single vector is pGT23, pGT24, pGT25, pGT26, pGT27, pGT28, pGT29, or pGT30. In yet another preferred aspect, the single vector is pGT54, pGT57, pGT713, pGT15, or pGT16. In yet another aspect, the single vector is pGT615, pGT616, pGT617 or pGT618.

A TM of the present invention can be an isolated DNA, RNA, or protein, or variant thereof, encoded by a nucleic acid sequence and having a therapeutic activity. More particularly, a TM of the present invention can be a modified, synthetic, or recombinant DNA, RNA or protein. In another aspect of the present invention, the encoded TM is a nucleic acid, e.g., a DNA or RNA, having a therapeutic activity. In one aspect of the present invention, the encoded TM is an RNA e.g., an siRNA or shRNA. In another aspect of the present invention, the encoded TM is a protein having a therapeutic activity and, preferably, a human protein or variant thereof. In one aspect the encoded TM is a monoclonal antibody having a therapeutic activity. In one aspect, the encoded TM is the monoclonal antibody, CAMPATH®. In another aspect, the nucleic acid sequence encoding such a protein is a gene or gene fragment. In one aspect, the encoded TM is a granulocyte macrophage colony stimulating factor (GMCSF) or variant of GMCSF (e.g., Leukine®). In another aspect, the encoded TM is an interferon, e.g., interferon-alpha (IFN-α) or interferon-beta (IFN-β), and more particularly, is IFN-β-1a.

An RM of the present invention can be a naturally-occurring molecule or variant thereof, or an isolated molecule. In some aspects, an RM of the present invention is a synthetic or recombinant molecule. For example, in some aspects, an RM of the present invention is a chemical compound, DNA, RNA, or protein. Further, in some aspects, an RM of the present invention is a modified molecule. In one aspect, the RM is a humanized protein. In another aspect, the RM is a human protein or variant thereof. For example, in one aspect, the RM is a transcriptional activator, e.g., a steroid receptor and, more particularly, a progesterone receptor. In one aspect, the RM comprises a transactivation domain (e.g., a VP16 or p65 transactivation domain). In another aspect, the RM comprises a ligand-binding domain (LBD). Further, in one aspect, an AM binds to the LBD of the RM, thereby activating the RM such that the presence of the activated RM regulates TM expression and/or activity. In another aspect, the RM comprises a DBD, e.g., a GAL-4 DBD. In one aspect, the RM comprises a DBD that binds to a functional sequence (e.g., a promoter sequence) operably linked to a nucleic acid encoding a TM, thereby regulating TM expression (e.g., inducing TM expression).

In another aspect, an RM of the present invention is activated and thereby TM expression and/or activity is regulated in the presence of the activated RM. In one aspect, an RM of the present invention is expressed or present in cells of a subject in an inactivated form, and is activated in the presence of an AM, thereby, TM expression and/or activity is regulated by the activated RM. In one aspect, the AM is a biomarker. In a further aspect, the AM is a biomarker for a disease or condition and, more particularly, is a biomarker for a

disease state or condition, or symptom thereof. In one aspect, the AM activates the RM by promoting or inhibiting conformational change, enzymatic processing or modification, specific binding, or dimerization of the RM. In a preferred aspect, the AM activates the RM by promoting homodimerization of the RM.

5 An AM of the present invention can be a naturally-occurring molecule or variant thereof, or an isolated molecule. In some aspects, the AM of the present invention is a synthetic or recombinant molecule. For example, in some aspects, the AM of the present invention is a chemical compound, DNA, RNA, or protein. Further, in some aspects, the AM of the present invention is a modified molecule. In one aspect, the AM is a humanized protein.

10 In another aspect, the AM is a human protein or variant thereof. In one aspect, the AM is a chemical compound, e.g., an antiprogesterin. In a preferred aspect, the AM is mifepristone.

 In another aspect, an RM of the present invention is inactivated and thereby TM expression and/or activity is regulated in the presence of an inactivated RM. In one aspect, an RM of the present invention is expressed or present in cells of a subject in an activated

15 form, and is inactivated in the presence of an IM, thereby, TM expression and/or activity is regulated by the inactivated RM. In one aspect, the IM is a biomarker. In a further aspect, the IM is a biomarker for a disease or condition and, more particularly, is a biomarker for a disease state or condition, or symptom thereof. In one aspect, the IM inactivates the RM by promoting or inhibiting conformational change, enzymatic processing, specific binding, or

20 dimerization of the RM. In a preferred aspect, the IM inactivates the RM by inhibiting homodimerization of the RM.

 An IM of the present invention can be a naturally-occurring molecule or variant thereof, or an isolated molecule. In some aspects, the IM of the present invention is a synthetic or recombinant molecule. For example, in some aspects, the IM of the present

25 invention is a chemical compound, DNA, RNA, or protein. Further, in some aspects, the IM of the present invention is a modified molecule. In one aspect, the IM is a humanized protein. In another aspect, the IM is a human protein or variant thereof. In a preferred aspect, the IM is a chemical compound.

 The expression of a TM, RM, AM, or IM of the present invention can be constitutive or

30 transient. In some aspects, expression of a TM, RM, AM, or IM is regulated or tissue-specific (e.g. muscle-specific). Examples of a regulated RM include, but are not limited to, an RM that is activated by an AM or inactivated by an IM. In one aspect, the expression of a TM, RM, AM, or IM of the present invention is driven by a regulated promoter or a tissue-specific promoter. In a further aspect, the regulated or tissue-specific promoter is regulated in the

35 presence of an RM and, more particularly, by the binding of the RM to the promoter. For example, in one aspect, an RM of the present invention binds to a promoter operably linked to a nucleic acid sequence encoding a TM and thereby, regulates the expression of the encoded TM as described herein, in the cells of a subject. In one aspect, the promoter that is operably linked to a nucleic acid encoding the TM, comprises at least one GAL-4 DNA-binding site

40 (DBS), and preferably comprises 3-18 GAL-4 DBS. In another aspect, the promoter is a Pol II

or Pol III promoter. In one aspect, the promoter is the Pol II promoter U6H1. In another aspect, the promoter is a Pol II promoter selected from a group consisting of: a muscle creatine kinase promoter (MCK), a promoter comprising hypoxia responsive element (HRE promoter), endothelial leukocyte adhesion molecule (ELAM) promoter, chimeric promoter (e.g., CMV/actin chimeric promoter), cyclin A promoter, and cdc6 promoter.

The present invention also provides pharmaceutical compositions and methods for treatment of disease or condition comprising the improved regulated expression system of the present invention as described herein. In particular aspects, the present invention provides pharmaceutical compositions and methods for treating a disease or condition; regulating the expression of a TM; administering a TM; 4) delivering a TM; or expressing a TM in cells of a subject, where the methods comprise contacting the cells with a regulated expression system of the present invention, such that the encoded TM is expressed in the cells, and such TM expression is regulated in the presence of an RM. In one aspect, the present invention provides pharmaceutical compositions and methods for treatment of leukemia, melanoma, hepatitis, and cardiomyopathy. In a preferred embodiment, the encoded TM of the regulated expression system of the present invention is an IFN, e.g., an IFN- α or an IFN- β , for treatment of leukemia, melanoma, hepatitis, or cardiomyopathy.

The pharmaceutical compositions of the present invention comprise at least one of the expression systems described herein, particularly, at least one of the TM and RM of the present invention, more particularly, at least one of the vectors of the present invention (e.g., pGT23, pGT24, pGT25, pGT26, pGT27, pGT28, pGT29, pGT30, pGT54, pGT57, pGT713, pGT715, pGT716, pTR-m IFN- β , pTR-hIFN- β or pBRES-GMCSF). In some aspects, the pharmaceutical compositions of the present invention comprise at least one AM or IM of the present invention. In one aspect, a pharmaceutical composition of the present invention comprises one or more vectors encoding at least one TM and/or RM. The TM, RM, AM, and IM of the present invention can be administered to a subject separately or together and ex vivo or in vivo, using any suitable means of administration described herein or known in the art. Examples of such suitable means of administration include, but are not limited to injection (e.g., subcutaneous injection), oral administration, and electroporation. In one aspect, a TM and RM of the present invention are present in a single vector, and separately administered from an AM that activates the RM (and thereby, the presence of the activated RM regulates TM expression and/or activity). In a further aspect, the AM is a compound (e.g., mifepristone) administered orally, and the single vector encoding a TM and RM is a single vector administered by injection or electroporation to cells of a subject (e.g., skeletal muscle cells).

The present invention further provides vectors and kits comprising the improved regulated expression system of the present invention. In some aspects, the improved regulated expression system of the present invention comprises one or more vectors, and each vector comprises one or more expression cassettes. In one aspect, the improved regulated expression system of the present invention comprises a single vector having at least one expression cassette and, more preferably, at least two expression cassettes. In a

preferred aspect, the improved regulated expression system of the present invention comprises a single vector comprising a first expression cassette having at least one cloning site for insertion of a first nucleic acid sequence encoding a TM, and a second expression cassette having at least one cloning site for insertion of a second nucleic acid sequence encoding an RM. In another aspect, the vector is a vector that is used for producing virus, e.g., an adeno-associated virus (AAV) shuttle plasmid and, more particularly, an AAV-1 shuttle plasmid. In one aspect, the vector of the present invention is a nonviral vector (i.e., a vector that does not produce virus), e.g., a plasmid vector that does not produce virus. In a preferred aspect, the vector is a plasmid vector of the present invention comprising a cloning site for insertion of a nucleic acid sequence comprising a sequence encoding a TM. Examples of such plasmid vectors of the present invention include, but are not limited to, pGT1, pGT2, pGT3, pGT4, pGT11, pGT12, pGT13, or pGT14.

The expression cassettes of the present invention comprise functional sequences for expression of an encoded molecule of the present invention, e.g., a TM, RM, AM, or IM. In some aspects, the expression cassette comprises at least one functional sequence operably linked to a nucleic acid sequence encoding a molecule of the present invention. Examples of a functional sequence are, but not limited to, a 5' or 3' untranslated region (e.g., UT12), intron (e.g., IVS8), poly(A) site (e.g., SV40 or hGH poly(A) site), or a DNA-binding site (DBS) (e.g., GAL-4 DBS). In one aspect, the functional sequence comprises at least one GAL-4 DBS and preferably comprises multimers of a GAL-4 DBS (e.g., 3-18 GAL-4 DBS). Such functional sequences also include, for example, sequences encoding a regulated promoter or tissue-specific promoter that promotes the regulated or tissue-specific expression, respectively, of a molecule encoded by a nucleic acid sequence operably linked to such functional sequences in an expression cassette of the present invention. In another aspect, the expression cassettes of the present invention comprise at least one cloning site and, more preferably, a multiple cloning site (MCS), for the insertion of a nucleic acid sequence encoding a molecule of the present invention, e.g., a TM, RM, AM, or IM.

In one aspect, a first expression cassette of the present invention comprises an MCS for insertion of a first nucleic acid sequence encoding a TM, an inducible promoter comprising at least one DBS (e.g., 3-18 GAL-4 DBS), 5' untranslated region (e.g., UT12), an intron (e.g., IVS8), and hGH poly(A) site, such that when the first nucleic acid sequence is inserted at the MCS, these functional sequences are operably linked to this sequence. In another aspect, a second expression cassette of the present invention comprises an MCS for insertion of a second nucleic acid sequence encoding a regulated RM and SV40 poly(A) site, such that when the second nucleic acid sequence is inserted at the MCS, these functional sequences are operably linked to this sequence. In a preferred aspect, the first and second expression cassettes are present in a single vector.

The kits of the present invention comprise at least one of the expression systems of the present invention described herein and, more particularly, at least one of the

pharmaceutical compositions, vectors, or molecules (e.g., TM, RM, AM, or IM) of the present invention.

In one aspect, the present invention is directed to a method of treating an anti-inflammatory disease or condition. The method comprises administering a regulated gene expression system comprising at least a vector comprising a first gene expression cassette and a second gene expression cassette. The first gene expression cassette comprises: i) a first nucleic acid sequence encoding a therapeutic molecule (TM) having a therapeutic activity, and ii) a first promoter and a first poly(A) site operably linked to the first nucleic acid sequence, wherein the TM is expressed in cells of a subject in a therapeutically effective amount and is an interferon molecule (IFNM) or a variant thereof, and the TM expression or activity is induced in the presence of an activated regulator molecule (RM). The second gene expression cassette comprises: i) a second nucleic acid sequence encoding the RM, and ii) a second promoter and a second poly(A) site operably linked to the second nucleic acid sequence, wherein the RM is expressed in the cells and activated in the presence of an activator molecule (AM), thereby inducing the TM expression or activity in a dose dependent manner. In a further aspect, the anti-inflammatory disease is multiple sclerosis.

In another aspect of the method, the vector and/or AM is administered one or more times. In a further aspect, the vector and/or AM is administered daily, every other day, three times per week, twice per week, weekly, biweekly, monthly, bimonthly, every three months, quarterly, semiannually, annually, or some combination thereof. In some embodiments, there is a lag time between the administration of the vector encoding the TM and the time in which an AM is imposed. For example, in one embodiment, the lag time between administration of the vector encoding the TM and the induction of the expression of the TM by an AM is for example, 5 days, 12 days, 20 days or 55 days.

In another aspect, the AM is formulated as an oral dosage or an injectable dosage. In a further aspect, the AM is formulated as an immediate release dosage or a controlled release dosage. In a further aspect, the AM is formulated as a controlled release dosage. In another aspect, the AM is mifepristone. In a further aspect, the mifepristone is administered at a dosage from about 0.01 mg/kg to about 10 mg/kg, from about 0.05 mg/kg to about 5 mg/kg or about 0.01 mg/kg. In a further aspect, the mifepristone is administered at a dosage from 0.01 mg/kg to 10 mg/kg, from 0.05 mg/kg to 5 mg/kg or 0.01 mg/kg.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

Figure 1 illustrates unlimiting examples of a regulated expression system of the present invention. **Figure 1A** illustrates an unlimiting example of a regulated expression system of the present invention comprising: 1) a first expression cassette comprising a first nucleic acid sequence encoding a therapeutic molecule (TM) and a first promoter sequence

encoding a DNA-binding site (DBS) and TATA sequence operably linked to the first nucleic acid sequence; 2) a second expression cassette comprising a second nucleic acid sequence encoding a regulator molecule (RM) and a second promoter sequence operably linked to the second nucleic acid sequence; 3) the expressed RM that is a fusion or chimeric protein comprising a DNA-binding domain (DBD), ligand-binding domain (LBD), and regulatory domain (RD); and 4) an activator or inactivator molecule (A/IM) that activates the RM or inactivates the RM, respectively. In one embodiment, an activator molecule (AM) binds to the RM and activates the RM and, thereby, the activated RM binds to the DBS of the promoter sequence operably linked to the TM sequence, resulting in the induction of TM expression in cells (e.g., mammalian cells). In another embodiment, the first and second expression cassettes are present in a single vector.

Figure 1B illustrates an unlimiting example of a regulated expression system of the present invention comprising: 1) a first expression cassette comprising a first nucleic acid sequence encoding a TM and a first promoter sequence encoding a DBS and TATA sequence operably linked to the first nucleic acid sequence; 2) a second expression cassette comprising a second nucleic acid sequence encoding a regulator molecule (RM) and a second promoter sequence operably linked to the second nucleic acid sequence; 3) the expressed RM that is a fusion or chimeric protein comprising a DBD, LBD, and activation domain (AD); and 4) an activator or inactivator molecule (A/IM). In one embodiment, an activator molecule (AM) binds to the RM and activates the RM, and thereby, the activated RM forms a homodimer that binds to the DBS of the promoter operably linked to the TM sequence, resulting in the induction of TM expression, in cells (e.g., mammalian cells). In another embodiment, the first and second expression cassettes are present in a single vector.

Figure 2 illustrates murine IFN- β and human IFN- β plasmid vectors for generation of recombinant protein. Figures 2 A and B illustrate murine IFN- β expression vectors for generation of recombinant protein (**A**, pGER90 (pCEP4/mIFN) and for gene-based delivery studies (**B**, pGER101 (pgWiz/mIFN)). The CMV promoter and enhancer present in pGER90 extends from -831 bp to +1 bp relative to the transcription start site, with no 5' UTR or intron. The CMV sequences present in pGER101 include the promoter, enhancer, 5' UTR, and natural Intron A from -674 bp to +942 bp. Figure 2 C and D illustrate human IFN- β expression vectors for generation of recombinant protein (**C**, pGER123 (pCEP4/hIFN) and for gene-based delivery studies (**D**, pGER125 (pgWiz/hIFN)).

Figure 3 illustrates the pharmacokinetic profile following injection of human IFN- β 1a protein in mice. C57BL/6 mice were administered either 25 ng (Low Dose) or 250 ng (High Dose) of recombinant hIFN- β 1a protein by either i.v. or i.m. injection. Human IFN- β levels were determined by ELISA (Toray-Fugi Bio, Biosource International) in serum samples obtained following terminal bleeding of mice at the indicated time points post-injection (n=4 mice per time point). Each data point represents the mean value +/- the standard deviation.

Figure 4 illustrates the pharmacokinetic profile following intramuscular injection of AAV-1-hIFN- β in mice. C57BL/6 mice (n=6 per group) were injected i.m. with 0.5×10^{10} ,

1.0x10¹⁰, or 5.0x10¹⁰ viral particles of AAV-1-hIFN- β . Blood samples were taken at the indicated time points following injection and hIFN- β serum levels were determined by ELISA. Each data point represents the mean value +/- the standard deviation.

Figure 5 illustrates Mx1 RNA induction in vitro (in L929 cells) by mIFN- β . L929 cells were seeded at 5x10⁵ cells in 6 well plates and stimulated with increasing amounts of purified recombinant mIFN- β protein. Four hours after treatment the cells were harvested, RNA isolated, and Mx1 RNA quantitated by TaqMan analysis. Mx1 RNA expression is plotted as the fold increase relative to GAPDH RNA.

Figure 6 illustrates Mx1 RNA induction following i.v. (A) or i.m. injection (B) of mIFN- β protein. C57BL/6 mice were administered 15, 150, or 500 ng of purified recombinant mIFN- β protein (specific activity-2.0x10⁸ units/mg) by either i.v. (via tail vein) or i.m. injection (n=3 mice per group). At the specified time points post-injection mice were bled, and RNA was isolated from PBMCs. Mx1 RNA was measured by quantitative RT-PCR. The fold increase in Mx1 RNA is expressed relative to GAPDH values measured in the same samples. The controls include naive mice (N), and mice injected with the vehicle buffer only followed by Mx1 analysis at 2 hours (V2h) or 4h (V4h) post-injection. Each column represents the mean value +/- standard deviation.

Figure 7 illustrates induction levels of IP-10 (A) and JE (B) following i.v. or i.m. injection of murine IFN- β protein. C57BL/6 mice were administered 15, 150, or 500 ng of purified recombinant mIFN- β protein (specific activity = 2.0x10⁸ units/mg) by either i.v. (via tail vein) or i.m. injection (n=3 mice per group). The mice were bled at 2, 4, 6, 12, 24, and 48 hours post-injection, and the plasma levels of IP-10 and JE were measured by ELISA (R&D Systems).

Figure 8 illustrates the induction of IP-10 following intramuscular injection of AAV-1-mIFN- β DNA or mIFN- β plasmid DNA with electroporation (EP) in mice. Normal mice (C57BL/6) were injected i.m. with either AAV-1-mIFN- β (5x10⁹ viral particles), or mIFN- β plasmid DNA (150 ug) with electroporation. Mice were bled at the indicated time points and IP-10 levels in plasma were determined by ELISA. Each column represents the mean value +/- the standard deviation (n=5 mice per group).

Figure 9 illustrates the induction of Mx1 mRNA following intramuscular injection of mIFN- β plasmid DNA. Mice were injected i.m. into the hind limb gastrocnemius and tibialis muscles with different amounts of plasmid DNA encoding mIFN- β (62.5, 125, 250, or 500 ug) followed by electroporation (n=5 per group). Mice were bled at the specified time points post-injection, RNA isolated from PBMCs, and Mx1 expression was determined by quantitative RT-PCR. Mx1 RNA levels were normalized to GAPDH expression and are shown as fold induction over background measured at day 0 compared to untreated controls (Controls, n=4). Each column represents the mean value +/- the standard deviation.

Figure 10 illustrates the induction of Mx1 mRNA following intramuscular injection of AAV-1-mIFN- β virus or mIFN- β plasmid DNA with electroporation in mice. Normal mice (C57BL/6) were injected i.m. with either AAV-1-mIFN- β (5x10¹⁰ viral particles), or mIFN- β

plasmid DNA (150 ug) with electroporation. Controls included PBS injected mice (i.m. control), and mice injected with SEAP plasmid (pSEAP) or AAV-1 expressing SEAP (AAV-SEAP). Mice were bled at the indicated time points and Mx1 RNA levels were determined by quantitative RT-PCR in RNA isolated from PBMCs. Mx1 RNA expression was normalized to GAPDH expression and is shown as fold induction over background measured at day 0 in PBS injected control mice. Each column represents the mean value $\pm$ the standard deviation (n=5 mice per group).

Figure 11 illustrates the efficacy of IFN- β protein in a mouse acute EAE model (as described in Example 5 and Material and Methods subsection A). Mice treated with 100K units of IFN- β developed significantly decreased clinical scores of EAE compared with vehicle treated mice (p=0.0046). Mice treated with 30K units of IFN- β also developed decreased clinical scores compared to vehicle treated mice, although this decrease did not reach statistical significance. The positive controls in this study, Mesopram and Prednisolone, also significantly decreased clinical scores.

Figure 12 illustrates the efficacy of gene-based delivery of mIFN- β in a murine acute EAE model. Female SJL mice were immunized with proteolipid peptide (PLP)/pertussis toxin on day 1 as fully described in the Materials and Methods. Groups of mice (n=10 per group) were injected with either PBS, a null plasmid (pNull) plus electroporation (EP) (pNull + EP, 120 ug), or plasmid DNA encoding mIFN- β (pmIFN- β) plus EP (pmIFN- β + EP, 120 ug) on day 2 of the study. For protein delivery, recombinant mIFN- β protein (100,000 units) was administered to another group of animals by s.c. injection every other day beginning on day 1 of the study. A significant decrease in disease severity was observed with pmIFN- β +EP versus the pNull+EP control group (p=0.0171). The results of the study are fully described in the Materials and Methods.

Figure 13 illustrates the efficacy of IFN- β protein in a mouse acute EAE model as fully described in Example 5 and Materials and Methods.

Figure 14 illustrates plasmid vectors pGT1, pGT2, pGT3, and pGT4 (A, B, C, D, respectively), which are unlimiting examples of one-plasmid regulated expression vectors of the present invention. In these examples, the regulated expression vectors of the present invention contain, in a single plasmid vector: 1) a first expression cassette with a multiple cloning site (MCS) for insertion of a nucleic acid encoding a therapeutic molecule (TM); and 2) a second expression cassette with a cloning site for insertion of a nucleic acid encoding a regulator molecule (RM). These four vectors each provide a different orientation of the first and second expression cassettes relative to each other as described and illustrated. In the first expression cassette, the skeletal muscle promoter (sk actin pro), untranslated region 12 (UT12), intervening sequence 8 (IV8) from the plasmid pLC1674 are located upstream of the MCS and human growth hormone poly (A) site (hGH polyA). A nucleic acid comprising a therapeutic molecule (TM) of interest, e.g., a transgene, can be inserted at the MCS.

Figure 15 illustrates unlimiting examples of regulated expression plasmid vectors of the present invention for gene-based delivery of murine IFN- β (pGT23, pGT24, pGT25, and

pGT26) (A), or human IFN- β (pGT27, pGT28, pGT29, and pGT30) (B). In these examples, the regulated expression vectors of the present invention contain, in a single plasmid vector:

- 1) a first expression cassette with a multiple cloning site (MCS) and a nucleic acid inserted at the MCS encoding either a human IFN- β gene or a murine IFN- β gene; and 2) a second

expression cassette with a cloning site and a nucleic acid inserted at the site encoding a regulator molecule (RM) that contains the modified LBD of the progesterone receptor (e.g., comprising the amino acid sequence of SEQ ID NO: 22 or encoded by the nucleic acid sequence of SEQ ID NO: 21). These vectors each provide a different orientation of the first and second expression cassettes relative to each other as described and illustrated as fully described in the Materials and Methods, subsection F.

Figure 16 illustrates the in vitro validation of hIFN- β regulated expression plasmid vectors of the present invention in murine skeletal muscle cells as fully described in Example 6, subsection C. Constitutive (pGER125) and inducible (pGT27, pGT28, pGT29, and pGT30) hIFN- β plasmid vectors were transfected into mouse muscle C2C12 cells, treated with MFP (10 nM), and media collected. Media was assayed for hIFN- β by ELISA. The average of two independent transfections are shown. Plasmid vectors pGS1694 + pGER129 is a two-plasmid system of Valentis in which the present inventors inserted the hIFN- β gene. The regulated expression vectors of the present invention were constructed with the hIFN- β gene in either the forward (hIFN, $\rightarrow$) or reverse (hIFNr, $\leftarrow$) direction, either upstream or downstream of the RM cassette.

Figure 17 illustrates the in vitro validation of mIFN- β regulated expression plasmid vectors of the present invention in murine skeletal muscle cells as fully described in Example 6, subsection C. Constitutive (pGER101) and inducible (pGT23, pGT24, pGT25, and pGT26) mIFN- β expression plasmids were transfected into mouse muscle C2C12 cells. Media was replaced 24 hours (hr) after transfection with fresh media with or without MFP (10 nM). Media was collected 24 hr later and assayed for mIFN- β by a reporter gene assay. The chart shows the average of three independent transfections. pGS1694 + pGER127 is a two-plasmid system of Valentis in which the present inventors inserted the mIFN- β gene. The regulated expression vectors of the present invention were constructed with the mIFN- β gene in either the forward (mIFN, $\rightarrow$) or reverse (mIFNr, $\leftarrow$) direction, either upstream or downstream of the RM cassette.

Figure 18 illustrates Mx1 RNA induction in vivo using a pBRES mIFN- β regulated expression system of the present invention. Constitutive (pGER101) and inducible regulated expression (pGT26) mIFN- β plasmid vectors were injected and electroporated into the tibialis and gastrocnemius muscles of mice (150 μ g per animal). Blood was collected at 7 days after injection. Mice were treated with MFP (0.33 mg/kg) by oral gavage once per day 7-10 days after injection. Blood was collected at 11 and 18 days after injection. PBMCs were isolated from the blood and RNA was prepared from PBMCs and assayed by RT-PCR to determine the level of Mx1 RNA. Mx1 expression levels were normalized to GAPDH. The results are shown as the mean (n=5 animals per group) $\pm$ the standard deviation, and show little or no

activity of Mx1 RNA using pBRES -mIFN in the absence of MFP at 7 days, and strong induction to levels higher than with CMV-mIFN in the presence of MFP at 11 days, as fully described in the Materials and Methods, subsection C. At 18 days, in the absence of MFP, the Mx1 RNA decreased nearly to baseline.

5 **Figure 19** illustrates IP-10 and JE induction with a pBRES mIFN- β regulated expression system of the present invention. Constitutive (pGER101) and inducible pBRES (pGT26) mIFN expression plasmids were injected and electroporated into hind limb muscles of C57BL/6 mice. Animals were bled and the plasma was assayed for the chemokines IP-10 and JE by ELISA on day 7 (absence of MFP), day 11 (following four consecutive days of oral
10 administration of MFP, and day 18. The results are shown as the mean (n=5 animals per group) +/- the standard deviation, and show little or no activity of chemokines (IP-10 and JE) using pBRES-mIFN in the absence of MFP at 7 days, and strong induction to levels higher than with CMV-mIFN in the presence of MFP at 11 days, as fully described in the Materials and Methods, subsection C. At 18 days, in the absence of MFP, the chemokine levels
15 returned to baseline.

Figure 20 illustrates plasmid vectors pbSER189 (A) and pgWIZ (B) used in the construction of plasmid vector pGER (pgWiz/mIFN) (C), as fully described in the Materials and Methods, subsection F.

20 **Figure 21** illustrates the plasmid vector pGER125 (pgWiz/hIFN) as fully described in the Materials and Methods, subsection F.

Figure 22 illustrates the plasmid vector pGene/V5-HisA as fully described in the Materials and Methods, subsection F.

Figure 23 illustrates the plasmid vector pGene-mIFN (pGER127) as fully described in the Materials and Methods, subsection F.

25 **Figure 24** illustrates the plasmid vector pGene-hIFN (pGER129) as fully described in the Materials and Methods, subsection F.

Figure 25 illustrates the plasmid vector pSwitch (Invitrogen) as fully described in the Materials and Methods, subsection F.

30 **Figure 26** illustrates the plasmid vector pGS1694 as fully described in the Materials and Methods, subsection F.

Figure 27 illustrates the plasmid vector pLC1674 as fully described in the Materials and Methods, subsection F.

Figure 28 illustrates the pGT-hGMCSF and pGT-mGMCSF shuttle plasmids and construction thereof, as fully described in the Materials and Methods, subsection F.

35 **Figure 29** illustrates the pZac2.1-RM-hGMCSF and pZac2.1-RM-mGMCSF (A) and pZac2.1-CMV-hGMCSF (pGT713) and pZac2.1-CMV-mGMCSF (pGT714) (B) shuttle plasmids and construction thereof, as fully described in the Materials and Methods, subsection F.

Figure 30 illustrates the pORF-hGMCSF and pORF9-mGMCSF used in the construction of pZac2.1-RM-hGMCSF and pZac2.1-RM-mGMCSF, respectively, as fully described in the Materials and Methods, subsection F.

Figure 31 illustrates the pGT715 (A) and pGT716 (B) shuttle plasmids, as fully described in the Materials and Methods, subsection F.

Figure 32 illustrates IP-10 induction in vivo with mIFN- β regulated expression plasmid vectors of the present invention. Inducible (pGT23, pGT24, pGT25, and pGT26) mIFN- β expression plasmids were injected and electroporated into hind limb muscles of C57BL/6 mice. Animals were bled and the serum was assayed for the chemokine IP-10 by ELISA on day 7 (absence of MFP), day 11 (following four consecutive days of oral administration of MFP, and day 18. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 33 illustrates hIFN induction in vivo with hIFN- β regulated expression plasmid vectors of the present invention. Constitutive (pGER125) and inducible (pGT27, pGT28, pGT29, and pGT30) hIFN- β expression plasmids were injected and electroporated into hind limb muscles of C57BL/6 mice. Animals were bled and the serum was assayed for hIFN by ELISA on day 7 (absence of MFP), day 11 (following four consecutive days of oral administration of MFP), and day 18. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 34A illustrates hEPO induction in vivo with hEPO regulated expression plasmid vectors of the present invention. Inducible two-plasmid (pGS1694 + pEP1666) and one-plasmid pBRES (pGT27, pGT28, pGT29, and pGT30) hEPO expression plasmids were injected and electroporated into hind limb muscles of C57BL/6 mice. Five animals of each group were administered MFP by i.p. injection for four consecutive days (7-10) and all bled 6 hr after the last MFP injection. The remaining five animals of each group were bled on day 10 in the absence of MFP treatment. Serum was assayed for hEPO by ELISA. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 34B illustrates induction of hematocrit count in vivo with hEPO regulated expression plasmid vectors of the present invention. Inducible two-plasmid (pGS1694 + pEP1666) and one-plasmid pBRES (pGT27, pGT28, pGT29, and pGT30) hEPO expression plasmids were injected and electroporated into hind limb muscles of C57BL/6 mice and animals were treated with MFP or left untreated and bled as above. Blood was clotted and centrifuged in microcapillary tubes and the % red blood cells (RBC) was measured. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 35 illustrates long-term, persistent, multiple hIFN inductions in vivo with a hIFN- β regulated expression AAV vector of the present invention. The inducible hIFN- β expression AAV vector AAV-1-GT58 was injected into hind limb muscles of C57BL/6 mice. Animals were bled and the serum was assayed for hIFN by ELISA in the absence or presence of MFP (four consecutive days of i.p. injections) as indicated. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 36 illustrates long-term, persistent, multiple IP-10 inductions in response to increasing dosages of MFP in vivo with repeated administrations of a mIFN- β regulated expression plasmid vector of the present invention. The inducible mIFN- β expression plasmid pGT26 was injected and electroporated on day 0 into hind limb muscles of C57BL/6 mice.

5 Animals were administered MFP at various concentrations by i.p. injection for four consecutive days (day 7-10 and 63-66) and then bled the following day (day 11 and 67). Plasmid DNA was re-injected on day 77 and 189. MFP treatments after plasmid re-injection were on day 84-87 and 196-199, respectively. Bleeds were taken on day 88 and 200, respectively. Serum was assayed for the chemokine IP-10 by ELISA. The results are shown as the mean (n=5 animals per group).

Figure 37A illustrates the kinetics of hIFN induction in vivo with a hIFN- β regulated expression AAV vector of the present invention. The inducible hIFN- β expression AAV vector AAV-1GT58 was injected into hind limb muscles of C57BL/6 mice. Animals were administered MFP by i.p. injection for four consecutive days and then bled at various times after the first MFP injection as indicated in the chart. Serum was assayed for hIFN by ELISA. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 37B illustrates the kinetics of hIFN de-induction in vivo with a hIFN- β regulated expression AAV vector of the present invention. The inducible hIFN- β expression AAV vector AAV-1GT58 was injected into hind limb muscles of C57BL/6 mice. Animals were administered MFP by i.p. injection for four consecutive days and then bled at various times after the last MFP injection as indicated in the chart. Serum was assayed for hIFN by ELISA. The results are shown as the mean (n=5 animals per group) +/- the standard deviation.

Figure 37C illustrates the kinetics of mIFN induction and de-induction, response to pulsatile or chronic MFP treatment, and the persistence of gene expression over several months with a mIFN- β regulated expression plasmid vector of the present invention. Constitutive (pGER101) and inducible (pGT26) mIFN expression plasmids were injected with electroporation into the hind limb muscles mice, and animals were bled at various time points before, during, or after MFP treatment as indicated on the chart. Serum was assayed for the chemokine IP-10 by ELISA. The results are shown as the mean (n=5 animals per group).

Figure 38 illustrates Mx-1 induction in vivo with a mIFN- β regulated expression plasmid vector of the present invention. The inducible mIFN- β expression plasmid pBRES mIFN (pGT26) or pBRES Null-MFP (control) plasmid was injected and electroporated into hind limb muscles of SJL mice with acute EAE. Mice were treated with MFP (0.33 mg/kg) by i.p. injection once per day (d) or every third day (etd) after plasmid injection. Blood was collected at day 5 after injection. PBMCs were isolated from the blood and RNA was prepared from and assayed by RT-PCR to determine the level of Mx1 RNA. Mx1 expression levels were normalized to GAPDH. The results are shown as the mean +/- the standard deviation.

Figure 39A illustrates the effect of treatment of mice with induced EAE neuroinflammation with mIFN- β protein by SC injection every other day for 25 days. SJL mice

were immunized on day 1 with proteolipid peptide (PLP) amino acids 139-151/pertussis according to the protocol described in Material and Methods. Groups of mice (n = 10 per group) were injected SC on day 1 with mIFN- β protein, 100,000 units (500 ng) or 300,000 units (1500 ng), or vehicle. Animals were scored for EAE disease progression and the average clinical scores are provided with the standard error of the mean score (SEM). Murine IFN β treated animals displayed significantly reduced disease compared to vehicle treated animals (p = 0.04, p = 0.01).

Figure 39B illustrates the delay in the onset of disease and decrease in the severity of disease following administration of pBRES mIFN- β as compared to the null vector control group. SJL mice were immunized on day 1 with PLP 139-151/pertussis according to the protocol described. Groups of mice (n = 10 per group) were injected IM on day -7 with 200 μ g of either pBRES null plasmid DNA or pBRES mIFN- β plasmid DNA with electroporation. One group of mice were treated daily with Prednisolone by IP injection and served as the positive control for the study. Plasmid treated mice were administered one mifepristone (MFP) containing slow release pellets by SC injection on day -3. Animals were scored for EAE disease progression and the average clinical scores are provided with the standard error of the mean score (SEM). Animals treated with the pBRES mIFN- β plasmid (+ MFP) displayed significantly reduced disease scores (p = 0.05) as well as a significant delay in the onset of disease (p = 0.02) compared to animals treated with the pBRES null plasmid (+ MFP).

Figure 40 illustrates the use of ELISA for detection of murine GM-CSF. BalbC mice (n=5/group) were injected i.m. with 5 or 10 μ g non-pegylated, or 5 μ g pegylated mGM-CSF. Mice received either a single injection or 4 daily injections. Serum was obtained 1, 3, 6, and 12 hours following the single injection or the fourth injection. mGM-CSF levels in serum were measured using the ELISA from R&D (Minneapolis).

Figure 41 shows that higher transgene expression levels were achieved using transfection by electroporation compared to a PINC formulation, but with similar kinetics. 200 μ g of pmGMCSF was injected into the murine gastrocnemus and tibialis muscles either formulated with PINC or followed by electroporation. Mice (N=5) were sacrificed at day 1, 2, 3, 4, 7 or 14. Serum was used to determine levels of mGM-CSF and muscle tissue was harvested for DNA and RNA analysis. The levels of mGM-CSF in serum were measured by ELISA.

Figure 42 shows data from plasmid dose optimization for the expression of GM-CSF. Various doses of pmGMCSF were injected into the murine gastrocnemus and tibialis muscles, followed by electroporation. Serum samples were obtained at day 1, 2, 4 and 7. Levels of mGM-CSF were measured by ELISA.

Figure 43A demonstrates the DSS-induced murine colitis model. Disease was induced in BalbC mice (N=10/group) by DSS (5%) applied daily in the drinking water. Mesopram, Metronidazole, or yeast expressed non-pegylated respectively pegylated mGM-CSF, was administered daily over a period of 8 days. Clinical scores (Daily Activity Index

(DAI), a cumulative index of: weight loss, stool consistency, and rectal bleeding) were recorded daily.

Figure 43B illustrates the efficacy of pCMV-GMCSF treatment in comparison with other treatments. Two concentrations of pCMV-mGMCSF were tested side by side with a control plasmid (pNull) and two mGM-CSF protein formulations, non-pegylated and pegylated in the DSS-induced colitis model (N=10/group). A single plasmid DNA injection was administered at day 0 followed by electroporation. Murine GM-CSF protein was injected s.c. daily. Disease was induced with 7% DSS in the drinking water and clinical scores (DIA) were recorded daily.

Figure 44A demonstrates MFP induction on GM-CSF expression in vitro in the pBRES backbone in all four orientations. The four plasmids containing mGM-CSF (pGT615, pGT616, pGT617 or pGT618) were tested in the C1C12 cells. Cells were transfected at day 0 and treated with MFP at day 1. Supernatants were tested for mGM-CSF at day 1 and 3 following MFP treatment. pGT618, which showed lowest basal activity and highest induction, was used for further in vivo characterization.

Figure 44B demonstrates MFP induction on GM-CSF expression in vivo. C57BL/6 mice (n=5/group) were injected with pGT618 followed by electroporation at day 0. MFP treatment was administered by intra peritoneal injection at day 4, 5 and 6. Blood samples were collected by tail vein bleeding 1, 2, 4, 6, and 8 hours after MFP treatment at day 4 and 6. Serum samples were used to determine levels of mGM-CSF.

Figure 45 is a schematic of the pBRES plasmids containing mGM-CSF in four orientations.

DETAILED DESCRIPTION OF THE INVENTION

The references cited herein, including e.g., patents, patent applications, journals, books, and Web-site publications, are incorporated herein by reference, in their entirety.

Abbreviations

AAV (adeno-associated virus)

AAV-1 (adeno-associated virus, serotype 1)

AAV-2 (adeno-associated virus, serotype 2)

AM (activator molecule)

AMP (ampicillin)

bp (base pairs)

BRES (Berlex Regulated Expression System)

BGH (bovine growth hormone)

CD (Crohn's disease)

CMV (cytomegalovirus)

DAI (Daily Activity Index)

DBD (DNA-binding domain)

DNA (deoxyribonucleic acid)

	EAE (Experimental Allergic Encephalomyelitis)
	enh (enhancer)
	E1b TATA (Adenovirus E1b gene promoter TATA box)
	EBNA-1 (Epstein-Barr virus Nuclear Antigen)
5	EDTA (ethylene diamine tetraacetic acid)
	EF-1 α (elongation factor-1 α)
	ELAM (endothelial leukocyte adhesion molecule)
	ELISA (enzyme-linked immunosorbent assay)
	EP (electroporation)
10	EPO (erythropoietin)
	GAL-4 (yeast GAL-4 protein)
	6x GAL-4 (six copies of the GAL-4 DNA binding site)
	GAPDH (glyceraldehyde 3-phosphate dehydrogenase)
	GMCSF or GM-CSF (granulocyte macrophage stimulating factor)
15	hGMCSF (human granulocyte macrophage colony-stimulating factor)
	hIFN (human interferon)
	hIFN- β (human interferon- β)
	hr (hour)
	HR (hormone receptor)
20	HRE (hypoxia responsive element)
	hGH (human growth hormone)
	hPR (human progesterone receptor)
	HTLV (human T-cell lymphotropic virus)
	HSV (herpes simplex virus)
25	Hygro (hygromycin)
	IBD (inflammatory bowel disease)
	IFN- β (interferon- β)
	IFN- β 1a (interferon- β 1a)
	IFN β 1b (interferon- β 1b)
30	IFN sig seq (interferon signal sequence)
	IFNM (IFN molecule)
	IgK (immunoglobulin kappa)
	i.m. or IM (intramuscular)
	inj. (injected)
35	INR or inr (transcription initiator element)
	IP-10 or IP-10 (interferon-alpha inducible protein 10)
	ITR (inverted terminal repeats)
	i.p. or IP (intraperitoneal)
	IVS8 (intervening sequence or intron 8)
40	i.v. or IV (intravenous)

	JE (murine analog of MCP-1)
	kDA (kilodalton)
	kan (kanamycin)
	KanR (Kanamycin resistance gene)
5	LBD (ligand-binding domain)
	MCP-1 (monocyte chemoattractant protein)
	MCS (multiple cloning site)
	MFP (mifepristone)
	mg (milligram)
10	mGMCSF (mouse granulocyte macrophage colony-stimulating factor)
	mIFN (murine interferon)
	mIFN- β (murine interferon-beta)
	ml (milliliter)
	min (min)
15	MCK (muscle creatine kinase)
	Mx1 (murine homologue of MxA)
	MxA (human myxovirus protein)
	ng (nanogram)
	ORF (open reading frame)
20	Ori (origin of replication)
	OriP (replication origin of Epstein Barr Virus)
	pBRES (plasmid Berlex Regulated Expression System)
	p65 (transcription regulatory domain of NFkappaB p65 protein)
	PBS (phosphate buffered saline)
25	PEG (polyethylene glycol)
	PINC (Protective Interacting Non-Condensing polymer)
	pg (picogram)
	pk (pharmacokinetics)
	PLP (proteolipid peptide)
30	PLP139-151 (proteolipid peptide amino acids 139-151)
	polyA or poly(A) (polyadenylation site)
	PR (progesterone receptor)
	pro (promoter)
	PTK (promoter of the Herpes Simplex Virus thymidine kinase gene)
35	pUC ori (replication origin of pUC plasmids)
	r (reverse)
	RM (regulator molecule)
	RNA (ribonucleic acid)
	rpm (revolutions per minute)
40	RT (room temperature)

	s.c. or SC (subcutaneous)
	SEAP (Secreted Alkaline Phosphatase)
	SEM (standard error of the mean)
	SHR (steroid hormone receptor)
5	shRNA (short hairpin RNA)
	siRNA (small interfering RNA)
	sk actin pro (skeletal muscle promoter)
	SkM or Sk (skeletal muscle)
	SV40 (simian virus 40)
10	TK (thymidine kinase)
	TKpA (thymidine kinase poly A)
	TM (therapeutic molecule)
	UbiB (Ubiquitin B)
	ug (microgram)
15	UC (ulcerative colitis)
	5'UTR (5' untranslated region)
	UT12 (untranslated region 12)
	VP-16 (herpes virus VP-16 transactivation domain)
	vol. (volume)
20	WPRE (Woodchuck Post-Transcriptional Regulator Element)

Technical and Scientific Terms

Technical and scientific terms used herein have the meanings commonly understood by one of ordinary skill in the art to which the present invention pertains, unless otherwise defined. Reference is made herein to various methodologies known to those of ordinary skill in the art. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full. Standard reference works setting forth the general principles of recombinant DNA technology include Sambrook, J., *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Plainview, N. Y.; McPherson, M. J., Ed. (1991) *Directed Mutagenesis: A Practical Approach*, IRL Press, Oxford; Jones, J. (1992) *Amino Acid and Peptide Synthesis*, Oxford Science Publications, Oxford; Austen, B. M. and Westwood, O. M. R. (1991) *Protein Targeting and Secretion*, IRL Press, Oxford. Any suitable materials and/or methods known to those of ordinary skill in the art can be utilized in carrying out the present invention; however, preferred materials and/or methods are described. Materials, reagents and the like to which reference is made in the following description and examples are obtainable from commercial sources, unless otherwise noted.

Regulated Expression System

The improved regulated, expression system of the present invention is a highly innovative technology which provides for nucleic acids that encode a therapeutic molecule (TM) that can be delivered to and expressed in the cells of a subject, such that the expression and/or activity of the expressed TM is regulatable and provides a therapeutic benefit to the subject, for the treatment of disease. An advantage of the regulated expression system of the present invention is that it provides for the tightly modulated expression of a therapeutic molecule (TM), e.g., a protein or nucleic acid, in cells of a subject. A further advantage of the present invention is that it provides for the expression and/or activity of a TM, in the cells of a subject, in a dose-dependent or orientation-dependent manner (as described herein), e.g., depending on the amount of a regulator molecule (RM) present in or administered to a subject, or the orientation of a nucleic acid encoding a TM, respectively. Consequently, another advantage of the compositions and methods of the present invention is that it can be used to optimize therapy in a manner specific to a disease or disease state of a subject. A further advantage of the present expression system is that it can comprise a single nucleic acid vector, which can be administered to a subject via a single injection. Thus, the present expression system provides significant advantages over known nucleic acid-based therapy or bolus protein-based therapy.

In particular, the expression system of the present invention provides for the regulated, long-term expression of a TM (e.g., a protein or nucleic acid) in the cells of a subject, resulting in therapeutic efficacy while minimizing dose-limiting side effects. More particularly, gene therapy, using the expression system of the present invention, can provide regulated, long-term expression of a protein and thereby minimize dose-limiting side effects and maximize therapeutic efficacy of the protein for the treatment of disease in a subject. For example, Interferon beta (IFN- β) has been shown to be an effective protein drug for subjects with multiple sclerosis (MS) in reducing the severity of the disease and slowing its progression. However, IFN- β is known to have a short half-life in circulation. Further, frequent, local administration of the protein may cause dose-dependent side effects. However, using the regulated expression system of the present invention, a nucleic acid encoding an IFN- β (e.g., IFN- β -1a) can be administered to the cells of a subject, and the expression of the encoded IFN- β in the cells can be regulated long-term, and optimized, to achieve maximum therapeutic efficacy and minimum dose-limiting side effects of the IFN- β drug, for treatment of MS.

Likewise, GM-CSF has been shown to have a therapeutic effect on chronic inflammation of the gastrointestinal tract, such as ulcerative colitis (UC), Crohn's disease (CD) and inflammatory bowel disease (IBD). However, using GM-CSF protein might hold some intrinsic problems including the source of the recombinant protein, injection schedule, half life time and bioavailability. Therefore, using the regulated expression system of the present invention, a nucleic acid encoding a GM-CSF molecule or variant thereof can be administered to the cells of a subject, and the expression of the encoded GM-CSF in the cells can be

regulated long-term, and optimized, to achieve maximum therapeutic efficacy and minimum dose-limiting side effects of the GM-CSF drug, for treatment of UC, CD and/or IBD.

In one embodiment, an AM that is a small molecule activator, in the form of an orally available pill, controls promoter induction and subsequent expression of a TM encoded by a nucleic acid sequence of the regulated, expression system of the present invention. In this manner the level of the expressed TM (e.g., a protein or nucleic acid) in circulation in a subject can be tightly regulated in an on/off manner and/or in a dose-dependent manner. An AM of the present invention can directly or indirectly control expression of a TM. For example, in one embodiment, the AM activates an RM, and the presence of the activated RM thereby modulates (e.g., induces) expression of the TM in the cells of a subject. Thus, another advantage of the regulated expression system of the present invention is that it allows for the option for continuous versus pulsatile therapy of a TM expressed in the cells of a subject (e.g., a protein or nucleic acid), and the modulation of expression levels of the TM, in order to optimize therapeutic efficacy of the TM while minimizing any side effects thereof. In particular, the regulated expression system of the present invention allows for the first time the option for continuous and durable, versus pulsatile, IFN- β protein therapy in MS subjects. Further, another advantage of the present invention is that it can provide renewable expression of a TM in the cells of a subject, by repeated administration of a nucleic acid vector encoding the TM.

More particularly, the present regulated expression system allows for the subject-specific or disease-specific therapy, by modulating and optimizing the expression level of a TM in the cells of a subject, to achieve maximum therapeutic efficacy and minimum side effects. As used herein, "subject-specific" or "disease-specific" therapy refers to treatment that is specific to a subject having a specific disease, stage of disease, or disease condition or symptom. For example, using the regulated expression system of the present invention, the level of IFN- β expressed in the cells of a subject having MS can be modulated and optimized to achieve maximum therapeutic efficacy and minimum side effects, for treatment of a specific condition, symptom, or stage of MS (e.g., relapsing remitting, primary progressive, or secondary progressive); or according to a subject's response or tolerance to IFN- β . In another example, the cells of a subject having UC, CD or IBD can be contacted with the regulated expression system of the present invention to express modulated and optimized levels of a GM-CSF molecule to achieve maximum therapeutic efficacy and minimum side effects.

More specifically, the present invention provides an improved regulated gene expression system, and pharmaceutical compositions and methods thereof for treatment of disease. The encoded TM can be a nucleic acid or protein that provides a therapeutic benefit to a subject having, or susceptible to, a disease. As used herein, "therapeutic benefit" or "therapeutic activity" includes, but is not limited to, the amelioration, modulation, diminution, repression, stabilization, or prevention, delay, or slowing of the onset or progression of a disease or symptom or condition of a disease. As used herein, "subject" refers to a mammal

(e.g., a human), and more particularly, refers to a mammal in need of treatment for a disease. "Treatment", "treating", "treat", or grammatical equivalents thereof, refers to providing a therapeutic benefit to a subject for a disease, including a stage, symptom or condition of a disease. "Disease" as used herein encompasses a stage, symptom, condition, or pathology of a disease, or genetic predisposition for a disease. Such diseases can be autoimmune or inflammatory diseases. In some embodiments the disease is a cancer. In some
5 embodiments, the disease is e.g., multiple sclerosis, leukemia, melanoma, hepatitis, cardiomyopathy ulcerative colitis, Crohn's disease or inflammatory bowel disease. Further, the improved regulated expression system of the present invention provides a novel approach
10 for engineering changes in an animal genome (e.g., a murine genome) so that gene function in an animal model can be accurately analyzed and credible animal models (e.g., murine models) of human diseases can be generated. In particular, the improved regulated expression system of the present invention provides an invaluable tool for biomedical
15 research because using the present system, expression of a target molecule e.g., a target gene in an animal genome (or other molecule of the present invention) can be regulated temporally and in a spacial-specific manner.

Further, the improved regulated expression system of the present invention provides a novel approach for the selective or unique expression of target shRNA both in vitro and in vivo. For example, using the regulated expression system of the present invention, a
20 polymerase II (POL II) based expression system can be modified to generate a target shRNA selectively or uniquely. For example to uniquely generate a target shRNA, the present regulated, expression system can be modified and used to generate the shRNA by operably linking a POL II promoter to an intron-containing gene, and the resulting spliced intron processed by the inclusion of MIR sequences to express the target shRNA. Also for example,
25 the RM protein-targeted GAL-4 binding sites of the present vectors and expression cassettes described herein could be inserted upstream of a U6 promoter to create an RM-reponsive system, with the additional potential modification of exchanging the p65 transactivator with a polymerase III (POL III) activator (e.g., Oct-2^Q).

In one embodiment, the present invention provides an improved regulated expression
30 system comprising at least a first expression cassette having a nucleic acid sequence encoding a TM, such that, when delivered to cells of a subject, the encoded TM is expressed, and the expression and/or activity of the TM is regulated in the presence of a regulator molecule (RM). "Regulation" of the activity and/or expression of a molecule of the present invention (e.g., a TM) as used herein refers to the modulation of the expression and/or an
35 activity of the molecule resulting in e.g., the induction, repression, increase, or decrease of an activity and/or the expression of such a molecule. Further examples of such regulation include, but are not limited to, the modulation of an amount, conformation, signal transduction, binding specificity, half-life, stability, or other cellular modification or processing of a molecule of the present invention (e.g., a TM). In preferred embodiments, the TM of the present
40 expression system is regulated. However, examples of other molecules that are suitable for

regulation in the present expression system, include, but are not limited to an RM, activator molecule (AM), and inactivator molecule (IM) as described herein.

In one embodiment of the present invention, the expression and/or activity of the TM is regulated in a dose-responsive or dose-dependent manner, e.g., according to the amount of a RM present in the cells of the subject or administered to the subject. In other embodiments, the expression and/or activity of the TM is regulated in a dose-responsive or dose-dependent manner, e.g., according to the amount of an activator molecule (AM), or inactivator molecule (IM) present in the cells of the subject or administered to the subject. In one embodiment, the expression and/or activity of the TM is regulated in a dose-responsive or dose-dependent manner according to the amount of the same TM or different TM present in the cells of the subject or administered to the subject. As used herein "dose-responsive" or "dose-dependent" refers to the correlation of the expression and/or activity of a molecule of the present invention (e.g. a TM), with the presence in, or administration to, the cells of a subject, a particular dose or amount of a second molecule. Examples of such a second molecule include, but are not limited to, an RM, AM, IM, or TM. Further examples, of a second molecule include a cellular molecule e.g., a biomarker (e.g., a biomarker associated with a disease).

As used herein "cells of a subject" refer to autologous cells from a subject, or heterologous cells (or donor cells) that are not from a subject but are delivered or administered to a subject as described herein. Preferably, the autologous cells are present in a subject, and the heterologous cells are delivered to and present in a subject. In preferred embodiments, a composition of the present invention, e.g., a vector encoding a TM and/or RM, is delivered in vivo to autologous cells of a subject, such that the encoded molecule is expressed in cells present in the subject. In one embodiment, a composition of the present invention, e.g., a vector encoding a TM and/or RM, is delivered ex vivo to autologous or heterologous cells of a subject and then the treated cells are delivered to the subject, such that the encoded molecule is expressed in cells present in the subject.

In another embodiment of the present invention, the expression and/or activity of the TM is orientation-dependent. As used herein "orientation-dependent" refers to the the 5' to 3' orientation of an expression cassette encoding a TM of the present invention, or the 5' to 3' direction of transcription or translation of an encoded TM of the present invention, and in some embodiments the orientation is: with respect to a vector comprising the expression cassette or encoding the TM; with respect to the orientation of another expression cassette on the same vector; or with respect to the orientation of the expression of another molecule encoded by the same vector. For example, in one embodiment, the expression and/or activity of the TM in cells is modulated with respect to the 5' to 3' orientation of the expression cassette encoding the TM, or with respect to the 5' to 3' orientation of the transcription or translation of the encoded TM. Consequently, TM expression and/or activity can be modulated by selection of a particular orientation of the expression cassette encoding the TM or the orientation of transcription or translation of the TM.

The regulated expression system of the present invention comprises at least one expression cassette encoding a TM and can comprise additional expression cassettes encoding one or more of the molecules of the present invention, e.g., a TM, RM, AM, or IM. Further, one or more expression cassettes can be present in a single vector, or in more than one vector. Further, the present invention is not limited to a single TM, RM, AM, or IM and encompasses embodiments having one or more or multiples of a TM, RM, AM, or IM of the present invention, which can be present alone or together in a single vector or in more than one vector. As used herein, "vector" refers to a nucleic acid suitable for inserting and expressing in cells a nucleic acid sequence encoding one or more molecules of the present invention, e.g., a TM, RM, AM, or IM. "Expression cassette", as used herein refers to a nucleic acid encoding the requisite components or functional sequences for the expression in cells of a molecule of the present invention (e.g., a protein or nucleic acid TM, RM, AM, or IM), where the molecule is encoded by a nucleic acid sequence operably inserted into the expression cassette (e.g., at a cloning site in the expression cassette) and operably linked to the functional sequences of the expression cassette. "Operably linked" or "operably inserted" sequence or sequences, as used herein, refers to a sequence or sequences fused, joined, attached or otherwise brought together with another sequence such that the respective sequences function as intended, known, and/or to achieve a particular outcome (e.g., a promoter sequence operably linked to a gene sequence to promote transcription of the encoded gene).

Figures 1A and 1B illustrate unlimited examples of a regulated expression system of the present invention comprising: 1) a first expression cassette comprising a first nucleic acid sequence encoding a therapeutic molecule (TM) and a first promoter sequence encoding a DNA-binding site (DBS) and TATA sequence operably linked to the first nucleic acid sequence; 2) a second expression cassette comprising a second nucleic acid sequence encoding a regulator molecule (RM) and a second promoter sequence operably linked to the second nucleic acid sequence, wherein the RM comprises a DNA-binding domain (DBD), ligand-binding domain (LBD), and regulatory domain (RD) and more specifically, in Figure 1B, an activation domain (AD); and 4) an activator or inactivator molecule (A/IM) that activates the RM or inactivates the RM, respectively, such that the presence of the activated or inactivated RM regulates the expression and/or activity of the TM.

In one embodiment, a first expression cassette comprises the following operably linked functional sequences: 6x GAL-4 DBS, E1b TATA, and transcription start site (e.g., SEQ ID NO: 1), 5' untranslated region UT12 (e.g., SEQ ID NO: 2), synthetic intron IVS8 (e.g., SEQ ID NO: 3), multiple cloning site (e.g., SEQ ID NO: 4 or SEQ ID NO: 5), human growth hormone (hGH) polyadenylation (poly(A)) site (e.g., SEQ ID NO: 6); and a second expression cassette comprises the following operably linked functional sequences: chicken skeletal muscle alpha-actin promoter (e.g., SEQ ID NO: 7) and SV40 poly(A) site (e.g., SEQ ID NO: 8). In a preferred embodiment, the first and second expression cassettes are present in a single vector (e.g., as schematically illustrated in Figures 1A-B). In another preferred

embodiment, the vector is a single plasmid vector e.g., pGT1 (comprising the sequence of SEQ ID NO: 9 and SEQ ID NO: 4), pGT2 (comprising the sequence of SEQ ID NO: 10 and SEQ ID NO: 4), pGT3 (comprising the sequence of SEQ ID NO: 11 and SEQ ID NO: 4), or pGT4 (SEQ ID NO: 12), wherein each vector comprises a multiple cloning site (SEQ ID NO: 4) located 3' of the IVS8 and 5' of the hGH poly(A) site (e.g., as schematically depicted in Figures 14A-D), or pGT11 (comprising the sequence of SEQ ID NO: 9 and SEQ ID NO: 5), pGT12 (comprising the sequence of SEQ ID NO: 10 and SEQ ID NO: 5), pGT13 (comprising the sequence of SEQ ID NO: 11 and SEQ ID NO: 5), or pGT14 (comprising the sequence of SEQ ID NO: 12 and SEQ ID NO: 5), wherein each vector comprises a multiple cloning site (SEQ ID NO: 5) located 3' of the IVS8 and 5' of the hGH poly(A) site.

Further, in preferred embodiments, the TM is an IFN- β or GMCSF. For example, in one embodiment, the first nucleic acid sequence encodes a TM that is human IFN- β 1a comprising the amino acid sequence of SEQ ID NO: 13, and is encoded by the nucleic acid sequence of SEQ ID NO: 14. In another embodiment, the first nucleic acid sequence encodes a TM that is a mouse IFN- β comprising the amino acid sequence of SEQ ID NO: 15, and is encoded by the nucleic acid sequence of SEQ ID NO: 16. In another embodiment, the first nucleic acid sequence encodes a TM that is a human GMCSF comprising the amino acid sequence of SEQ ID NO: 17, and is encoded by the nucleic acid sequence of SEQ ID NO: 18. In another embodiment, the first nucleic acid sequence encodes a TM that is a mouse GMCSF comprising the amino acid sequence of SEQ ID NO: 19, and is encoded by the nucleic acid sequence of SEQ ID NO: 20. Further, in preferred embodiments, the RM is a variant of a wild-type or naturally-occurring progesterone receptor (PR). For example, in one embodiment, the second nucleic acid sequence encodes an RM that is a mutated PR comprising the amino acid sequence of SEQ ID NO: 22, and is encoded by the nucleic acid sequence of SEQ ID NO: 21.

In preferred embodiments, the nucleic acid sequence encoding a TM is inserted or cloned into the Spe I and Not I restriction enzyme sites of an MCS of a first expression cassette, of a single plasmid vector. In one embodiment, the single plasmid vector comprising a nucleic acid sequence encoding a TM is e.g., pGT23 (SEQ ID NO: 23), pGT24 (SEQ ID NO: 24), pGT25 (SEQ ID NO: 25), or pGT26 (SEQ ID NO: 26), where the encoded TM is a mouse IFN- β (e.g., comprising the amino acid sequence of SEQ ID NO: 15 and/or encoded by a nucleic acid sequence comprising SEQ ID NO: 16), and the 5'-3' orientation of transcription of the encoded TM and of the inserted nucleic acid sequence is schematically illustrated in Figure 15A (see arrow). In another embodiment, the single plasmid vector comprising a nucleic acid sequence encoding a TM is e.g., pGT27 (SEQ ID NO: 27), pGT28 (SEQ ID NO: 28), pGT29 (SEQ ID NO: 29), or pGT30 (SEQ ID NO: 30), where the encoded TM is a human IFN- β (e.g., comprising the amino acid sequence of SEQ ID NO: 13 and/or encoded by a nucleic acid sequence comprising SEQ ID NO: 14), and the orientation of transcription of the encoded TM and of the inserted nucleic acid sequence is schematically illustrated in Figure 15B (see arrow).

Further, in one embodiment, the single plasmid vector comprises the nucleic acid sequence of a vector backbone (e.g., SEQ ID NO: 12), MCS (e.g., SEQ ID NO: 31), and SpeI-NotI fragment (SEQ ID NO: 31), wherein the fragment encodes a TM that is a mouse IFN- β , has an SpeI sequence at the 5' end and NotI sequence at the 3' end compatible for insertion of the fragment at the SpeI-NotI site in the MCS, and is inserted at the SpeI-NotI site of the MCS. Further, in one embodiment, the single plasmid vector comprises the nucleic acid sequence of a vector backbone (e.g., SEQ ID NO: 12), MCS (e.g., SEQ ID NO: 32), and SpeI-NotI fragment (SEQ ID NO: 31), wherein the fragment encodes a TM that is a human IFN- β , has an SpeI sequence at the 5' end and NotI sequence at the 3' end compatible for insertion of the fragment at the SpeI-NotI site in the MCS, and is inserted at the SpeI-NotI site of the MCS.

Further, in one embodiment, an AM binds to the RM and activates the RM, thereby, the activated RM binds to the DBS of the promoter sequence operably linked to the TM sequence, resulting in the induction of TM expression and/or activity, in cells (e.g., mammalian cells). However, in another embodiment, an inactivator molecule (IM) binds to the RM and inactivates the RM, thereby, the inactivated RM does not bind to the DBS of the TM promoter, resulting in the repression or in the lack of induction of TM expression and/or activity. In one embodiment of the example illustrated in Figure 1B, an activator molecule (AM) binds to the LBD of the RM and activates the RM, thereby, the activated RM forms a homodimer that binds to the DBS of the promoter operably linked to the TM sequence, resulting in the induction of TM expression and/or activity, in cells (e.g., mammalian cells). In another embodiment of the examples illustrated in Figures 1A and 1B, the first and second expression cassettes are present in a single vector. In another preferred embodiment, a first expression cassette encoding a TM and a second expression cassette encoding an RM of the present invention are present in a single vector (e.g., pGT23 (SEQ ID NO: 23), pGT24 (SEQ ID NO: 24), pGT25 (SEQ ID NO: 25), pGT26 (SEQ ID NO: 26), pGT27 (SEQ ID NO: 27), pGT28 (SEQ ID NO: 28), pGT29 (SEQ ID NO: 29), pGT30 (SEQ ID NO: 30), pGT615 (SEQ ID NO: 59), pGT616 (SEQ ID NO: 60), pGT617 (SEQ ID NO: 61), or pGT618 (SEQ ID NO: 62).

A "therapeutic molecule" or "TM" as used herein refers to a molecule having a therapeutic activity or providing a therapeutic benefit. A TM of the present invention can be an isolated DNA, RNA, or protein, or variant thereof, encoded by a nucleic acid sequence and having a therapeutic activity. "Variants" as used herein, include muteins, e.g., muteins of an isolated DNA, RNA, protein, or chemical compound. More particularly, a TM of the present invention can be a modified, synthetic, or recombinant DNA, RNA or protein. "Modified" as used herein, encompasses molecules modified chemically, synthetically, or by recombinant technology, including e.g., mutated, fusion, or chimeric molecules. In one embodiment, the encoded TM is a protein that is expressed and cleaved or processed in the cells of a subject and thereby results in multiple TMs, or an activated TM, or a TM that differs from the expressed and uncleaved or unprocessed TM. In another embodiment of the present invention, the encoded TM is a nucleic acid (e.g., an RNA) having a therapeutic activity. In

one embodiment, the encoded RNA encodes multiple splice sites that are multiply or differentially spliced in the cells of a subject. In some embodiments, the multiply- or differentially-spliced RNAs encode for different or variant proteins, or comprise different or variant RNAs, having a similar or separate therapeutic activity. In some embodiments the multiply- or differentially-spliced RNAs are spliced in response to the presence of a specific factor, disease, condition, or tissue.

In another embodiment of the present invention, the encoded TM is a protein having a therapeutic activity and, preferably, a human protein or variant thereof. In a further embodiment, the nucleic acid sequence encoding such a protein is of a gene or gene fragment. In one embodiment, the TM is a granulocyte macrophage colony stimulating factor (GMC-SF), and more particularly, is mGM-SCF or hGM-CSF. In another embodiment, the TM is an interferon, e.g., interferon-beta (IFN- β), and more particularly, is IFN- β 1a or IFN- β 1b. In some embodiments the encoded TM is an antibody, and preferably a monoclonal antibody (e.g., CAMPATH). Suitable sequences encoding a monoclonal antibody can be identified and made using methods known in the art, and inserted into a vector of the regulated expression system of the present invention as described herein. The therapeutic activity of monoclonal antibodies has been reported (see e.g., Gatto, B. (2004) 4:411-414; Groner et al. (2004) 4:539-547).

"Regulator molecule" or "RM" as used herein refers to a molecule that regulates the expression and/or activity of a TM of the present invention. Examples of such regulation by an RM of the present invention, include, but are not limited to, the modulation of TM expression and/or activity, and more particularly, an increase, decrease, activation (or induction), or inactivation (or repression) of TM expression and/or activity, by an RM of the present invention. Further such modulation of TM expression and/or activity by an RM of the present invention can be direct (e.g., by direct contact of an RM with a TM) or indirect (e.g., where the RM effects a molecule in a signal transduction pathway that results in the modulation of TM expression and/or activity). Further examples of RMs suitable for use in the regulated, expression system of the present invention include, but are not limited to, molecules that effect cellular expression, activity, or processing of a TM of the present invention. Examples of such suitable RMs, include, but are not limited to, transcriptional regulatory molecules (e.g., that activate, inactivate, decrease, or increase transcription of an RNA of an expressed TM); RNA processing molecules (e.g., molecules that activate, inactivate, decrease, or increase RNA processing such as RNA splicing, polyadenylation, or cleavage of an RNA of an expressed TM); or molecules that effect protein translation or post-translational processing of a protein (e.g., enzymes that activate, inactivate, decrease, or increase the phosphorylation, cleavage, or formation of a particular conformation or multimeric form of a protein of an expressed TM).

An RM of the present invention can be a naturally-occurring or isolated molecule, or variant thereof. In some embodiments, an RM of the present invention is a synthetic or recombinant molecule. For example, in some embodiments, an RM of the present invention

is a chemical compound, DNA, RNA, or protein. Further, in some embodiments, an RM of the present invention is a modified molecule. In one embodiment, the RM is a humanized protein. In another embodiment, the RM is a human protein or variant thereof. For example, in one embodiment, the RM is a transcriptional activator e.g., a steroid receptor and, more particularly, a progesterone receptor. In one embodiment, the RM comprises a transactivation domain (e.g., a VP16 or p65 transactivation domain, see e.g., Schmitz et al. (1991) EMBO J 10:3805-3817; Moore et al. (1993) Molec and Cell Biol 13:1666; Blair et al. (1994) Molec and Cell Biol 14:7226-7234), and/or other functional domain (e.g., a basal factor interaction domain) of a co-activator (e.g., p300/CBP), a basal transcription factor (e.g. TFIIB), or a histone acetyltransferase (e.g. p300/CBP or P/CAF, Latchman, D. (2004) Eukaryotic Transcription Factors, Elsevier Academic Press, London; Goodman et al. (2000) Genes & Devl 14:1553-1577; Shikama et al. (1997) Trends in Cell Bio 7:230-236). In another embodiment, the RM comprises a ligand-binding domain (LBD). Further, in one embodiment, an AM binds to the LBD of the RM, thereby activating the RM such that the presence of the activated RM regulates TM expression and/or activity. In another embodiment, the RM comprises a DBD, e.g., a GAL-4 DBD. In one embodiment, the RM comprises a DBD that binds to a functional sequence (e.g., a promoter sequence) operably linked to a nucleic acid encoding a TM, thereby regulating TM expression (e.g., inducing TM expression).

In another embodiment, an RM of the present invention is activated by an activator molecule (AM) and, thereby, TM expression and/or activity is regulated in the presence of the activated RM. "Activator molecule" or "AM" as used herein refers to a molecule that induces or increases the expression and/or activity of an RM of the present invention. Examples of such activation by an AM include, but are not limited to the induction or increase in expression and/or activity of an RM of the present invention. Further such activation in RM expression and/or activity by an AM of the present invention can be direct (e.g., by direct contact of an AM with a RM) or indirect (e.g., where the AM affects a molecule in a signal transduction pathway that results in the modulation of RM expression and/or activity). Further examples of AMs suitable for use in the regulated expression system of the present invention include, but are not limited to, molecules that effect cellular processing of an RM of the present invention (examples of such cellular processing are described herein, e.g., above).

In one embodiment, the AM is a biomarker. In a further embodiment, the AM is a biomarker for a disease or condition and, more particularly, is a biomarker for a disease state or condition, or symptom thereof. In one embodiment, the AM activates the RM by promoting or inhibiting conformational change, enzymatic processing or modification, specific binding, or dimerization of the RM. In a preferred embodiment, the AM activates the RM by promoting homodimerization of the RM. In one embodiment, the AM activates the RM by binding to the RM and, more particularly, to a functional domain of the RM, e.g., an AD of the RM.

An AM of the present invention can be a naturally-occurring or isolated molecule, or variant thereof. In some embodiments, the AM of the present invention is a synthetic or recombinant molecule. For example, in some embodiments, the AM of the present invention

is a chemical compound, DNA, RNA, or protein. Further, in some embodiments, the AM of the present invention is a modified molecule. In one embodiment, the AM is a humanized protein. In another embodiment, the AM is a human protein or variant thereof. In one embodiment, the AM is a chemical compound, e.g., an antiprogesterin. In a preferred embodiment, the AM is mifepristone.

In one embodiment, the regulated expression system of the present invention comprises: 1) a first expression cassette having a first nucleic acid sequence encoding a TM, and at least one GAL-4 DNA-binding site (DBS) and, more particularly, six GAL-4 DBS (6XGAL-4 DBS), located upstream and operably linked to the first nucleic acid sequence; 2) a second expression cassette having a second nucleic acid sequence encoding an RM that is a modified progesterone receptor comprising a VP-16 AD or p65 AD (e.g., a p65 AD comprising the nucleic acid sequence of SEQ ID NO: 39 or amino acid sequence of SEQ ID NO: 40), progesterone (PR) LBD, and GAL-4 DBD, and an actin promoter sequence located upstream and operably linked to the second nucleic acid sequence; and 3) an AM that is a small molecule inducer, e.g., mifepristone (MFP) that when orally administered to a subject, activates the expressed RM in the cells of the subject and, thereby, the activated RM forms a dimer that binds to the 6XGAL-4 DBS and induces expression of the encoded TM. In a preferred embodiment, the first and second expression cassettes are present in a single vector.

In another embodiment, the RM of the present invention is a transcriptional regulator and more particularly, a mutated steroid receptor. In one embodiment, the RM is a mutated human PR (hPR) and comprises a mutated hPR receptor LBD, (e.g., having a C-terminal deletion of about 19-66 amino acids), wherein the RM is activated in the presence of an AM that is an antagonist of the wild-type PR from which the mutant PR was derived. In another embodiment, the RM of the present invention comprises a regulatory domain (RD), e.g., an activation domain (AD), and more particularly, a transactivation domain (TD). Examples of suitable regulatory domains for use in the RM of the present invention, include, but are not limited to, those known in the art or described herein (e.g., TAF-1, TAF-2, TAU-1, and TAU-2).

In another embodiment, an RM of the present invention is inactivated and thereby TM expression and/or activity is regulated in the presence of an inactivated RM. "Inactivator molecule" or "IM" as used herein refers to a molecule that inactivates the expression and/or activity of an RM of the present invention. Examples of such inactivation by an IM include, but are not limited to the repression or decrease in expression and/or activity of an RM of the present invention. Further such inactivation in RM expression and/or activity by an IM of the present invention can be direct (e.g., by direct contact of an IM with a RM) or indirect (e.g., where the IM affects a molecule in a signal transduction pathway that results in the inactivation of RM expression and/or activity). Further examples of IMs suitable for use in the regulated, expression system of the present invention include, but are not limited to,

molecules that effect cellular processing of an RM of the present invention (examples of such cellular processing are described herein, e.g., above).

In one embodiment, an RM of the present invention is expressed or present in cells of a subject in an activated form, and is inactivated in the presence of an inactivator molecule (IM), thereby, TM expression and/or activity is regulated by the inactivated RM. In another embodiment, the IM is a biomarker. In a further embodiment, the IM is a biomarker for a disease or condition and, more particularly, is a biomarker for a disease state or condition, or symptom thereof. In one embodiment, the IM inactivates the RM by promoting or inhibiting conformational change, enzymatic processing, specific binding, or dimerization of the RM. In a preferred embodiment, the IM inactivates the RM by inhibiting homodimerization of the RM. In one embodiment, the IM inactivates the RM by binding to the RM and, more particularly, to a functional domain of the RM, e.g., an AD of the RM.

An IM of the present invention can be a naturally-occurring or isolated molecule, or variant thereof. In some embodiments, the IM of the present invention is a synthetic or recombinant molecule. For example, in some embodiments, the IM of the present invention is a chemical compound, DNA, RNA, or protein. Further, in some embodiments, the IM of the present invention is a modified molecule. In one embodiment, the IM is a humanized protein. In another embodiment, the IM is a human protein or variant thereof. In a preferred embodiment, the IM is a chemical compound.

The expression of a TM, RM, AM, or IM of the present invention can be constitutive or transient. In some embodiments, expression of a TM, RM, AM, or IM is regulated or tissue-specific (e.g. muscle-specific). Examples of a regulated RM include, but are not limited to, an RM that is activated by an AM or inactivated by an IM. In one embodiment, the expression of a TM, RM, AM, or IM of the present invention is driven by a regulated promoter or a tissue-specific promoter. In a further embodiment, the regulated or tissue-specific promoter is regulated in the presence of an RM and, more particularly, by the binding of the RM to the promoter. In one embodiment, the tissue-specific promoter is a muscle-specific promoter and, more particularly, an actin promoter. In one embodiment, an RM of the present invention binds to a promoter operably linked to a nucleic acid sequence encoding a TM and thereby regulates the expression of the encoded TM as described herein, in the cells of a subject.

The TM, RM, AM, or IM of the present invention can be isolated, produced, and modified using known methods and assays for nucleic acids, proteins, and chemical compounds, as described herein, e.g., below.

Pharmaceutical Compositions And Treatment Methods

The present invention also provides pharmaceutical compositions and methods for treatment of a variety of diseases comprising the improved regulated expression system of the present invention as described herein.

In particular embodiments, the present invention provides pharmaceutical compositions and methods for treating a disease or condition; regulating the expression of a

TM; administering a TM; delivering a TM; or expressing a TM in cells of a subject, where the methods comprise contacting the cells of a subject with a regulated expression system of the present invention, such that the encoded TM is expressed in the cells of the subject, and such TM expression is regulated in the presence of an RM.

5 The pharmaceutical compositions of the present invention comprise at least one TM, RM, AM, or IM of the present invention present and, in some embodiments, the nucleic acid sequence encoding such molecules are present alone or together in a single vector or in more than one vector. In other embodiments, the pharmaceutical compositions of the present invention can comprise more than one of each TM, RM, AM, or IM, and more than one kind thereof (e.g., a first and second TM, RM, AM, and/or IM). More particularly, the
10 pharmaceutical compositions of the present invention can comprise nucleic acid sequences encoding more than one of each TM, RM, AM, or IM, and more than one kind thereof. In one embodiment, a pharmaceutical composition of the present invention comprises at least one of the vectors of the present invention (e.g., pGT23 (SEQ ID NO: 23), pGT24 (SEQ ID NO: 24),
15 pGT25 (SEQ ID NO: 25), pGT26 (SEQ ID NO: 26), pGT27 (SEQ ID NO: 27), pGT28 (SEQ ID NO: 28), pGT29 (SEQ ID NO: 29), pGT30 (SEQ ID NO: 30), , pGT615 (SEQ ID NO:59), pGT616 (SEQ ID NO:60), pGT617 (SEQ ID NO:61), or pGT618 (SEQ ID NO:62).

 In some embodiments, a pharmaceutical composition of the present invention comprises at least one AM or IM of the present invention. In one embodiment, a
20 pharmaceutical composition of the present invention comprises one or more vectors encoding at least one TM and/or RM. The TM, RM, AM, and IM of the present invention can be administered to a subject separately or together, and ex vivo or in vivo, using any suitable means of administration described herein or known in the art. Examples of such suitable means of administration include, but are not limited to injection (e.g., intramuscular or
25 subcutaneous injection), oral administration, and electroporation. In one embodiment, a TM and RM of the present invention are present in a single vector, and separately administered from an AM that activates the RM (and thereby, the presence of the activated RM regulates TM expression and/or activity). In a further embodiment, the AM is a compound (e.g., mifepristone) administered orally to a subject, and the single vector encoding a TM and RM is
30 a single vector administered by injection or by electroporation to cells of a subject (e.g., skeletal muscle cells). Further examples of a suitable means for administering a composition of the present inventions include the ex vivo delivery of the composition, e.g., a nucleic acid vector encoding a TM and/or RM, to cells of a subject and then the delivery of the treated cells to the subject, such that the encoded molecule is expressed in cells in the subject (see
35 e.g., Studeny et al. (2004) J Natl Cancer Inst 96(21):1593-1603; Studeny et al. (2002) Cancer Res 62(13):3603-3608).

 In one embodiment, the regulated expression system of the present invention comprises a nucleic acid sequence encoding a therapeutic gene (e.g., IFN- β gene or GM-CSF gene) that is administered to a subject by injection. As used herein "therapeutic gene"
40 refers to a gene encoding a TM, e.g., a protein having a therapeutic activity (e.g., which may

be an interferon molecule (IFNM) such as IFN- β 1a or 1b or GM-CSF). In a particular embodiment, the gene is IFN- β 1a or GM-CSF and is administered as a single intramuscular injection periodically, e.g., 3 to 6 months, using a vector of the present invention. In other embodiments, a therapeutic gene is administered every 1-3 months, 3 to 6 months, 6 to 9 months, or 9 to 12 months. In another embodiment, the regulation of the circulating levels of the expressed protein is achieved by controlled induction of a promoter driving expression of the encoded protein in the target subject cells or tissue.

In a preferred embodiment, the RM is a small molecule activator, in the form of an orally available pill, that controls promoter induction and subsequent expression of a TM and, more particularly, a therapeutic gene. In this manner the level of expressed TM (e.g., a protein or nucleic acid), in the circulation can be tightly regulated in an on/off manner and/or in a dose-dependent manner. Thus, the regulated, expression system of the present invention allows for the first time the option for continuous versus pulsatile therapy of a TM (e.g., a protein or nucleic acid), and the modulation of expression levels of a TM, in order to optimize therapeutic efficacy of a TM while minimizing any side effects thereof. In particular, the regulated expression system of the present invention allows for the first time the option for continuous versus pulsatile TM therapy in subjects and, more particularly, allows for subject-specific therapy by modulating and optimizing expression levels of a TM in cells of the subject to achieve maximum therapeutic efficacy and minimum side effects, for treatment of a disease.

Using the regulated expression system of the present invention, nucleic acids encoding a TM (e.g., a protein or nucleic acid) can be delivered to target cells of a subject, for treatment of disease. More particularly, using the regulated, expression system of the present invention, nucleic acids encoding a TM can be delivered to target cells of a subject, such that the expressed TM is provided in a therapeutically effective dose or amount. As used herein, a "therapeutically effective dose" or "therapeutically effective amount" of a TM of the present invention is a dose or amount that, when present in the cells of a subject in need of treatment of a disease, results in a therapeutic benefit to the subject (i.e., results in treatment of the disease). Further, a suitable amount or dose of a nucleic acid encoding a TM administered to a subject or present in the cells of a subject; or an amount or dose of an RM, AM, or IM, or nucleic acid encoding an RM, AM, or IM, that is administered to and/or present in the cells of a subject, that results in the presence of a TM in the cells of a subject and/or a therapeutically effective amount of the TM, can be determined empirically by one skilled in the art. For example, in one embodiment a suitable dose or amount of an RM or a nucleic acid encoding an RM, administered to a subject, is a dose or amount that regulates (e.g., induces) the expression and/or activity of a TM in the cells of a subject such that a therapeutically effective dose is achieved.

Factors influencing the amount of TM that constitutes a therapeutically effective dose include, but are not limited to, the severity and history of the disease to be treated, and the age, health, and physical condition of the subject undergoing therapy. A therapeutically

effective dose of a TM of the present invention can also depend upon the dosing frequency and severity of the disease in the subject undergoing treatment. The dosing regimen of a TM of the present invention can be continued for as long as is required to achieve the desired effect, *i.e.*, for example, prevention and/or amelioration of the disease, symptoms associated with the disease, disease severity, and/or periodicity of the recurrence of the disease, as described herein. In one embodiment, the dosing regimen is continued for a period of up to one year to indefinitely, such as for one month to 30 years, about three months to about 20 years, about 6 months to about 10 years. Further, the TM may be administered daily, every other day, three times per week, twice per week, weekly, biweekly, monthly, bimonthly, every three months, quarterly, semiannually, annually, or any combination thereof.

Examples of suitable nucleic acids for use in the regulated expression system of the present invention include, but are not limited to, those nucleic acids encoding a gene for a hormone, growth factor, enzyme, cytokine, receptor, or MHC molecule having a therapeutic activity. Additionally, suitable genes for use in the compositions and methods of the present invention, include nucleic acid sequences that are exogenous or endogenous to cells into which the nucleic acid encoding the gene of interest can be introduced. Of particular interest and suitability for use in the compositions and methods of the present invention for treatment of disease are those genes encoding a polypeptide that is either absent, produced in diminished quantities, or produced in a mutant form in those subjects having or are susceptible to a genetic disease. Examples of such genetic diseases include, but are not limited to, retinoblastoma, Wilms tumor, adenosine deaminase deficiency (ADA), thalassemias, cystic fibrosis, Sickle cell disease, Huntington's disease, Duchenne's muscular dystrophy, Phenylketonuria, Lesch-Nyhan syndrome, Gaucher's disease, and Tay-Sach's disease.

Also of particular interest and suitability for use in the compositions and methods of the present invention for treatment of disease are nucleic acids encoding a tumor suppressor gene. Examples of such suitable tumor suppressor genes include, but are not limited to, retinoblastoma, GM-CSF, G-CSF, M-CSF, human growth hormone (HGH), TNF, TGF- β , TGF- α , hemoglobin, interleukins, co-stimulatory factor B7, insulin, factor VIII, factor IX, PDGF, EGF, NGF, EPO, and β -globin, as well as biologically or therapeutically active muteins of the proteins encoded by such genes. Suitable genes for delivery to target cells can be from any species, but preferably a mammalian species, and more preferably a human. Further, preferred species, as sources of suitable genes, are those species into which the gene of interest is to be delivered using the methods and compositions of the present invention, *e.g.*, a mammalian species and preferably a human.

Further examples of suitable nucleic acids for use in the compositions and methods of the present invention include, but are not limited to, those that encode a protein or nucleic acid TM having an antiinflammatory, antiviral, or anticancer activity. Examples of such suitable nucleic acids include, but are not limited to, those encoding a granulocyte macrophage stimulating colony factor (GMCSF) or variant thereof (*e.g.*, Leukine[®] or human

GMCSFLeu²³Asp²⁷Glu³⁹), having an anticancer activity (see e.g., the GMCSF mutants of US Patent No.s 5,032,676; 5,391,485; and 5,393,870). Also, for example, suitable nucleic acids include, but are not limited to, those encoding an interferon having an antiinflammatory or antiviral activity, e.g., an inteferon, particularly IFN- β , and more particularly, an IFN- β 1a or IFN- β 1b.

In a preferred embodiment, the compositions and methods of the present invention are used to treat MS by delivering to a subject in need of treatment, a nucleic acid encoding a TM that is an IFN- β and, more particularly, is IFN- β 1a, such that the IFN- β is expressed in the cells of the subject and the expression and/or activity of the IFN- β is regulated by an RM, as described herein.

MS is a chronic and severe disease characterized by focal inflammation in the central nervous system (CNS) (see e.g., Hemmer *et al.* (2002) *Neuroscience* 3: 291-301; Keegan *et al.* (2002) *Ann. Rev. Med.* 53: 285-302; Young, V. Wee (2002) *Neurology* 59: 802-808; Goodin *et al.* (2001) *Am. Academy of Neurology* 58: 169-178). An associated loss of the insulating myelin sheath from around the axons of the nerve cells (demyelination) and a degeneration of the axons are also prominent features of the disease. Resulting from the focal inflammation, an astrocytotic gliosis leads to the formation of sclerotic lesions in the white matter (see e.g., Prineas (1985) *Demyelinating Diseases*, Elsevier: Amsterdam; Raine (1983) *Multiple Sclerosis*, Williams and Wilkins: Baltimore; Raine *et al.* (1988) *J. Neuroimmunol.* 20: 189-201; and Martin (1997) *J. Neural Transmission (Suppl)* 49: 53-67).

There are two major types of MS subject populations at the onset of the disease: those subjects with relapsing-remitting MS and those subjects with primary progressive MS. Relapsing-remitting MS is characterized by episodes (the so called relapses or exacerbation) where new neurologic deficits emerge or preexisting neurologic deficits worsen and periods of remission where the clinical symptoms are stabilized or diminished, whereas, primary progressive MS subjects suffer from progressive neurological deterioration without exacerbations. A large proportion of subjects with relapsing-remitting MS also experience during the course of their disease a worsening of neurologic symptoms independent of relapses, with or without superimposed relapses. Once this stage of the disease is reached, it is called secondary progressive MS.

The clinical symptoms of MS are thought to result from a focal breakdown in the blood-brain barrier (BBB) which permits the entry of inflammatory infiltrates into the brain and spinal cord. Further, these infiltrates are thought to consist of various lymphocytes and macrophages that lead to demyelination, axonal degeneration and scar tissue formation, and the degeneration of oligodendrocytes imperative to CNS myelin production (see e.g., Martin (1997) *J. Neural Transmission (Suppl)* 49:53-67). Consequently, the nerve-insulating myelin and the ability of oligodendroglial cells to repair damaged myelin are seriously compromised (see e.g., *Scientific American* 269(1993):106-114). These symptoms of MS include pain and tingling in the arms and legs, localized and generalized numbness, muscle spasm and

weakness, difficulty with balance when standing or walking, difficulty with speech and swallowing, cognitive deficits, fatigue, and bowel and bladder dysfunction.

Although there is no known cure for MS, immunomodulatory therapy with interferons has proven to be successful in reducing the severity of the underlying disease in subjects with MS. Interferons are important cytokines characterized by antiviral, antiproliferative, and immunomodulatory activities. These activities form a basis for the clinical benefits that have been observed in the treatment of subjects with multiple sclerosis. The interferons are divided into the type I and type II classes. IFN- β belongs to the class of type I interferons, which also includes interferons alpha, tau and omega, whereas interferon gamma is the only known member of the distinct type II class.

Human IFN- β is a regulatory polypeptide with a molecular weight of 22 kDa consisting of 166 amino acid residues. The polypeptide can be produced by most cells in the body, in particular fibroblasts, in response to viral infection or exposure to other biologics. Further, IFN- β binds to a multimeric cell surface receptor, and productive receptor binding results in a cascade of intracellular events leading to the expression of IFNB inducible genes which in turn produces effects which can be classified as antiviral, antiproliferative and immunomodulatory.

Human IFN- β is a well-characterized polypeptide. The amino acid sequence of human IFN- β is known (see e.g., Gene 10:11-15, 1980, and in EP 83069, EP 41313 and U.S. Pat. No. 4,686,191). Also, crystal structures have been reported for human and murine IFN- β , respectively (see e.g., Proc. Natl. Acad. Sci. USA 94:11813-11818, 1997. J. Mol. Biol. 253:187-207, 1995; reviewed in Cell Mol. Life Sci. 54:1203-1206, 1998). In addition, protein-engineered variants of IFN- β have been reported (see e.g., WO 9525170, WO 9848018, U.S. Pat. No. 5,545,723, U.S. Pat. No. 4,914,033, EP 260350, U.S. Pat. No. 4,588,585, U.S. Pat. No. 4,769,233, Stewart et al, DNA Vol. 6 No. 2 1987 pp. 119-128, Runkel et al, 1998, Jour. Biol. Chem. 273, No. 14, pp. 8003-8008). Also, the expression of IFN- β in CHO cells has been reported (see e.g., U.S. Pat. No. 4,966,843, U.S. Pat. No. 5,376,567 and U.S. Pat. No. 5,795,779). Further, IFN- β fusion proteins are reported, e.g., in WO 00/23472.

Commercial preparations of IFN- β are approved for the treatment of subjects with MS and are sold under the names Betaseron® (also termed Betaferon® or IFN- β 1b_{ser17}, which is non-glycosylated, produced using recombinant bacterial cells, has a deletion of the N-terminal methionine residue and the C17S mutation), Avonex® and Rebif® (also termed IFN- β 1a, which is glycosylated, produced using recombinant mammalian cells. Further, a comparison of IFN- β 1a and IFN- β 1b with respect to structure and function has been presented in Pharm. Res. 15:641-649, 1998).

IFN- β is the first therapeutic intervention shown to delay the progression of MS. In addition, the approved dose of IFN- β has been shown to be effective in reducing the exacerbation rate of MS, and more subjects remain exacerbation-free for prolonged periods of time as compared with placebo-treated subjects. Furthermore, the accumulation rate of disability is reduced (see e.g., Neurol. 51:682-689, 1998).

IFN- β has inhibitory effects on the proliferation of leukocytes and antigen presentation. Furthermore, IFN- β may modulate the profile of cytokine production towards an anti-inflammatory phenotype. Finally, IFN- β can reduce T-cell migration by inhibiting the activity of T-cell matrix metalloproteases. Such IFN- β activities are likely to act in concert to account for the beneficial effect of IFN- β in the treatment of subjects with MS (see *e.g.*, Neurol. 51:682-689, 1998).

In a preferred embodiment, the compositions and methods of the present invention are for use in the treatment of subjects suffering from various clinically recognized forms of MS, including but not limited to, relapsing-remitting MS, different types of progressive MS (including, but not limited to, *e.g.*, primary and secondary progressive MS, progressive-relapsing MS) and, also, clinically isolated syndromes suggestive of MS.

As used herein, "relapsing-remitting" MS is a clinical course of MS that is characterized by clearly defined, sporadic exacerbations or relapses, during which existing symptoms become more severe and/or new symptoms appear. Such exacerbations or relapses, may be followed by partial recovery, or full recovery and remission. The length of time between these sporadic exacerbations or relapses may be months or years, during which time inflammatory lesions, demyelination, axonal loss, and scar formation may still proceed. Relapsing-remitting MS is the most common beginning phase of MS, and it has been reported that about 50% of the cases have progression within 10 to 15 years, and another 40% within 25 years of onset.

As used herein, "primary-progressive" MS is a clinical course of MS that is characterized from the beginning by progressive disease, with no plateaus or remissions, or an occasional plateau and very short-lived, minor improvements. As the disease progresses, the subject may experience difficulty in walking, the steadily decline in motor skills, and an increase in disabilities over many months and years, generally, in the absence of those distinct inflammatory attacks characteristic of relapsing-remitting MS.

As used herein, "secondary-progressive" MS is a clinical course of MS that initially is relapsing-remitting and then becomes progressive at a variable rate independent of relapses. Although subjects experiencing this type of MS may continue to experience inflammatory attacks or exacerbations, eventually the exacerbations and periods of remission may diminish, with the disease taking on the characteristic decline observed with primary-progressive MS.

As used herein "progressive-relapsing" MS is a clinical course of MS that may show permanent neurological deterioration from the onset of the disease, but with clear, acute exacerbations or relapses that look like relapsing-remitting MS. For these subjects, lost functions may never return. It has been reported that this type of MS has a high mortality rate if untreated.

Clinically isolated syndromes suggestive of MS include, but are not limited to, early onset multiple sclerosis and monosymptomatic MS. For purposes of the present invention, the term "multiple sclerosis" is intended to encompass each of these clinical manifestations of

the disease and clinically isolated syndromes suggestive of MS unless otherwise specified. For example, a subject having MS or symptoms associated with MS is a subject in need of treatment of MS or associated symptoms of MS. In one embodiment, when a subject suffering from MS undergoes treatment in accordance with the pharmaceutical compositions and methods of the present invention, treatment can result in the prevention and/or amelioration of MS disease symptoms, disease severity, and/or periodicity of recurrence of the disease, *i.e.*, treatment of MS using the compositions and methods of the present invention can result in lengthening the time period between episodes in which symptoms flare, and/or can suppress the ongoing immune or autoimmune response associated with the disease, which, left untreated, can enhance disease progression and disability.

In another embodiment, the compositions and methods of the present invention are used to treat UC, CD or IBD by delivering to a subject in need of treatment, a nucleic acid encoding a TM that is a GM-CSF molecule and, more particularly, is human or murine GM-CSF or variant thereof, such that the GM-CSF is expressed in the cells of the subject and the expression and/or activity of the GM-CSF is regulated by an RM, as described herein. Ulcerative colitis (UC) and inflammatory bowel disease (IBD) and Crohn's disease (CD), are serious, life long disorders. IBD, including CD, is characterized by chronic inflammation through the GI tract with alternating periods of active disease and remission. These diseases are characterized by a persistent over-stimulation of the specific immune response, leading to chronic inflammation. Current treatments are difficult to tolerate and may not provide therapeutic benefit. Treatment may include drugs, nutrition supplements, surgery, or a combination of these options. The goals of traditional treatment is to control inflammation, correct nutritional deficiencies, and relieve symptoms like abdominal pain, diarrhea, and rectal bleeding. At this time, treatment can help control the disease by lowering the number of times a person experiences a recurrence, but there is no cure.

Anti-Inflammation Drugs. Most people are first treated with drugs containing mesalamine, a substance that helps control inflammation. Sulfasalazine is the most commonly used of these drugs. Patients who do not benefit from it or who cannot tolerate it may be put on other mesalamine-containing drugs, generally known as 5-ASA agents, such as Asacol, Dipentum, or Pentasa. Possible side effects of mesalamine-containing drugs include nausea, vomiting, heartburn, diarrhea, and headache.

Cortisone or Steroids. Cortisone drugs and steroids, called corticosteroids, provide very effective results. Prednisone is a common generic name of one of the drugs in this group of medications. In the beginning, when the disease is at its worst, prednisone is usually prescribed in a large dose. The dosage is then lowered once symptoms have been controlled. These drugs can cause serious side effects, including greater susceptibility to infection.

Immune System Suppressors. Drugs that suppress the immune system are also used to treat Crohn's disease. Most commonly prescribed are 6-mercaptopurine or a related drug, azathioprine. Immunosuppressive agents work by blocking the immune reaction that

contributes to inflammation. These drugs may cause side effects like nausea, vomiting, and diarrhea and may lower a person's resistance to infection. When patients are treated with a combination of corticosteroids and immunosuppressive drugs, the dose of corticosteroids may eventually be lowered. Some studies suggest that immunosuppressive drugs may enhance the effectiveness of corticosteroids.

Infliximab (Remicade®). This drug is the first of a group of medications that blocks the body's inflammation response. The U.S. Food and Drug Administration approved the drug for the treatment of moderate to severe Crohn's disease that does not respond to standard therapies (mesalamine substances, corticosteroids, immunosuppressive agents) and for the treatment of open, draining fistulas. Infliximab, the first treatment approved specifically for Crohn's disease is a TNF substance. Additional research will need to be done in order to fully understand the range of treatments Remicade may offer to help people with Crohn's disease.

GM-CSF is a cytokine that stimulates white blood cell production, in particular granulocytes and macrophages. The protein has been used to stimulate the immune system through the production of these immune cells. GM-CSF may be used to strengthen the innate immune systems of UC, CD and IBD patients against microbes, thus reducing the specific immune response and subsequent chronic inflammation. Clinical studies using Sagramostim, which is the generic name for Leukine®, containing the granulocyte-macrophage colony-stimulating factor (GM-CSF) as the active ingredient have shown encouraging results. However, protein therapies are not ideal due to the requirement for daily administration, short half life, bioavailability, and possible side effects.

In one embodiment, the regulated gene expression systems, vectors, compositions and methods of the present invention may be used to treat patients suffering from the clinically recognized symptoms of UC, IBD and CD. These diseases may be active or in remission.

Further, a subject can be pre-treated with a pharmaceutical composition or can be a naive subject who has not been pre-treated with a pharmaceutical composition, prior to treatment using a pharmaceutical composition or method of the present invention. For example, for the treatment of MS, a pre-treated subject can be one who has been pretreated with an IFN- β protein drug (e.g., IFN- β 1a) or IFN- β variant (e.g., IFN- β 1b), prior to treatment with the compositions or methods of the present invention. For example, an approved dose of Betaseron®, Avonex®, or Rebif® can be used to pre-treat subjects. In another example, subjects suffering from UC, IBD and/CD may be pretreated with a GM-CSF protein drug, such as Leukine®. Thus, the pharmaceutical compositions and methods of the present invention are suitable for use in the treatment of pre-treated and naive subjects.

The pharmaceutical compositions and methods of the present invention can also be used to block or reduce the physiological and pathogenic deterioration associated with a disease, e.g., inflammatory response in the brain and other regions of the nervous system, breakdown or disruption of the blood-brain barrier, appearance of lesions in the brain, tissue

destruction, demyelination, autoimmune inflammatory response, acute or chronic inflammatory response, neuronal death, and/or neuroglial death. Beneficial effects of the pharmaceutical compositions and methods of the present invention include, but are not limited to, preventing the disease, slowing the onset of an established disease, ameliorating symptoms of a disease, reducing an exacerbation rate, slowing the progression of the disease, and postponing or preventing disability including cognitive decline, loss of employment, hospitalization, and finally death. The episodic recurrence of a particular type of disease (e.g. MS, UC, CD or IBD), can be treated, e.g., by decreasing the severity of the symptoms (such as the symptoms described above) associated with the episode, or by lengthening the time period between the occurrence of episodes, e.g., by days, weeks, months, or years, where the episodes can be characterized by the flare-up and exacerbation of disease symptoms, or preventing or slowing the appearance of brain inflammatory lesions e.g. in MS (see, e.g., Adams (1993) Principles of Neurology, page 777, for a description of a neurological inflammatory lesion) or chronic inflammation of the GI tract in UC, CD and/or IBD.

Further, suitable nucleic acids for use in the compositions and methods of the present invention, can encode a TM that is a fusion or chimeric protein, or a fusion or chimeric nucleic acid (e.g., RNA). In some embodiments, a TM of the present invention can regulate expression of a gene product or block one or more steps in a biological pathway (e.g., a sepsis pathway) and, thereby, provide a therapeutic benefit. Further, the nucleic acid can encode a toxin fused to a TM (e.g., a receptor ligand gene or an antibody that directs the fused toxin to a target such as a tumor cell or a virus) and, thereby, have a therapeutic effect. Standard methods for operably inserting and/or fusing, nucleic acid sequences, or inserting and/or amino acid sequences into amino acid sequences, of the present invention are described herein and in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989); Ausubel et al. (eds.), Current Protocols In Molecular Biology, John Wiley and Sons (1987)).

Adverse effects due to some disease treatment regimens are known in the art (see, e.g., Munschauer *et al.* (1997) Clinical Therapeutics 19(5): 883-893; Walther *et al.* (1999) Neurology 53: 1622-1627; Lublin *et al.* (1996) 46: 12-18; Bayas *et al.* (2000) 2: 149-159; Ree *et al.* (2002) 8: 15-18; Walther *et al.* (1998) 5(2): 65-70). For example, some of the adverse effects due to treatment of MS include, but are not limited, e.g., flu-like symptoms; increased spasticity or deterioration of neurological symptoms; menstrual disorders; laboratory abnormalities (e.g., abnormal blood count/value for hemoglobin, leukocytes, granulocytes, lymphocytes, or thrombocytes); abnormal laboratory value for liver enzymes (e.g. bilirubin, transaminases, or alkaline phosphatases); injection site reactions, (e.g., inflammation, pain, or erythema); cutaneous or subcutaneous necroses; and depression. Suitable co-medications and the use of these co-medications, in conjunction with the compositions and methods of the present invention, for treating adverse effects due to treatment of a disease (e.g., MS, UC, IBD or CD), can be determined according to co-

medications generally known in the art for treatment of such effects (see, e.g., Munschauer *et al.* (1997) *Clinical Therapeutics* 19(5): 883-893; Walther *et al.* (1999) *Neurology* 53: 1622-1627; Lublin *et al.* (1996) 46: 12-18; Bayas *et al.* (2000) 2: 149-159; Ree *et al.* (2002) 8: 15-18; Walther *et al.* (1998) 5(2): 65-70). Doses and dosing regimens for such co-medications are also generally known. Such co-medications are well known in the art and may include, but are not limited to, e.g., those that help alleviate or mitigate adverse effects due to a disease or due to treatment of a disease. Examples of such co-medications include, but are not limited to, analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), and steroids.

Other suitable examples of co-medications also include, but are not limited to, e.g., ibuprofen, acetaminophen, acetylsalicylic acid, prednisone, pentoxifylline, baclofen, steroids, antibacterial agents, and antidepressants (see e.g., Walther *et al.* (1999) *Neurology* 52: 1622-1627). For example, flu-like symptoms can be treated with NSAIDs (e.g., ibuprofen or acetylsalicylic acid) or with paracetamol or with pentoxifylline; increased spasticity or deterioration of neurological symptoms can also be treated with NSAIDs and/or muscle relaxants (e.g., baclofen); menstrual disorders can be treated with oral contraceptives; injection site reactions can be treated with systemic NSAIDs and/or steroids (e.g., hydrocortisone); cutaneous or subcutaneous necrosis can be treated with antibacterial agents and depression can be treated with antidepressants (see e.g., Walther *et al.* (1999) *Neurology* 53: 1622-1627).

Combination therapies with other drugs, which are effective in the treatment of a particular disease and have a different adverse event profile may increase the treatment effect and level out the adverse event profile. For treatment of MS, examples of combination therapies include, but are not limited to, e.g., glatiramer acetate (Copaxone), mitoxantrone, cyclophosphamide, cyclosporine A, cladribine, monoclonal antibodies (e.g., Campath-H1® or Antegren®/Natazulimab®), and statins. Likewise, combination therapies may be used for UC, IBD and/or CD as well.

Effective treatment of disease in a subject using the methods of the invention can be examined in several alternative ways including, for example, EDSS (extended disability status scale) score, Functional Composite Score, cognitive testing, appearance of exacerbations, or MRI e.g., for the treatment of MS. The EDSS is a means to grade clinical impairment due to MS (see e.g., Kurtzke (1983) *Neurology* 33:1444). Eight functional systems, the walking range, the ability to walk, and the ability to maintain self-care functions are evaluated for the type and severity of neurologic impairment. For example, prior to treatment, impairment in the following systems is evaluated: pyramidal, cerebellar, brainstem, sensory, bowel and bladder, visual, cerebral, and other. Together with the assessment of the walking range, of the ability to walk with or without assistive devices, and of the ability to maintain self-care functions the final EDSS score is calculated. Follow-up scores are then obtained at defined intervals of treatment. The grade scale may range, e.g., from 0 (normal) to 10 (death due to MS). An increase of one full step (or a one-half step at the higher baseline EDSS scores)

may define disease progression (see e.g., Kurtzke (1994) *Ann. Neurol.* 36:573-79, Goodkin (1991) *Neurology.* 41:332.).

For treatment of MS, exacerbations can be defined as the appearance of a new symptom that is attributable to MS and accompanied by an appropriate new neurologic abnormality (see e.g., IFN- β MS Study Group). Exacerbations typically last at least 24 hours, and are preceded by stability or improvement for at least 30 days or a separation of at least 30 days from onset of the last event. Standard neurological examinations may result in the exacerbations being classified as either mild, moderate, or severe according to changes in a Neurological Rating Scale (see e.g., Sipe *et al.* (1984) *Neurology* 34:1368), and/or changes in EDSS score or evaluating physician opinion. An annual exacerbation rate (or other measures for the frequency of relapses, like e.g., a hazard ratio for recurrent relapses), the proportion of exacerbation-free subjects, and other relapse-based measures for disease activity are then determined, and the effectiveness of therapy is assessed between the treated group and the placebo group, for any of these measurements.

Similarly, disease progression may be monitored in UC, IBD and CD subjects using the daily activity index (DAI), a cumulative index of: weight loss, stool consistency, and rectal bleeding.

Further, suitable vectors for use in the compositions and methods of the present invention for the treatment of disease are those having minimal immunological toxicity, e.g., plasmid or AAV vectors. For example, plasmid vectors encoding either TGF- β or IL-4, under control of a CMV promoter, reportedly protect mice from myelin basic protein (MBP) induced EAE with minimal immunological toxicity (see e.g., C.A. Piccirillo and G.J. Prud'homme (1999) *Human Gene Therapy* 10: 1915-22)). Further, a variety of vectors and target tissues are reportedly suitable for use for expressing cytokines in an EAE model, including a non-replicative herpes simplex (HSV) type-1 vector expressing IL-4, IL-10, or IL-1 antagonist following intrathecal administration (see e.g., G. Martino *et al.* (2000) *J. Neuroimmunol* 107: 184-90).

A number of new technologies can also be used to diagnose and manage treatment of disease (e.g., MS). For example, magnetic resonance imaging (MRI) scanning can be used as a concomitant indicator of disease and disease activity, and can also be used as a diagnostic tool (see e.g., Paty *et al.* (1993) *Neurology* 43: 662-667; Frank *et al.* (1994) *Ann. Neurol.* 36(suppl.): S86-S90; (1995) *Neurology* 45: 1277-1285; Filippi *et al.* (1994) *Neurology* 44: 635-641). For example, for the treatment of MS, MRI can be used to measure active lesions using, e.g., gadolinium-DTPA-enhanced T1-weighted imaging (see e.g., McDonald *et al.* (2001) *Ann. Neurol.* 50: 121-127) or the location and extent of lesions using T2-weighted and T1-weighted techniques. Baseline MRIs can be obtained and thereafter, the same imaging plane and subject position can be used for each subsequent study. For MS, areas of lesions can be outlined and summed slice by slice for total lesion area, and various criteria may be examined, e.g.: 1) evidence of new lesions; 2) rate of appearance of active or new lesions; and 3) change in lesion area or lesion volume (see e.g., Paty *et al.* (1993)

Neurology 43:665). Thus, improvement due to therapy may then be established, e.g., when there is a statistically significant improvement in an individual subject compared to baseline or in a treated group versus a placebo group.

5 **Formulation and Administration of Compositions**

In preferred embodiments, the nucleic acid compositions of the present invention are formulated for administration or delivery to the cells of a subject. In some embodiments, the nucleic acid compositions of the present invention are formulated with non-ionic and/or anionic polymers. Such polymers can enhance transfection efficiency and expression of
10 molecules encoded by the nucleic acid, and protect the nucleic acid from degradation. Thus, in some embodiments where transfection efficiency or expression is enhanced using formulated nucleic acid compositions of the present invention, lower amounts of the nucleic acid composition (e.g., a vector encoding a molecule of the present invention, e.g., a TM and/or RM of the present invention) can be used. As used herein, "biodegradable polymers",
15 refers to polymers that can be metabolized or cleared in vivo by a subject and having no or minimal toxic effects or side effects on the subject.

"Anionic polymers" as used herein refers to polymers having a repeating subunit which includes, for example, an ionized carboxyl, phosphate or sulfate group having a net negative charge at neutral pH. Examples of anionic polymers suitable for use in the present
20 invention include, but are not limited to, poly-amino acids (e.g., poly-glutamic acid, poly-aspartic acid and combinations thereof), poly-nucleic acids, poly-acrylic acid, poly-galacturonic acid, and poly-vinyl sulfate. In some embodiments, where the polymer is a polymeric acid, the polymer is utilized as a salt form. Examples of other polymers include, but are not limited to PVP, PVA, and chitosan. As used herein, "poly-L-glutamic acid" is used
25 interchangeably herein with "poly-L-glutamic acid, sodium salt", "sodium poly-L-glutamate" and "poly-L-glutamate." "Poly- L-glutamate" as used herein refers to a sodium salt of poly-L-glutamic acid. Further, in preferred embodiments the L stereoisomer of polyglutamic acid is used in the compositions of the present invention, but, in other embodiments, other stereoisomer or racemic mixtures of isomers are suitable for use in the compositions of the
30 present invention. Further, in some embodiments other salts of anionic amino acid polymers are suitable for use in the compositions of the present invention.

The term "anionic amino acid polymers" as used herein refers to polymeric forms of a given anionic amino acid, for example, a poly-glutamic acid or poly-aspartic acid. In some
35 embodiments, polymers formed of a mixture of anionic amino acids, for example glutamic acid and aspartic acid, may be equally suitable for use in compositions of the present invention.

Methods for formulating pharmaceutical compositions are generally known in the art. For example, see Remington's Pharmaceutical Sciences 18. sup. ed.: Mack Pub. Co.:
Eaton, Pa. 1990, for a thorough discussion on the formulation and selection of
40 pharmaceutically acceptable carriers, stabilizers, and isomolytes (also see, e.g., U.S. Patent

No.s 4,588,585; 5,183,746; 5,795,779; and 5,814,485; U.S. Application No.s 10/190,838, 10/035,397; and PCT International Application No.s PCT/US02/21464 and PCT/US01/51074).

5 A pharmaceutically acceptable carrier and other components (e.g., co-medications) may be used in the pharmaceutical compositions and methods of the present invention. As used herein, "pharmaceutically acceptable carrier" is a carrier or diluent that is conventionally used in the art to facilitate the storage, administration, and/or the desired effect of the therapeutic ingredients of the pharmaceutical composition. A carrier may also reduce undesirable side effects of administering or delivering to a subject a pharmaceutical
10 composition of the present invention. A suitable carrier is preferably stable, e.g., incapable of reacting with other ingredients in the formulation. Further, a suitable carrier preferably does not produce significant local or systemic adverse effect in a subject at the doses and concentrations employed for therapy. Such carriers are generally known in the art.

Suitable pharmaceutically acceptable carriers are, e.g., solvents, dispersion media,
15 antibacterial and antifungal agents, microcapsules, liposomes, cationic lipid carriers, isotonic and absorption delaying agents and the like which are not incompatible components of the pharmaceutical compositions of the present invention. The use of such media and agents for therapeutically effective or active substances is well known in the art. Supplementary active ingredients may also be incorporated into the pharmaceutical compositions of the present
20 invention and used in the methods of the present invention.

Additional examples of pharmaceutically suitable carriers for use in the pharmaceutical compositions of the present invention are large stable macromolecules such as albumin, gelatin, collagen, polysaccharide, monosaccharides, polyvinylpyrrolidone, polylactic acid, polyglycolic acid, polymeric amino acids, fixed oils, ethyl oleate, liposomes,
25 glucose, sucrose, lactose, mannose, dextrose, dextran, cellulose, mannitol, sorbitol, polyethylene glycol (PEG), heparin alginate, and the like. Slow-release carriers, such as hyaluronic acid, may also be suitable.

Stabilizing agents such as human serum albumin (HSA), mannitol, dextrose, trehalose, thioglycerol, and dithiothreitol (DTT), may also be added to the pharmaceutical
30 compositions of the present invention to enhance their stability. Suitable stabilizing agents include but are not limited to ethylenediaminetetracetic acid (EDTA) or one of its salts such as disodium EDTA; polyoxyethylene sorbitol esters e.g., polysorbate 80 (TWEEN 80), polysorbate 20 (TWEEN 20); polyoxypropylene-polyoxyethylene esters e.g., Pluronic F68 and Pluronic F127; polyoxethylene alcohols e.g., Brij 35; semethicone; polyethylene glycol e.g.,
35 PEG400; lysophosphatidylcholine; and polyoxyethylene-p-t-octylphenol e.g., Triton X-100. Stabilization of pharmaceutical compositions by surfactants is generally known in the art (see e.g., Levine *et al.* (1991) J. Parenteral Sci. Technol. 45(3):160-165).

Other acceptable components of the pharmaceutical compositions of the present invention may include, but are not limited to, buffers that enhance isotonicity such as water,
40 saline, phosphate, citrate, succinate, acetic acid, aspartate, and other organic acids or their

salts. Preferably, pharmaceutical compositions of the present invention comprise a non-ionic tonicifying agent in an amount sufficient to render the compositions isotonic with body fluids.

The pharmaceutical compositions of the present invention can be made isotonic with a number of non-ionic tonicity modifying agents generally known to those in the art, e.g.,
5 carbohydrates of various classifications (see, e.g., Voet and Voet (1990) Biochemistry (John Wiley & Sons, New York); monosaccharides classified as aldoses (e.g., glucose, mannose, arabinose), and ribose, as well as those classified as ketoses (e.g., fructose, sorbose, and xylulose); disaccharides (e.g., sucrose, maltose, trehalose, and lactose); and alditols (acyclic polyhydroxy alcohols) e.g., glycerol, mannitol, xylitol, and sorbitol. In a preferred
10 embodiment, non-ionic tonicifying agents are trehalose, sucrose, and mannitol, or a combination thereof.

Preferably, the non-ionic tonicifying agent is added in an amount sufficient to render the formulation isotonic with body fluids. In one embodiment, when incorporated into a pharmaceutical composition of the present invention (including, e.g., an HSA-free
15 pharmaceutical composition), the non-ionic tonicifying agent is present at a concentration of about 1 % to about 10 %, depending upon the agent used (see e.g., U.S. Application No.s 10/190,838, 10/035,397; and PCT International Application No.s PCT/US02/21464 and PCT/US01/51074).

Further, preferred pharmaceutical compositions of the present invention may
20 incorporate buffers having reduced local pain and irritation resulting from injection, or improve solubility or stability of a component of the pharmaceutical compositions of the present invention (e.g., comprising and/or encoding a TM, RM, AM, and/or IM). Such buffers include, but are not limited to, e.g., low-phosphate, aspartate, and succinate buffers.

The pharmaceutical compositions of the present invention may additionally comprise
25 a solubilizing compound or formulation that is capable of enhancing the solubility of the components of the compositions. Suitable solubilizing compounds include, e.g., compounds containing a guanidinium group, preferably arginine. Additional examples of suitable solubilizing compounds include, but are not limited to, e.g., the amino acid arginine, or amino acid analogues of arginine that retain the ability to enhance the solubility of an IFN- β mutein
30 of the present invention. Examples of such amino acid analogues include but are not limited to, e.g., dipeptides and tripeptides that contain arginine. Further examples of suitable solubilizing compounds are discussed in, e.g., U.S. Patent No.s 4,816,440; 4,894,330; 5,005,605; 5,183,746; 5,643,566; and in Wang *et al.* (1980) J. Parenteral Drug Assoc. 34: 452-462).

35 In preferred embodiments, the pharmaceutical compositions of the present invention (e.g., comprising and/or encoding a TM, RM, AM, and/or IM) are formulated in a unit dosage and in an injectable form such as a solution, suspension, or emulsion, or in the form of lyophilized powder, which can be converted into solution, suspension, or emulsion prior to administration. The pharmaceutical compositions of the present invention may be sterilized

by membrane filtration, which also removes aggregates, and stored in unit-dose or multi-dose containers such as sealed vials, ampules or syringes.

In another embodiment, an AM or IM of the present invention is formulated for oral administration. In one embodiment, the nucleic acids encoding a TM and/or RM of the present invention are formulated for administration by injection or electroporation, and an AM and/or IM of the present invention is formulated for oral administration. For example, in a preferred embodiment, the regulated expression system of the present invention comprises a single vector encoding at least a TM and an RM formulated for delivery by injection or electroporation to the cells of a subject, and an AM that is formulated for oral administration to the subject, such that the presence of the AM in the cell of the subject activates the RM and thereby the RM induces expression of the TM in the cells of the subject.

Liquid, lyophilized, or spray-dried pharmaceutical compositions of the present invention may be prepared as known in the art, e.g., as an aqueous or nonaqueous solution or suspension for subsequent administration to a subject in accordance with the methods of the present invention. Each of these pharmaceutical compositions may comprise a therapeutically or prophylactically effective or active component. As used herein, a therapeutically or prophylactically "effective" or "active" component is an amount of a molecule of the present invention (e.g., comprising and/or encoding a TM, RM, AM, and/or IM) that is included in the pharmaceutical composition of the present invention to bring about a desired therapeutic or prophylactic response with regard to treatment, prevention, or diagnosis of a disease or condition in a subject in need of treatment, using the pharmaceutical compositions and methods of the present invention. Preferably the pharmaceutical compositions of the present invention comprise appropriate stabilizing agents, bulking agents, or both to minimize problems associated with loss of biological or therapeutic activity during preparation and storage.

Formulation of the pharmaceutical compositions of the present invention are preferably stable under the conditions of manufacture and storage and preserved against the contaminating action of microorganisms such as bacteria and fungi. Methods of preventing microorganism contamination are well known, and can be achieved e.g., through the addition of various antibacterial and antifungal agents.

Suitable forms of the pharmaceutical composition of the present invention may include sterile aqueous solutions or dispersions, and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Suitable forms are preferably sterile and fluid to the extent that they can easily be taken up and injected via a syringe. Typical carriers may include a solvent or dispersion medium containing, for example, water buffered aqueous solutions (*i.e.*, biocompatible buffers), ethanol, polyols such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants, or vegetable oils. Sterilization can be accomplished by any art-recognized technique, including but not limited to filtration or addition of antibacterial or antifungal agents, for example, paraben, chlorobutanol,

phenol, sorbic acid or thimerosal. Further, isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions.

Production of sterile injectable solutions containing a pharmaceutical composition of the present invention may be accomplished by incorporating the composition in the desired amount, in an appropriate formulation with various ingredients (e.g., those enumerated
5 herein) as desired, and followed by sterilization. To obtain a sterile powder, the above solutions can be vacuum-dried or freeze-dried as necessary.

The pharmaceutical compositions of the present invention can thus be compounded for convenient and effective administration in pharmaceutically effective amounts with a suitable pharmaceutically acceptable carrier in a therapeutically effective dose. The precise
10 therapeutically effective amount of the compositions and methods of the present invention for application to humans can be determined by the skilled artisan with consideration of individual differences in age, weight, extent of cellular infiltration by inflammatory cells and condition of the MS subject.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.
15

The principal active ingredients may be compounded for convenient and effective administration in therapeutically effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as described herein. Further, the co-medications are contained in a unit dosage form in amounts generally known in the art. In the case of compositions containing supplementary active ingredients, e.g. co-medications, the dosages
20 may be determined, e.g., by reference to the known dose and manner of administration of the ingredients.

The pharmaceutical compositions of the present invention may be administered in a manner compatible with the dosage formulation and in such an amount as will be therapeutically effective. Further, the pharmaceutical compositions of the present invention may be administered in any way which is medically acceptable and which may depend on a specific type or stage of disease or associated symptoms being treated. Possible administration routes include injections, by parenteral routes such as intravascular, intravenous, intra-arterial, subcutaneous, intramuscular, intratumor, intraperitoneal, intraventricular, intraepidural or others, as well as oral, nasal, ophthalmic, rectal, topical, or by
30 inhalation. In a preferred embodiment, the administration route is intramuscular. In a preferred embodiment, the pharmaceutical composition of the present invention is administered intramuscularly, every 3-12 months. In another preferred embodiment, the intramuscular administration is via automated or manual injection (e.g., using a syringe) of the pharmaceutical composition. Sustained release administration is also contemplated, e.g.,
35 using erodible implants.

In particular, the nucleic acid pharmaceutical compositions of the present invention can be delivered to cells of a subject using any means described herein or known in the art, including e.g., by injection or other suitable means. For example, known methods of delivery of nucleic acids to cells by physical means are suitable for use and include, but are not limited to, electroporation, sonoporation, and pressure. In some embodiments, delivery of a nucleic acid composition of the present invention is by electroporation and comprises the application of a pulsed electric field to create transient pores in the cellular membrane and, thereby, an exogenous molecule, e.g., a nucleic acid composition of the present invention, is delivered to the cell. It is known that adjusting the electrical pulse generated by an electroporetic system, nucleic acid molecules can find their way through passageways or pores in the cell that are created during such a procedure (see e.g., U. S. Patent 5, 704,908, U.S. Patent 5,704,908).

As used herein, "pulse voltage device", or "pulse voltage injection device" refers to an apparatus that is capable of causing or causes uptake of nucleic acid molecules into the cells of a subject by emitting a localized pulse of electricity to the cells, thereby, causing the cell membrane to destabilize and result in the formation of passageways or pores in the cell membrane. Conventional devices of this type are suitable for use for the delivery of a nucleic acid composition of the present invention. In some embodiments, the device is calibrated to allow one of ordinary skill in the art to select and/or adjust the desired voltage amplitude and/or the duration of pulsed voltage and therefore. A pulse voltage nucleic acid delivery device can include, for example, an electroporetic apparatus as described e.g. in U.S. Patent No. 5,439,440, U.S. Patent No. 5,704,908, U.S. Patent No. 5,702,384, PCT No. WO96/12520, PCT No. WO 96/12006, PCT No. WO 95/19805, or PCT No. WO 97/07826.

Packaging material used to contain the active ingredient of a pharmaceutical composition of the present invention can comprise glass, plastic, metal or any other suitable inert material and, preferably, is packaging material that does not chemically react with any of the ingredients contained therein. In one embodiment, the pharmaceutical composition is packaged in a clear glass, single-use vial; and a separate vial containing diluent is included for each vial of drug. In another preferred embodiment, the diluent is provided in a syringe (i.e., the syringe is pre-filled with the diluent). In yet another preferred embodiment, the pharmaceutical composition of the present invention is provided in solution in a syringe (i.e., the syringe is pre-filled with the pharmaceutical composition in solution) and is ready for use. In one embodiment, the pharmaceutical composition of the present invention can be stored under refrigeration, between 2° to 8°C (36° to 46°F). In a preferred embodiment, the pharmaceutical composition is stored at room temperature.

Vectors And Kits

The present invention further provides vectors and kits comprising the improved regulated expression system of the present invention for treatment of disease. In some embodiments, the improved regulated expression system of the present invention comprises one or more vectors, and each vector comprises one or more expression cassettes. In one

embodiment, the improved regulated expression system of the present invention comprises a single vector having at least one expression cassette and, more preferably at least two expression cassettes.

Suitable vectors for use in the regulated, expression system of the present invention, include, but are not limited to, those that are capable of expressing an encoded TM, and/or other encoded molecule of the present invention (e.g., RM, AM, or IM), when administered to the cells of a subject. Examples of suitable vectors include, but are not limited to, those described herein and those known in the art, including vectors for producing virus and nonviral vectors (vectors that do not produce virus). For example, one class of suitable vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. Further, known vectors for producing virus (see e.g., Wang, et al., Gene Therapy, 4: 432- 441,1997; Oligino, et al., Gene Therapy, 5: 491-496,1998) may be modified and adapted for use in the regulated expression system of the present invention. Further, the vectors of the present invention can be modified to include additional functional and operably linked sequences for optimal expression of an encoded molecule. Examples of suitable functional sequences include, but are not limited to, splicing, polyadenylation and other types of RNA processing sequences; and transcriptional promoter, enhancer, and termination sequences. Suitable cDNA expression vectors incorporating such functional sequences include those described by Okayama, H., *Mol. Cell. Biol.* 3:280 (1983), and others.

"Plasmid" as used herein refers to a composition comprising extrachromosomal genetic material, usually of a circular duplex of DNA, that can replicate independently of chromosomal DNA. Plasmids may be used as vectors, as described herein. "Vector" as used herein refers to a composition (e.g., a nucleic acid construct) comprising genetic material designed to direct transformation or transfection of a targeted cell. Further, a vector may contain multiple functional sequences positionally and sequentially oriented with respect to other sequences of the vector such that an encoded molecule of the present invention can be transcribed and when necessary translated in the transfected or transformed cells. Where a vector or expression cassette encodes a molecule of the present invention (e.g., a TM, RM, AM or IM), it comprises the essential components (e.g., promoter, poly(A) site, transcription start and stop sites) for expression of the encoded molecule in a heterologous cell (e.g., cells of a subject) according to the regulated expression system of the present invention, described herein.

In a preferred embodiment, the improved regulated expression system of the present invention comprises a single vector comprising a first expression cassette having at least one cloning site for insertion of a first nucleic acid sequence encoding a TM, and a second expression cassette having at least one cloning site for insertion of second nucleic acid sequence encoding an RM. In another embodiment, the vector is a vector for producing virus encoding a molecule of the present invention (e.g., a TM and/or RM) for delivery to cells of a

subject as described herein, e.g., a shuttle plasmid and more particularly, an AAV-1 shuttle plasmid (see e.g., Table 8).

In another preferred embodiment, the vector is a single plasmid vector e.g., pGT1 (comprising the sequence of SEQ ID NO: 9 and SEQ ID NO: 4), pGT2 (comprising the sequence of SEQ ID NO: 10 and SEQ ID NO: 4), pGT3 (comprising the sequence of SEQ ID NO: 11 and SEQ ID NO: 4), or pGT4 (SEQ ID NO: 12), wherein each vector comprises a multiple cloning site (SEQ ID NO: 4) located 3' of the IVS8 and 5' of the hGH poly(A) site (e.g., as schematically depicted in Figures 14A-D), or pGT11 (comprising the sequence of SEQ ID NO: 9 and SEQ ID NO: 5), pGT12 (comprising the sequence of SEQ ID NO: 10 and SEQ ID NO: 5), pGT13 (comprising the sequence of SEQ ID NO: 11 and SEQ ID NO: 5), or pGT14 (comprising the sequence of SEQ ID NO: 12 and SEQ ID NO: 5), wherein each vector comprises a multiple cloning site (SEQ ID NO: 5) located 3' of the IVS8 and 5' of the hGH poly(A) site.

The expression cassettes of the present invention comprise functional sequences for expression of an encoded molecule of the present invention, e.g., a TM, RM, AM, or IM. In some embodiments, the expression cassette comprises at least one functional sequence operably linked to a nucleic acid sequence encoding a molecule of the present invention. As used herein "functional sequence" refers to a nucleic acid or amino acid sequence having a function or activity in a cell, e.g., a function or activity relating to the cellular expression, processing, or cloning of a molecule, or to the biological or cellular activity or function of a molecule. Examples of a functional sequence include a sequence encoding a molecule of the present invention (e.g., a TM, RM, AM, or IM), promoter, protein or nucleic acid binding site, splice site, transcription stop site, regulatory domain (e.g., activation domain), transcription start site, protein or nucleic acid stabilization site, intervening sequence, restriction enzyme site or cloning site, viral packaging signal, or other cellular, protein, or nucleic acid processing or regulatory signal (e.g., a signal transduction sequence or tissue-specific sequence). Further examples of a functional sequence are, but not limited to, a 5' or 3' untranslated region (e.g., UT12, SEQ ID NO: 2), intron (e.g., IVS8, SEQ ID NO: 3), polyadenylation (poly(A)) site (e.g., SV40, SEQ ID NO: 8 or hGH poly(A) site, SEQ ID NO: 6), or a DNA-binding site (DBS) (e.g., SEQ ID NO: 49).

Such functional sequences also include, for example, sequences encoding a regulated promoter or tissue-specific promoter that promotes the regulated or tissue-specific expression, respectively, of a molecule encoded by a nucleic acid sequence operably linked to such functional sequences in an expression cassette of the present invention. Examples of suitable promoters include, but are not limited to, a CMV promoter, muscle-specific promoter (e.g., actin promoter or muscle creatine kinase (MCK) promoter), condition-specific (e.g., hypoxia or inflammation) promoter or element (e.g., ELAM promoter or HRE), constitutive promoter (e.g., ubiquitin, PGK, or EF1 α promoter), synthetic or chimeric promoter (e.g., CMV/actin promoter), cell-cycle specific promoter (e.g., cyclin A or cdc6 promoter). In one embodiment, the promoter is a physiologically responsive promoter, e.g., a promoter

responsive to inflammation and, preferably responsive to the presence of cytokines or chemokines or other cellular or biological molecules indicative of the onset or presence of a disease or condition. Further examples of suitable promoters are provided in Table 1 below.

Table 1

Promoter	Plasmid/Source	Description	Reference(s)
EF-1 α	pdrive-chef1 (InvivoGen)	Chimpanzee EF-1 α promoter	e.g., KimDW, et al.1990 Gene. 91(2):217-23; Guo ZS, et al. 1996 Gene Ther. 3(9):802-810
EF-1 α /RU5	pdrive-chef1ru5 (InvivoGen)	Chimpanzee EF-1 α promoter +HTLV 5'UTR	e.g., KimDW, et al. 1990 Gene. 91(2):217-23; Guo ZS, et al. 1996 Gene Ther. 3(9):802-810; Takebe Y. et al 1998 Mol Cell Bio. 8(1):466-472
UbiB	pdrive-hubib (InvivoGen)	Human Ubiquitin B promoter	e.g., Ciechanover A.and Schwartz AL. 1998 PNAS 95(6):2727-30; Yew NS, et al. 2001 Mol Ther. 4(1):75-82
Sk promoter	pGS1694 (Valentis)	Skeletal muscle actin promoter+UT12 5'UTR+IVS8 intron	e.g., PCT application no. PCT/US01/30305
MCK promoter	Mouse genomic DNA	Mouse Muscle Creatine Kinase	e.g., Jaynes JB et al, 1986 Mol Cell Biol. Aug;6(8):2855-64. Hauser et al 2000, Mol Ther 2:16-25
EF-1 α /RU5	pORF-htrail (InvivoGen)	Human EF-1 α + HTLV 5'UTR and bacterial promoter	e.g., KimDW, et al. 1990 Gene. 91(2):217-23; Guo ZS, et al. 1996 Gene Ther. 3(9):802-810; Takebe Y. et al 1998 Mol Cell Bio. 8(1):466-472
TK promoter	phRL-TK (Promega)	HSV Thymidine Kinase promoter	e.g., Wagner EF et al, 1985 EMBO J (4) 663-6; Stewart CL et al, 1987 EMBO J (6) 383-8

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In one embodiment, the regulated expression system of the present invention comprises one or more vectors comprising at least one expression cassette having a CMV promoter sequence that is operably linked to a nucleic acid sequence encoding a molecule of the present invention that is a fusion or chimeric protein, and the promoter drives the expression of the encoded molecule (e.g., TM, RM, AM, or IM). In some embodiments, a suitable promoter would be one that would provide a durable level of expression of the encoded molecule in the cells of a subject. In one embodiment, a nucleic acid vector encodes a molecule of the present invention (e.g., TM, RM, AM, or IM) that is operably linked to a promoter that provides durable expression in the cells of a subject, and the nucleic acid vector is administered to the cells of the subject via electroporation, such that the molecule is expressed in the cells, preferably, at the site of administration. In a preferred embodiment, the encoded molecule is a TM.

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In another embodiment, the regulated expression system of the present invention comprises one or more vectors comprising a first expression cassette having a promoter sequence comprising at least one GAL-4 DBS, operably linked to a nucleic acid sequence encoding a TM of the present invention. In one embodiment, the promoter sequence
5 comprises multimers of a GAL-4 DBS, e.g., 3-18 GAL-4 DBS.

Further, the expression cassettes of the present invention can be suitably modified to comprise cloning sites for the insertion of a desired nucleic sequence. In another embodiment, the expression cassettes of the present invention comprise at least one cloning site and, more preferably a multiple cloning site (MCS), for the insertion of a nucleic acid
10 sequence encoding a molecule of the present invention, e.g., a TM, RM, AM, or IM. As used herein, "cloning site" refers to an enzyme site or other site in a nucleic acid wherein a nucleic acid sequence can be inserted, operably linked, or otherwise attached using conventional methods known in the art e.g., such that the sequence functions for its intended purpose. In one embodiment, a first expression cassette of the present invention comprises an MCS for
15 insertion of a first nucleic acid sequence encoding a TM, an inducible promoter comprising at least one DBS (e.g., 3-18 GAL-4 DBS), 5' untranslated region (e.g., UT12, SEQ ID NO: 2), an intron (e.g., IVS8, SEQ ID NO: 3), and hGH poly(A) site (e.g., SEQ ID NO: 6), such that when the first nucleic acid sequence is inserted at the MCS (e.g., SEQ ID NO: 4, or SEQ ID NO: 5), these functional sequences are operably linked to the first nucleic acid sequence. In another
20 embodiment, a second expression cassette of the present invention comprises an MCS for insertion of a second nucleic acid sequence encoding a regulated RM and SV40 poly(A) site (e.g., SEQ ID NO: 8), such that when the second nucleic acid sequence is inserted at the MCS, these functional sequences are operably linked to the second nucleic acid sequence. In a preferred embodiment, the first and second expression cassettes are present in a single
25 vector.

The kits of the present invention comprise at least one of the expression systems of the present invention described herein and, more particularly, at least one of the pharmaceutical compositions, vectors, or molecules (e.g., TM, RM, AM, or IM) of the present
30 invention.

Isolation and Construction of Compositions

The compositions of the present invention include, for example, chemical compounds, proteins, and nucleic acids (e.g., DNA or RNA molecules), particularly, nucleic acids encoding a protein or RNA. The chemical, nucleic acid, and protein compositions of the
35 present invention can be isolated, constructed, and/or tested using conventional methods and assays, as described herein or in the art.

"Protein" or "amino acid molecule" as used herein refers to a peptide, full-length protein, or fragment or portion of a full-length protein. Further, a protein of the present invention can be a fused, chimeric, modified, isolated, synthetic, or recombinant amino acid
40 molecule. In particular, examples of proteins suitable for use in the compositions and

methods of the present invention, include, but are not limited to, a wild-type, full-length protein (including a secreted form thereof), or an analog, derivative, or variant thereof having a biological or therapeutic activity. More particularly, protein variants of the present invention can be muteins i.e., comprising a mutation e.g., a single or multiple amino acid substitution, deletion, or addition such that the variant retains or has a biological or therapeutic activity. Sequences encoding a protein may include, e.g., codon-optimized versions of wild-type protein sequences, or humanized sequences. Optimal codon usage in humans can be identified from codon usage frequencies for expressed human genes and may be determined by methods known in the art e.g., program "Human High.codN" from the Wisconsin Sequence Analysis Package, Version 8.1, Genetics Computer Group, Madison, WI. For example, codons that are most frequently used in highly expressed human genes may be optimal codons for expression in the cells of a human subject, and, thus, can be used as a basis for constructing a synthetic coding sequence.

"Nucleic acid" as used herein with reference to a molecule of the present invention, refers to a nucleic acid molecule, e.g., a DNA or RNA, or fused, chimeric, modified, isolated, synthetic, or recombinant form thereof. In particular, examples of nucleic acids suitable for use in the compositions and methods of the present invention, include, but are not limited to, a wild-type, full-length DNA or RNA (e.g., mRNA) encoding a protein, or other nucleic acid molecule having a biological or therapeutic activity (e.g., shRNA, siRNA, ribozyme, antisense RNA or DNA, RNA or DNA oligonucleotide), or an analog, derivative, or variant thereof. More particularly, nucleic acid variants of the present invention can be muteins i.e., comprising a mutation, e.g., a single or multiple nucleic acid substitution, deletion, or addition such that the variant retains or has a biological or therapeutic activity.

Further, modifications of the regulated, expression system of the present invention can be carried out and tested using conventional methods and assays, as described herein or in the art.

"Modified" as used herein, with reference to a molecule of the present invention (e.g., comprising and/or encoding a TM, RM, AM, and/or IM) refers to any reaction or manipulation resulting in a change or alteration of a reference nucleic acid, amino acid, or chemical molecule to arrive at a desired composition or molecule of the present invention (e.g., mutation of a wild-type protein or nucleic acid to arrive at a desired variant thereof having a specific biological and/or therapeutic activity; mutation of a protein or nucleic acid sequence to arrive at a desired humanized sequence; or mutation of a chemical compound to arrive at a desired chemical structure, and/or biological and/or therapeutic activity). For example, the functional domains or functional sequences of a molecule of the present invention, including any sequence operably linked to or encoding a molecule of the present invention (including e.g., a vector sequence or transcription control sequence), can be modified to arrive at a desired composition or molecule of the present invention.

In particular, the regulated expression system of the present invention can be modified or optimized to achieve a particular specificity (e.g., specific to a particular tissue,

condition, disease, or biomarker or other molecule), stringency, or amount of regulation, expression, and/or activity for use in the treatment of disease, as described herein. For example, the regulated, expression system of the present invention can be modified or optimized to achieve such objectives by isolating or constructing: 1) novel or variant AMs and
5 IMs having a desired binding specificity for the LBD of an RM; 2) RMs having a novel or variant AD, LBD (e.g., that binds a novel or variant AM or IM), and/or DBD (e.g., that binds a novel or variant promoter sequence); 3) promoters having activity that is highly specific and responsive to the presence of a particular RM (e.g., that are specifically activated or inactivated in the presence of, e.g., by the binding of, a particular RM); 4) fully humanized
10 sequences (e.g., modifying sequences encoding a GAL-4 DBD and GAL-4 DBS such that the sequences are fully humanized); 5) an expression cassette for expression of an RNA (e.g., shRNA, siRNA, ribozyme, or antisense RNA), particularly, a RNA TM; and 6) modifying promoter or other functional sequences to reduce non-specific expression, particularly, of a TM encoded by a sequence operably linked to the promoter or other functional sequences.

15 In one embodiment, the regulated expression system of the present invention is modified such that the basal expression of a TM is significantly reduced in order to increase reliance on administration of an RM and, thereby, provide an increased margin of safety by virtue of extrinsically controlled TM expression rather than through dependence on the dose of plasmid administered. For example, a promoter sequence operably linked to a nucleic
20 acid coding sequence encoding a TM, may be modified and optimized for the number of copies of a GAL-4 DBS, such that the responsiveness of the promoter (and resulting TM expression) can be modulated by the presence of (e.g., binding of) an RM having a GAL-4 DBD. Also for example, a minimal promoter can be constructed and modified using standard methods and operably linked to a TM to reduce the basal expression of the TM.

25 Further, in some embodiments, the TM is encoded by a nucleic acid sequence that when delivered to and/or is present in the cells of a subject, the TM is expressed at a low level. In some embodiments, it is preferable to regulate the level of TM expression by inherent properties of the nucleic acid encoding the TM that is delivered to and/or present in the cells of the subject. For example, in some embodiments, as the level of basal TM
30 expression in the cells of a subject increases with an increasing amount of nucleic acid encoding the TM, it may be desirable to reduce the amount of expressed TM protein in the cells of the subject by utilizing a weak promoter.

Utilizing unique restriction endonuclease sites in the promoter region, different regions of the promoter and 5' UTR can be modified to delete sequences that may have an
35 effect on the expression of a TM in the presence or absence of an RM of the present invention. For example, in another embodiment a deletion is made in a sequence encoding the transcription initiation region (inr) such that the intrinsic activity of an inducible promoter that is operably linked to a sequence encoding a TM, is modified, e.g., the activity is decreased or is increased. In another embodiment, downstream of the transcription initiation

region (inr), is an operably-linked sequence encoding the UT12 (5' untranslated region of CMV, +1 to +112).

In another embodiment, the TM is encoded by a nucleic acid sequence that is operably linked to an inducible promoter sequence (e.g., SEQ ID NO: 1) having 6X GAL-4 DBS operably linked to a TATA box sequence. The sequence from -33 to -22, which contains the TATA box from the Elb region of Adenovirus type 2 (residues 1665-1677 of NCBI accession no. J01917) is suitable for use in such an embodiment of the present invention. In another embodiment, the promoter sequence comprises 6X GAL-4 DBS operably linked to an Ad Elb TATA box sequence and a CMV sequence that contains the putative initiator (inr) region of the CMV promoter (Macias et al., Journ. of Virol. 70(6):3628 (1996)), such that these functional sequences are operably linked to a nucleic acid encoding a TM.

In some embodiments, the TM is encoded by a nucleic acid sequence that is operably linked to multiple copies of a GAL-4 DBS comprising a 17 nucleotide sequence 5'-TGGAGTACTGTCCTCCG-3' or 5'-CGGAGTACTGTCCTCCG-3' (e.g., of the consensus GAL-4 DBS of SEQ ID NO: 49). In one embodiment, the TM is encoded by a nucleic acid sequence that is operably linked to 4 copies of a GAL-4 DBS each comprising the 17 nucleotide sequence 5'-CGGAGTACTGTCCTCCG-3' separated by a 10 nucleotide spacer having the nucleotide sequence 5'-AGTTTAAAAG-3' as in e.g., SEQ ID NO: 50. In another embodiment, the TM is encoded by a nucleic acid sequence that is operably linked to 6 copies of a GAL-4 DBS arranged in two groups with 3 copies each of a GAL-4 DBS, and wherein: 1) in each group (containing 3 copies of a GAL-4 DBS) the second copy of the GAL-4 DBS comprises the 17 nucleotide sequence 5'-TGGAGTACTGTCCTCCG-3' and the first and third copy of the GAL-4 DBS each comprise the 17 nucleotide sequence 5'-CGGAGTACTGTCCTCCG-3; 2) each copy within the group of 3 copies is separated by two nucleotides 5'-AG-3'; and 3) between the two groups of 3 copies there is a longer spacer sequence 5'-AGTCGAGGGTCTGAAG-3' (e.g., the sequence of SEQ ID NO: 51 comprising 6 copies of a GAL-4 DBS as described).

In some embodiments, where the expression in a particular tissue is desired, the regulated expression system of the present invention may be modified to comprise tissue-specific promoters. For example, if the target tissue for TM expression is muscle, the nucleic acid sequence encoding a TM may be operably linked to a muscle-specific promoter, e.g., an actin promoter sequence. Tissue-specific promoters, (e.g., muscle-specific promoters) may increase the fidelity of expression of the encoded TM. In some embodiments, tissue-specific promoters may provide the advantage of reduced expression in dendritic and other antigen-presenting cells, thus avoiding immune responses to the expressed TM (e.g., protein or nucleic acid).

In some embodiments, there is a lag time between the administration of the vector encoding the TM and the time in which an AM is imposed. For example, in one embodiment, the lag time between administration of the vector encoding the TM and the induction of the expression of the TM by an AM is for example, 5 days, 12 days, 20 days or 55 days.

Further, the AM may be administered one or more times over a period of time of one day to more than one year. The AM may be administered daily, every other day, three times per week, twice per week, weekly, biweekly, monthly, bimonthly, every three months, quarterly, semiannually, annually, or any combination thereof.

5 In one embodiment, the regulated expression system is modified such that the specificity, selectivity, precise timing, and/or level of TM expression and/or activity is modulated in the presence of an RM. In a further embodiment, the RM has a rapid clearance in a subject administered an RM of the present invention.

In one embodiment, the RM is a protein and is modified such that it is activated in the
10 presence of a specific or cognate ligand and, thereby, the presence of the activated RM regulates the expression and/or activity of a TM. Further, the specificity and stringency of activation of the RM can be optimized by mutation of the GAL-4 DBD of the RM to minimize any propensity to form dimers in the absence of an AM. More specifically, to minimize any non-specific activation and/or dimer formation of the RM (e.g., in the absence of an AM), the
15 RM can be modified by mutation of the GAL-4 domain by deleting or otherwise mutating the C-terminal portion of the GAL-4 DBD (e.g., 20 C-terminal residues) and, thereby, reducing the length of a coiled-coil structure that is predicted to contribute to GAL-4 homodimer formation.

In some embodiments, the GAL-4 DNA Binding Domain ("GAL-4 DBD") comprises a portion or fragment of amino acids 1-93 of the N-terminal DBD of GAL-4 (where e.g., the
20 sequence of amino acids 2-93 is SEQ ID NO: 37 and amino acid 1 is a methionine). For example, in some embodiments, the GAL-4 DBD comprises amino acids 2-93 of the N-terminal DBD of GAL-4 (e.g., comprising the amino acid sequence of SEQ ID NO: 38, or encoded by the nucleic acid sequence of SEQ ID NO: 37). In one embodiment, the GAL-4 DBD comprises amino acids 2-93 of the N-terminal DBD of GAL-4 and an operably linked N-
25 terminal peptide sequence e.g. as in SEQ ID NO: 46 (or as encoded by the nucleic acid sequence of SEQ ID NO: 45), wherein, e.g., the N-terminal peptide sequence is immediately followed by amino acids 2-93 of the N-terminal DBD of GAL-4. In other embodiments, the GAL-4 DBD comprises amino acids 2-74 of the N-terminal DNA binding domain of GAL-4 (e.g., comprising the amino acid sequence of SEQ ID NO: 48, or encoded by the nucleic acid
30 sequence of SEQ ID NO: 47). In some embodiments, a suitable GAL-4 DBD has a modification in a nucleic acid sequence or amino acid sequence that results in a mutation of the GAL-4 DBD such that it retains the ability to bind to a canonical 17-mer binding site, CGGAAGACTCTCCTCCG, but has a reduced ability to form a helical tertiary structure needed for autodimerization. In some embodiments, mutations or deletions are made to the
35 region spanning amino acids 75 to 93 and/or 54 to 74 of the GAL-4 DBD sequence. For example, in one embodiment, a deletion is made of the amino acids 54 to 64 or 65 to 75 of the GAL-4 DBD sequence, such that autodimerization is minimized through the coiled-coil region of an RM comprising the mutated GAL-4 DBD.

In one embodiment, the nucleic acid sequence of the RM is modified to encode a
40 fusion or chimeric protein comprising one or more functional domains, e.g., a DNA binding

domain (DBD), ligand-binding domain (LBD), and/or regulatory domain (RD) (e.g., an activation domain). Examples of suitable functional domains for use in the fusion or chimeric proteins of the present invention include, but are not limited to the GAL-4 DBD, human progesterone receptor (hPR) LBD, and NFκB p65 AD.

5 Examples of suitable regulatory domains (RD) for use in an RM of the present invention, include, but are not limited to, NFκBp65, VP-16, TAF- 1, TAF-2, TAU-1, TAU-2, ORF-10, TEF-1, and any other nucleic acid or amino acid sequences having a regulatory function (e.g., regulates the expression and/or activity of a molecule of the present invention) and, more particularly, a transcriptional regulatory function (see e.g., Pham et al. (1992) 6(7)
10 :1043-50 Mol. Endocrinol.; Dahlman-Wright et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1619-1623; Milhon et al. (1997) Mol. Endocrinol. 11(12) :1795-805; Moriuchi et al. (1995) Proc. Natl. Acad. Sci. USA 92(20):9333-7; Hwang et al. (1993) EMBO J 12(6):2337-48). In one embodiment, the preferred RD is a human transactivation domain (e.g., NFκB p65). In another embodiment, the RM, particularly a functional domain of the RM (e.g., an RD), is
15 humanized.

 In one embodiment, the LBD of an RM of the present invention is derived from an amino acid sequence correlating to a wild-typed LBD of a receptor in the steroid-receptor family, e.g., a progesterone receptor (PR) and more particularly, a human progesterone receptor (hPR). In another embodiment, the RM is a steroid receptor, and the amino acid
20 sequence of the LBD of the steroid receptor (e.g., a PR, and more particularly an hPR) is mutated to result in a mutated steroid-receptor LBD (e.g., a mutated hPR LBD) that selectively binds to an AM that is an antiprogesterone instead of progesterone. Thus, in this embodiment, an RM that is a mutated steroid-receptor LBD (e.g., a mutated hPR LBD) can be selectively activated by an AM that is an antiprogesterone, instead of a naturally-occurring
25 progesterone. In particular, in one embodiment, the antiprogesterone binds to a natural PR, but acts as an antagonist.

 In one embodiment, the progesterone binds to a wild-type PR and acts as an agonist, and does not bind to a truncated or mutated PR. In another embodiment, a mutated PR retains the ability to bind antiprogesterones, but responds to them as agonists. In a preferred
30 embodiment, when the antiprogesterone binds to a mutated PR that is an RM, the mutated PR protein is activated and forms a dimer. In this embodiment, the dimer-antiprogesterone complex then binds to the DBS of a promoter sequence and, thereby, induces transcription of a nucleic acid sequence encoding a TM, where the nucleic acid sequence is operably linked to the promoter.

35 In one embodiment, the presence of the anti-progesterone MFP (RU486), the chimeric RM binds to a 17-mer GAL-4 DBS operably linked to a nucleic acid sequence encoding a TM, and results in an efficient ligand-inducible transactivation of TM expression. The modified steroid-hormone LBD of the RM may also be modified by deletion of carboxy terminal amino acids, preferably, from about 1 to 120 carboxy terminal amino acids. The extent of deletion
40 desired can be obtained using standard molecular biological techniques to achieve both

selectivity for the desired ligand and high inducibility when the ligand is administered. In one embodiment, the mutated steroid hormone receptor LBD is mutated by deletion of about 1 to about 60 carboxy terminal amino acids. In another embodiment 42 carboxy terminal amino acids are deleted. In yet another embodiment, having both high selectivity and high inducibility, 19 carboxy terminal amino acids are deleted.

In one embodiment, the nucleic acid sequence of an RM comprises a sequence encoding a truncated GAL-4 DBD, a mutated progesterone receptor having a C-terminal deletion of 19 amino acids, and a p65 transactivation domain (e.g., SEQ ID NO: 39). In another embodiment, the nucleic acid sequence of an RM comprises a sequence encoding a chimeric receptor having a mutated progesterone-receptor ligand-binding domain, a truncated GAL-4 DNA binding domain, and a VP16 or p65 transregulatory domain, where the p65 transregulatory domain is part of the activation domain of the human p65 protein; a component of the NFkappaB complex. By replacing VP16 with a variety of human-derived activation domains, e.g., residues 286-550 of the human p65, the potent inducibility of the chimeric receptor can be retained while "humanizing" the protein or reducing the potential for a foreign protein immune response due to the viral VP16 component.

A DBD of an RM of the present invention is not limited to a modified GAL-4 DBD as described herein. For example, in some embodiments, a suitable DBD is one that has been modified to remove sequences that are not essential for recognition of binding sites but may be predicted to contribute to autodimerization by virtue of their secondary structure. Other DBDs that may be so modified and suitable, include e.g., the known DBD of a member of the steroid-receptor family (e.g., glucocorticoid receptor, progesterone receptor, retinoic acid receptor, thyroid receptor, androgen receptor, ecdysone receptor) or other cellular DNA-binding proteins such as the cAMP Response Element Binding protein (CREB) or zinc finger DNA binding proteins, such as SP1.

The steroid-receptor family of gene regulatory proteins is also suitable for the construction of an RM of the present invention. Steroid receptors are ligand-activated transcription factors whose ligands can range from steroids to retinoids, fatty acids, vitamins, thyroid hormones, and other presently unidentified small molecules. These compounds bind to receptors and either up-regulate or down-regulate the expression of steroid-regulated genes. The compounds are reportedly cleared from the body by existing mechanisms and are usually non-toxic. In the present invention, a ligand of a steroid receptor may be any compound or molecule that activates the steroid receptor e.g., by binding to, or otherwise interacting with, the LBD of the steroid receptor.

The term "steroid-hormone receptor" as used herein refers to steroid-hormone receptors in the superfamily of steroid receptors. Representative examples of the steroid-hormone receptor family, include, but are not limited to, the estrogen, progesterone, glucocorticoid, mineralocorticoid, androgen, thyroid hormone, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors. The receptors for hormones in the steroid/thyroid/retinoid supergene family, for example, are transcription factors that bind to

target sequences in the regulatory regions of hormone-sensitive genes to enhance or suppress their transcription. These receptors have evolutionarily conserved similarities in a series of discrete structural domains, including a ligand binding domain (LBD), a DNA binding domain (DBD), a dimerization domain, and one or more transactivation domain(s).

5 Various mutations or changes in the amino acid sequences of the different structural domains may be generated to form a variant steroid receptor or more specifically a mutated steroid receptor. In one embodiment, the mutated steroid receptor is capable of preferentially binding to a non-natural or non-native ligand rather than binding to the wild-type or naturally-occurring hormone receptor ligand. In one embodiment, a mutated hormone
10 receptor is generated by deletion of amino acids at the carboxy terminal end of a reference hormone receptor (e.g., a wild-type or naturally-occurring hormone receptor) e.g., a deletion of from about 1 to about 120 amino acids from the carboxy terminal end of the reference hormone receptor. In another embodiment, a mutated progesterone receptor of the present invention comprises a carboxy terminal deletion of from about 1 to about 60 amino acids of a
15 reference progesterone receptor (e.g., a wild-type or naturally-occurring hormone receptor). In another embodiment, a mutated progesterone receptor comprises a carboxy terminal deletion of 19 amino acids of a reference progesterone receptor (e.g., a wild-type or naturally-occurring hormone receptor).

Further examples of modified or mutated steroid-hormone receptors for modification
20 and/or use in the compositions and methods of the present invention are described in, for example: (1) "Adenoviral Vector-Mediated Delivery of Modified Steroid Hormone Receptors and Related Products and Methods" International Patent Publication No. WO0031286 (PCTAJS99/26802); (2) "Modified Glucocorticoid Receptors, Glucocorticoid Receptor/
Progesterone Receptor Hybrids" International Patent Publication No. WO9818925
25 (PCTAJS97/19607); (3) "Modified Steroid Hormones for Gene Therapy and Methods for Their Use" International Patent Publication No. WO9640911 (PCT/US96/0432); (4) "Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy" International Patent Publication No. WO 9323431 (PCTAJS93/0439); (5) "Progesterone Receptors Having C-Terminal Hormone Binding Domain Truncations", U.S. Patent No.
30 5,364,791; (6) "Modified Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy" U.S. Patent No. 5,874,534; and (7) "Modified Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy" U.S. Patent No. 5,935,934.

Furthermore, a mutated steroid-hormone receptor LBD may be selected based on the
35 ability of an antagonist of a wild-type steroid-hormone receptor to activate the mutant receptor even in the presence of an agonist for the wild-type receptor. For example, in one embodiment, progesterone is the normal ligand for the progesterone receptor and functions as a strong agonist for the receptor. The anti-progestin, MFP (RU486), is a non-natural or non-native ligand for the progesterone receptor. MFP is considered an "anti-progestin"
40 because, although it is able to exert an agonist effect on the wild-type progesterone receptor,

MFP inhibits the agonistic effects of progesterone. Thus, MFP may be considered an "antagonist" for the wild-type progesterone receptor when in the presence of the normal agonist, i.e., when both MFP and progesterone are together in the presence of the wild-type progesterone receptor. However, in one embodiment of the present invention, the mutated progesterone steroid-hormone receptor is not activated by progesterone (agonist for the wild type receptor) but is activated in the presence of MFP ("antagonist" for the wild type receptor). In addition, in one embodiment, progesterone does not block the activation of the mutated steroid-hormone receptor by MFP. Thus, the mutated receptor may be characterized as activated when bound to an antagonist (e.g, MFP) for the wild-type receptor even in the presence of an agonist (e.g., progesterone) for the wild-type progesterone receptor.

In one embodiment, the mutated steroid receptor activates the transcription of a desired TM in the presence of an antagonist for a wild-type steroid hormone receptor. In some embodiments, the antagonist is a non-naturally-occurring or non-wild-type ligand that acts as an antagonist of a wild-type steroid receptor (e.g., a wild-type steroid hormone receptor). In one embodiment, an antagonist of a wild-type steroid hormone receptor is a molecule that interacts with or binds to the wild-type steroid hormone receptor and blocks the activity of an agonist of the receptor. In another embodiment, an agonist of a wild-type steroid hormone receptor is a molecule that interacts with the wild-type steroid hormone receptor to regulate the expression and/or activity of a TM in the cells of a subject. Examples of such agonists include, but are not limited to, progesterone or progestin for the progesterone receptor, where progesterone binds to a wild-type progesterone receptor to activate the transcription of progesterone-regulated genes.

In one embodiment, suitable progesterone receptor agonists are chemical compounds that mimic progesterone. For example, Mifepristone (MFP) or otherwise known as RU486 is a non-natural ligand that also binds to the wild-type progesterone receptor and competes with progesterone for binding. In one embodiment, in the presence of progesterone and the wild-type progesterone receptor, MFP exerts an antagonistic effect on the receptor by blocking the activation of the receptor by progesterone.

The therapeutic dose of MFP for use as an abortifacient in humans is 600 mg, or approximately 10 mg/kg. The present MFP dose response studies in animals have shown that the EC₅₀ for induction of transgene expression using the pBRES regulated expression system in vivo is approximately 0.50 mg/kg, two orders of magnitude below the abortifacient dose. This value has been reported by other laboratories using the two plasmid GeneSwitch (Abruzzesse et al. (2002) *Methods Mo. Med.* 69:109-122). A large safety database exists demonstrating the safety of low doses of MFP administered over several months in humans (Sitruk-Ware and Spitz. (2003) *Contraception* 68:409-420). It is known that MFP at low doses does have a pharmacological effect as a contraceptive in women. However, this effect would not pose a problem with its use as an AM of the present invention in female MS patients because women patients receiving IFN- β protein therapy are advised not to bear children during IFN- β treatment.

The progesterone receptor (PR) may be modified, e.g. in the LBD of the progesterone receptor, such that it only binds to MFP and not to progesterone. For example, mutation of the LBD of the progesterone receptor may be such that binding of the MFP activates the progesterone receptor. In one embodiment, the mutated PR LBD, or more generally any other mutated steroid receptor LBD, is fused with a particular DBD (e.g., the GAL-4 DBD), such that binding of MFP selectively activates the RM to transactivate TM expression and/or activity that is driven by a promoter recognized by the DBD of the PR. Thus, in some embodiments, the mutated steroid receptor of the present invention is not activated in the presence of agonists for the wild-type steroid receptor, but instead the mutated steroid receptor is activated in the presence of non-natural ligands.

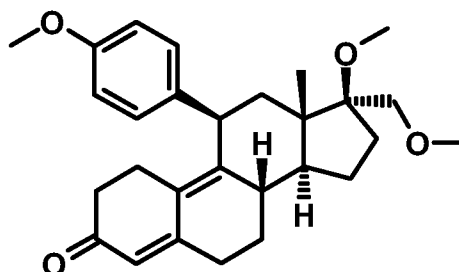
The term "non-natural ligands" or "non-native ligands" refers to compounds which are non-wild-type or not naturally-occurring ligands that bind to the ligand binding domain of a receptor. Examples of non-natural ligands are Selective Progesterone Receptor Modulators (SPRMs) or mesoproggestins (see e.g., Chwalisz et al. (2002) *Ann NY Acad Sci* 955:373-388; Elger et al. (2000) *Steroids* 65(10-11):713-723; Chwalisz et al. (2004) *Semin Reprod Med* 22(2):113-119; DeManno et al. (2003) 68(10-13):1019-1032; Fuhrmann et al. (2000) *J Med Chem* 43(26):5010-5016). Examples of SPRMs or mesoproggestins are described and illustrated in Table 2 below (compounds 1-16).

Table 2

1. name: 17 β -Methoxy-17 α -(methoxymethyl)-11 β -methoxyphenyl-4,9-estra-dien-3-one

structure:

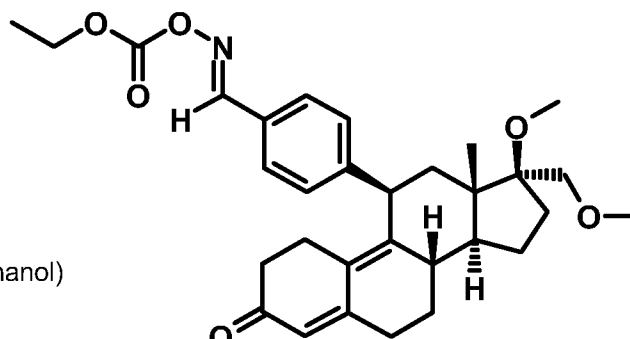
formula: C₂₈ H₃₆ O₄
 molecular mass: 436,60 g/mol
 mp.: 184 – 186 ° C (dichloromethane)



2. name: 4-[17 β -Methoxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1(E)-[O-(ethoxy)carbonyl]oxime

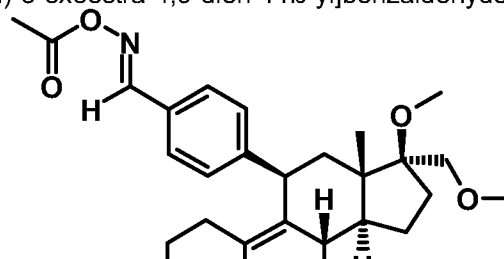
structure:

formula: C₃₁ H₃₉ N O₆
 molecular mass: 521,66 g/mol
 mp.: 143-151 ° C (decomp. methanol)



3. name: 4-[17 β -Methoxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1(E)-oxime acetate

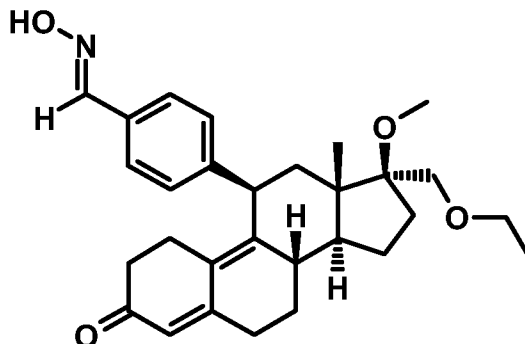
structure:



formula: $C_{30}H_{37}NO_5$
molecular mass: 491,63 g/mol
mp.: 110-119 °C (ethyl acetate)

4. name: 4-[17 α -(Ethoxymethyl)-17 β -methoxy-3-oxoestra-4,9-dien-11 β -yl] benzaldehyde-1(E)-oxime

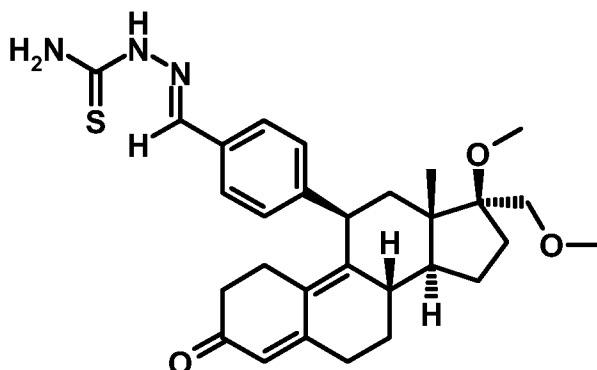
structure:



formula: $C_{29}H_{37}NO_4$
molecular mass: 463,62 g/mol
mp.: 90- 95 °C (methyl *tert.* butyl ether)
purity HPLC: 98,9 area % (264 nm)

5. name: 4-[17 β -Methoxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-thiosemicarbazone

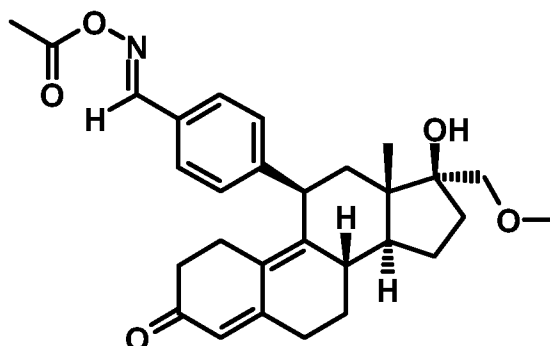
structure:



formula: $C_{29}H_{37}N_3O_3S$
molecular mass: 507,69 g/mol
mp.: 217-236 °C (methanol)

6. name: 4-[17 β -Hydroxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1(E)-oxime acetate

structure:



formula: $C_{29}H_{35}NO_5$

molecular mass: 477,61

m.p.: 112 °C (acetone, decomp.), $\alpha_D = +209^\circ$ (CHCl₃)

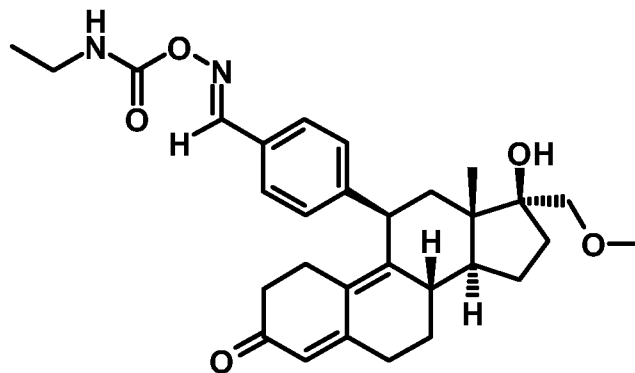
5

7. name: 4-[17 β -Hydroxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1-(E)-[O-(ethylamino)carbonyl]oxime

structure:

10

15



20

formula: C₃₀ H₃₈ N₂ O₅

molecular mass: 506,65 g/mol

mp.: 184-190 °C (methylenechloride / ethyl acetate)

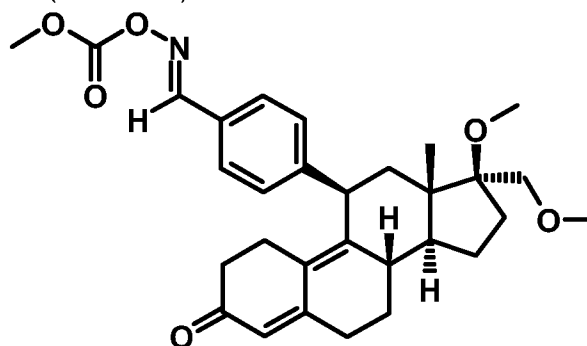
25

8. name: 4-[17 β -Methoxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1-(E)-[O-(methoxy)carbonyl]oxime (ZK280993)

structure:

30

35



40

formula: C₃₀ H₃₇ N O₆

molecular mass: 507,63 g/mol

mp.: 110 °C (mtbe, decomp.)

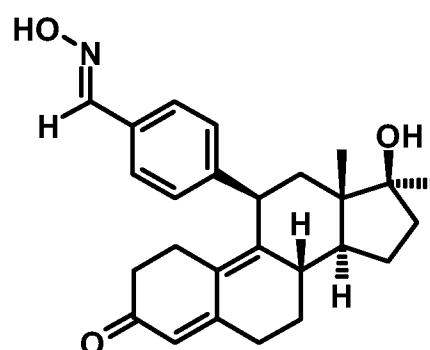
45

9. name: 4-(17 β -Hydroxy 17 α -methyl-3-oxoestra-4,9-dien-11 β -yl) benzaldehyde-1(E)-oxime (ZK280999)

structure:

50

55



60

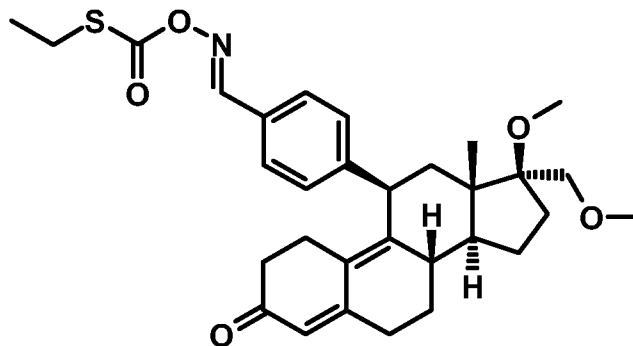
formula: C₂₆ H₃₁ N O₃

molecular mass: 405,53 g/mol

mp.: 163-165 °C (ethanol/water)

10. name: 4-[17 β -Methoxy-17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1(E)-[O-(ethylthio)carbonyl]oxime (ZK190425)

structure:



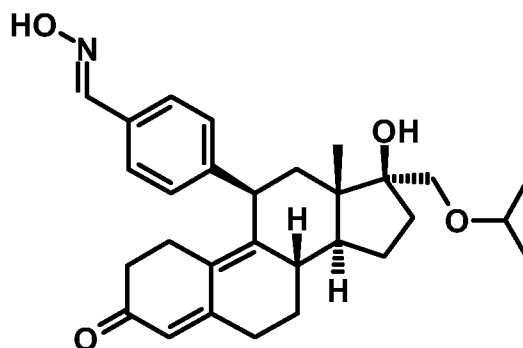
formula: C₃₁ H₃₉ N O₅ S

molecular mass: 537.72 g/mol

mp.: 148 – 155 °C decomp. (acetone/ n-hexane)

11. name: 4-[17 β -Hydroxy-17 α -(2-propoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1(E)-oxime (ZK281100)

structure:



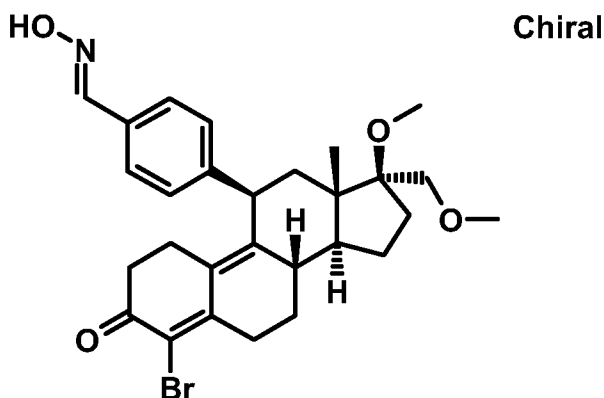
formula: C₂₉ H₃₇ N O₄

molecular mass: 463,62 g/mol

mp.: 192-196 °C (diethylether, decomp.)

12. name: 4-(4'-Brom-17 β -methoxy 17 α -(methoxymethyl)-3-oxoestra-4,9-dien-11 β -yl]benzaldehyde-1(E)-oxime (ZK281117)

structure:



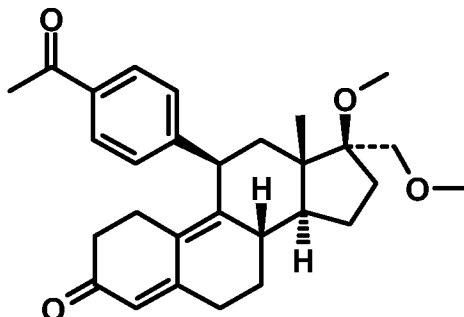
formula: C₂₈ H₃₄ Br N O₄

molecular mass: 528,48 g/mol

mp.: 138-141 °C (diethylether/n-hexane)

13. name: 4-[17β-Methoxy-17α-(methoxymethyl)-3-oxoestra-4,9-dien-11β-yl]acetophenon
11β-(4-Acetylphenyl)17β-methoxy-17α-(methoxymethyl)-4,9-estradien-3-one
(ZK139905)

structure:



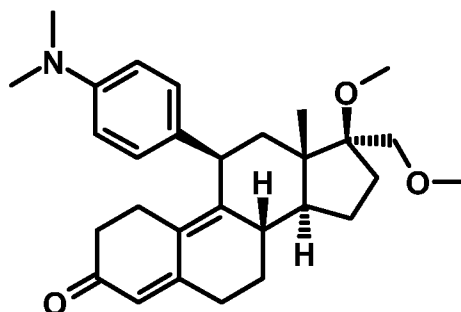
formula: $C_{29}H_{36}O_4$

molecular mass: 448,61 g/mol

mp.: 133-137 °C (diethylether/methylenechloride)

14. name: 11β-[4-(Dimethylamino)phenyl]-17β-methoxy-17α-(methoxymethyl)-4,9-estradien-3-one
(ZK281317)

structure:



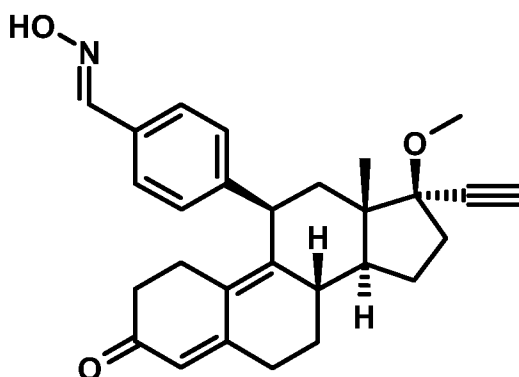
formula: $C_{29}H_{39}NO_3$

molecular mass: 449,64

foam (hexane), $\alpha_D = +184^\circ$ ($CHCl_3$)

15. name: 4-[17α-Ethynyl-17β-methoxy-3-oxoestra-4,9-dien-11β-yl]
benzaldehyde-1(E)-oxime (ZK281527)

structure:



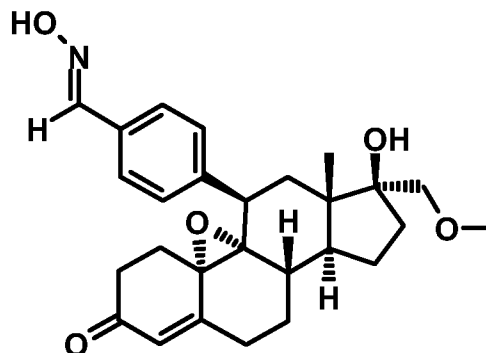
formula: $C_{28}H_{31}NO_3$

molecular mass: 429,56 g/mol

mp.: 149-157 °C (acetone)

16. name: 4-[9 α ,10 α -epoxy-17 β -hydroxy-17 α -(methoxymethyl)-3-oxoestr-4-en-11 β -yl]
benzaldehyde-(E)-oxime (ZK234965)

structure:



formula: C₂₇H₃₃NO₅

molecular mass: 451,57

mp.: 110° C decomp. (acetone / methyl tert.-butyl ether)

Also, examples of non-natural ligands and non-native ligands are anti-hormones that may include the following: 11-(4-dimethylaminophenyl)-17-hydroxy-17c-propynyl-4, 9-estradiene-3-one (RU38486 or Mifepristone); 11-(4-dimethylaminophenyl)-17 α -hydroxy-17-(3-hydroxypropyl)-13-methyl-4, 9-gonadiene-3-one (ZK98299 or Onapristone); 11-(4-acetylphenyl)-17-hydroxy-17c-(1-propynyl)-4,9-estradiene-3-one (ZK112993); 11-(4-dimethylaminophenyl)-17-hydroxy-17(z-(3-hydroxy-1(Z)-propenyl-estra-4,9-diene-3-one (ZK98734); (7, 11, 17)-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro [ester-4,9-diene-17,2' (3'H)-furan]-3-one (Org31806); (11, 14, 17c)-4', 5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4, 9-diene-17,2' (3'H)-furan]-3-one (Org31376); 5-c-pregnane-3,2-dione, Org 33628 (Kloosterboer et al. (1995) Ann N Y Acad Sci Jun 12;761:192-201), Org 33245 (Schoonen et al. (1998) J Steroid Biochem Mol Biol Feb;64(3-4): 157- 70). A further example of such ligands are non-steroidal progesterone receptor-binding ligands e.g., that act as an inducer of an RM of the present invention.

Using standard methods for amino acid or nucleic acid modifications (including, e.g., deletions, insertions, point mutations, fusions), a protein domain (e.g., AD, LBD, DBD) or functional nucleic acid sequence (e.g., sequence encoding a protein, RNA, promoter, splice site, intron, DNA binding site, or poly(A) site) of a molecule of the present invention (e.g., a TM, RM, AM, IM, or nucleic acid encoding a TM, RM, AM, or IM) can be modified or optimized for such regulation, expression, and/or activity. Further, a molecule of the present invention (e.g., a TM, RM, AM, IM, or nucleic acid encoding a TM, RM, AM, or IM) can be modified such that the expression and/or activity of the molecule is transient or constitutive, and/or is regulated by the presence of a particular condition, disease, biomarker or other molecule, or is self-regulated. More particularly, the primary, secondary, or tertiary structure of a nucleic acid or amino acid molecule, or chemical compound, of the present invention can be modified to achieve a particular stringency, specificity, or amount of binding, activation, inactivation, or

conformation (e.g., to form a homo- or hetero-dimer or other multimer, or bind a specific or cognate ligand or site).

For example, a transcribed portion of an expression cassette of the present invention can be modified to include post-transcriptional elements (e.g., a UTR, splice site, intron, and/or poly(A) signal) that optimize or improve the specificity, level and fidelity of expression and/or activity of an operably linked, encoded, and expressed molecule (e.g., a TM, RM, AM, or IM). Further, the promoter sequence of an operably linked sequence encoding a molecule of the present invention can be modified such that the expression of the encoded molecule is, e.g., transient or constitutive, inducible or repressible, and/or modulated or otherwise regulated by the presence of a specific condition or molecule.

Various functional sequences of an expression cassette of the present invention can be modified to optimize for the expression and/or activity of an encoded molecule (e.g., TM, RM, AM, or IM). For example, intron sequences can be modified to optimize for the highly efficient and accurate splicing of RNA transcripts from such sequences. Thereby, cryptic splicing can be minimized and expression can be maximized of the desired molecule (e.g., TM, RM, AM, or IM) encoded by a nucleic acid of the present invention. Examples of suitable synthetic introns for use in the compositions of the present invention include, but are not limited to, consensus sequences for a 5' splice site, 3' splice site, and/or branch point. The 5' splice site is reported to pair with U1 snRNA. A suitable 5' splice site consensus sequence is one that is optimized to minimize the free energy of helix formation between U1 RNA and the synthetic 5' splice site e.g., 5' splice site sequence comprising 5'-CAGGUAAGU-3'. Further, the branch point (BP) sequence, except for a single bulged A residue, is reported to pair with U2 snRNA. Thus, a branch point sequence can be optimized to minimize the free energy of helix formation between U2 RNA and the sequence. The BP is typically located 18-38 nts upstream of the 3' splice site. In one embodiment, the BP sequence of the synthetic intron is located 24 nts upstream from the 3' splice site and is e.g., BP sequence comprising 5' UACUAC 3'. Further, the polypyrimidine tract of the consensus sequence for 3' splice sites can be optimized for 3' splice site function. For example, it has been reported that at least 5 consecutive uracil residues are optimal for 3' splice site function, and thus, in some embodiments the polypyrimidine tract of a synthetic intron of the present invention, has 7 consecutive uracil residues.

Similarly, the length of an intron can be optimized. For example, it is known that naturally-occurring introns may be 90-200 nt in length. In one embodiment, the length of the resultant internal exons are less than 300 nucleotides. In one embodiment, a synthetic intron is IVS8 (e.g., SEQ ID NO: 3) and comprises restriction enzyme sites, BbsI and Earl (located within the synthetic intron), and PstI and NheI. The restriction enzyme BbsI may be used to cleave the DNA precisely at the 5' splice site, and Earl may be used to cleave the DNA near the 3' splice site. Further, a synthetic intron may be inserted at multiple locations of a nucleic acid sequence encoding a molecule of the present invention. For example, in some

embodiments, a nucleic acid sequence encoding a molecule of the present invention is modified to comprise multiple introns.

In another embodiment, in addition to the synthetic intron, IVS8 (e.g., SEQ ID NO: 3) an expression cassette of the present invention is modified to comprise a nucleic acid sequence encoding a CMV 5' UTR termed UT12, an expression control element (e.g., SEQ ID NO: 2). In another embodiment, an expression cassette of the present invention is modified to comprise a nucleic acid sequence encoding a SV40 poly(A) signal (e.g., SEQ ID NO: 8). In yet another embodiment, an expression cassette of the present invention is modified to comprise a nucleic acid sequence encoding a human growth hormone ("hGH") poly (A) signal (e.g., SEQ ID NO: 6). These and other modifications described herein may be employed to optimize the level and fidelity of expression of an encoded molecule (e.g., TM, RM, AM, IM) that is encoded by a nucleic acid sequence of the present invention (e.g., encoded by a nucleic acid sequence of an expression cassette, or vector), as described herein.

The term "intron" as used herein refers to a sequence encoded in a DNA sequence that is transcribed into an RNA molecule by RNA polymerase but is removed by splicing to form the mature messenger RNA. A "synthetic intron" refers to a sequence that is not initially replicated from a naturally-occurring intron sequence and generally will not have a naturally-occurring sequence, but will be removed from an RNA transcript during normal post-transcriptional processing. Such synthetic introns can be designed to have a variety of different characteristics, in particular such introns can be designed to have a desired strength of splice site and a desired length. In a preferred embodiment of the present invention, both the molecular switch expression cassette and the therapeutic gene expression cassette include a synthetic intron. The synthetic intron includes consensus sequences for the 5' splice site, 3' splice site, and branch point. When incorporated into eukaryotic vectors designed to express therapeutic genes, the synthetic intron will direct the splicing of RNA transcripts in a highly efficient and accurate manner, thereby minimizing cryptic splicing and maximizing production of the desired gene product.

Further, using known methods, a functional sequence encoding a domain of a protein can be modified to optimize for the activity of the protein. For example, using molecular modeling a truncation mutant can be designed such that there is lower dimerization potential while retaining sequence-specific DNA binding activity of a protein having a GAL-4 DBD. The GAL-4 DBD is reported to bind as a dimer to the palindromic 17-mer GAL-4 DBS (CGGAAGACTCTCCTCCG) and such dimer binding reportedly results in the activation of an inducible promoter having the GAL-4 DBS. Thus, the nucleic acid sequence encoding a protein having a GAL-4 DBD can be modified such that the tertiary structure of the GAL-4 DBD is optimized to reduce any unregulated (e.g., AM-independent) or undesired dimerization, resulting in activation of an inducible promoter.

The structure and function of the GAL-4 DBD sequences are known (e.g., see PCT/US01/30305). For example, the cysteine (C) may be involved in chelating zinc; the

coiled-coil structures that form the dimerization elements comprise residues 54-74 and 86-93; the generally hydrophobic amino acids are reportedly at the first and fourth positions of each heptad repeat sequence; residue Ser 47 and Arg 51 are reported to form a hydrogen bond between the protein chains forming the dimer; and residues 8-40 reportedly form the Zn binding domain or the DNA recognition unit. This DNA recognition unit has two alpha helical domains that form a compact globular structure and in the presence of Zn resulting in a structure that reportedly is a binuclear metal ion cluster rather than a zinc finger, i.e., the cysteine-rich amino-acid sequence (CysXa-Xaa2-Cys14-Xaaa-CysZ1-Xaa6-CysZS-Xaa2-Cys31-Xaaa-Cys38) binds two Zn(II) ions (Pan and Coleman (1990) PNAS 87: 2077-81).

The Zn cluster may be responsible for making contact with the major groove of the 3 bp at extreme ends of the 17-met binding site; and a proline at 26 (cis proline) reportedly forms the loop that joins the two alpha-helical domains of the zinc cluster domain and is also critical for this function. Further, residues 41-49 reportedly join the DNA recognition unit and the dimerization elements, residues 54-74 and 86-93.

Once dimerized, residues 47-51 of the dimer can also interact with phosphates of the DNA target. Residues 50-64 may be involved in weak dimerization. The dimers consist of a short coiled-coil that forms an amphipathic alpha-helix and wherein two alpha-helices are packed into a parallel coiled-coil similar to a leucine zipper. In addition to hydrophobic interactions of 3 pairs of leucines and a pair of valines found within residues 54-74, there are two pairs of Arg-Glu 2O salt links, and hydrogen bonds between Arg 51 of one monomer to Ser 47 of the other monomer. Residues 65-93 may form a strong dimerization domain. The structure of residues 65-71 is a continuation of the coiled-coil structure for one heptad repeat. Residues 72-78 contain a proline and therefore disrupt the amphipathic helix. Residues 79-99, however, contain three more potentially alpha-helical heptad sequences (Marmorstein et al (1992) Nature 356: 408-414). Further, the Kd for binding of GAL-4 residues 1-100 is reported to be 3 nM (Reece and Ptashne (1993) Science 261: 909-911).

In view of the structure and function of the GAL-4 DBD sequences a number of possible modifications can be made to the regions of the GAL-4 domain. In some embodiments, the GAL-4 regions are modified to optimize for the elimination or reduction of any basal expression and retention of sequence-specific DNA binding. More particularly, in some embodiments, the length of the region that contains the interacting coiled-coil sequences of the GAL-4 DBD (e.g., residues 54-74 and residues 86-93) can be shortened by deletion e.g., by deleting amino acid sequence 54-64, 65-74, 54-74, or 86-93. Also, GAL-4 mutants with only one coiled-coil region can be constructed by deleting one of the coiled-coil regions. In addition, mutant or artificial sequences may be inserted into the GAL-4 domain using unique restriction sites positioned at, e.g., the junctions of each of the alpha-helical heptad sequences. Thus, modified versions of the GAL-4 domain can be produced that have progressively reduced alpha-helical heptad sequences.

In some embodiments, the native GAL-4 sequence is modified to remove the N-terminal methionine and additional amino acids are added to the N-terminal end of the

sequence. In these embodiments, the modifications to the N-terminal amino acids of the native GAL-4 sequence are not of consequence as long as they do not affect the tertiary structure of residues 8-40 of the Zn binding domain. Further, in some embodiments, the specific binding of a small molecule AM, e.g. MFP, to a mutated hPR LBD of a protein having a GAL-4 DBD (e.g., an RM) triggers a conformational change in the protein so as to initiate dimerization of the protein. Additionally, in some embodiments, an expression cassette of the present invention comprises a nucleic acid sequence encoding residues 2-93 of the GAL-4 DBD sequence of SEQ ID NO: 37. Further, in one embodiment, the DNA recognition sequence of the GAL-4 DBD comprises residues 9-40 of the GAL-4 DBD sequence of SEQ ID NO: 37.

In one embodiment of the present invention, the GAL-4 domain is truncated by deletion of 19 amino acids at the C- terminal portion of the GAL-4 DBD and comprises residues 75-93 of the GAL-4 DBD sequence of SEQ ID NO: 37. In one embodiment, an RM of the present invention is a chimeric protein comprising a mutated progesterone receptor comprising residues 2-74 of the GAL-4 DBD sequence of SEQ ID NO: 37 and a mutated progesterone receptor LBD that is specifically activated in the presence of an AM. Further, in the absence of the AM there is little or no RM activation and resulting induction or activation of transcription of a nucleic acid sequence operably linked to a promoter having a GAL-4 DBS.

As mentioned, nucleic acids encoding variants of a native molecule (e.g., protein or nucleic acid) are also suitable for use in the compositions and methods of the present invention. For example, a variant of IFN- β (e.g., IFN- β 1b) is suitable for use as a TM in the compositions and methods of the present invention, particularly, for the treatment of MS. Preferably, the IFN- β variant is a variant of a native human IFN- β . Variants of native human IFN- β , which may be naturally-occurring (e.g., allelic variants that occur at the IFN- β locus) or recombinantly or synthetically produced, have amino acid sequences that are similar to, or substantially similar to a mature native IFN- β sequence. Nucleic acids encoding a native human IFN- β (e.g., comprising the amino acid sequence of SEQ ID NO: 13) are suitable for use in the compositions and methods of the present invention e.g., IFN- β 1a (e.g., SEQ ID NO: 14). Also, nucleic acids encoding a human IFN- β variant are suitable for use in the compositions and methods of the present invention e.g., IFN- β 1b (see e.g., US Patent Ser. No.s 4,588,585, 4,737,462, and 4,959,314). Variants also encompass nucleic acids encoding fragments or truncated forms of a native molecule (e.g., protein or nucleic acid) that retain a biological or therapeutic activity. For example, nucleic acids encoding these biologically active fragments or truncated forms of a native protein. Further, in some embodiments, the expressed protein of the present invention may be glycosylated or not glycosylated.

Further, suitable protein or nucleic acid variants for use in the compositions and methods of the present invention can be variants of a native or wild-type protein or nucleic acid, respectively, of any mammalian species including, but not limited to, avian, canine, bovine, porcine, equine, and human. Non-limiting examples of IFN- β variants encompassed

by the present invention (e.g., encoded by a nucleic acid, e.g., Nagata *et al.* (1980) *Nature* 284:316-320; Goeddel *et al.* (1980) *Nature* 287:411-416; Yelverton *et al.* (1981) *Nucleic Acids Res.* 9:731-741; Streuli *et al.* (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78:2848-2852; EP028033B1, and EP109748B1. See also, e.g., U.S. Pat. No.s 4,518,584; 4,569,908; 5 4,588,585; 4,738,844; 4,753,795; 4,769,233; 4,793,995; 4,914,033; 4,959,314; 5,545,723; and 5,814,485. These citations also provide guidance regarding residues and regions of the IFN- β protein that can be altered without the loss of biological activity.

Changes or modifications of expressed proteins and nucleic acids (e.g., RNA) of the present invention can be introduced by mutation into the nucleotide sequences encoding them, thereby leading to changes in the amino acid sequence of the expressed protein or 10 nucleic acid sequence without altering the biological or therapeutic activity of the expressed molecule. For example, an isolated nucleic acid molecule encoding a variant protein having a sequence that differs from the amino acid sequence for a reference or starting protein can be created by introducing one or more nucleotide substitutions, additions, or deletions into the 15 corresponding nucleotide sequence (for IFN- β variants, see, e.g., U.S. Pat. No. 5,588,585, U.S. Pat. No. 4,959,314; 4,737,462; L. Lin (1998) *Dev. Biol. Stand.* 96: 97-104), such that one or more amino acid substitutions, additions or deletions are introduced into the sequence encoding a reference or starting protein and thereby resulting in a variant protein when the encoding protein is expressed. For example, mutations can be introduced by standard 20 techniques for modifying nucleic acid or amino acid sequences, such as site-directed mutagenesis and PCR-mediated mutagenesis.

Further, nucleic acid sequences encoding a protein can be modified to encode conservative amino acid substitutions at one or more predicted, preferably nonessential amino acid residues. As used herein, a "nonessential" amino acid residue is a residue that 25 can be altered from a reference sequence of a protein without altering its biological or therapeutic activity, whereas an "essential" amino acid residue is required for such activity. As used herein, a "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include 30 amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, 35 phenylalanine, tryptophan, histidine). In preferred embodiments, such substitutions are not made for conserved amino acid residues, or for amino acid residues residing within a conserved motif.

Further, the nucleotide sequences of a variant molecule can be made by introducing mutations randomly along all or part of the coding sequence of a reference molecule, such as 40 by saturation mutagenesis, and the resultant mutants can be screened for biological or

therapeutic activity. Following mutagenesis, the encoded protein can be expressed recombinantly, and the activity of the protein can be determined using standard assay techniques described herein or known in the art. In preferred embodiments, biologically or therapeutically active protein variants have at least 80 %, more preferably about 90 % to about 95 % or more, and most preferably about 96 % to about 99 % or more amino acid sequence identity to the amino acid sequence of a reference protein, which serves as the basis for comparison or reference. As used herein "sequence identity" is the same amino acid residues that are found within a variant protein and a protein molecule that serves as a reference when a specified, contiguous segment of the amino acid sequence of the variant is aligned and compared to the amino acid sequence of the reference molecule.

For the optimal alignment of two sequences for the purposes of sequence identity determination, the contiguous segment of the amino acid sequence of the variant may have additional amino acid residues or deleted amino acid residues with respect to the amino acid sequence of the reference molecule. The contiguous segment used for comparison to the reference amino acid sequence will comprise at least 20 contiguous amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the amino acid sequence of the variant can be made by assigning gap penalties. Methods of sequence alignment are well known in the art.

For example, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. One preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is *e.g.*, the algorithm of Myers and Miller (1988) *Comput. Appl. Biosci.* 4:11-7. Such an algorithm is utilized in the ALIGN program (version 2.0), which is part of the GCG alignment software package. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. Another preferred, non-limiting example of a mathematical algorithm for use in comparing two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 90:5873-5877, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-410. BLAST amino acid sequence searches can be performed with the XBLAST program, score=50, wordlength=3, to obtain amino acid sequence similar to the protein of interest.

To obtain gapped alignments for comparison purposes, gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. Alternatively, PSI-BLAST can be used to perform an integrated search that detects distant relationships between molecules (see *e.g.*, Altschul *et al.* (1997) *supra.*). When utilizing BLAST, gapped BLAST, or PSI-BLAST programs, the default parameters can be used (see *e.g.*, www.ncbi.nlm.nih.gov). Also see the ALIGN program (Dayhoff (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3, National Biomedical Research Foundation, Washington, D.C.) and programs in the Wisconsin Sequence Analysis Package, Version 8 (available from

Genetics Computer Group, Madison, Wis.), for example, the GAP program, where default parameters of the programs are utilized.

When considering percentage of amino acid sequence identity, some amino acid residue positions may differ as a result of conservative amino acid substitutions, which do not affect properties of protein function. In these instances, percent sequence identity may be adjusted upwards to account for the similarity in conservatively substituted amino acids. Such adjustments are well known in the art (see, e.g., Myers and Miller (1988) *Comput. Appl. Biosci.* 4:11-17).

Further, in embodiments where the RM, AM, or IM is a protein, the protein can be covalently linked with, e.g., polyethylene glycol (PEG) or albumin. These covalent hybrid molecules can have certain desirable properties such as an extended serum half-life after administration to a subject. Methods for creating PEG-IFN adducts involve chemical modification of monomethoxypolyethylene glycol to create an activated compound that will react with a protein of the present invention. Methods for making and using PEG-linked proteins are reported, e.g., in Delgado *et al.* (1992) *Crit. Rev. Ther. Drug. Carrier Syst.* 9:249-304 (and as described herein in the Background). Methods for creating albumin fusion proteins involve fusion of the coding sequences for the protein of interest and albumin and are reported, e.g., in U.S. Pat. No. 5,876,969.

Biologically or therapeutically active protein or nucleic acid variants encompassed by the invention preferably retain or have a biological or therapeutic activity. In some embodiments, the variant retains at least about 25 %, about 50 %, about 75 %, about 85 %, about 90 %, about 95 %, about 98 %, about 99 % or more of the biological or therapeutic activity of the reference molecule (e.g., protein or nucleic acid). Variants whose activity is increased in comparison with the activity of the reference molecule (e.g., protein or nucleic acid) are also encompassed. The biological or therapeutic activity of variants can be measured by any method known in the art (see e.g., assays described in Fellous *et al.* (1982) *Proc. Natl. Acad. Sci USA* 79:3082-3086; Czerniecki *et al.* (1984) *J. Virol.* 49(2):490-496; Mark *et al.* (1984) *Proc. Natl Acad. Sci. USA* 81:5662-5666; Branca *et al.* (1981) *Nature* 277:221-223; Williams *et al.* (1979) *Nature* 282:582-586; Herberman *et al.* (1979) *Nature* 277:221-223; Anderson *et al.* (1982) *J. Biol. Chem.* 257(19):11301-11304).

Generally, for cloning, testing, and other uses described herein, the nucleic acid, protein and chemical compositions of the present invention can be produced or synthesized using methods known in the art. For example, proteins can be produced by culturing a host cell transformed with an expression vector comprising a nucleotide sequence that encodes a protein or nucleic acid of the present invention. The host cell is one that can transcribe the nucleotide sequence and produce the desired protein or nucleic acid, and can be prokaryotic (see, e.g., *E. coli*) or eukaryotic (e.g., a yeast, insect, or mammalian cell). Examples of recombinant production of IFN- β , including suitable expression vectors, are provided in, e.g., Mantei *et al.* (1982) *Nature* 297:128; Ohno *et al.* (1982) *Nucleic Acids Res.* 10:967; Smith *et*

al. (1983) *Mol. Cell. Biol.* 3:2156, and U.S. Pat. No. 4,462,940, 5,702,699, and 5,814,485; U.S. Pat. No. 5,795,779).

Further, genes have been cloned using recombinant DNA ("rDNA") technology and can be produced and tested in e.g., animal or plant cells, or transgenic animals (see e.g., Nagola *et al.* (1980) *Nature* 284:316; Goeddel *et al.* (1980) *Nature* 287:411; Yelverton *et al.* (1981) *Nuc. Acid Res.* 9:731; Streuli *et al.* (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78:2848). Proteins may also be produced with rDNA technology, e.g., by extracting poly-A-rich 12S messenger RNA from virally induced human cells, synthesizing double-stranded cDNA using the mRNA as a template, introducing the cDNA into an appropriate cloning vector, transforming suitable microorganisms with the vector, harvesting the microorganisms, and extracting the protein therefrom (see, e.g., European Patent Application No.s 28033 (published May 6, 1981); 32134 (published Jul. 15, 1981); and 34307 (published Aug. 26, 1981)),

Also, proteins can be synthesized chemically and tested, by any of several techniques that are known to those skilled in the peptide art (see e.g., Li *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:2216-2220, Steward and Young (1984) *Solid Phase Peptide Synthesis* (Pierce Chemical Company, Rockford, Ill.), and Baraney and Merrifield (1980) *The Peptides: Analysis, Synthesis, Biology*, ed. Gross and Meinhofer, Vol. 2 (Academic Press, N. Y., 1980), pp. 3-254, discussing solid-phase peptide synthesis techniques; and Bodansky (1984) *Principles of Peptide Synthesis* (Springer-Verlag, Berlin) and Gross and Meinhofer, eds. (1980), *The Peptides. Analysis, Synthesis, Biology*, Vol. 1 (Academic Press, New York, discussing classical solution synthesis). A protein of the present invention can also be chemically prepared e.g., by the method of simultaneous multiple peptide synthesis. See, for example, Houghten (1984) *Proc. Natl. Acad. Sci. USA* 82:5131-5135; and U.S. Pat. No. 4,631,211.

Further, one skilled in the art would know how to test the compositions of the present invention for the treatment of disease using accepted and appropriate animal models and methods known in the art. For example, it has been reported that gene delivery systems can be used to deliver cytokines in several animal autoimmune disease models, e.g., including experimental allergic encephalomyelitis (EAE), arthritis, lupus, and NOD diabetes models (see e.g., G.C. Tsokos and G.T. Nepom (2000) *Clin. Invest.* 106: 181-83; G.J. Prud'homme (2000) *J. Gene Med.* 2: 222-32). EAE is a model of central nervous system inflammation that ensues after immunization with certain CNS auto-antigens, for example brain derived proteolipid or myelin basic protein. Its course and clinical manifestations are similar to multiple sclerosis (MS) in humans and it has become an accepted model to study MS. There have been several reports that describe the testing of Type I IFN's, delivered as a protein (15-20) and by a vector (see e.g., K. Triantaphyllopoulos *et al.* (1998) *Gene Therapy* 5: 253-63; J.L. Croxford *et al.* (1998) *J. Immunol.* 160: 5181-87), in murine and rat EAE models. Yu *et al.* have shown that mice administered mIFN- β protein, at the time of EAE disease induction, exhibit delayed progression to disability (as measured by clinical score), delayed onset of

relapse, and a decrease in exacerbation frequency compared to normal mice (see e.g., M. Yu et al. (1996) J. Immunol. 64: 91-100). This result closely resembles the human results with IFN- β treatment. Plasmid based vectors were used by Triantaphyllopoulos et al. in a gene therapy-based approach to deliver IFN- β to the CNS under the control of a neuron specific promoter (see e.g., K. Triantaphyllopoulos et al. (1998) Gene Therapy 5: 253-63). These vectors were injected intra-cranially into mice during the effector phase of EAE, and reduced or prevented the clinical signs of disease. The results of the studies performed to date using IFN- β in the murine EAE model demonstrate that IFN is an effective therapy in delaying the onset, and reversing the manifestations of disease in the EAE model. Gene therapy methods for delivering IFN- β in this model (local intra-cranial administration) have been shown to be effective.

Likewise, the DSS-induced mouse colitis model is useful for evaluating therapies for UC, IBD and CD. Disease is induced in mice by daily administration of 5% or 7% dextran sodium sulfate (DSS) in the drinking water over 8 days. Disease progression is monitored by the Daily Activity Index (DAI), a cumulative index of: weight loss, stool consistency, and rectal bleeding, which mimics the symptoms of UC, IBD and/or CD.

EXAMPLES

The following examples are offered by way of illustration and are not intended to limit the invention in any way.

As described in the following Examples, human IFN- β (hIFN- β) and murine IFN- β (mIFN- β) expression vectors were constructed, assays developed to measure IFN- β directly in serum, and biomarkers were identified that correlate with IFN- β expression *in vivo*. Further, pharmacokinetic studies were performed in normal mice comparing gene-based delivery of IFN- β with bolus protein delivery and a superior pharmacokinetic profile was demonstrated using intramuscular injection of non-viral plasmid DNA or an adeno-associated viral (AAV) vector encoding IFN- β . In addition, long term, stable expression of hIFN- β was observed for nearly 6 months with an AAV-1 vector expressing hIFN- β . Also, single intramuscular injection of plasmid DNA encoding mIFN- β was shown to be efficacious in a murine model of experimental allergic encephalomyelitis (EAE), and equally as effective as an every-other-day injection of mIFN- β protein. As described below, examples of the regulated expression system of the present invention were constructed and tested. For example, regulated expression of IFN- β was demonstrated in normal mice using a regulated expression system of the present invention, where a TM and RM are contained in a single plasmid vector.

The data from the studies described in these Examples demonstrate the potential of the regulated, expression system of the present invention for delivery of a nucleic-acid encoded TM, for treatment of disease. In particular, these Examples demonstrate the potential for the use of the regulated expression system of the present invention for delivery of

a nucleic acid encoding IFN- β (e.g., IFN- β 1a) for long term, regulated expression of the protein for the treatment of MS. In one embodiment, the delivery vector is a single plasmid vector comprising a first and second expression cassette encoding a TM (e.g., IFN- β) and RM, respectively which provides persistent (e.g., greater than 3 months) and renewable
 5 expression through oral administration of an AM e.g., a small molecule inducer (e.g., MFP) and, further, the vector is capable of repeat administration by intramuscular injection.

EXAMPLE 1: Construction of Vectors for Use in IFN- β or GMCSF Gene Therapy

A. Plasmid Vectors: The murine IFN- β (mIFN- β) gene from the bacterial
 10 expression vector pbSER189 was PCR amplified, with immunoglobulin kappa (IgK) (for protein purification) or mIFN- β (for gene therapy) signal sequence added on the 5' primer. The PCR products were inserted downstream of the cytomegalovirus (CMV) promoter in the expression vectors pCEP4/WPRE, to generate pGER90 (Figure 2A) for recombinant protein expression and purification, and pgWiz, to generate pGER101 (Figure 2B) for
 15 gene therapy.

The human IFN- β gene from the bacterial expression vector pbSER178 was PCR amplified by the same procedure as the mIFN- β gene (except with the hIFN signal sequence for the gene therapy vector) and inserted into pCEP4/WPRE to generate pGER123 (Figure 2C) for recombinant protein expression and purification, and pgWiz to
 20 generate pGER125 (Figure 2D) for gene therapy.

The construction of plasmid vectors is fully described in the Methods and Materials section, subsection F.

B. AAV-1 Vectors: An AAV-1-hIFN- β shuttle plasmid encoding hIFN- β was constructed by inserting the blunted HincII/NotI fragment of pGWIZ/hIFN- β encoding
 25 hIFN- β into the blunted AgeI-SalI site of the AAV-1 vector, pTReGFP. The resulting shuttle plasmid was named pGT62 (SEQ ID NO: 44) and used to produce AAV-1 virus encoding hIFN- β . Two batches of the AAV-1 virus were prepared as described herein using standard methods and used in pharmacokinetic studies (Figure 4). The expression levels of hIFN- β in these two viral batches were validated by ELISA.

Also, AAV-1-GMCSF shuttle plasmids pGT714 and pGT713 (Figure 30B),
 30 encoding mGMCSF or hGMCSF, were constructed by inserting a fragment encoding mGMCSF or hGMCSF into the vector pGENE/V5HisA (Invitrogen). The resulting vectors were named pGT723-GENE/hGMCSF and pGT724-GENE/mGMCSF. A fragment encoding mGM-CSF was then excised from pGT724-GENE/mGMCSF by digesting the
 35 vector with KpnI-XbaI. Similarly, a fragment encoding hGM-CSF was excised from pGT723-GENE/hGMCSF by digesting the vector with KpnI-XbaI. The vector pZac2.1 was digested with KpnI-XbaI and treated with calf intestinal phosphatase (CIP) and then the excised fragment encoding either mGMCSF or hGMCSF was inserted into pZac2.1 at the KpnI-XbaI site. The resulting shuttle plasmids were named pGT713 (pZac2.1-CMV-hGMCSF) and pGT714 (pZac2.1-CMV-mGMCSF) (Figure 30B).
 40

The construction of the vectors of the present invention is fully described in the Methods and Materials section, subsection F.

EXAMPLE 2: Pharmacokinetic Studies of IFN- β Gene Delivery

5 **A. Pharmacokinetic Studies with Human IFN- β :** Pharmacokinetic studies were performed in normal mice to compare bolus protein versus gene-based delivery of human IFN- β (hIFN- β).

1) Human IFN- β 1a Protein Pharmacokinetic Study: A pharmacokinetic study was carried out in C57BL/6 mice using bolus injection of recombinant hIFN- β 1a delivered either by intramuscular (i.m.) or intravenous (i.v.) injection and using a commercially available ELISA to detect serum levels of hIFN- β . Figure 3 shows the pharmacokinetic profile of hIFN- β 1a protein in serum of mice following a single i.m. or i.v. injection of either 25 ng (1 ug/kg) or 250 ng (10 ug/kg) of hIFN- β 1a protein. Following i.v. injection, hIFN- β 1a was detected in serum in a dose dependent manner at the first time point (30 min), and was rapidly cleared such that the levels were near the limit of detection (LOD) of the assay (LOD = 12.5 pg/ml) by 6 hours. Following i.m. injection of recombinant hIFN- β 1a protein, the hIFN- β 1a serum level reached a maximum level at 2 hours post-injection and then decreased by approximately 10-fold by 6 hours. The amount of hIFN- β 1a remaining in the serum at 6 hours was higher with the i.m. injection compared to the i.v. injection. With both the i.v. and i.m. injections, a 10-fold difference in serum hIFN- β 1a level was seen between the high and low dose. The highly transient kinetics displayed following bolus injection of recombinant hIFN- β 1a is very similar to the results previously reported in humans and in other animal species (Buchwalder, P-A et al. (2000) J Interferon Cytokine Res 20: 57-66; Pepinsky, RB et al. (2001) J Pharm Exp Ther 297: 1059-55).

2) Pharmacokinetic Study of Gene-Based Delivery of AAV-1-CMV hIFN- β 1a: An AAV-1 vector was constructed to constitutively express human IFN- β (AAV-1-CMV hIFN- β 1a) and delivered by i.m injection at three doses (0.5×10^{10} , 1.0×10^{10} , or 5.0×10^{10} viral particles) into C57BL/6 mice. The results are shown in Figure 4. Human IFN- β 1a expression in the serum of mice was low on day 2 but increased rapidly up to day 10 at which time serum levels in all three dose-groups reached a plateau or gradually increased. A clear dose response was observed with increasing amounts of AAV-1-CMV hIFN- β 1a vector administered. At the two higher doses steady levels of hIFN- β 1a expression was detected in the serum 171 days post-injection. In contrast to the study using bolus i.m. injection of recombinant hIFN- β 1a protein, this study using gene-based delivery of an AAV-1 vector encoding hIFN- β 1a, demonstrates long-term expression of hIFN- β 1a in serum after a single injection of the vector.

EXAMPLE 3: Identification and Use of IFN- β Biomarkers for Gene Therapy

A. Development of mIFN- β Biomarkers: For higher sensitivity in detection of murine IFN- β (mIFN- β) activity in vivo, biomarkers for mIFN- β activity were identified in mice after injection of mIFN- β protein or mIFN- β encoded gene therapy vectors.

Biomarkers can be used to follow human IFN- β activity in clinical samples from patients treated with Betaseron (IFN- β 1b) (see e.g., Arnason, BG (1996) *Clin Immunol Immunopathol* 81: 1-11; Deisenhammer, F et al. (2000) *Neurology* 54: 2055-60; Knobler, RL et al. (1993) *J Interferon Res* 13: 333-40.; Kracke, A et al. (2000) *Neurology* 54: 193-9). One of the primary biomarkers used in the IFN- β clinical studies is MxA (see e.g., Kracke, A et al. (2000) *Neurology* 54: 193-9; Bertoloto, A et al. (2001) *J Imm Meth* 256: 141-152) since it is specifically induced by type I IFN's (see e.g., von Wussow, P et al (1990) *J Imm* 20:2015-19). In the present study, the expression of the MxA mouse homologue, Mx1, (see e.g., Hug, H et al. *Mol Cell Biol* 8: 3065-79; Pavlovic, J (1993) *Ciba Found Symp* 176: 233-43) isolated from murine peripheral blood monocytes (PBMC's) was used to detect the presence of biologically active mIFN- β .

A quantitative PCR/RT-PCR assay was developed to quantitate the levels of Mx1 mRNA in murine PBMC's. Specifically, peripheral blood mononuclear cells (PBMCs) were isolated from mice treated with either mIFN- β protein or following gene-based delivery of the mIFN- β gene. Blood samples obtained from treated mice were centrifuged on a ficoll cushion for 25 minutes at 2,000 rpm. Purified PBMCs were pelleted and RNA for the Mx1 assay was purified using the "RNAeasy" mini extraction kit from Qiagen. The RNA was stored in H₂O at -80 °C. Mx1 RT-PCR was performed using TaqMan[®] chemistry and analysis was done on the Applied Biosystems (ABI) PRISM[®] 7700 Sequence Detection instrument. For reverse transcription of the RNA and amplification of cDNA, the "One-step TaqMan[®] RNA" kit from ABI was used. RNA was reverse transcribed for 30 minutes at 48 °C and the amplification was done in 40 cycles with a denaturation step at 95 °C for 30 seconds, and an annealing/elongation step at 60 °C for 1 minute. The samples were analyzed with an Mx1-specific probe/primer combination. Mx1 expression was normalized to GAPDH expression measured in parallel using a standard assay from ABI.

The assay was validated *in vitro* by examining Mx1 RNA induction in murine L929 cells treated with purified recombinant mIFN- β protein (Figure 5). A dose dependent increase in the expression of Mx1 RNA was observed with an EC₅₀ of approximately 50 pg/ml mIFN- β resulting in a 100-fold increase in the level of Mx1 RNA.

B. Bolus Injection of mIFN- β Protein Induces Transient Biomarker Response *In Vivo*: The biomarker response after bolus injection of purified recombinant mIFN- β was measured in these studies. Induction of Mx1 RNA expression (25- to 50-fold relative to vehicle injected mice) was observed with each mIFN- β concentration tested whether administered i.v. or i.m. (Figure 6). The highest Mx1 expression levels were measured 2 hours after injection. At that time point a clear dose response was observed when mIFN- β was delivered i.m. Mx1 expression dropped rapidly and beyond 12 hours

after injection no Mx1 RNA levels above background were detected. When mIFN- β was injected i.v., the highest induction was observed with 150 ng. At the 500 ng dose there was a diminished level of Mx1 RNA induction. This saturating effect has been observed in other studies in which high IFN- β levels lead to an apparent down-regulation of the bioresponse, perhaps due to down regulation of the IFN type I receptor (see e.g., Mager, DE and Jusko, WJ (2002) *Pharm Res* 19:1537-43). Mx1 RNA expression in the i.v. injected mice also peaked at approximately 2 hours post-injection and dropped rapidly thereafter.

This is the first time that the expression of Mx1 RNA has been used as a biomarker to follow the activity of mIFN- β in mice. The results clearly show that Mx1 RNA is expressed constitutively in mouse PBMC's at a low level and can be strongly upregulated by mIFN- β treatment. However, the upregulation is short term and rapidly drops from high expression levels to background within 12-24 hours. The rapid kinetics correspond with the short half-life time reported for type I interferons in humans (see e.g., Salmon, P et al. (1996) *J Interferon Cytokine Res* 16: 759-64; Buchwalder, P-A et al. (2000) *J Interferon Cytokine Res* 20: 57-66) and other animals species (Pepinsky, RB et al. (2001) *J Pharm Exp Ther* 297: 1059-55; Mager, DE et al. (2003) *J Pharm Exp Ther* 306: 262-70).

C. Chemokines IP-10 and JE: During the analysis of plasma samples from mice treated with mIFN- β protein, two murine chemokines, IP-10 and JE the murine homologue of MCP-1 (monocyte chemoattractant protein, see e.g., Yoshimura, T (1989) *FEBS Lett* 244: 487-93), were also identified to have a similar response and activation to mIFN- β as Mx1 RNA (Figures 7 and 8). A strong induction of IP-10 and JE was seen 2 hours after administration of mIFN- β protein either i.v. or i.m. IP-10 levels increased 3000-fold at the high dose delivered i.v. With each of the three mIFN- β doses tested, a rapid drop to background in the plasma levels of IP-10 and JE was observed by 24 hours. A clear dose response for IP-10 and JE was observed with both routes of administration.

Such a strong dose dependent induction of IP-10 and JE by IFN- β has not previously been known until demonstrated by the present inventors, as described herein. Although IP-10 is known as a biological marker for IFN- γ by virtue of the interferon responsive element (ISRE) in the promoter region (see e.g., Luster, AD et al. (1985) *Nature* 315: 672-76), it is has not previously been shown to be a specific biomarker for mIFN- β in mice.

D. Long-term Biomarker Response Following mIFN- β Gene Delivery *In*

Vivo: These studies demonstrate the measurement of the induction of mIFN- β biomarkers in mice following intramuscular delivery of plasmid DNA or an AAV-1 vector encoding mIFN- β .

EXAMPLE 4: Delivery of mIFN- β Gene

A. Plasmid Delivery of mIFN- β Gene: For plasmid delivery different doses of plasmid DNA encoding mIFN- β were injected i.m. into mice followed by electroporation of the injected muscle. Mx1 expression was measured from PBMCs isolated from each individual animal and expressed as the fold increase over background levels of the control group (Figure 9). There was a strong up-regulation of Mx1 RNA (40- to 130-fold induction) in all four groups receiving mIFN- β plasmid DNA at day 2 post-injection. Mx1 expression in all four groups was significantly above background ($p < 0.002$). The Mx1 expression data showed that there is a dose response with 250 μ g as the optimal DNA concentration. There was an initial peak at the first day after electroporation followed by a drop in expression and at later time points the expression levels increased again. This variation in biomarker response was also reflected in the levels of the chemokines IP-10 and JE (data not shown) and appears to be a reproducible phenomenon in other studies using plasmid delivery of IFN- β . Significant levels of Mx1 induction were observed out to day 49 of the study.

B. AAV-1 Delivery of mIFN- β Gene: C57BL/6 mice were injected with the DNA of pGT61 encoding mIFN- β , or with the virus produced from pGT61 encoding mIFN β , or the DNA of pGER75 encoding SEAP (see Materials and Methods, subsection G).

The mice were bled at days 2, 10, 14 and 17 post-injection. Mice that received the pGT61 DNA showed an approximately 15-fold induction of Mx1 RNA over background at day 2 (Figure 10). Mx1 expression continued to increase to greater than 100-fold over background by day 10. By day 17 Mx1 RNA expression level was about 180-fold above background. No increased Mx1 expression was observed in the control group that received the pGER75 DNA.

The Mx1 RNA expression levels in the mice injected with the virus produced from pGT61 were about 5-fold higher on day 10, 14 and 17 than in mice that received the pGT61 DNA. This was supported by IFN- β RNA RT-PCR analysis performed on the injected muscles. At day 17 when the animals were sacrificed the mIFN- β RNA expression in the muscle was 9.0×10^5 copies/ μ g RNA in the DNA-injected muscles compared with 2.0×10^6 copies/ μ g RNA in the virus-injected muscles (data not shown).

The plasma samples were also analyzed for IP-10 and JE (Figures 7 and 8). The results obtained were very similar to that obtained for Mx1 RNA induction. At day 2 the mice that received the DNA by electroporation showed higher IP-10 plasma level compared to the mice that were injected with the AAV-1-mIFN- β expressed virus. However, by day 10 the IP-10 levels in the mice injected with AAV-1-mIFN- β showed a strong increase and averaged approximately 5- to 10- fold greater than plasmid mIFN- β injected mice.

C. Summary and Conclusions: The pharmacokinetic profile following bolus injection of human IFN- β 1a protein is very similar to previously published reports of studies using hIFN- β 1a administered to normal human volunteers and patients (see e.g.,

Buchwalder, P-A (2000) J Interferon Cytokine Res 20: 57-66). Human IFN- β 1a protein injected i.v. is rapidly cleared, and by 6 hours the serum levels are below the detection limit of the assay. Following i.m. injection of the protein the peak values are lower but the serum half-life is prolonged. However, the kinetics are still very rapid and serum levels fall below the limit of detection within hours. Recent pharmacokinetic studies in mice, rats and monkeys using a PEGylated form of IFN- β 1a show that the attachment of a 20-kDa polymer of polyethylene glycol (PEG) extends the half-life ($t_{1/2}$) from approximately 1 hour to 10 hours (see e.g., Pepinsky, RB et al. (2001) J Pharm Exp Ther 297: 1059-66).

Attempts to measure serum levels of hIFN- β following plasmid DNA delivery have been unsuccessful presumably due to low expression of the transgene, even though detectable levels of hIFN- β protein have been measured by ELISA in lysates of the injected muscles (data not shown). However, using an AAV-1 vector encoding hIFN- β , very high serum levels of hIFN- β protein were detected by the hIFN- β ELISA in a dose dependent manner following i.m. injection. Moreover, very stable and persistent levels were measured out to nearly 6 months post-injection. It is interesting to note the lack of an apparent immunogenic response to hIFN- β expression as a foreign transgene in this model, though the blood was not analyzed for the presence of anti-hIFN- β antibodies. It has been reported that bolus protein delivery of hIFN- β is highly immunogenic in other animal models (e.g. monkeys, see ref. 33). The results suggest that i.m. administration of an AAV-1 vector encoding hIFN- β could be developed as a platform to achieve high transgene expression over extended periods of time.

Three mIFN- β biomarkers were identified and validated to perform pharmacokinetic studies using murine IFN- β protein or gene delivery. A highly sensitive quantitative RT-PCR assay was developed to measure the induction of Mx1 RNA isolated from PBMC's of mice administered mIFN- β . Two murine chemokines, IP-10 and JE, were also identified as sensitive IFN- β biomarkers and commercial ELISA's allowed the means to rapidly quantitate and support the results obtained with the Mx1 TaqMan assay. Two different types of gene delivery vectors were tested, plasmid DNA (plus electroporation) and AAV-1. Administration of bolus mIFN- β protein either i.v. or i.m. resulted in a strong but transient induction of all three biomarkers, with a T_{max} of approximately 2 hours. Biomarker levels rapidly dropped to background within 12 to 24 hours post-injection. A dose response was observed for Mx1, IP-10 and JE when mIFN- β was injected i.m. The rapid drop in the biomarker levels directly reflects the rapid systemic clearance of IFN- β following bolus protein administration.

Gene-based delivery of mIFN- β using plasmid plus electroporation or an AAV-1 vector resulted in biomarker responses that were greater than those observed when mIFN- β protein was injected. With plasmid DNA a biomarker response was measured out to 49 days and was down to background levels at day 63. These data demonstrated for the first time that a mIFN- β expression plasmid is capable of expressing biologically active mIFN- β for at least 7 weeks. The kinetics of the biomarker response was slightly

different when mIFN- β DNA was delivered by an AAV-1 vector. The response was relatively low early after infection and increased over the first week. Stabilization in the biomarker response at a high level was observed in the second and third week after infection.

5 In summary, a superior pharmacokinetic profile has been demonstrated for gene-based delivery for both human and murine IFN- β compared to bolus protein administration in mice. First, the level of IFN- β expressed is equal to or greater than the levels achieved with protein delivery, as measured directly in the serum or as reflected by the induction of IFN- β biomarkers. Second, the duration of IFN- β expression from a single
10 injection of an IFN- β vector is far longer (stable expression for weeks to months) compared to the transient kinetics observed with protein administration (hours).

EXAMPLE 5: Efficacy Studies using Gene-based Delivery of mIFN- β

These studies demonstrate that gene-based delivery is efficacious in an animal
15 model of MS. The rodent EAE model is an accepted model of MS and there are several reports in which IFN- β has been shown to be active in these models (Yu, M et al. (1996) J Imm 64: 91-100). There have also been reports that gene-based delivery of IFN-B is efficacious in some of these models (see e.g., Triantaphyllopoulos, K et al., (1998) Gene Therapy 5: 253-63). The results of these studies validate a murine EAE model with mIFN- β
20 protein; and compare gene-based delivery and protein delivery of mIFN- β in the model.

A. Efficacy of mIFN- β Protein in Mouse Acute EAE Model: Eight-week old female SJL mice were immunized with proteolipid protein (PLP) on day 1 and then treated every other day through the course of the study with subcutaneous (s.c.) injections of different doses (10,000, 20,000, 30,000, or 100,000 units per group) of purified recombinant murine
25 IFN- β protein. The positive controls used for this study were Mesopram and Prednisolone, administered intraperitoneally (i.p.), twice daily.

Specifically, the gene encoding mIFN- β was cloned into a pCEP4 expression vector (Invitrogen). The expression plasmid encoding mIFN- β was transiently transfected into 293E cells (Edge Biosystems) using X-tremeGene Ro-1539 Transfection Reagent (Roche). Murine
30 IFN- β protein was purified from the medium by ion-exchange chromatography and by hydrophobic-interaction chromatography. The product was sialyzed and concentrated against dilution buffer (50 mM sodium acetate, pH 5.5, 150 mM sodium chloride, and 5% polypropylene glycol) and sterile filtered. Aliquots of the purified protein were stored at -80 °C. The activity of the purified protein was assessed using a luciferase reporter gene assay
35 (Hardy et al. (2001) Blood 97:473-482), using a commercial mIFN- β reference standard from Access Biomedical (San Diego, CA). The specific activity of the purified protein was 2×10^8 units/mg.

For animal studies, purified mIFN- β was diluted to 100 ug/mL in dilution buffer. Immediately prior to injection of the animals, the mIFN- β stock solution was diluted to the

desired concentration of mIFN- β . The vehicle control used in these studies was the dilution buffer minus mIFN- β .

The results of the study are shown in Figure 11. Mice treated with 100,000 units of IFN- β (approximately 500 ng, 20 ug/kg) developed significantly decreased clinical scores of EAE compared with vehicle treated mice ($p=0.0046$). Mice treated with 30,000 units of IFN- β also demonstrated decreased clinical scores compared to vehicle treated mice, although this decrease did not reach statistical significance. The positive controls in this study, Mesopram and Prednisolone, also significantly decreased clinical scores.

B. Gene-based Delivery of mIFN- β is Efficacious in Murine Acute EAE Model:

Based upon the results of the previous study demonstrating that mIFN- β protein is efficacious in the murine Acute EAE model a second study was performed to test and compare plasmid delivery of mIFN- β with protein delivery. As in the first study, the mice were injected on day 1 with PLP. For gene delivery, the mice received an intramuscular injection on day 2 of the study with either PBS, null plasmid DNA (pNull) with electroporation (EP), mIFN- β plasmid DNA (plus EP), or mIFN- β plasmid DNA formulated with a polymer formulation called "PINC" (Mumper, RJ et al (1998) J Controlled Release 52: 191-203). For protein delivery mice were injected every other day with murine IFN- β protein (100,000 units, s.c. injection) or vehicle.

The results of this study are shown in Figure 12. As in the previous study the mice treated with 100,000 Units of mIFN- β protein had significantly decreased clinical scores compared to the vehicle control treated mice ($p=0.045$). Gene delivery of the mIFN- β + EP also significantly decreased clinical scores, compared to gene delivery of pNull & EP ($p=0.0171$). Gene delivery using the PINC formulation of IFN- β did not statistically decrease clinical scores compared to pNull (data not shown). The full results of this study are described in the Materials and Methods, subsection A, and in Figure 13.

C. Summary and Conclusions: A murine acute EAE model has been validated using recombinant mIFN- β protein by demonstrating that every other day injection of 100,000 units of mIFN- β during the course of the study significantly decreased the severity of the disease compared to a vehicle control. Gene-based delivery of a plasmid encoding mIFN- β with electroporation was shown to significantly decrease the clinical scores in diseased mice. A single injection of the plasmid on day 2 of the study was as effective in reducing the scores as an every other day injection of IFN- β protein. These results demonstrate that gene-based delivery of IFN- β is efficacious in an animal model of MS.

EXAMPLE 6: Regulated Expression of IFN- β In Vivo Using a Regulated Expression System

A. Design, Construction and *In Vitro* Validation: The regulated expression systems of the present invention has advantages over known expression systems. In a preferred embodiment, the system of the present invention solves several development and manufacturing issues by having in a single vector a first expression cassette encoding a

therapeutic molecule of interest (TM) (e.g., an IFN- β transgene) and a second expression cassette encoding a regulator molecule (RM) that regulates the expression of the TM.

As an example of one preferred embodiment, the present inventors provide a new and improved regulated expression system. In this embodiment, the expression cassettes of the regulated, expression system of the present invention are present in a single plasmid vector called pBRES. The pBRES single vector has a number of versatile features incorporated into its design, including multiple cloning sites (MCS) for the insertion of different transgenes as well as different promoters to drive expression of the regulatory protein. In addition, the size of the pBRES expression cassettes is compatible with many different delivery vectors, including plasmid and AAV vectors.

B. Construction of mIFN- β and hIFN- β Inducible Expression Vectors for Gene

Therapy: The mIFN- β and hIFN- β genes from pGER101 and pGER125, respectively, were transferred to a series of four pBRES vectors (Figure 14A-D). The resulting plasmids have either the expression cassette encoding the murine or human IFN- β gene and the expression cassette encoding the RM gene in four different orientations relative to each other (Figure 15A-B). The resulting pBRES plasmids encoding the mIFN- β were designated as pGT23, pGT24, pGT25, and pGT26 (Figure 15A), and the resulting pBRES plasmids encoding the hIFN- β were designated as pGT27, pGT28, pGT29, and pGT30 (Figure 15B). See the Materials and Methods, subsection F for a complete description of the construction of these plasmids.

C. *In Vitro* Validation of IFN- β Expression Vectors:

Constitutive (pGER125) and inducible (pGT27, pGT28, pGT29, and pGT30) hIFN- β expression plasmids were transfected into murine muscle C₂C₁₂ cells. The cells were treated with the inducer, MFP, and the media was assayed for hIFN- β by ELISA (Figure 16). The results indicate little hIFN- β expression in the absence of MFP. Human IFN- β expression from the pBRES-hIFN- β plasmids is induced by MFP approximately 20- to 90-fold, to levels up to about 50% of that expressed from the CMV promoter (pGER125). Compared to a two-plasmid expression system (pGS1694 plus pGER129), all four plasmid orientations of the BRES system displayed comparable basal activity in the absence of MFP and induced activities equal to or greater than the two-plasmid in the presence of MFP. A similar *in vitro* study was performed with the mIFN- β pBRES plasmids (Figure 17) and the results were very similar to those described with the hIFN- β pBRES plasmids.

D. Regulated Expression of IFN- β In Vivo:

A study was performed in naïve C57BL/6 mice using the mIFN- β pBRES plasmid vector pGT26 which was constructed by digestion of pGER101 with Sal I, blunt-ending by filling in the 5' overhang with Klenow DNA polymerase, ligation of Spe I linkers, digestion with Spe I and Not I, and insertion of the resulting fragment carrying the mIFN gene between the Spe I and Not I sites of pGT4.

The plasmid vector pGT26 was used to test whether the expression of mIFN- β could be regulated in an off/on/off pulsatile manner through oral administration of the inducer, MFP. Constitutive and inducible pBRES mIFN- β expression plasmids were injected with electroporation into the hind limb muscles of mice. Mice were treated with MFP for four

consecutive days, beginning on day 7 after plasmid injection. Blood was collected at days 11 and 18 post-injection, PBMCs were isolated and Mx1 RNA levels were determined by RT-PCR. Plasma samples were also assayed for the chemokines IP-10 and JE. The results of the study for biomarker analysis of Mx1 RNA and chemokine analysis are shown in Figures 18 and 19, respectively. In the absence of MFP little or no biomarker induction is observed at 7 days. Following oral administration of MFP, all biomarkers were strongly induced, to levels higher than with CMV-mIFN- β at day 11. By day 18 the chemokine levels had returned to baseline and the Mx1 RNA level had decreased nearly to baseline (see Materials and Methods, subsection G below for description of the study and controls).

E. Efficacy of pBRES mIFN- β gene delivery in EAE model

The efficacy of IFN- β in an EAE model was demonstrated using the pBRES plasmid, in which mIFN- β expression was controlled by MFP induction. **Fig. 39a** shows the validation of the murine active EAE model in SJL mice in which the animals were injected with PLP/pertussis on day 1 to initiate disease and then treated with mIFN- β protein (100,000 or 300,000 units) by SC injection every other day throughout the course of the 25 day study. Based upon the clinical scores, both concentrations of mIFN- β protein significantly reduced the severity of the disease compared to vehicle treated animals. For mIFN- β gene delivery the pBRES plasmid (either Null vector or IFNb) was administered by IM injection to the hind limbs of the animals on day -7 and slow release MFP pellets were implanted on day -3 of the study. **Fig. 39b** shows the results of the study demonstrating that the pBRES mIFN- β plasmid treated animals displayed a significant delay in the onset of disease as well as a significant decrease in the overall severity of disease scores compared to the null vector control group. At the conclusion of the study, mRNA analysis of the plasmid injected muscles confirmed highly elevated levels of mIFN- β RNA in the pBRES mIFN- β treated animals (data not shown).

Experimental Design: Eight-week old female SJL mice (Jackson Labs, Bar Harbor, ME) were immunized on day 1 of the study with a 0.1 ml subcutaneous (SC) injection containing 150 ug proteolipid Protein (PLP) 139-151 in Complete Freund's Adjuvant (CFA) supplemented with 200 ug *M. tuberculosis* H37Ra. Immediately after immunization, all mice received a 0.1 ml IP injection of pertussis toxin. Two days after immunization, all mice received a second IP injection of pertussis toxin. Mice were housed under standard conditions, with food and water available *ad libitum* and a schedule of 12 hours of light and 12 hours of darkness. The mice were handled according to protocols and experimental design approved by the Berlex Biosciences Institutional Animal Care and Use Committee.

The positive control used for this study was Prednisolone, 2.5 mg/kg, (Sigma, St. Louis) administered by IP injection twice daily, beginning on day 1, until the end of the study. For mIFN- β protein delivery, the mice were given SC injections of purified recombinant mIFN- β protein every other day, starting on day 1 of the study (100,000 or 300,000 IU per animal per injection in 100 ul vehicle buffer). Recombinant mIFN- β protein was purified according to Schaefer et al. ((2006) *J. Interferon Cytokine Res.*, 26:449-454) and stored at at -80°C in

vehicle buffer (50 mM sodium acetate, pH 5.5, 150 mM sodium chloride, and 5% propylene glycol) prior to use. The specific activity of the purified protein was 2×10^8 international units (IU) per mg.

For mIFN- β gene delivery, mice received an IM injection on day -7 of either pBRES null vector plasmid DNA with electroporation (EP), or pBRES mIFN- β vector plasmid DNA with EP. Each animal was injected bilaterally with PBS or a total of 200 μ g plasmid DNA (100 μ g per limb) in 120 μ l PBS (20 μ l into the tibialis muscle and 40 μ l into the gastrocnemius muscle of each hind limb). Immediately following injection the muscles were electroporated with a caliper electrode (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse) using a BTX ECM 830 EP instrument supplied by Genetronics (San Diego, CA). On day -3 the null vector and mIFN- β plasmid treated mice were each administered a single MFP pellet (2.5 mg/ 60 day release pellet, Innovative Research of America, Sarasota, FL) by SC injection.

The mice were scored daily based on the following scoring system: 0 = normal; 1 = limp tail; 2 = difficulty righting; 3 = incomplete paralysis of one or both hind limbs; 4 = complete paralysis of one or both hind limbs, or hind limbs mobile but drag; 5 = complete paralysis of both hind limbs and weakness/paralysis of forelimbs, moribund, or dead. Moribund mice were euthanized. Statistical analysis was performed by applying a Mann-Whitney U test on the cumulative clinical scores obtained from each animal in each group of the study (n = 10 animals per group).

F. Summary and Conclusions: The present inventors have identified two vectors, a non-viral plasmid DNA and an adeno-associated virus type 1 (AAV-1), that are suitable for delivery of a therapeutic molecule (TM), e.g. an IFN- β gene, to treat a chronic disease e.g., MS. Further, the present inventors have shown that both vectors can be delivered to skeletal muscle by intramuscular injection and generate IFN- β expression levels that are measurable and persistent in murine animal models. In the case of plasmid DNA the present inventors have demonstrated that a single injection of a mIFN- β encoded plasmid (with electroporation) is efficacious and as effective as an every other day injection of mIFN- β protein in an animal model of MS.?? The present inventors developed biomarkers of mIFN- β to show that plasmid encoded mIFN- β expression persists for at least 45 days following a single plasmid injection. In the case of the AAV-1 vector the present inventors have shown that a single intramuscular injection of a human IFN- β encoded AAV-1 vector results in high serum levels of the human IFN- β protein that persists for 6 months. Both plasmid and AAV-1 vectors have been shown to be compatible with the BRES regulated expression system of the present invention. For example, the present inventors demonstrated the regulated expression of IFN- β in mice using a single-plasmid vector pBRES regulated expression system of the present invention.

In one embodiment of the new and improved BRES regulated expression system, the expression cassettes are present in a single vector, e.g., a single plasmid vector. In this embodiment, the pBRES single plasmid vector contains the expression cassettes for both the Regulator molecule (RM) (e.g., a transcriptional activator such as a modified steroid hormone

receptor) and the therapeutic molecule (TM) (e.g., human or murine IFN- β) on a single shuttle plasmid expression vector. The single vector of the BRES regulated expression system contains multiple cloning sites (MCS) to simplify the insertion or replacement of promoters, regulatory elements and transgenes into the plasmid backbone. As demonstrated herein by the present inventors, pBRES mIFN- β and pBRES hIFN- β single-plasmid vectors were constructed and tested *in vitro*, and shown to have low background activity in the absence of the activator molecule (AM), the small molecule inducer MFP, and showed high inducibility (comparable to a two-plasmid system) in the presence of MFP.

An *in vivo* study was conducted in normal mice using a pBRES mIFN- β plasmid vector, and oral administration of MFP. Using biomarkers to monitor mIFN- β expression levels the results showed low background expression of mIFN- β biomarkers in the absence of MFP, strong induction following MFP administration, and a return to basal levels of expression upon withdrawal of MFP (Figures 18 and 19). Based upon these results the present inventors have achieved the regulated expression of IFN- β *in vivo* using the regulated expression system of the present invention.

Thus, an outcome of these studies and the compositions and methods of the present invention is a gene-based delivery system for IFN- β that will provide long-term, regulated expression of IFN- β for the treatment of a disease or condition, e.g., an anti-inflammatory disease or condition, and more preferably MS. The gene therapy vectors of the present invention can incorporate one or more expression cassettes for delivery of a therapeutic molecule (TM) of interest (e.g., IFN- β) for treatment of a disease or condition. In one embodiment, the regulated expression system as described herein can provide long-term, renewable expression through oral administration of the small molecule inducer, MFP. The single-vector BRES system is capable of repeat administration, e.g., by intramuscular injection, and will allow the testing of continuous versus pulsatile IFN- β therapy in the clinic.

EXAMPLE 7: Selection of Candidate Vector

EXAMPLE 7: BRES Regulated expression system

A. BRES Orientation: The *in vivo* studies performed utilized one of the four pBRES orientations that were constructed as described in Figure 15. As described herein, this was based upon *in vitro* data that showed that the construct, pGT26, had the highest level of transgene expression in the presence of MFP. These four pBRES orientations can be tested *in vivo* using the protocols described herein to determine which one provides the best "window" of transgene expression (e.g., the lowest basal expression level minus MFP, and highest induced expression level plus MFP).

i. Orientation-dependent effects on target gene expression in pBRES plasmids: A study was performed in naïve C57BL/6 mice using the mIFN- β pBRES plasmid vectors pGT23, 24, 25, and 26, to determine the level of mIFN expression as assayed by the level of the chemokine IP-10, which serves as a biomarker for mIFN expression. pGT23, pGT24, pGT25, and pGT26 were injected with electroporation into the hind limb muscles of

mice, with 15 animals per group. Five mice from each group were bled at day 7 in the absence of MFP. The remaining 10 mice in each group were treated with MFP for four consecutive days, beginning on day 7 after plasmid injection. Blood was collected at days 11 and 18 post-injection, and plasma samples were assayed for IP-10 (See "Experimental Design" for details). The results show that pGT26 offered the best combination of low expression – MFP and high expression + MFP, consistent with the in vitro results (Figure 32). pGT24 had the highest induction of IP-10 expression, but the IP-10 levels in the absence of MFP were higher than the other orientations. pGT25 had lower IP-10 levels both – and + MFP, and pGT23 had IP-10 levels + MFP about the same as pGT26. The IP-10 levels with pGT24 – MFP, however, were considerably higher than for pGT26. These results in total indicate an orientation-dependent effect on both basal and induced target gene expression.

Experimental Design: In vivo transfection of pBRES/mIFN plasmids and comparison of the four orientations of pBRES/mIFN was performed as follows. Normal C57BL/6 mice were injected and electroporated with single-vector pBRES mouse IFN expression plasmids. mIFN expression was monitored by biomarker response and mIFN RNA analysis. The mice went through one off/on/off cycle of MFP treatment.

DNA solutions: Each mouse in all injected groups received 250 ug of plasmid DNA in 150 ul PBS.

Table 3

Group	plasmid	description	n*
1	pGT23	mIFN-RM	15
2	pGT24	mIFN rev-RM	15
3	pGT25	RM-mIFN	15
4	pGT26	RM-mIFN rev	15

*n=number of animals

DNA delivery: Adult male C57BL/6 mice were injected bilaterally on day 0 with 250 ug plasmid DNA in 150 ul PBS. The DNA solution was injected 25 ul into the tibialis muscle and 50 ul into the gastrocnemius muscle of each hind leg, followed by electroporation with a caliper (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse).

MFP treatment: Groups 1-4 (all injected mice) were administered MFP by oral gavage at 0.33 mg/kg (100 ul of 0.083 mg/ml in sesame oil, made fresh) as indicated in the table below.

Table 4

Group/ plasmid	n/ mouse #	day 0	day 7	day 7-10	day 11	day 18
1 pGT23	15 101-115	inject 101-115	Terminal bleed + muscles 101-105	MFP 106-115	Terminal bleed + muscles 106-110. Tail bleed 111-115	Terminal bleed + muscles 111-115
2 pGT24	15 201-215	inject 201-215	Terminal bleed + muscles 201-205	MFP 206-215	Terminal bleed + muscles 206-210. Tail bleed 211-215	Terminal bleed + muscles 211-215

3 pGT25	15 301-315	inject 301-315	Terminal bleed + muscles 301-305	MFP 306-315	Terminal bleed + muscles 306-310. Tail bleed 311-315	Terminal bleed + muscles 311-315
4 pGT26	15 401-415	inject 401-415	Terminal bleed + muscles 401-405	MFP 406-415	Terminal bleed + muscles 406-410 Tail bleed 411-415.	Terminal bleed + muscles 411-415
5 uninjecte d	5 501-515		Terminal bleed + muscles 501-505			

Day 7: Compares baseline level of expression in the absence of MFP.

Day 11: Compares induced level of expression after MFP treatment.

Day 18: Compares expression after 7 days without MFP treatment.

- 5 **Blood collection and Endpoint Analysis/Assay Procedure:** Mice were bled by tail nick or cardiac puncture (terminal bleed) at the time points indicated in the table above. Blood was collected into Microtainer tubes (containing EDTA) at RT (room temperature). Plasma was collected from the tail bleeds for IP-10 assays. PBMC's were separated and collected from the terminal bleeds, and the remaining plasma was assayed for
- 10 IP-10 by ELISA.

ii. Orientation-dependent effects on target gene expression in pBRES

- plasmids:** pBRES/hIFN plasmids were also tested in a similar manner as described above. Constitutive (pGER125) or inducible pBRES/hIFN (pGT27, pGT28, pGT29, and pGT30) plasmids were injected with electroporation into the hind limb muscles of naive C57BL/6 mice.
- 15 Mice were bled at day 4 in the absence of MFP, then treated with MFP for four consecutive days, beginning on day 7 after plasmid injection. Blood was collected at days 11 and 18 post-injection. Serum was collected from clotted blood and samples were assayed for hIFN by ELISA (See "Experimental Design" below for details).

- The results show that pGT28 offered the best combination of low expression
- 20 – MFP and high expression + MFP, consistent with the in vitro results (Figure 33). Expression of hIFN – MFP at day 4 was undetectable for all pBRES/hIFN plasmids. Expression of hIFN + MFP at day 7 was less than with the CMV promoter for pGT27, but was higher for pGT28, 29, and 30. Expression of hIFN from pGT28 was as much as 5-fold higher as that from CMV. Expression had fallen to nearly undetectable for all pBRES/hIFN plasmids
- 25 by day 18, with pGT28 having the lowest expression at that point. These results in total indicate an orientation-dependent effect on both basal and induced target gene expression.

- Experimental Design:** In vivo transfection of pBRES/hIFN plasmids and comparison of the four orientations of pBRES/hIFN was performed as follows. Normal adult C57BL/6 mice were injected with plasmid vectors carrying the pBRES/hIFN or CMV-hIFN
- 30 expression cassettes. Human IFN expression was assayed through one off/on/off cycle of MFP treatment.

Plasmid DNA solutions: Group 2-6 mice (plasmid) received 250 ug DNA per mouse in a volume of 150 ul.

Table 5

Group	vector	description	n*
2	pGT27	hIFN-RM in plasmid	5
3	pGT28	hIFN rev-RM in plasmid	5
4	pGT29	RM-hIFN in plasmid	5
5	pGT30	RM-hIFN rev in plasmid	5
6	pGER125	CMV-hIFN in plasmid	5

*n=number of animals

DNA delivery: For Groups 2-6 (plasmid), 25 ul of the DNA solution was injected into the tibialis muscle and 50 ul into the gastrocnemius muscle of each hind leg, followed by electroporation with a caliper (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse).

MFP treatment: Groups 2-5 were administered MFP by i.p. injection at 0.33 mg/kg (100 ul of 0.083 mg/ml in sesame oil, made fresh) on day 7-10, as indicated in the table below.

Table 6: Cycle 1

Group/ plasmid	n/ mouse #	day 0	day 4	day 7-10	day 11	day 18
2 pGT27	5 201-205	inject and EP plasmid	tail bleed	MFP	tail bleed	terminal bleed + muscles
3 pGT28	5 301-305	inject and EP plasmid	tail bleed	MFP	tail bleed	terminal bleed + muscles
4 pGT29	5 401-405	inject and EP plasmid	tail bleed	MFP	tail bleed	terminal bleed + muscles
5 pGT30	5 501-505	inject and EP plasmid	tail bleed	MFP	tail bleed	terminal bleed + muscles
6 pGER125	5 601-605	inject and EP plasmid	tail bleed		tail bleed	terminal bleed + muscles
7 uninjected	5 701-705					terminal bleed + muscles

Blood collection and Endpoint Analysis/Assay Procedure: Mice were bled by tail nick or cardiac puncture (terminal bleed) at the time points indicated in the table above. Blood was collected in Microtainer tubes (no anti-coagulant). Serum was separated and collected from the blood, and then assayed for hIFN by ELISA.

iii. Orientation-dependent effects on target gene expression in pBRES

plasmids: pBRES/hEPO plasmids were also tested in a similar manner as above. Inducible one-plasmid pBRES/hEPO (pGT15, pGT16, pGT17, and pGT18) or two-plasmid (pGS1694 + pEP1666) vectors were injected with electroporation into the hind limb muscles of naive C57BL/6 mice, with 10 animals per group. Five mice from each group were treated with MFP for four consecutive days, beginning on day 7 after plasmid injection, and five mice from each group did not receive MFP. Blood was collected at day 10 post-injection, 6 hr after the last MFP treatment. Serum was collected from clotted blood and samples were assayed for hEPO by ELISA (See Experimental Design below for details). The results show that the induced expression levels are higher than the two plasmid system for 3 of the 4 pBRES/EPO plasmids and that the expression levels vary with orientation, consistent with the mIFN and

hIFN pBRES plasmids (Figure 34A). The results show a good correlation between EPO expression and hematocrit levels (Figure 34B), demonstrating a physiological effect of inducible EPO gene expression. These results in total indicate an orientation-effect on both basal and induced target gene expression.

5 **Experimental Design:** In vivo transfection of pBRES/hEPO plasmids and comparison of the four orientations of pBRES/hEPO with the two-plasmid regulated expression system was performed as follows. Normal adult C57BL/6 mice were injected with pBRES/hEpo plasmid vectors or the two-plasmid regulated expression system with Epo as the GS-responsive target gene. Human Epo expression was assayed in the absence and
10 presence of MFP treatment. The following protocol consists of five groups of mice (N=10, where "N" is the number of animals). For each group, all ten mice were injected/electroporated with one of the 4 pBRES plasmids or the two plasmids. An additional group of N=10 (Group 6) was uninjected for negative controls.

 For each tail bleed or terminal bleed below, 10 ul of blood was immediately
15 collected for a hematocrit assay, and serum from the remainder of the blood was also collected. See below under "Blood Collection and Endpoint Analysis/Assay Procedure". For each of groups 1-5, at day 7 five mice were injected with MFP days 7-10 in the morning, and tail bled on day 10 in the afternoon, about 6 hrs after the last MFP injection (induced samples). The remaining five mice in each group were not induced with MFP, and were
20 terminally bled at day 11 (terminal bleeds were necessary for accurate uninduced levels). Group 6 was terminally bled at day 11.

 Thus, in this experiment, the pBRES and two plasmid systems were compared as to their MFP- induced and uninduced levels, and also compared was their constantly-induced levels over time.

25 **Plasmid DNA solutions:** Group 1 mice received 100 ug each plasmid per mouse in a volume of 150 ul. Group 2-5 mice received 200 ug DNA per mouse in a volume of 150 ul.

Table 7

Group	vector	description	n*
1	pGS1694 pEP1666	actin pro-GS GS-responsive Epo	10
2	pGT15	hEpo-RM	10
3	pGT16	hEpo rev-RM	10
4	pGT17	RM-hEpo	10
5	pGT18	RM-hEpo rev	10

*n=number of animals

DNA delivery: 25 ul was injected into the tibialis muscle and 50 ul was injected into the gastrocnemius muscle of each hind leg, followed by electroporation with a caliper (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse).
40

MFP treatment: Mice were administered MFP by i.p. injection of 100 ul of MFP solution (0.083 mg/ml in sesame oil).

Table 8

Group/	n*/	day 0	day 7-10	day 10
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plasmid	mouse #			
1) pGS1694 + pEP1666	10 101-110	inject and EP plasmid	MFP i.p. 101-105	tail bleed 101-105 terminal bleed 106-110
2) pGT15	10 201-210	inject and EP plasmid	MFP i.p. 201-205	tail bleed 201-205 terminal bleed 206-210
3) pGT16	10 301-310	inject and EP plasmid	MFP i.p. 301-305	tail bleed 301-305 terminal bleed 306-310
4) pGT17	10 401-410	inject and EP plasmid	MFP i.p. 401-405	tail bleed 401-405. terminal bleed 406-410
5) pGT18	10 501-510	inject and EP plasmid	MFP i.p. 501-505	tail bleed 501-505. terminal bleed 506-510
6 uninjected	10 601-610			terminal bleed 601-610

*n=number of animals

Blood collection and Endpoint Analysis/Assay Procedure: Mice were bled by tail nick or cardiac puncture (terminal bleed) at the time points indicated in the table above.

- 5 Blood was collected in Microtainer tubes (no anti-coagulant), allowed to clot, centrifuged, and the serum collected. The serum was assayed for hEpo by ELISA.

- Hematocrit:** Approximately 10 ul blood was collected (aspirated) directly from a tail nick in a capillary tube, sealed with clay, and centrifuged ~5 min at ~10,000 g within 10 minutes after collection. The blood was separated in the capillary tube into 3 layers, i.e.: RBC's at the bottom (40-50% total volume), a small "buffy layer" (WBC and platelets) and the remainder plasma. A sliding gauge was used to read the hematocrit (percentage of RBC to total blood).

- B. BRES Backbone:** Any of the plasmid backbone modifications of the pBRES vectors of the present invention, as described herein, that demonstrate a significant increase in the level and/or duration of transgene expression (as determined by the methods described herein) can be incorporated in the pBRES vectors of the present invention. Additional modifications to the pBRES vectors of the present invention may include the use of a stronger promoter. This type of modification is relatively easy to test due to the modular design of the pBRES system.

- C. pBRES/Vector Testing:** Pharmacokinetics and efficacy studies by the present inventors have employed IFN- β expression cassettes utilizing the CMV promoter enhancer. The BRES expression system of the present invention can be tailored to suit a particular therapeutic need as described herein. For example, changes or modifications of the vectors or expression cassettes of the present invention can be tested in C57BL/6 naïve mice. These studies can be conducted, e.g., to: 1) determine the optimal dose of vector and activator molecule (AM) (e.g., small molecule inducer MFP) necessary to achieve therapeutic levels of a therapeutic molecule (TM) (e.g., IFN- β) systemically either for continuous or pulsatile treatment paradigms, and 2) determine the duration of transgene expression over time, as well as define the optimal time for repeat administration of the vector and inducer. Once these parameters have been established in mice (or other suitable animals) then a superior pharmacokinetic profile of gene-based delivery versus protein delivery (e.g., level of

expression, persistence of expression, renewable expression) can be established using these optimized delivery conditions in another species, preferably non-human primates.

EXAMPLE 8: Selection of Candidate Vector

The selection of the type of vector can be determined by specific studies. For example, for plasmid DNA it can be determined whether electroporation is a desirable component of intramuscular injection to obtain a therapeutic level of the therapeutic molecule (TM), e.g., a therapeutic level of IFN- β . If administration by electroporation of the gene therapy vector of the present invention is desirable, then as described herein and additionally from what is known in the art, a device and protocol that can be clinically feasible and acceptable can be designed. For example, for AAV-1 vectors of the present invention, it can be determined whether repeat administration of the vector is desirable based on potential immunogenic properties reported for AAV vectors (see e.g., Chirmule, N et al. (2000) J Virol 74: 2420-25).

A. Methods to Improve Transfection of Skeletal Muscle using Plasmid DNA:

Plasmid DNA is a desirable vector for gene-based delivery because it is simple, non-immunogenic, and easy to produce and manufacture. Several different methods have been developed to enhance skeletal muscle (SkM) transfection efficiency of plasmid DNA by intramuscular injection. These methods include the use of enzymes such as hyaluronidase to treat the muscle and surrounding extracellular matrix prior to delivery (see e.g., Mennuni, C et al. (2002) Hum Gene Ther 13: 355-65), various polymer formulations (see e.g., Mumper, RJ et al. (1998) J Controlled Release 52: 191-203; Nicol, F et al. (2002) Gene Ther 9: 1351-58), as well as devices such as electroporation (see e.g., Aihara, H et al. (1998) Nat. Biotech 16: 867-70; Bloquel, C et al (2004) J Gene Med 6: S11-S23) or ultrasound (see e.g., Schratzberger, P et al. (2002) Mol Ther 6: 576-83). Intravascular delivery of plasmid DNA to limb SkM using high pressure and large volumes ("cuff" method) has also been shown to be effective in achieving high transfection and broad distribution of plasmid DNA to this target tissue (see e.g., Budker, V et al. (1998) Gene Ther 5: 272-76). Of all of these methods electroporation and intravascular delivery are reported to be the most effective and have been shown in several animal models to enhance the transfection of plasmid DNA into SkM by two to three orders of magnitude over naked DNA alone (see e.g., Bloquel, C et al (2004) J Gene Med 6: S11-S23; Qian, HS et al. (2004) Mol Ther 9: Supp 1, S91). The present inventors using plasmid DNA have shown that detectable levels of IFN- β in the serum (measurable IFN- β protein in the serum, bio-marker response, or efficacy) are achieved when electroporation has been employed with the delivery of plasmid DNA.

Suitable electroporation devices for clinical use in the delivery of IFN- β plasmid DNA can be evaluated and determined by testing in rabbits and other larger animals using methods described herein or known in the art. Electroporation devices that may be suitable for such testing and use may include those developed by Inovio AS, Ichor Medical Systems, Genetronics, Inc. Genetronics has reported testing of a device in humans (unpublished

presentation at Gordon Research Conference on Bioelectrochemistry, July 25-30, NH). Inovio has also reported the results of testing electroporation technology in human volunteers (see e.g., Kjelen, R et al. (2004) Mol Ther 9: Supp1, S60). Ichor Medical Systems has recently reported the development of an electroporation device suitable for the delivery of therapeutic DNA (see e.g., Evans, CF et al. (2004) Mol Ther 9: Supp 1, S56).

Using a plasmid encoding a LacZ reporter gene the present inventors have demonstrated high transfection efficiency to rat hind limb skeletal muscle using intra-arterial delivery of a plasmid solution. Mirus has recently reported an intra-venous delivery method for delivery of plasmid DNA with decreased volume under decreased pressure which can be tested for plasmid delivery to SkM using methods described herein or known in the art (see e.g., Hagstrom, JE et al. (2004) Mole Ther 10: 386-98).

Methods other than electroporation or intravascular delivery to enhance the uptake of plasmid DNA to SkM and the subsequent expression of the transgene can be tested and their suitability for delivery of plasmid DNA determined using methods described herein or known in the art. In this regard, certain chemical agents that have been reported to enhance vector uptake to SkM can be tested and may be suitable for use in the delivery of the plasmid vectors of the present invention, including polymer formulations and antennopodia peptides (AP). For example, "F68" is a poloxamer formulation that can be used to formulate and deliver plasmid DNA and has been reported to enhance the delivery of plasmid DNA to SkM by approximately 10-fold (see e.g., Mumper, RJ et al. (1998) J Controlled Release 52: 191-203; Qian, HS et al. (2004) Mol Ther 9: Supp 1, S91). The Antennapedia (AP) peptides and other peptides of similar composition have been reported to facilitate the transport of large macromolecules across the cell membrane (see e.g., Bucci, M et al. (2000) Nat. Med 6: 1362-67; Gratton, J-P et al. (2003) Nat Med 9: 357-62).

B. Methods to Increase the Level and Duration of IFN- β Expression from Plasmid DNA Vectors: In addition to evaluating certain chemical agents that enhance plasmid DNA uptake to SkM, various approaches can be used to increase the level and duration of transgene expression from the plasmid DNA vectors of the present invention. For example, it has been reported that the removal of bacterial DNA sequences from plasmid DNA to create circular plasmids containing only the expression cassette ("minicircle DNA") results in 10- to 100-fold higher transgene expression (using factor IX and alpha1-antitrypsin as transgenes) compared to standard plasmid DNA following transfection of liver in mice (see e.g., Chen, Z-Y et al. (2003) Mol Ther 8: 495-500). The removal of bacterial DNA sequences that are enriched in CpG regions has been shown to decrease transgene expression silencing and result in more persistent expression from plasmid DNA vectors (see e.g., Ehrhardt, A et al. (2003) Hum Gene Ther 10: 215-25; Yet, NS (2002) Mol Ther 5: 731-38; Chen, ZY et al. (2004) Gene Ther 11: 856-64). The regulated expression systems of the present invention can be modified using such approaches to increase and prolong the level of transgene expression using plasmid DNA vectors. In a preferred embodiment, a pBRES plasmid vector encoding IFN- β is modified to increase and prolong the level of transgene expression.

In one embodiment, expression of the IFN- β transgene in the regulated expression system of the present invention was driven by the strong cytomegalovirus (CMV) promoter to constitutively express IFN- β . It has been reported that gene-based expression using the CMV promoter undergoes silencing through extensive methylation of the promoter region *in vivo* (see e.g., Brooks, A et al. (2004) J Gene Med 6: 395-404). In addition the results from the *in vivo* study by the present inventors using the pBRES gene therapy plasmid vector pGT26- β IFN- β showed higher IFN- β levels using the pBRES expression cassette than the levels achieved using the CMV driven expression cassette (see e.g., Example 6). Given these results the IFN- β BRES expression system of the present invention may generate significantly higher and more persistent expression levels than what has thus far been observed using CMV driven plasmid DNA expression cassettes and therefore it is suitable for examining the delivery of the pBRES plasmid vector without electroporation.

In a preferred embodiment, the method of administration is by intramuscular injection of an IFN- β plasmid solution in the absence of electroporation. Detectable levels of IFN- β in serum can be tested by administering plasmid vector by intramuscular injection to naive mice. A complete characterization of the expression level and persistence of the pBRES expression cassette can be performed and compared with the CMV vectors previously used. Plasmid formulations including F68 poloxamer and Antennapedia peptides can be tested for their ability to enhance plasmid transfection of SkM and subsequent IFN- β transgene expression. Lastly, modifications of the plasmid vector backbone (e.g., removal of bacterial sequences) can be explored as a means to increase and prolong transgene expression.

C. AAV-1 Vectors: Adeno-associated virus (AAV) is a single stranded DNA virus (parvovirus) that was initially isolated as a contaminant in adenoviral isolates from humans. AAV has a number of features that make it particularly attractive as a gene therapy vector. In addition to its non-pathogenic and replication deficient nature in the absence of a helper virus it contains a very simple genome consisting of only two genes, *rep* and *cap*. These genes are replaced in recombinant AAV vectors with the desired transgene flanked by characteristic 5' and 3' inverted terminal repeats (ITR's) of approximately 135 base pairs each. The ITR's are the only remaining components of AAV derived DNA required for vector delivery. Studies to date have shown that recombinant AAV vectors without the *rep* gene do not integrate *in vivo* but rather form large concatameric structures that remain episomal in non-dividing cells (see e.g., Duan, D et al. (1998) J Virol 72: 8568-77; Vincent-Lacaze, N et al. (1999) J Virol 73: 1949-55; Schnepp, BC et al. (2003) J Virol 77: 3495-3504).

AAV has a relatively small capacity for DNA, approximately 4.5 kb, but this is usually sufficient to accommodate all but the largest therapeutic transgenes. AAV2 has been tested in human gene therapy trials and has shown to provide long term expression and minimal inflammation (see e.g., Silwell, JL and Samulski, RJ (2003) BioTechniques 34: 148-59). Recently, alternative AAV serotypes have been shown to have excellent transfection efficiency to SkM in addition to long term expression characteristic of this vector system (see e.g., Grimm, D and Kay, MA (2003) Curr Gene Ther 3: 281-304). The studies by the present

inventors using an AAV-1 vector expressing luciferase under a CMV promoter have shown high expression persisting beyond 12 months following intramuscular injection into the hind limb of mice (see e.g., Qian, HS et al. (2004) Mol Ther 9: Supp 1, S60). Persistent expression of hEPO out to five years in non-human primates has been reported (see e.g.,
5 Xiao, W et al. (1999) J Virol 73: 3994-4003).

As described herein the present inventors have tested AAV-1 IFN- β expressing vectors (constitutive expression using the CMV promoter/enhancer) to demonstrate robust levels of hIFN- β protein as well as mIFN- β biomarker responses following intramuscular injection into the hind limbs of C57BL/6 mice.

10 In choosing a viral-based delivery vector for treating a chronic disease such as MS the regulated expression systems of the present invention can be tested, using methods as described herein or as known in the art, for their ability to demonstrate not only long term expression of the therapeutic transgene but also the ability to re-administer the gene (see e.g., Chirmule, N et al. (2000) J Virol 74: 2420-25). In order to determine if AAV-1 is a
15 suitable vector for re-administration, studies can be performed e.g., using the candidate vector, AAV-1, and AAV2, the serotype that is believed to be the most prevalent in the human population. Such an approach can be used to determine: 1) whether pre-existing antibodies to AAV2 or AAV-1 will affect the ability to deliver and express genes encoded in an AAV-1 vector, and 2) whether an AAV-1 vector can be re-administered at a dose sufficient to
20 maintain therapeutic levels of IFN- β in mice. Vectors expressing either the reporter gene luciferase or the therapeutic gene, murine IFN- β , in either AAV-1 or AAV2 vectors can be administered i.m. at different doses and transgene expression monitored. After four weeks the vector can be re-administered and again transgene expression can be monitored, Neutralizing antibodies can be measured ex vivo by the ability of immunized mouse serum to
25 inhibit viral uptake in a cell-based assay.

i. In vivo activity of a pBRES-hIFN AAV vector: A study was performed in naïve C57BL/6 mice using the hIFN- β pBRES AAV vector AAV-1GT58 injected into the hind limb muscles of mice. Mice were bled at day 4 in the absence of MFP, then treated with MFP for four consecutive days, beginning on day 7 after plasmid injection. Blood was
30 collected at days 11 and 18 post-injection. Serum was collected from clotted blood and samples were assayed for hIFN by ELISA (See "Experimental Design" below for details). Mice were then subjected to seven more cycles of MFP treatments and bleeds spaced about six to eight weeks apart. Each cycle consisted of a bleed 3 days before MFP treatment, then 4 days of MFP, then a bleed the day after the last day of MFP, then another bleed 7 days
35 after that. The results show very high inducible hIFN expression, peaking at about 3 months after injection at a level as much as 75-fold higher than what was obtained with the strongest pBRES/hIFN plasmid (Figure 35). hIFN expression decreased gradually over time, and background expression – MFP remained low throughout the course of the experiment. This demonstrates long-term, persistent (see also Example 9B), inducible expression of a
40 therapeutic target gene from an AAV vector.

Experimental Design: Normal adult C57BL/6 mice were injected with an AAV vector carrying the pBRES/hIFN expression cassettes, as follows. Human IFN expression was assayed through multiple off/on/off cycles of MFP treatment.

Viral solution: Group 1 mice (AAV) received 5×10^{10} viral particles (vp) per mouse in a volume of 75 μ l.

Table 9

Group	vector	description	n*
1	AAV-1-GT58	RM-hIFN rev in AAV-1	5

*n=number of animals

MFP treatment: Group 1 was administered MFP by i.p. injection at 0.33 mg/kg (100 μ l of 0.083 mg/ml in sesame oil, made fresh) on day 7-10, as indicated in the tables below.

Table 10: Cycle 1-3: 1-12 weeks

Group	n*/ mouse #	day 0	day 4	day 7-10	day 11	day 18
AAV-1-GT58	5/ 101-105	inject virus	tail bleed	MFP	tail bleed	tail bleed

Group	n*/ mouse #	day 39	day 42-45	day 46	day 53
AAV-1-GT58	5/ 101-105	tail bleed	MFP	tail bleed	tail bleed

Group	n*/ mouse #	day 81	day 84-87	day 88	day 95
AAV-1-GT58	5/ 101-105	tail bleed	MFP	tail bleed	tail bleed

Table 11: Cycle 4-8: 4-11 months

Group	n*/ mouse #	day 123	day 126-129	day 130	day 137
AAV-1-GT58	5/ 101-105	tail bleed	MFP	tail bleed	tail bleed

Group	n*/ mouse #	day 165	day 168-171	day 172	day 178
AAV-1-GT58	5/ 101-105	tail bleed	MFP	tail bleed	tail bleed

Group	n*/ mouse #	day 221	day 224-227	day 228	day 235
AAV-1-GT58	5*/ 101-105	tail bleed	MFP	tail bleed	tail bleed

Group	n*/ mouse #	day 284	day 287 - 290	day 291	day 298
AAV-1-GT58	5/ 101-105	tail bleed	MFP	tail bleed	tail bleed
Group/ plasmid	n*/ mouse #	day 340	day 343 – 346	day 347	day 354
AAV-1-GT58	5/ 101-105	tail bleed	MFP	tail bleed	tail bleed

*n=number of animals

Blood collection and Endpoint Analysis/Assay Procedure: Mice were bled by tail nick at the time points indicated in the table above. Blood was collected in
5 Microtainer tubes (no anti-coagulant) and centrifuged for separation and collection of serum. The serum was assayed for hIFN by ELISA.

Example 9: Gene Therapy Using a Regulated Expression System

A. Dose/Response and Kinetics Studies: Dose/response studies can be performed to determine the amount of gene therapy vector of the present invention for
10 delivery, whether delivered by plasmid or by AAV-1, that is necessary to achieve therapeutic levels of the therapeutic molecule (TM) (e.g., IFN- β) in mice (or other suitable animals). In one embodiment, the therapeutic level is defined as the induced level of the therapeutic molecule (TM), e.g. a transgene encoding a therapeutic protein, achieved systemically by the vector that is equivalent to the level of therapeutic protein achieved by a therapeutic amount of bolus
15 of the therapeutic protein given in humans on a mg/kg basis. For example, in a preferred embodiment, the therapeutic level is defined as the induced level of IFN- β achieved systemically by a gene therapy vector of the present invention that is equivalent to the level of IFN- β achieved by a therapeutic amount of bolus IFN- β protein given in humans on a mg/kg basis. In humans the current single dose of IFN- β 1a is either 30 ug or 44 ug (Rebif).

20 In addition, a complete pharmacokinetic profile of the expression of the therapeutic molecule (TM) can be performed using an activator molecule (AM) to determine the AM dose/response, as well as the kinetics of induction of TM expression following AM administration. For example, in a preferred embodiment, a complete pharmacokinetic profile of IFN- β expression can be performed with the inducer MFP to determine the MFP
25 dose/response, as well as the kinetics of IFN- β induction following MFP administration.

i. MFP dose/response and plasmid re-injection with a pBRES/mIFN plasmid in vivo: A study was performed in naïve C57BL/6 mice using the mIFN- β pBRES plasmid vector pGT26 to determine the level of mIFN expression in response to various doses of MFP, as assayed by the level of the chemokine IP-10. pGT26 was injected with
30 electroporation into the hind limb muscles of mice, and the animals were treated with MFP at 0.0033 mg/kg to 1.0 mg/kg on day 7-10 after plasmid injection. Blood was collected at days 11 post-injection, and serum samples were assayed for IP-10 (see "Experimental Design" below for details). The results show an MFP dose-dependent increase in IP-10 levels (Figure 36). There was no increase over background IP-10 levels in the absence of MFP or at 0.0033

mg/kg. IP-10 levels then increase from 0.01 to 1.0 mg/kg MFP. A second and third cycle of MFP induction was performed at the same concentrations at day 39 and 67. By day 67 the level of IP-10 had decreased several-fold from the day 11 level, so the plasmid DNA was re-injected at day 77. Another cycle of MFP treatment was performed and the mice were bled on day 88. The results show renewed induction of IP-10 expression to the same levels as on day 11. Two more MFP cycles on day 102 and 137 were performed, and the MFP dose-response was still observed, with gradually decreasing IP-10 levels over time. Subsequently, the plasmid DNA was injected for a third time on day 189, and the following MFP cycle and bleed on day 200 once again showed a strong MFP dose-response with IP-10 expression renewed to about the same levels as on day 11 and 88. This demonstrates both a positive correlation of target gene expression with inducer dose, and also persistence and renewal of gene expression with repeat administration of the plasmid vector (see Example 9B below).

For safety studies in humans, a complete characterization of the rate at which the activator molecule (AM) and TM expression can be turned "off" following withdrawal of the AM can be performed. The frequency of AM dosing necessary to achieve steady state levels of the TM can be evaluated. In a preferred embodiment, a complete characterization of the rate at which MFP and IFN- β expression can be turned "off" following withdrawal of the inducer can be evaluated. Further, the frequency of MFP dosing necessary to achieve steady state levels of IFN- β can be evaluated.

Experimental Design: Normal C57BL/6 mice were injected and electroporated with a single-vector pBRES murine IFN expression plasmid and treated with various doses of MFP, and mIFN expression was assayed by biomarker response, as follows.

Table 12

Group	MFP (mg/kg)	n*
1	0	5
2	0.0033	5
3	0.01	5
4	0.033	5
5	0.10	5
6	0.33	5
7	1.00	5

Table 13

Group/ plasmid	n*/ mouse #	day 0	day 7-10	day 11	day 18
1 pGT26	5/ 101-105	inject DNA	100 ul sesame oil	tail bleed	tail bleed
2 pGT26	5/ 201-205	inject DNA	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed	tail bleed
3 pGT26	5/ 301-305	inject DNA	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed	tail bleed
4 pGT26	5/ 401-405	inject DNA	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed	tail bleed
5 pGT26	5/ 501-505	inject DNA	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed	tail bleed
6 pGT26	5/ 601-605	inject DNA	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed	tail bleed
7 pGT26	5/ 701-705	inject DNA	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed	tail bleed
8 uninjected	5/ 801-805		100 ul sesame oil	tail bleed	tail bleed
9 uninjected	5/ pool			terminal bleed	

Table 14

Group	plasmid	description	n*
1-7	pGT26	pBRES/mIFN rev	35

*n=number of animals

DNA solutions: Each mouse received 250 ug of plasmid DNA in 150 ul PBS.

DNA delivery: Adult male C57BL/6 mice were injected bilaterally on day 0

with 250 ug plasmid DNA per mouse in 150 ul PBS. The DNA solution was injected 25 ul into

the tibialis muscle and 50 ul into the gastrocnemius muscle of each hind leg, followed by

electroporation with a caliper (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse).

MFP treatment: As indicated in the tables above and below, Groups 1-7

received 100 ul sesame oil alone or with MFP at various concentrations by i.p. injection on

days 7-10.

Table 15: Cycle 1

Group/ plasmid	n*/ mouse #	day 0	day 7-10	day 11	day 18
1 pGT26	5/ 101-105	inject DNA	100 ul sesame oil	tail bleed	tail bleed
2 pGT26	5/ 201-205	inject DNA	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed	tail bleed
3 pGT26	5/ 301-305	inject DNA	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed	tail bleed
4 pGT26	5/ 401-405	inject DNA	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed	tail bleed
5 pGT26	5/ 501-505	inject DNA	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed	tail bleed
6 pGT26	5/ 601-605	inject DNA	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed	tail bleed
7 pGT26	5/ 701-705	inject DNA	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed	tail bleed

8 uninjected	5/ 801-805		100 ul sesame oil	tail bleed	tail bleed
9 uninjected	5/ pool			terminal bleed	

*n=number of animals

Table 16: Cycle 2: Same MFP concentrations as Cycle 1

Group/ plasmid	n*/ mouse #	day 35-38	day 39
1 pGT26	5/ 101-105	100 ul sesame oil	tail bleed
2 pGT26	5/ 201-205	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed
3 pGT26	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed
4 pGT26	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed
5 pGT26	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed
6 pGT26	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
7 pGT26	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed
8 uninject.	5/ 801-805	100 ul sesame oil	tail bleed

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Table 17: Cycle 3: Same MFP concentrations as Cycles 1 and 2

Group/ plasmid	n*/ mouse #	day 63-66	day 67
1 pGT26	5/ 101-105	100 ul sesame oil	tail bleed
2 pGT26	5/ 201-205	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed
3 pGT26	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed
4 pGT26	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed
5 pGT26	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed
6 pGT26	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
7 pGT26	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed
8 uninjected	5/ 801-805	100 ul sesame oil	tail bleed

*n=number of animals

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Re-injection of DNA on Day 77

Each mouse received 250 ug of plasmid DNA in 150 ul PBS.

Table 18

Group	plasmid	n*
2-8	pGT26	35

*n=number of animals

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Table 19: Cycle 4 Same MFP concentrations as Cycles 1-3 for Groups 2-7. Control groups (1 and 8) treated with 0.33 mg/kg MFP.

Group	Day 0/Day 77 pGT26	n*/ mouse #	day 84-87	day 88
1	+/-	5/ 101-105	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
2	+/+	5/ 201-205	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed
3	+/+	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed
4	+/+	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed
5	+/+	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed
6	+/+	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
7	+/+	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed
8	-/+	5/ 801-805	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed

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Table 20: Cycle 5 Same MFP concentrations as Cycles 1-5 for Groups 2-7. Control groups (1 and 8) treated with 0.33 mg/kg MFP.

Group T	Day 0/Day 77 pGT26	n*/ mouse #	day 98-101	day 102
1	+/-	5/ 101-105	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
2	+/+	5/ 201-205	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed
3	+/+	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed
4	+/+	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed
5	+/+	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed
6	+/+	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
7	+/+	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed
8	-/+	5/ 801-805	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed

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Table 21: Cycle 6 Same MFP treatments as in Cycle 5.

Re-injection of DNA on Day 189: Each mouse received 250 ug of plasmid DNA in 150 ul PBS. Group 9 were new control mice, where the age was matched as closely as possible. Group 1 was also injected with plasmid.

Group	Day 0/Day 77 pGT26	n*/ mouse #	day 133-136	day 137
1	+/-	5/ 101-105	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
2	+/+	5/ 201-205	MFP 100 ul of 0.00083 mg/ml = 0.0033 mg/kg	tail bleed
3	+/+	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed
4	+/+	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed
5	+/+	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed
6	+/+	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
7	+/+	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed
8	-/+	5/ 801-805	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed

Table 22

Group	plasmid	n*
3-9	pGT26	35

Table 23: Cycle 7 Same MFP concentrations as Cycles 4-6 for Groups 3-7.

Control groups (1, 2, 8, and 9) were treated with 0.33 mg/kg MFP.

Group	Day 0/77/189 pGT26	n*/ mouse #	day 196-199	day 200
1	+/-/+	5/ 101-105	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
2	+/+/-	5/ 201-205	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
3	+/+/+	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	tail bleed
4	+/+/+	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	tail bleed
5	+/+/+	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	tail bleed
6	+/+/+	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
7	+/+/+	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	tail bleed
8	-/+/+	5/ 801-805	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed
9	-/-/+	5/ 901-905	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	tail bleed

Table 24: Cycle 8 Same MFP concentrations as Cycle 7. Groups 3-7 were terminally harvested, and RNA and DNA prepared from muscle.

Group	Day 0/77/189 pGT26	n*/ mouse #	day 224-227	day 228
3	+/+/+	5/ 301-305	MFP 100 ul of 0.0025 mg/ml = 0.01 mg/kg	terminal bleed and muscles
4	+/+/+	5/ 401-405	MFP 100 ul of 0.0083 mg/ml = 0.033 mg/kg	terminal bleed and muscles
5	+/+/+	5/ 501-505	MFP 100 ul of 0.025 mg/ml = 0.1 mg/kg	terminal bleed and muscles
6	+/+/+	5/ 601-605	MFP 100 ul of 0.083 mg/ml = 0.33 mg/kg	terminal bleed and muscles
7	+/+/+	5/ 701-705	MFP 100 ul of 0.25 mg/ml = 1.0 mg/kg	terminal bleed and muscles

*n=number of animals

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Blood collection and Endpoint Analysis/Assay Procedure: Mice were bled by tail nick or cardiac puncture and the blood was collected in Microtainer tubes (no anti-coagulant). The serum was then separated from the blood, collected, and assayed for IP-10 by ELISA.

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ii. Determination of induction and de-induction kinetics of hIFN from a pBRES AAV vector in vivo: A study was performed in naïve C57BL/6 mice using the hIFN- β pBRES AAV vector AAV-1GT58 to examine the kinetics of induction and de-induction. AAV-1GT58 was injected into hind limb muscles of C57BL/6 mice, the animals were administered MFP by i.p. injection for four consecutive days, during which time they bled at various times after the first MFP injection to determine the induction kinetics. They were then bled at various times after the last MFP injection to determine the de-induction kinetics (see “Experimental Design” below for details). The serum was assayed for hIFN by ELISA. The results show that the induction and de-induction kinetics are rapid, with peaks levels of hIFN reached by 48-72 hr after the first MFP treatment (Figure 37A), and diminishing to background levels within 96 hr after MFP treatment (Figure 37B). This demonstrates that the pBRES system can be rapidly turned on and off in vivo.

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Experimental Design: Normal adult C57BL/6 mice were injected with an AAV1 vector carrying the pBRES/hIFN expression cassette, as follows. For each group, five animals (n=5) were sacrificed at the indicated “Harvest Times” in the table below. Human IFN expression was determined in the serum by ELISA in the absence of MFP, or after single/multiple MFP administration.

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Table 25

Group	N*	Vector	Treatment (MFP)	Harvest Time
1	10	PBS	None	24, 96 hours (post-injection)
2	30	RM-hIFN AAV1	None	7, 14, 21, 28, 35, 42 days (post-injection)
3	25	RM-hIFN AAV1	Day 17 only	1, 3, 6, 12, 24 hours (post-MFP administration)
4	5	RM-hIFN AAV1	Days 16, 17	24 hours (post-MFP administration)
5	5	RM-hIFN AAV1	Days 15, 16, 17	24 hours (post-MFP administration)
6	35	RM-hIFN AAV1	Days 18, 19, 20, 21	6, 12, 24, 36, 48, 72, 96 hours (post-MFP administration)

Virus solutions and delivery: Group 1 mice received 75 ul PBS and Groups 2-6 mice received 5×10^{10} viral particles (vp) per mouse in a volume of 75 ul PBS in one hind leg (right leg). 25 ul was injected into the tibialis muscle and 50 ul injected into the gastrocnemius muscle.

Table 26

Group	vector	Description	n*
1	None		10
2	AAV-1-GT58	RM-hIFN rev in AAV-1	30
3	AAV-1-GT58	RM-hIFN rev in AAV-1	25
4	AAV-1-GT58	RM-hIFN rev in AAV-1	5
5	AAV-1-GT58	RM-hIFN rev in AAV-1	5
6	AAV-1-GT58	RM-hIFN rev in AAV-1	35

*n=number of animals

MFP treatment: Groups 3-6 were administered MFP by i.p. injection at 0.33 mg/kg (100 ul of 0.083 mg/ml in sesame oil, made fresh) according to the following schedule: Group 3 = day 17, Group 4 = days 16 + 17, Group 5 = days 15-17, and Group 6 = days 18-21.

Blood collection and Endpoint Analysis/Assay Procedure: Mice were bled by cardiac puncture (terminal bleed) at the time points indicated in the Table 27. Blood was collected in Microtainer tubes (no anti-coagulant) and the serum was separated from the blood, collected and assayed for hIFN by ELISA.

B. Persistence of Expression and Repeat Administration Studies: The data generated thus far has shown that expression using CMV promoter-based constructs persists for constitutive IFN- β at least 49 days using plasmid DNA and 6 months using an AAV-1

vector. The plan is to repeat and extend these studies using the candidate vector with the pBRES system to demonstrate both persistent, as well as regulatable expression of IFN- β for at least 3 months. These studies can be conducted in naïve C57BL/6 mice.

The pBRES vectors of the present invention can be tested in C57BL/6 mice (or other suitable animals) for repeat biomarker endpoints in response to ELISA or administration using administration of the activator molecule (AM). The presence of neutralizing antibodies against the pBRES vector, expressed therapeutic molecule (e.g., a transgene), regulator molecule (RM) or therapeutic protein, can be monitored. For example, the activator molecule (AM) can be administered chronically as well as in a pulsatile manner to evaluate the ability to maintain expression levels of the therapeutic molecule (TM) over time as well as provide renewable expression levels in an on/off manner with AM dosing.

In one preferred embodiment, the IFN- β pBRES vector of the present invention can be tested in C57BL/6 mice (or other suitable animal) for repeat biomarker endpoints in response to MFP administration or administration of IFN- β . The presence of neutralizing antibodies against the vector, IFN molecule (IFNM) (e.g., a IFN- β transgene), regulator molecule (RM), or IFN- β protein, can be monitored. MFP can be administered chronically as well as in a pulsatile manner to evaluate the ability to maintain IFN- β expression levels over time as well as provide renewable expression levels in an on/off manner with MFP dosing.

i. Kinetics of mIFN induction and de-induction from pBRES plasmid, pulsatile and chronic MFP treatment: A study was performed in naïve C57BL/6 mice using pGT26 to examine the kinetics of induction and de-induction with the mIFN- β pBRES plasmid vector, compare mIFN gene expression in response to pulsatile or chronic administration of MFP, and examine the persistence of gene expression over several months. Constitutive (pGER101, CMV) or inducible (pGT26, pBRES) mIFN expression plasmids were injected with electroporation into the hind limb muscles of 20 mice per group, and five mice of each group were bled at day 7 in the absence of MFP. All 20 of the mice that received pGT26 were treated with MFP on day 7-10 after plasmid injection. To examine the kinetics of de-induction, blood was collected from five mice of each group at each of days 11, 12, 14, 16, and 18 days post-injection. All 20 pGT26 mice then received MFP on days 21-24, and to examine the kinetics of induction, blood was collected from five mice of each group at each of days 22, 23, and 25. (See "Experimental Design" below for details). To examine target gene expression in response to continuous treatment with MFP, mice that received pGT26 were injected with MFP i.p. every day from day 35-50, and five mice from each group were bled during this period on day 35 (before MFP treatment), 39, 42, 45, and 51. To examine gene expression after 3.5 months, mice were bled on day 105, then treated with MFP on day 105-108, and bled on day 109 and 116. Serum samples were assayed for IP-10 as a biomarker for mIFN expression.

The results (Figure 37C) show that induction of IFN expression from the pBRES plasmid upon MFP treatment occurred within 24 hr, and expression decreased to baseline 24-48 hr following peak induction. Expression of mIFN from the BRES system was

higher than that driven by the CMV promoter. All three cycles of mIFN expression over the course of two to three months were at high levels. Continuous MFP treatment resulted in sustained high-level expression of mIFN over two weeks. IP-10 levels after 3.5 months were about one-half to two-thirds less than in earlier MFP cycles, but was still well above the background IP-10 levels and also considerably higher than that generated by expression of mIFN from the CMV promoter, which also decreased over time with about the same kinetics (two-thirds of expression lost after 3.5 months). In total, this experiment demonstrates the capacity of the BRES system for continuous, high-level target gene expression over several months, with the ability to be rapidly turned off and on again multiple times.

Experimental Design: Normal C57BL/6 mice were injected and electroporated with single-vector pBRES and CMV promoter mouse IFN expression plasmids, as follows. IFN expression was monitored for at least several months, using IP-10 as the endpoint biomarker for mIFN activity. MFP treatment and bleeds are designed to determine the kinetics of the "on" and "off" responses.

DNA solutions: Each mouse in all injected groups received 250 ug of plasmid DNA in 150 ul PBS.

Table 27

Group	plasmid	Description	n*
1	pGER101	CMV-mIFN	10
2	pGT26	pBRES/mIFN	20

*n=number of animals

DNA delivery: Adult C57BL/6 mice were injected bilaterally on day 0 with 250 ug plasmid DNA per mouse in 150 ul PBS. 25 ul of the DNA solution was injected into the tibialis muscle and 50 ul was injected into the gastrocnemius muscle of each hind leg, followed by electroporation with a caliper (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse).

MFP treatment: Group 2 was administered MFP by i.p. injection at 0.33 mg/kg (100 ul of 0.083 mg/ml in sesame oil, made fresh) as indicated in the table below.

Schedule: Cycle 1 (day 7-18): Determine the "off" kinetics. Cycle 2 (day 21-25): Determine the "on" kinetics.

Table 28 Cycle 1

Group/ plasmid	n*/ mouse #	day 0	day 7	day 7-10	day 11	day 12
1 CMV-mIFN	10 101-110	inject DNA	tail bleed A (101- 105)		tail bleed B (106- 110)	
2 BRES1- mIFN	20 201-220	inject DNA	tail bleed A (201- 205)	MFP A-D (201-220)	tail bleed B (206- 210)	tail bleed C (211- 215)

*n=number of animals

Table 29 Cycle 2

Group/	day 14	day 16	day	day 21-	day	day	day
--------	--------	--------	-----	---------	-----	-----	-----

plasmid			18	24	22	23	25
1 CMV-mIFN	tail bleed A		tail bleed B		tail bleed A		tail bleed B
2 BRES1- mIFN	tail bleed D (216- 220)	tail bleed A	tail bleed B	MFP A-D	tail bleed C	tail bleed D	tail bleed A

Table 30: Cycle 3 Continuous MFP treatment

Group/ plasmid	day 35	day 35-50	day 39	day 42	day 45	day 51
1 CMV-mIFN	tail bleed B		tail bleed A		tail bleed B	terminal bleed and muscles A
2 BRES1- mIFN	tail bleed A	MFP A-D	tail bleed B	tail bleed C	tail bleed D	terminal bleed and muscles A

Blood collection and Endpoint Analysis/Assay Procedure: Mice were bled by tail nick or cardiac puncture (terminal bleed) at the time points indicated in the table above. Blood was collected in Microtainer tubes (no anti-coagulant) and centrifuged for separation and collection of serum. The serum from Groups 1 and 2 was assayed for IP-10 by ELISA.

C. Bioequivalence Studies: Pharmacokinetic studies can be conducted in normal mice and in one other species, preferably non-human primates, using the candidate vector and pBRES expression system, under delivery conditions, e.g., as established in the studies described above. "Bioequivalence" can be, but is not limited to, e.g., to demonstrate that the present regulated expression system of the present invention provides a superior pharmacokinetic profile for IFN- β gene-based delivery over IFN- β protein delivery in both animal models as defined, but not limited to, e.g., in the table below.

Table 31: Non-Limiting Examples of Bioequivalence Criteria

Criteria	Endpoint
Administration	Clinically feasible for IM delivery
Expression Level	Equal to or greater than *therapeutic level achieved with bolus protein
Persistence of Expression	Greater than 3 months
Repeat Administration	Renewable expression upon repeat administration of vector and inducer
Continuous/Pulsatile Expression	Optimal MFP dose necessary to achieve and maintain *therapeutic level over time; renewable with MFP dosing.

*In a nonlimiting embodiment, "therapeutic level" in an animal model is defined as the systemic level of IFN- β (as determined, e.g., by ELISA and/or biomarker induction) that is equivalent to the level achieved by a therapeutic amount of bolus IFN- β 1a protein administered in humans on a mg/kg basis.

D. Safety/Toxicity Studies: If AAV-1 is selected as the candidate vector

biodistribution studies following i.m. administration of the candidate vector can be performed. The endpoints will include vector DNA and expressed IFN- β RNA and protein distribution to target tissue (muscle), blood, lymph, heart, liver, kidney, lungs, male and female gonads (testis, ovary).

E. Gene-Based Delivery for Treatment of a Disease or Condition: An outcome

from these studies is a gene-based delivery system for delivery of a therapeutic molecule (TM), e.g. a transgene encoding a therapeutic protein, for treatment of a disease or condition. In a preferred embodiment, the present invention provides a regulated expression system of delivery of an IFN- β transgene that will provide long term, regulated expression of IFN- β for the treatment of MS. In a preferred embodiment, an outcome of these studies is that the pBRES vector of the present invention can provide persistent, renewable expression (e.g., greater than 3 months) through the oral administration of the small molecule inducer, MFP; and is capable of repeat administration by intramuscular injection.

i. Characterization of pBRES mIFN plasmid activity in EAE disease

mice: In order to show a biological effect in an animal disease model of MS, a study was performed in SJL mice with active EAE. Constitutive (pGER101, pmIFN) or inducible (pGT26, pBRES/mIFN) mIFN expression plasmids and null plasmid controls were injected with electroporation into the hind limb muscles of SJL mice. EAE was induced in the mice by injection of PLP 139-151/pertussis toxin the day before and after injection of pmIFN, and 7 and 9 days after the injection of pBRES/mIFN. Mice were treated with MFP (0.33 mg/kg) by i.p. injection once per day (d) or every third day (etd) after plasmid injection. Blood was collected at day 5 after injection. PBMCs were isolated from the blood and RNA was prepared from and assayed by RT-PCR to determine the level of Mx1 RNA (See "Experimental Design" below for details). The results show about an 8-fold increase in Mx1 RNA levels with pBRES/mIFN plus daily MFP injections, and no significant increase over a null vector in the absence of MFP (Figure 38). This demonstrates a biological response in an animal disease model similar to that which has been shown to be efficacious in this model.

Experimental Design: Female SJL mice (8 weeks old, Jackson Labs, ~20

g) were distributed into 12 groups (n=13 per group), and each group was injected on Days 1 and 3 with PLP 139-151/pertussis, according to the established murine A-EAE protocol, as follows.

Group 1, Untreated. No treatment control.

Groups 2-3, CMV plasmid plus Electroporation (EP): Two bilateral

intramuscular (im) injections of either Null (pgWiz) or mIFN (pGER101) CMV plasmid DNA into the tibialis (20 μ l) and gastrocnemius muscles (40 μ l), followed immediately by EP on Day 2, as administered.

Groups 4-9, BRES1 Plasmid plus EP: Two bilateral intramuscular (im)

injections of either pBRES Null (pGT4) or pBRES mIFN (pGT26) plasmid DNA into the tibialis (20 μ l) and gastrocnemius muscles (40 μ l), followed by EP one week (Day -7) prior to

initiation of disease, was administered. MFP (0.33 mg/kg) was administered daily or every third day (etd) by ip injection beginning on Day 1. The animals receiving MFP "etd" were dosed on Days 1, 4, 7, 10, 13, 16, 19, and 22.

Groups 10-11, Buffer Control, mIFN Protein: IFN protein buffer or mIFN protein (100,000 units = 500 ng) was administered every other day by sc injection, beginning on Day 1.

Group 12, Prednisolone: Prednisolone, daily bid was administered by intraperitoneal (ip) injection, beginning on Day 1.

Table 32

Group	# of mice	Agent	Dose	Volume/dose
1 Untreated	13	None	-----	-----
2. pNull/EP	13	plasmid	2 x 60 ug	2 x 60 ul im
3. pmIFN/EP	13	plasmid	2 x 60 ug	2 x 60 ul im
4. pBRES Null/EP (-MFP)	13	plasmid	2 x 60 ug	2 x 60 ul im
5. pBRES Null/EP (+MFP daily)	13	plasmid	2 x 60 ug	2 x 60 ul im
6. pBRES Null/EP (+MFP etd)	13	plasmid	2 x 60 ug	2 x 60 ul im
7. pBRES mIFN/EP (-MFP)	13	plasmid	2 x 60 ug	2 x 60 ul im
8. pBRES mIFN/EP (+MFP daily)	13	plasmid	2 x 60 ug	2 x 60 ul im
9. pBRES mIFN/EP (+MFP etd)	13	plasmid	2 x 60 ug	2 x 60 ul im
10. Buffer control	10	buffer	-----	100 ul/inj.* sc
11. mIFN ?protein	10	protein	100,000 U/inj.*	100 ul/inj.*
12. Prednisolone	10	compound	2.5 mg/kg/inj.*	100 ul/inj.* ip

*inj.=injection

Table 33

Group	plasmid	Injection day	DNA (mg)	mg/ml	ml	PBS (ml)	total vol (ml)
2	pNull (pgWiz)	2	1.8	5.32	0.34	1.46	1.8
3	pmIFN (pgWiz/mIFN) (pGER101)?	2	1.8	5.36	0.34	1.46	1.8
4, 5, 6	pBRES Null (pGT4)	-7	1.8 x 3 = 5.4	6.35	0.85	4.55	1.8 x 3 = 5.4
7, 8, 9	pBRES mIFN (pGT26)	-7	1.8 x 3 = 5.4	5.28	1.02	4.38	1.8 x 3 = 5.4

Group 11 mIFN protein :100,000 units (500 ng) /100 ul inj. x 13 animals x 15 injections = 1.95×10^7 units (97.5 ug)/19.5 ml. Stock mIFN solution = 100 μ g/mL or 2×10^7 units/mL. Dilution tubes contained 100 uL x 15 animals = 1.5 mL (a total of 15 dilution tubes were made containing 150 mM NaCl, 50 mM Sodium acetate, pH 5, 5% propylene glycol). 75 μ L of stock solution was added to each tube. Final concentration = 10^6 units/mL .

Endpoint Analysis (Mx1 RNA analysis): Three animals from groups 1-9 were sacrificed on Day 5 and terminally bled for Mx1 RNA analysis (using purple tubes containing EDTA), and the injected muscles harvested for IFN RNA analysis.

F. Production: The process for production of a gene therapy vector of the present invention comprising a pBRES hIFN- β expression cassette can be suitable for cGMP-manufacturing. Using methods described herein or known in the art, the pBRES vectors of the present invention can be made of sufficient purity, potency, and stability to perform preclinical development studies. The gene therapy vectors of the present invention, and preferably the pBRES vectors of the present invention, can be fully characterized with respect to the plasmid backbone, capsid (in the case of AAV as a delivery vector), transgene expression product (IFN- β), and inducer (MFP), using methods described herein or known in the art.

G. Pharmacology: Bioequivalence with protein delivery can be demonstrated in an animal model. Dose/response for vector and inducer or activator molecule (AM) (e.g., small molecule inducer MFP) can be characterized and optimized *in vivo*. Repeat administration and persistence of transgene expression can be fully characterized. Immunogenicity studies can be conducted with the candidate vector.

H. Pharmacokinetics/safety/toxicology: Pharmacokinetic studies of the expressed IFN- β transgene can be conducted using direct detection of the expressed transgene as well as measurement of IFN- β biomarkers. If AAV-1 is the selected candidate vector, biodistribution studies can be performed to examine the fate of the vector DNA and its expression products.

Example 10: Regulated GM-CSF Gene Therapy for Crohn's Disease

A. Vector Selection for Muscle Targeted GM-CSF Gene Delivery and Assay

Validation. Protein therapy for Crohn's disease is limited by the source of the recombinant protein, injection schedule and half life and bioavailability of the protein after injection. Gene delivery of GM-CSF can overcome many of these obstacles by providing controlled expression of the protein according to the needs of the patient. To develop this gene delivery system, plasmids encoding GM-CSF were delivered into mouse skeletal muscles, resulting in therapeutic levels of GM-CSF being expressed and reduced clinical disease symptoms.

i. GM-CSF ELISA validation. A commercially available ELISA (R&D, Minneapolis) was validated for the detection of murine (m)GM-CSF in serum. In a pharmacokinetic study two formulations of mGM-CSF protein, non-pegylated or pegylated, were injected i.m. and serum samples were assayed for mGM-CSF. Both mGM-CSF formulations could be measured in serum, but the kinetics were significantly different (Fig. 40). The levels of non-pegylated mGM-CSF dropped rapidly within 3 hours following administration, whereas pegylated mGM-CSF was detectable over a 12 hour period.

ii. Vector selection for skeletal muscle targeted GM-CSF gene delivery. For GM-CSF gene delivery two vehicles were tested, plasmid DNA and recombinant (r)AAV serotype 1. Both systems are favored for muscle targeted gene delivery (EPrud'homme, et al. (2006) *Curr. Gene Ther.* 6 :243-273; Xiao et al. (1999) *J. Virol.* 73:3994-4003; Liu et al. (2004) *Hum*

Gene Ther. 15:783-92.), and expression kinetics for IFN- β , luciferase and SEAP have been extensively described in the present application.

Plasmid and AAV1 vector systems were generated as previously described by cloning mGM-CSF into the plasmid vector pZac2.1 containing a CMV promoter for constitutive expression. This vector can be used for both the plasmid-based approach and to generate recombinant rAAV1. Briefly, AAV-1-GMCSF shuttle plasmids pGT714 and pGT713 (Figure 30B), encoding mGMCSF or hGMCSF, were constructed by inserting a fragment encoding mGMCSF or hGMCSF into the vector pGENE/V5HisA (Invitrogen). The resulting vectors were named pGT723-GENE/hGMCSF and pGT724-GENE/mGMCSF. A fragment encoding mGM-CSF was then excised from pGT724-GENE/mGMCSF by digesting the vector with KpnI-XbaI. Similarly, a fragment encoding hGM-CSF was excised from pGT723-GENE/hGMCSF by digesting the vector with KpnI-XbaI. The vector pZac2.1 was digested with KpnI-XbaI and treated with calf intestinal phosphatase (CIP) and then the excised fragment encoding either mGMCSF or hGMCSF was inserted into pZac2.1 at the KpnI-XbaI site. The resulting shuttle plasmids were named pGT713 (pZac2.1-CMV-hGMCSF) and pGT714 (pZac2.1-CMV-mGMCSF) (Figure 30B). Due to species specificity, only the murine GM-CSF (pGT714) was used for further efficacy studies in mice.

iii. Pharmacokinetic of mGM-CSF administered as a plasmid. To establish that measurable levels of mGM-CSF could be generated in vivo, pGT714 (pmGMCSF) was tested in C57BL/6 mice. 200 μ g of the plasmid was delivered either in combination with electroporation or formulated with PINC, a poloxamer that is described to be able to enhance transfection efficiency (Mumper et al. (1998) *J Controlled Release* 52:191-203). In summary, higher transgene expression levels were achieved by using electroporation compared to the PINC formulation, but the kinetics were similar (Fig. 41). After electroporation, mGM-CSF protein levels were high on day 1 and 2 but dropped to very low levels within a period of 1-2 weeks. The study measured mGM-CSF protein concentration in serum as well as the amount of pmGMCSF DNA and RNA expression in the muscle. The DNA and RNA kinetics followed the pmGMCSF protein kinetics in the serum, with higher levels following electroporation compared to PINC (data not shown).

Biomarker activities for GM-CSF, such as spleen weight (Pojda et al. (1989) *Exp Hematol.* 17:1100-4), number of neutrophils (Kato et al. (2006) *Int. J. Hematol.* 84:205-209), or Mac3 expression on macrophages (Burke et al. (2004) *Clin. Diag. Lab. Immunol.* 3:588-598), indicated the presence of biological active mGM-CSF following gene delivery (data not shown).

iv. Optimization of GM-CSF Expression In Vivo Various factors influence transgene expression, such as dose of the transfected plasmid, MFP treatment protocol, nature of DNA (such as plasmid backbone) and transfection conditions. The present constitutive and regulatable expression systems have been extensively studied expressing IFN- β and other transgenes as described previously. The same optimization experiments may be carried out using GM-CSF as the transgene.

Dose response: Various doses of pmGMCSF were injected in to the murine gastrocnemus and tibialis muscles, followed by electroporation. Serum samples were obtained at day 1, 2, 4 and 7. Levels of mGM-CSF were measured by ELISA as previously described (Fig 42). Although a dose response was shown correlating to the amount of plasmid administered, no difference in the duration of transgene expression was observed.

MFP treatment protocol: Properly timed MFP treatment protocol is critical for GM-CSF expression. It is known that electroporation causes short term inflammation. Among the factors that influence the strength of inflammation are the electroporation conditions, the type of vector DNA or the transgene itself. GM-CSF is known as part of the innate immune system activating neutrophils and macrophages. Using the constitutive CMV promoter GM-CSF expression was initiated directly after DNA injection, which may have impacted the strength of inflammation caused by electroporation. Regulated expression offers the possibility to unlink the innate immune response, caused by electroporation from the beginning of the GM-CSF expression. The data obtained so far indicate, that the time between electroporation and the onset of GM-CSF expression induced by MFP is critical for the expression level as well as the duration of expression. Therefore, a kinetic study to explore the optimal time point to start the MFP treatment and initiate GM-CSF expression is performed. Additionally, formulations of MFP that do not contain sesame oil, such as MFP pellets discussed previously, are tested to prevent adverse effects on the disease model.

Plasmid backbone: The pBRES plasmid containing GM-CSF is optimized in regard to the number of methylation sites that might impact levels and duration of GM-CSF expression. There is increasing evidence that plasmid DNA has an adjuvant activity, caused by DNA methylation, that might influence the duration of expression and lead eventually to lower expression levels (Kato et al. (2006) *Int. J. Hematol.* 84:205-209). The effect of methylated plasmid DNA on the expression kinetics is evaluated to determine the effect of an optimized plasmid backbone with a reduced number of methylation sites on the expression of GM-CSF.

v. Pharmacokinetic of mGM-CSF administered as an AAV vector. AAV1-mGM-CSF was produced by the Penn Vector Core of the University of Pennsylvania based on pGT714 as previous described. Biological activity of the recombinant AAV1 was confirmed in vitro, in HEK 293 cells (data not shown). The in vivo testing was done in the same way as described for the plasmid vector. AAV1-mGMCSF (5×10^{10} virus particles/mouse) were injected i.m., serum samples were obtained over a period of 3 months and tested for the presence of mGM-CSF. In contrast to the plasmid based delivery, following injection of AAV1-mGMCSF no mGM-CSF could be detected in the serum, neither at very early time points nor at later time points (data not shown).

vi. DSS-induced colitis model. Prior to the gene delivery study the model was established and validated in Balb/C mice using non-pegylated and pegylated mGM-CSF protein. Disease was induced in Balb/C mice with 5% dextran sodium sulfate (DSS), provided in the drinking water every day over a period of 8 days. As the main endpoint, the Disease

Activity Index (DAI, a cumulative index of: weight loss, stool consistency, and rectal bleeding) was recorded daily. Mesopram and Metronidazole were used as positive controls for the model, and were administered IP daily (50 mg/kg). mGM-CSF was also injected daily, using 5 µg of non-pegylated protein, and 5 or 10 µg of the pegylated protein. The DAI score was significantly reduced in mice that were treated with Mesopram or Metronidazole, as well as in those mice that were treated with the non-pegylated respectively pegylated mGM-CSF (Fig. 43A).

vii. Proof of concept study using pCMV-GMCSF plasmid DNA in the DSS-induced colitis model. In this study the efficacy of pGMCSF plasmid DNA was evaluated side by side with Mesopram, Metronidazole, non-pegylated or pegylated mGM-CSF. The disease was induced using 7% DSS, administered daily in the drinking water. Mesopram, Metronidazole and mGM-CSF protein were administered daily, using the same dosing regimen as described for the validation study. pGMCSF plasmid DNA was injected at day 0 followed by electroporation. An additional group was treated in the same way, but received a null-plasmid, without transgene (pNull) instead of pGMCSF.

At day 8, the final day of the study, mGM-CSF concentrations were measured in sera of all mice. GM-CSF could be measured in those mice that received the GM-CSF protein as well as in mice that received pGMCSF, but was not detectable in mice that received the pNull, Mesopram or Metronidazole (data not shown).

Significantly decreased disease scores were observed in mice that received pCMV-GMCSF at both concentrations evaluated, but not in those that were injected with the pNull control vector (Fig. 43B). Treatment was also efficacious in mice that were injected with Metronidazole or Mesopram, and mGM-CSF protein, non-pegylated or pegylated (Fig. 43A).

The efficacy measured following gene delivery was close to that determined for mice that received mGM-CSF protein, independent of the protein formulation. The p-values suggested that gene delivery using pCMV-GMCSF was slightly more efficacious than the protein treatment, although this difference did not reach statistical significance.

viii. Regulated GM-CSF expression. As described previously, regulatable pBRES plasmids encoding GM-CSF were constructed in all four possible orientations of the transgene relative to the promoter to determine the most effective orientation (Fig. 45). Activity of the four plasmids was confirmed in vitro (Fig. 44A), and the plasmid that showed the best ratio of peak versus basal expression level in vitro, pGT618, was tested in vivo. C57BL/6 mice were injected with the plasmid followed by electroporation and treated with the inducer MFP for 3 days, starting from day 4. Blood samples obtained at day 4 did not contain any mGM-CSF, whereas those samples obtained at day 6 contained approximately 180 pg/ml mGM-CSF in average (Fig. 44B). The experiment showed that mGM-CSF expression can be regulated using the pBRES system.

MATERIALS AND METHODS

A. Efficacy of Gene-Based Delivery of Murine IFN- β Protein in Mouse Acute

EAE: Seventy 8-week old female SJL mice from Jackson Labs were immunized with a 0.1 ml SC (divided between base of tail and upper back) injection containing 150 ug Proteolipid Protein (PLP)139-151 in Incomplete Freund's Adjuvant (IFA) supplemented with 200 ug *M. tuberculosis* H37Ra. This emulsion was obtained by mixing saline containing 3 mg/ml PLP 1:1 with IFA containing 4 mg/ml ground *M. tuberculosis*. Immediately after immunization, all mice received a 0.1 ml IP injection of pertussis toxin. Two days after immunization (day 3 of study), all mice received a second IP injection of pertussis toxin.

Mice treated with IFN- β protein or its vehicle (20 nM NaAc, pH 5.5, 150 mM NaCl, 5% propylene glycol) were dosed with 0.1 ml, SC once every other day beginning on the day of immunization until the end of the study. The positive controls used for this study were 9 mg/kg Mesopram (ZK-117137) and 2.5 mg/kg Prednisolone. Both controls use a dose volume of 0.1 ml/injection and are administered IP, twice daily, beginning on the morning of immunizations until the end of the study.

Experimental Groups (n=10):

1. Vehicle
2. 10 K units murine IFN- β
3. 20 K units murine IFN- β
4. 30 K units murine IFN- β
5. 100 K units murine IFN- β
6. Mesopram, 9 mg/kg IP
7. Prednisolone, 2.5 mg/kg IP

Clinical Scoring of Mouse Acute EAE: The mice were scored daily based on the following scoring system:

- 0 = normal
- 1 = limp tail
- 2 = difficulty righting
- 3 = incomplete paralysis of one or both hind limbs
- 4 = complete paralysis of one or both hind limbs, or hind limbs mobile but drag
- 5 = complete paralysis of both hind limbs & weakness/paralysis of forelimbs, moribund, or dead

Moribund mice were euthanized. One half scores are added to mice exhibiting borderline clinical symptoms. Mice treated with 100K units of IFN- β developed significantly decreased clinical scores of EAE compared with vehicle treated mice ($p=0.0046$). Mice treated with 30K units of IFN- β also developed decreased clinical scores compared to vehicle treated mice, although this decrease did not reach statistical

significance. The positive controls in this study, Mesopram and Prednisolone, also significantly decreased clinical scores. See Example 5 and Figure 13.

B. Efficacy of Gene-Based Delivery of Murine IFN- β in Mouse Acute EAE:

One hundred thirty 8-week old female SJL mice from Jackson Labs were immunized with a 0.1 ml SC (divided between base of tail and upper back) injection containing 150 ug Proteolipid Protein (PLP)139-151 in Incomplete Freund's Adjuvant (IFA) supplemented with 200 ug *M. tuberculosis* H37Ra. This emulsion was obtained by mixing saline containing 3 mg/ml PLP 1:1 with IFA containing 4 mg/ml ground *M. tuberculosis*. Immediately after immunization, all mice received a 0.1 ml IP injection of pertussis toxin. On day 2, mice in the plasmid + electroporation groups received appropriate intramuscular injections followed immediately by electroporation. Mice in the plasmid + PINC groups also received the appropriate intramuscular injections. Two days after immunization (day 3 of study) all mice received a second 0.1 ml IP injection of pertussis toxin. On day 5, mice in the plasmid + PINC groups received the same treatment as on day 2.

Mice treated with IFN- β protein or its vehicle (20 nM NaAc, pH 5.5, 150 mM NaCl, 5% propylene glycol) were dosed with 0.1 ml, sc once every other day beginning on the day of immunization until the end of the study. The positive controls used for this study were 9 mg/kg Mesopram (ZK-117137) and 2.5 mg/kg Prednisolone. Both controls use a dose volume of 0.1 ml/injection and both controls are administered IP, twice daily, beginning on the morning of immunizations until the end of the study.

There were a total of 10 groups in this study. Each group had 13 mice. The last 3 mice in each group were bled via tail nick on day 6 of the study for Mx 1 RNA analysis. The same 3 animals that were bled on day 6 were bled via cardiac puncture on day 13 of the study for Mx1 RNA analysis from PBMC's, and injected muscles were collected for analysis.

Experimental Groups (n=13):

1. PBS control
2. pNull + EP
3. pmIFN- β + EP
4. pNull + PINC
5. pmIFN- β + PINC
6. IFN- β protein (100K units) SC
7. Vehicle, SC
8. Mesopram, 9 mg/kg IP
9. Prednisolone, 2.5 mg/kg IP
10. Untreated

Clinical Scoring of EAE: The mice were scored daily based on the following scoring system:

0 = normal

1 = limp tail

2 = difficulty righting

3 = incomplete paralysis of one or both hind limbs

4 = complete paralysis of one or both hind limbs, or hind limbs mobile but drag

5 = complete paralysis of both hind limbs & weakness/paralysis of forelimbs, moribund, or dead

Moribund mice are euthanized. One half scores are added to mice exhibiting borderline clinical symptoms. Mice treated with 100K units of murine IFN- β protein had significantly decreased clinical scores of EAE, compared to the vehicle control treated mice ($p=0.045$). Gene delivery of the murine IFN- β + EP also significantly decreased clinical scores, compared to gene delivery of pNull & EP ($p=0.0171$). Gene delivery using the PINC formulation of IFN- β did not statistically decrease clinical scores compared to pNull & PINC. Both Mesopram and Prednisolone, the positive controls for the EAE model, significantly decreased clinical scores.

C. Regulated Expression of mIFN- β *In Vivo*

IFN15-GS5: In vivo transfection of pBRES/IFN plasmids: Mifepristone-regulated mIFN- β expression was demonstrated from a pBRES/mIFN- β plasmid electroporated into mouse muscle.

Experimental Design: Normal C57BL/6 mice can be injected and electroporated with a pBRES single vector of the present invention and control plasmid DNAs as described in Tables 34 and 35 below.

Table 34

plasmid	Description	date	ug/ul
pGT4	Empty pBRES vector. Negative control for pGT26.	4/23/04	6.35
pGT26	RM/mIFN- β reverse. Experimental pBRES/mIFN- β plasmid.	4/14/04 4/16/04	4.73 4.80
pGER101	pgWiz/mIFN- β (CMV/mIFN- β). Positive control for mIFN- β expression.	2/18/04	5.73
pGT31	SEAP/RM. Positive control for RM function.	4/23/04	5.78

Table 35 **Groups (n = 5/group)**

Group	plasmid	time points		
		day 7	day 11	day 18
1	none	– MFP		
2	pGT4	– MFP	+ MFP	
3	pGT26	– MFP		
4	pGER101	– MFP		
5	pGT26		– MFP	
6	pGT26		+ MFP	
7	pGER101		– MFP	
8	pGT26		– MFP	– MFP
9	pGT26		+ MFP	– MFP
10	pGER101		– MFP	– MFP
11	pGER101		+ MFP	
12	pGT31	– MFP	+ MFP	– MFP
13	none		– MFP	

mIFN- β expression can be assayed by biomarkers and RNA levels in muscle at 3 time points. On day 7 after DNA injection Groups 1, 3, and 4 can be terminally harvested and Group 2 can be tail bled to determine uninduced background mIFN- β expression and biomarker activity in mice receiving GS/mIFN – MFP (Group 3) in comparison to uninjected mice (Group 1) and mice receiving empty pBRES vector (Group 2). CMV/mIFN (Group 4) serves as a positive control. Group 12 can be tail bled to determine uninduced background levels of SEAP expression.

Mice in Groups 2, 6, 9, 11, and 12 can be treated with MFP on days 7-10 after DNA injection. On day 11, Groups 5-7 can be terminally harvested to determine induced mIFN expression and biomarker activity in mice receiving RM/mIFN + MFP (Group 6) in comparison to mice receiving CMV/mIFN (Group 7). Uninduced levels in mice receiving RM/mIFN – MFP (Group 5) will also be assayed. Group 2 (empty pBRES vector) can be terminally bled to determine whether MFP stimulates the biomarker response. Groups 8-10 can be tail bled to determine if biomarker activity is detectable from small volumes of blood and to provide an induced time point in the same mice with which to compare the uninduced levels on day 18. Group 11 (CMV/mIFN + MFP) can be terminally harvested to determine whether MFP affects mIFN expression or inhibits the biomarker response. Group 12 can be tail bled to determine induced levels of SEAP expression. Group 13 will provide a negative control for SEAP expression.

On day 18, eight days after the last MFP treatment, Groups 8-10 can be terminally harvested to determine if the mIFN- β RNA levels and biomarker activity in the RM/mIFN - /+/- MFP group (Group 9) have returned to baseline in comparison to RM/mIFN mice that never received MFP (Group 8). CMV/mIFN (Group 10) again serves as a positive control.

Group 12 can be terminally bled to determine if SEAP expression has returned to baseline.

D. Reagents

DNA solutions: Each mouse in Groups 2-11 can receive 250 ug of plasmid DNA in 150 ul PBS. Each mouse in Group 12 can receive 25 ug of plasmid DNA in 150 ul PBS (see Table 36 below).

Table 36: Prepare DNA solutions for 5 mice/group plus extra

Group	Plasmid	# of mice	Prep solution for	mg DNA	mg/ml	DNA	total ml	PBS
2	pGT4	5	8 mice	2.0	6.35	315 ul	1.2	0.89 ml
3, 5, 6, 8, 9	pGT26	25	32 mice	8.0	4.73 4.80	1.2 ml 0.48 ml	4.8	3.12 ml
4, 7, 10, 11	pGER10 1	20	25 mice	6.25	5.73	1.09	3.75	2.66 ml
12	pGT31	5	8 mice	0.2	5.78	35 ul	1.2	1.17 ml

E. Animal Procedure

1) DNA delivery (Groups 2-12): Adult male C57BL/6 mice (5 per group) can be injected bilaterally on day 0 with 250 ug (Groups 2-11) or 25 ug (Group 12) plasmid DNA per mouse in 150 ul PBS. The DNA solution can be injected 25 ul into the tibialis muscle and 50 ul into the gastrocnemius muscle of each hind leg, followed by electroporation with a caliper (8 pulses at 200 V/cm, 1 Hz, 20 msec/pulse).

2) MFP treatment (Groups 2, 6, 9, 11, and 12): Mice in Groups 2, 6, 9, 11, and 12 can be administered MFP by oral gavage at 0.33 mg/kg (100 ul of 0.083 mg/ml in sesame oil, made fresh) on days 7 through 10 post-injection as indicated in Table 37 below. Group 12 mice can be bled prior to MFP treatment on day 7.

Table 37

Group	day 7	day 8	day 9	day 10	day 11	day 18
1) uninjected	Terminal bleed + muscles					
2) empty vector (pGT4)	tail bleed, then MFP	MFP	MFP	MFP	terminal bleed + muscles	

3) RM/mIFN (pGT26) - MFP	Terminal bleed + muscles					
4) CMV/mIFN (pGER101)	Terminal bleed + muscles					
5) RM/mIFN (pGT26) - MFP					terminal bleed + muscles	
6) RM/mIFN (pGT26) -/+ MFP	MFP	MFP	MFP	MFP	terminal bleed + muscles	
7) CMV/mIFN (pGER101)					terminal bleed + muscles	
8) RM/mIFN (pGT26) - MFP					tail bleed	terminal bleed + muscles
9) RM/mIFN (pGT26) -/+/- MFP	MFP	MFP	MFP	MFP	tail bleed	terminal bleed + muscles
10) CMV/mIFN (pGER101)					tail bleed	terminal bleed + muscles
11) CMV/mIFN (pGER101) -/+ MFP	MFP	MFP	MFP	MFP	terminal bleed + muscles	
12) SEAP/RM (pGT31)* -/+/- MFP	tail bleed, then MFP	MFP	MFP	MFP	tail bleed	terminal bleed
13) uninjected					terminal bleed	

*pGT31 was constructed by digestion of pGER75 (CMV/SEAP) with Nhe I and Not I, and insertion of the resulting fragment carrying the SEAP gene between the Spe I and Not I sites of pGT1.

3) Harvest of blood and muscle (Groups 1-11): On the appropriate day after DNA injection as indicated in Table 6 above, mice can be tail bled or terminally bled. When mice are terminally bled, the injected muscles can be collected.

Blood. Blood can be collected into Microtainer tubes (containing EDTA) at RT and then PBMCs can be separated and collected. The leftover plasma can be stored at -20 °C for cytokine assays.

Muscle: The injected muscles of both legs can be harvested, pooled together, and cut into pieces no larger than 5 mm on one side. Approximately one-fourth of the chopped muscle can be placed into 1.5 ml of RNA-Later solution in a 2 ml tube. The remainder of the muscle can be stored at -70 °C. The DNA and RNA can be extracted from the muscle samples in RNA-Later solution. The samples can be stored at 4 °C for at least 24 h and then transferred to -20 °C if they can be stored for more than 5 days.

Blood (Groups 12 and 13): Mice in Group 12 can be tail bled on day 7 and day 11 and terminally bled on day 18 into yellow Microtainer tubes (no anti-coagulant). The mice can be bled prior to MFP treatment on day 7. Mice in Group 13 can be terminally bled on day 11 into yellow Microtainer tubes (no anti-coagulant).

4) Endpoint Analysis/Assay Procedure

Results of Biomarker Assays: The Mx1 RNA and both chemokines (IP-10 and JE) showed little or no activity with pBRES-mIFN- β (pGT26) in the absence of MFP at 7 days. All biomarkers were strongly induced, to levels higher than with CMV-mIFN- β , in the presence of MFP at 11 days. At 18 days, in the absence of MFP, the chemokine levels had returned to baseline and the Mx1 RNA had decreased nearly to baseline. See Figures 18 and 19.

Mx1 RNA from PBMC: RNA can be prepared from the separated PBMC's and assayed for Mx1 RNA by TaqMan.

JE and IP-10 protein from plasma: The plasma can be assayed for JE and IP-10 cytokines by ELISA.

mIFN- β RNA from muscle: RNA can be prepared from the injected muscles and assayed for mIFN- β RNA by TaqMan.

Plasmid DNA from muscle: DNA can be prepared from the injected muscles and assayed for plasmid DNA by TaqMan. Primers and probe specific for the CMV promoter can be used for DNA from Groups 4, 7, 10, and 11. Primers and probe specific for the GAL-4 DNA binding domain of the regulator protein can be used for Groups 2, 3, 5, 6, 8, and 9.

SEAP protein from serum: The serum can be assayed for SEAP expression by the chemiluminescent activity assay, using the serum from the Group 13 mice as a diluent.

F. Construction of Plasmid Vectors

pGER101 (pgWiz/mIFN): The mouse IFN- β (mIFN- β) gene was amplified by PCR from the plasmid vector pbSER189 (Figure 20A) with the mIFN signal sequence placed on the 5' primer and Sal I and Not I restriction enzyme sites added at the 5' and 3' ends. The

fragment was digested with Sal I and Not I and inserted into the Sal I and Not I sites of plasmid vector pgWIZ (Figure 20B) resulting in plasmid vector pGER101 (Figure 20C).

pGER125 (pgWiz/hIFN): The human IFN- β (hIFN- β) gene was amplified by PCR from plasmid vector pbSER178 with the hIFN signal sequence replaced on the 5' primer and Sal I and Not I restriction enzyme sites added at the 5' and 3' ends. The fragment was digested with Sal I and Not I and inserted into the Sal I and Not I sites of plasmid vector pgWIZ resulting in plasmid vector pGER125 (Figure 21).

pGene/V5-HisA: Plasmid vector was purchased from Invitrogen and contains 6 GAL-4 binding sites upstream of a minimal promoter (E1b TATA), a 5' untranslated region (UTR) that is UT12 derived from CMV, a synthetic intron 8 (IVS8), a multiple cloning site (MCS) and the bovine growth hormone (bGH) poly(A) site. Genes inserted at the MCS can be regulated by a regulator molecule (RM). For example, a gene inserted at the MCS can be induced by the activated form of the modified progesterone receptor (e.g. comprising the amino acid sequence of SEQ ID NO: 22 or encoded by the nucleic acid sequence of SEQ ID NO: 21) upon binding of the activated RM to the GAL-4 sites (Figure 22).

pGene-mIFN (pGER127): Plasmid vector pGER101 was digested with Sal I, filled in with Klenow, ligated to Hind III linkers, and digested with Hind III and Not I. The mIFN- β gene fragment was inserted into the Hind III and Not I sites of plasmid vector pGene/V5-HisA resulting in plasmid vector pGene-mIFN (Figure 23).

pGene-hIFN (pGER129): Plasmid vector pGER125 was digested with Sal I, filled in with Klenow, ligated to Hind III linkers, and digested with Hind III and Not I. The mIFN- β gene fragment was inserted into the Hind III and Not I sites of plasmid vector pGene/V5-HisA resulting in plasmid vector pGene-hIFN (pGER129) (Figure 24).

pSwitch: This plasmid vector was purchased from Invitrogen and encodes the modified progesterone receptor (e.g. comprising the amino acid sequence of SEQ ID NO: 22 or encoded by the nucleic acid sequence of SEQ ID NO: 21) linked to an autoinducible RM-responsive promoter (4XGAL-4 DNA binding sites and thymidine kinase (tk) promoter) upstream of 5' untranslated region 12 (UT12) derived from CMV and synthetic intron 8 (IVS8) driving expression of the gene for the RM protein (Figure 25).

pGS1694: Plasmid vector pGS1694 was provided by Valentis and contains the chicken skeletal muscle actin promoter (sk actin pro), 5' untranslated region 12 (UT12) and synthetic intron 8 (IVS8) driving expression of the gene encoding the modified progesterone receptor (e.g. comprising the amino acid sequence of SEQ ID NO: 22 or encoded by the nucleic acid sequence of SEQ ID NO: 21) (Figure 26).

pLC1674: Plasmid vector pLC1674 was provided by Valentis and contains a "RM-responsive" promoter (i.e., a promoter responsive to the activated form of the modified progesterone receptor (e.g. comprising the amino acid sequence of SEQ ID NO: 22 or encoded by the nucleic acid sequence of SEQ ID NO: 21), 5' untranslated region 12 (UT12) and synthetic intron 8 (IVS8) driving expression of the gene encoding the firefly luciferase gene (luc) (Figure 27).

G. Construction of Vectors for Producing Virus

Vectors for producing virus (e.g., shuttle plasmids) and methods of producing virus (e.g., AAV-1 virus) are known in the art and can be used to produce the virus of the present invention. In some embodiments, the viruses of the present invention are produced from shuttle plasmids (e.g., see Table 38) and used for the delivery and expression of a molecule of the present invention (e.g., a TM and/or RM encoded by a sequence contained in the vector) in the cells of a subject, for treatment of disease.

pGT2/mGMCSF and pGT/hGMCSF: Shuttle plasmids pGT2/mGMCSF and pGT/hGMCSF were constructed as follows (Figure 28). A fragment encoding mouse GMCSF (mGM-CSF) was excised from pORF9-mGMCSF (Figure 30) by digesting the vector plasmid with *AgeI* and *NheI*. This fragment was then blunted. Similarly a fragment encoding human (hGM-CSF) was excised from pORF-hGMCSF (Figure 30) by digesting with the vector plasmid with *SgrAI* and *NheI*. This fragment was then blunted. The excised and blunted fragment encoding either mGMCSF or hGMCSF was inserted into the *EcoRV* site of pGT2 vector plasmid. The orientation of the insert was then checked by restriction digest mapping. The resulting shuttle plasmids were named pGT2/mGMCSF (encoding mouse GMCSF) and pGT2/hGMCSF (encoding human GMCSF) (Figure 28).

pZac2.1-RM-hGMCSF and pZac2.1-RM-mGMCSF: Shuttle plasmids pZac2.1-RM-hGMCSF and pZac2.1-RM-mGMCSF were constructed as follows (Figure 29A). A fragment encoding a mouse GMCSF was excised from the plasmid pORF9-mGMCSF (Figure 30) by digesting the plasmid with *AgeI* and *NheI* and blunted, and the resulting blunted fragment inserted into the *EcoRV* site of the plasmid pGT2, resulting in the plasmid pGT2/mGMCSF. Similarly, a fragment encoding human GMCSF was excised from the plasmid pORF-hGMCSF (Figure 30) by digesting the plasmid with *SgrAI* and *NheI* and blunted, and the resulting blunted fragment inserted into the *EcoRV* site of the plasmid pGT2, resulting in the plasmid pGT2/hGMCSF. The inserts in the resulting vector plasmids were each checked and verified by restriction digest mapping.

The vector plasmids pGT2/hGMCSF and pGT2/mGMCSF were then each digested with *FseI* and *SrfI*. These pBRES-GMCSF fragments were then blunted. The plasmid vector pZac2.1 was digested with *BglII* and *ClaI*, and blunted. The blunted pBRES-GMCSF fragments were each ligated to a blunted pZac2.1 vector. Positive clones were verified by restriction digests. The resulting shuttle plasmids were named pZac2.1-RM-hGMCSF (encoding human GMCSF) and pZac2.1-RM-mGMCSF (encoding mouse GMCSF) (Figure 29A).

pZac2.1-CMV-mGMCSF and pZac2.1-CMV-hGMCSF: Shuttle plasmids pZac2.1-CMV-mGMCSF and pZac2.1-CMV-hGMCSF were constructed as follows (Figure 29B). A fragment encoding a human GMCSF and a fragment encoding a mouse GMCSF were each separately cloned into the plasmid vector pGENE/V5HisA (Invitrogen) resulting, respectively, in the plasmids pGT723-GENE/hGMCSF (encoding human GMCSF) and pGT724-GENE/mGMCSF (encoding mouse GMCSF). A fragment encoding mouse GMCSF was

- excised from pGT724/mGMCSF by digesting the plasmid with KpnI and XbaI, and a fragment encoding human GMCSF was excised from pGT723/hGMCSF by digesting the plasmid with KpnI and XbaI. The resulting fragments were each separately inserted into the KpnI/XbaI site of the vector pZac2.1 that had been digested with KpnI and XbaI, and treated with calf alkaline phosphatase (CIP). The resulting shuttle plasmids were named pGT713 (or pZac2.1-CMV-hGMCSF) encoding human GMCSF and pGT714 (or pZac2.1-CMV-mGMCSF) encoding mouse GMCSF (Figure 29B).

Additional shuttle plasmids were constructed as described in Table 38 below.

Table 38

Shuttle Plasmid	Description of Shuttle Plasmid	Construction of Shuttle Plasmid for Producing AAV-1 Virus
pGT61	AAV-1 shuttle plasmid encoding mIFN- β operably linked to a CMV promoter	The SpeI-Ascl fragment of pGT26 encoding mIFN- β gene was inserted into the NheI-MluI site of pZAC2.1, resulting in the shuttle plasmid pGT61 that produces the AAV-1GT61 virus.
pGT62	AAV-1 shuttle plasmid encoding hIFN- β operably linked to a CMV promoter	The SpeI-Ascl fragment of pGT30 encoding hIFN- β gene was inserted into NheI-MluI site of pZAC2.1, resulting in the shuttle plasmid pGT62 that produces the AAV-1GT62 virus.
pGT54	AAV-1 shuttle plasmid containing pBRES encoding mIFN- β	The Swal-SbfI fragment of pGT53 was ligated to the Swal-SbfI fragment of pGT26 containing the BRES sequence, resulting in the shuttle plasmid pGT54 that produces the AAV-1GT54 virus.
pGT57	AAV-1 shuttle plasmid containing pBRES encoding hIFN- β	The FseI-SwaI fragment of pGT53 was ligated to the FseI-SrfI fragment of pGT28 containing the BRES sequence, resulting in the shuttle plasmid pGT57 that produces the AAV-1GT57 virus.
pGT58	AAV-1 shuttle plasmid containing pBRES encoding hIFN- β	The Swal-SbfI fragment of pGT53 was ligated to the Swa-SbfI/ fragment of pGT30 containing BRES sequence, resulting in the shuttle plasmid pGT58 that produces the AAV-1GT58 virus.
pGT714	AAV-1 shuttle plasmid encoding mGMCSF operably linked to a CMV promoter	The fragment encoding mGMCSF from the plasmid vector pORF9-mGMCSF (Invitrogen) (Figure 30) was inserted into the multicloning site of pZAC2.1, resulting in the shuttle plasmid pGT714 (Figure 29B, pZac2.1-CMV-mGMCSF) that produces the AAV-1GT714 virus.
pGT713	AAV-1 shuttle plasmid encoding hGMCSF operably linked to a CMV promoter	The fragment encoding hGMCSF from the plasmid vector pORF9-hGMCSF (Invitrogen) (Figure 30) was inserted into the multicloning site of pZAC2.1, resulting in the shuttle plasmid pGT713 (Figure 29B, pZac2.1-CMV-

		hGMCSF) that produces the AAV-1GT713 virus.
pGT716	AAV-1 shuttle plasmid containing pBRES mGMCSF	A blunt ended fragment encoding mGMCSF was inserted into the EcoRV site of pGT2 resulting in the plasmid vector pGT712, and the FseI-SrfI fragment of pGT712 containing pBRES-mGMCSF was blunt ended and inserted into pZAC2.1, resulting in the shuttle plasmid pGT716 (SEQ ID NO: 42) that produces the AAV-1GT716 virus (see Figure 31B).
pGT715	AAV-1 shuttle plasmid containing pBRES hGMCSF	A blunt ended fragment encoding hGMCSF was inserted into the EcoRV site of pGT2 resulting in the viral vector pGT711, and the FseI-SrfI fragment or pGT711 containing pBRES-hGMCSF was blunt ended and inserted into pZAC2.1, resulting in the shuttle plasmid pGT715 (SEQ ID NO: 41) that produces AAV-1GT715 virus (see Figure 31A).
pTR-mIFN- β	AAV-1 shuttle plasmid encoding mIFN- β operably linked to a CMV promoter	The blunted HincII-BsrBI fragment of pgWIZ/WT IFN encoding mIFN- β was inserted into the blunted AgeI-Sall site of pTRReGFP, resulting in the shuttle plasmid pTR-mIFN- β (SEQ ID NO: 43) that produces the pTR-mIFN- β virus, resulting in the shuttle plasmid pTR-mIFN- β that produces AAV-1TR-mIFN- β virus.
pTR-hIFN- β	AAV-1 shuttle plasmid encoding hIFN- β operably linked to a CMV promoter	The blunted HincII/NotI fragment of pgWIZ/hIFNb encoding hIFN- β was inserted into the blunted AgeI-Sall site of pTRReGFP, resulting in the shuttle plasmid pTR-hIFN- β (SEQ ID NO: 44) that produces AAV-1TR-hIFN- β virus.
pGER75	AAV-1 shuttle plasmid encoding SEAP operably linked to CMV promoter	A fragment encoding SEAP was amplified via PCR using pSEAP2 DNA (Clontech) as a template and the amplified fragment was inserted into the NheI-XbaI site of the vector phRL-CMV (Promega), resulting in the shuttle plasmid pGER75 that produces AAV-1GER75 virus.

In Table 38 above, for the AAV-1-pBRES constructs, the pZAC2.1 shuttle plasmid was modified at the MCS resulting in shuttle plasmid pGT53, in order to enable the insertion of the fragment containing the BRES sequence into the vector. To insert the fragment containing the BRES sequence into pGT53, the appropriate pGT plasmid (as described above in Table 8) was digested with restriction enzymes that resulted in a fragment containing the entire BRES sequence encoding the respective IFN, and this fragment was inserted into a compatible site of pGT53. The resulting AAV-1 shuttle plasmids were used to make AAV-1 virus preps as described herein using standard methods for producing AAV-1 virus.

For the CMV-promoter-containing shuttle plasmids, a fragment encoding the respective IFN gene was isolated from the appropriate plasmid vector via restriction enzyme digestion and inserted into the pZAC2.1 plasmid vector at (a) compatible restriction site(s) as described in Table 8 above.

5 For the pBRES hGMCSF shuttle plasmids, an SrgAI/NheI fragment of pORF9-hGMCSF (Invitrogen) encoding hGMCSF was blunt-ended and inserted into the EcoRV site of pGT2 resulting in pGT711. The FseI/SrfI fragment of pGT712 containing the entire pBRES sequence was blunt-ended and inserted into pZAC2.1 resulting in pGT715 (Figure 31A).

10 For the pBRES mGMCSF shuttle plasmids, an AgeI/NheI fragment of pORF9-mGMCSF (Invitrogen) encoding mGMCSF was blunt-ended and inserted into the EcoRV site of the pGT2 vector resulting in pGT712. The FseI/SrfI fragment of pGT712 containing the entire BRES sequence was blunt-ended and inserted into pZAC2.1 resulting in pGT716 (Figure 31B).

15 The mouse and human GMCSF genes from the respective pORF9 plasmids (Invitrogen) were cloned through the plasmid pGENE/V5HisA plasmid (Invitrogen) so that they could each be excised with KpnI/XbaI and cloned into pZAC2.1.

20 One skilled in the art will readily appreciate that the compositions and methods of the present invention are well adapted to carry out the objects and obtain the ends and advantages described herein, as well as those inherent in the present invention. Changes to the compositions and methods of the present invention, and other uses, will occur to those skilled in the art and such changes are contemplated and encompassed herein as described and as claimed.

CLAIMS

What is claimed is:

1. A regulated gene expression system comprising a vector comprising:
 - a) a first gene expression cassette comprising
 - i) a first nucleic acid sequence encoding a GM-CSF; and
 - ii) a first promoter and first polyadenylation (polyA) site operably linked to said first nucleic acid sequence;wherein the expression of said first nucleic acid sequence and/or the activity of the expressed polypeptide is regulated in the presence of a regulator molecule (RM); and
 - b) a second gene expression cassette comprising
 - i) a second nucleic acid sequence encoding said RM; and
 - ii) a second promoter and a second polyA site operably linked to said second nucleic acid sequence;wherein said RM is expressed and regulates the expression and/or activity of said first nucleic acid sequence.
2. The regulated gene expression system of claim 1, wherein said RM is expressed and activated in the presence of an activator molecule (AM) and regulates the expression and/or activity of said first nucleic acid sequence.
3. The regulated gene expression system of claim 1, wherein expression of the first nucleic acid sequence is induced by the expression or activation of said RM.
4. The regulated gene expression system of claim 3, wherein the expression of said first nucleic acid sequence is transient.
5. The regulated gene expression system of claim 3, wherein the expression of said first nucleic acid sequence is constitutive.
6. The regulated gene expression system of claim 3, wherein said RM is activated or induced by a conformational change or modification of said RM..
7. The regulated gene expression system of claim 6, wherein an AM causes the conformational change or modification of said RM.
8. The regulated gene expression system of claim 1, wherein the expression of said first nucleic acid sequence is dose-dependent on the activation or induction of said RM.

9. The regulated gene expression system of claim 1, wherein said expression and/or activity of said first nucleic acid sequence is dependent on the 5' to 3' orientation of said first gene expression cassette.
- 5 10. The regulated gene expression system of claim 1, wherein said expression and/or activity of said first nucleic acid sequence is dependent on the 5' to 3' orientation of said second gene expression cassette.
11. The regulated gene expression system of claim 2, wherein said AM is selected from
10 the group consisting of:
- i) a naturally occurring molecule or variant thereof;
 - ii) a modified molecule;
 - iii) a synthetic molecule; and
 - iv) a chemical compound.
- 15 12. The regulated gene expression system of claim 11, wherein said AM is an antiprogesterone.
13. The regulated gene expression system of claim 12, wherein said antiprogesterone is
20 mifepristone.
14. The regulated gene expression system of claim 1, wherein said first promoter is a regulated promoter.
- 25 15. The regulated gene expression system of claim 1, wherein said second promoter is a regulated promoter.
16. The regulated gene expression system of claim 15, wherein said second promoter is
30 tissue specific.
17. The regulated gene expression system of claim 16, wherein said second promoter is muscle specific.
18. The regulated gene expression system of claim 17, wherein said second promoter is
35 an actin promoter.
19. The regulated gene expression system of claim 3, wherein the expression of said RM is constitutive.

20. The regulated gene expression system of claim 3, wherein the expression of said RM is transient.

21. The regulated gene expression system of claim 1, wherein said RM binds to said first promoter and induces expression of said first nucleic acid sequence.

22. The regulated gene expression system of claim 21, wherein said RM is activated or induced by an AM to induce expression of said first nucleic acid sequence.

23. The regulated gene expression system of claim 1, wherein said RM comprises a transactivation domain.

24. The regulated gene expression system of claim 23, wherein said transactivation domain is a VP16 or p65 transactivation domain.

25. The regulated gene expression system of claim 1, wherein said RM comprises a ligand binding domain (LBD).

26. The regulated gene expression system of claim 25, wherein an AM binds to said LBD to activate said RM.

27. The regulated gene expression system of claim 1, wherein said RM comprises a DNA binding domain (DBD).

28. The regulated gene expression system of claim 27, wherein said DBD comprises a Gal4 DBD.

29. The regulated gene expression system of claim 2, wherein said RM is selected from the group consisting of:

- i) a naturally occurring molecule or variant thereof;
- ii) a recombinant molecule; and
- iii) a synthetic molecule.

30. The regulated gene expression system of claim 29, wherein said RM is a protein.

31. The regulated gene expression system of claim 30, wherein said RM is a humanized protein.

32. The regulated gene expression system of claim 30, wherein said protein is a human protein or variant thereof.

33. The regulated gene expression system of claim 32, wherein said protein is a transcriptional activator.

5 34. The regulated gene expression system of claim 33, wherein said transcriptional activator is a nuclear steroid receptor.

35. The regulated gene expression system of claim 34, wherein said nuclear steroid is a progesterone receptor.

10

36. The regulated gene expression system of claim 27, wherein said first promoter comprises a binding site for said DBD of said RM.

15

37. The regulated gene expression system of claim 36, wherein said binding site comprises a Gal-4 binding site.

38. The regulated gene expression system of claim 37, wherein said binding site comprises multimers of said Gal-4 binding site.

20

39. The regulated gene expression system of claim 38, wherein said binding site comprises 6-18 Gal-4 binding sites.

40. The regulated gene expression system of claim 1, wherein said first polyA site is a human Growth Hormone (hGH) polyA site.

25

41. The regulated gene expression system of claim 1, wherein said second polyA site is a SV40 polyA site.

30

42. The regulated gene expression system of claim 1, wherein said first gene expression cassette comprises a first functional sequence operably linked to said first nucleic acid sequence.

43. The regulated gene expression system of claim 42, wherein said first functional sequence is a 5' untranslated region and/or intron.

35

44. The regulated gene expression system of claim 43, wherein said first functional sequence is the 5' untranslated region UT12.

40

45. The regulated gene expression system of claim 43, wherein said first functional sequence is the intron IVS8.

46. The regulated gene expression system of claim 1, wherein said GM-CSF molecule is selected from the group consisting of:

- i) a naturally-occurring molecule or variant thereof;
- 5 ii) a modified molecule; and
- iii) a recombinant molecule.

47. The regulated gene expression system of claim 46, wherein said GM-CSF molecule is a protein.

10

48. The regulated gene expression system of claim 47, wherein said GM-CSF is a human protein or variant thereof.

15

49. The regulated gene expression system of claim 48, wherein said GM-CSF comprises the amino acid sequence of SEQ ID NO:17.

50. The regulated gene expression system of claim 49, wherein said GM-CSF is encoded by a sequence comprising the nucleic acid sequence of SEQ ID NO:18.

20

51. The regulated gene expression system of claim 47, wherein said GM-CSF comprises the amino acid sequence of SEQ ID NO:19.

52. The regulated gene expression system of claim 51, wherein said GM-CSF is encoded by a sequence comprising the nucleic acid sequence of SEQ ID NO:20.

25

53. The regulated gene expression system of claim 1, wherein the first nucleic acid comprises the sequence of SEQ ID NO:18.

30

54. The regulated gene expression system of claim 1, wherein the first nucleic acid comprises the sequence of SEQ ID NO:20.

55. The regulated gene expression system of claim 1, wherein said RM comprises the amino acid sequence of SEQ ID NO:22.

35

56. The regulated gene expression system of claim 55, wherein said RM is encoded by a sequence comprising SEQ ID NO:21.

57. The regulated gene expression system of claim 1, wherein said vector is a plasmid.

58. The regulated gene expression system of claim 57, wherein said plasmid is pGT615 (SEQ ID NO:59), pGT616 (SEQ ID NO:60), pGT617 (SEQ ID NO:61), or pGT618 (SEQ ID NO:62).

5 59. A method for providing a therapeutic benefit to a subject comprising contacting the cells of the subject with the regulated gene expression system of claim 1 such that the cells express said GM-CSF molecule which is regulated by said RM.

60. The method of claim 59, wherein said subject has Crohn's disease, ulcerative colitis
10 or inflammatory bowel disease.

61. The method of claim 59, wherein said regulated gene expression system further comprises an AM.

15 62. The method of claim 61, wherein said RM is expressed or activated by said AM.

63. The method of claim 62, wherein the expression or activation of said RM results in the expression or activation of said GM-CSF molecule.

20 64. The method of claim 59, wherein said GM-CSF molecule is a protein with the sequence of SEQ ID NO:17 or SEQ ID NO:20.

65. The method of claim 62, wherein said RM is a progesterone receptor and said AM is mifepristone.
25

66. The method of claim 59, wherein said regulated gene expression system is administered as a plasmid vector.

67. The method of claim 66, wherein said plasmid vector is pGT615 (SEQ ID NO:59),
30 pGT616 (SEQ ID NO:60), pGT617 (SEQ ID NO:61), or pGT618 (SEQ ID NO:62).

68. The method of claim 66, wherein said vector is administered by contacting said cells in vivo or ex vivo.

35 69. The method of claim 68, wherein said contacting is by injection.

70. The method of claim 68, wherein said contacting is by electroporation.

40 71. The method of claim 68, wherein said vector is administered one or more times.

72. The method of claim 71, wherein said vector is administered daily, every other day, three times per week, twice per week, weekly, biweekly, monthly, bimonthly, every three months, quarterly, semiannually, annually, or some combination thereof.
- 5 73. The method of claim 61, wherein said AM is administered orally or by injection.
74. The method of claim 61, wherein said AM is administered one or more times.
75. The method of claim 62, wherein said AM is administered daily, every other day,
10 three times per week, twice per week, weekly, biweekly, monthly, bimonthly, every three months, quarterly, semiannually, annually, or some combination thereof.
76. A pharmaceutical composition comprising the vector of claim 1 and a carrier.
- 15 77. A kit comprising the regulated gene expression system of claim 1.
78. The kit of claim 77, further comprising an AM.

FIG. 1A

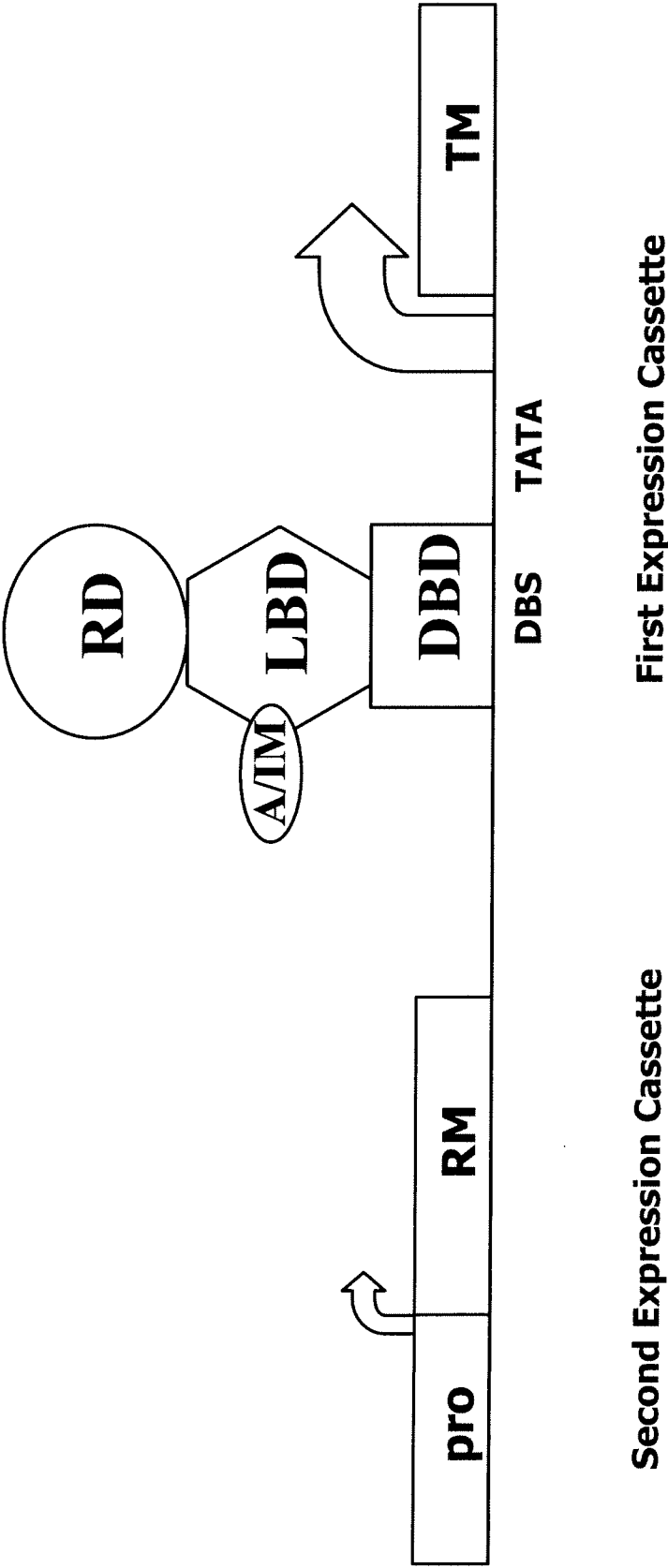


FIG. 1B

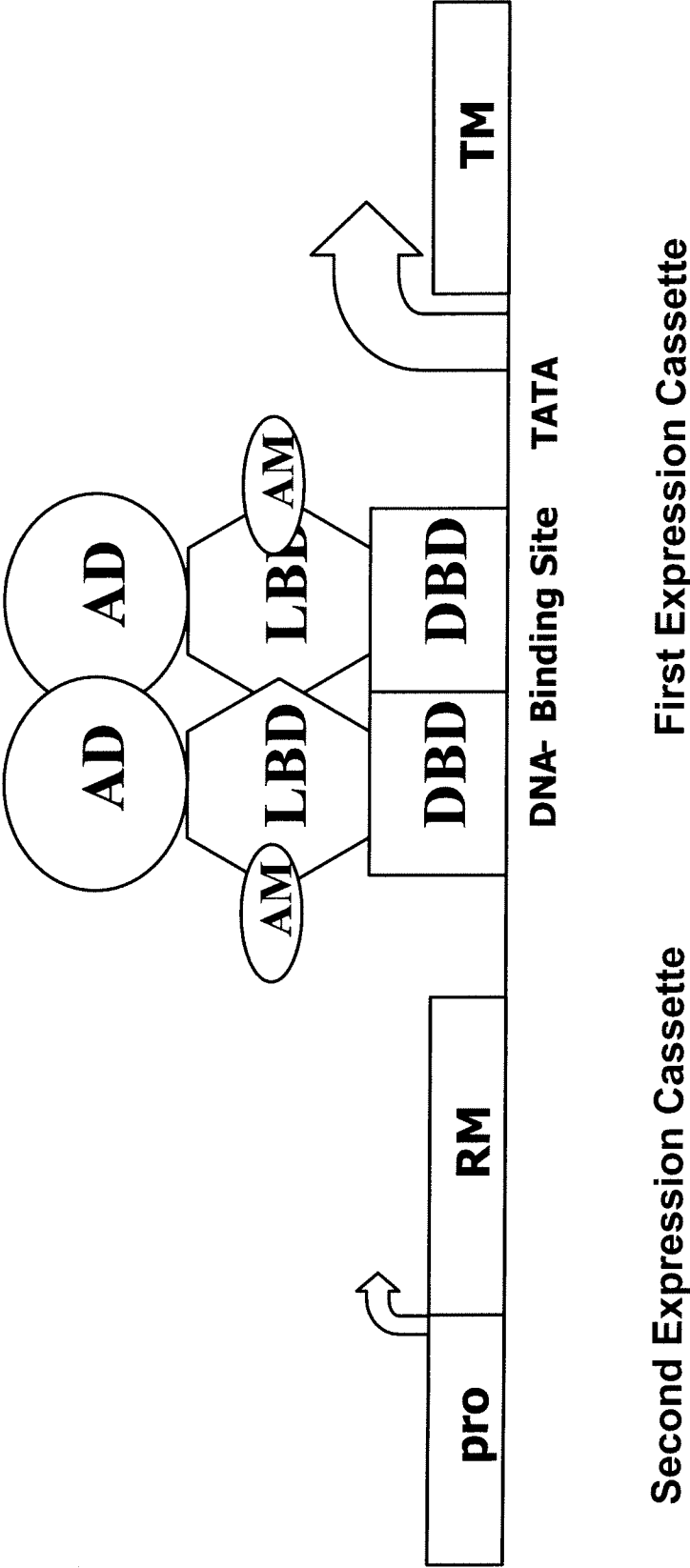


FIG. 2B

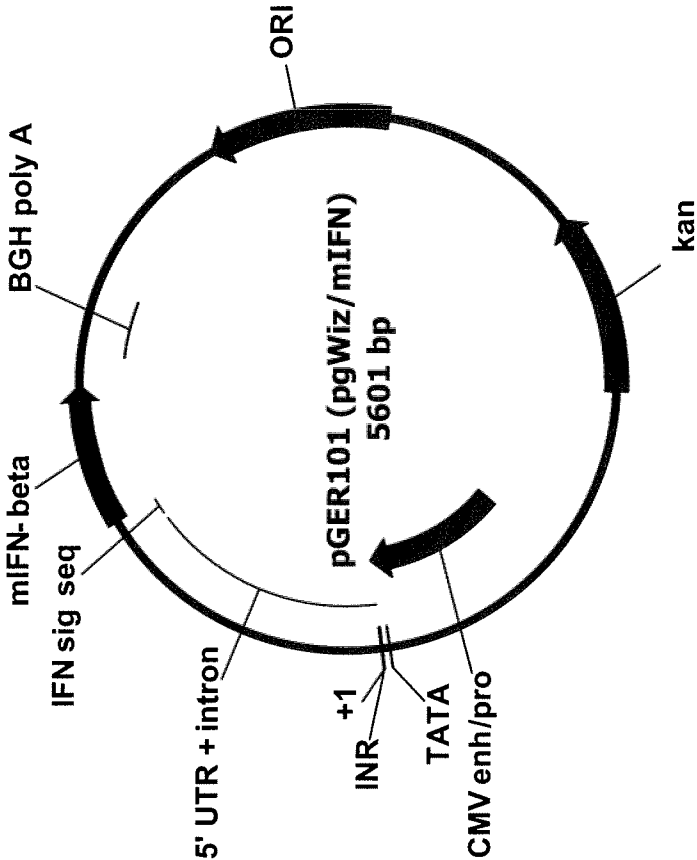


FIG. 2A

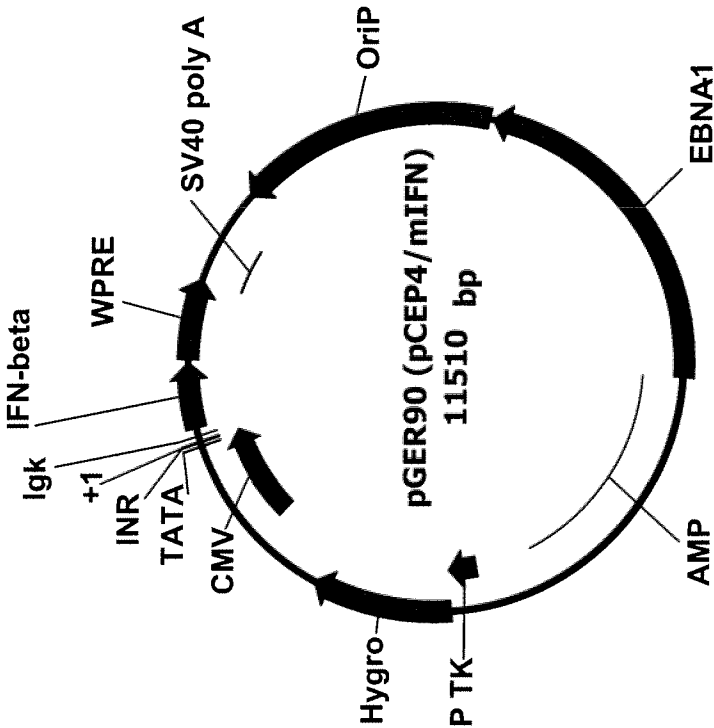


FIG. 2D

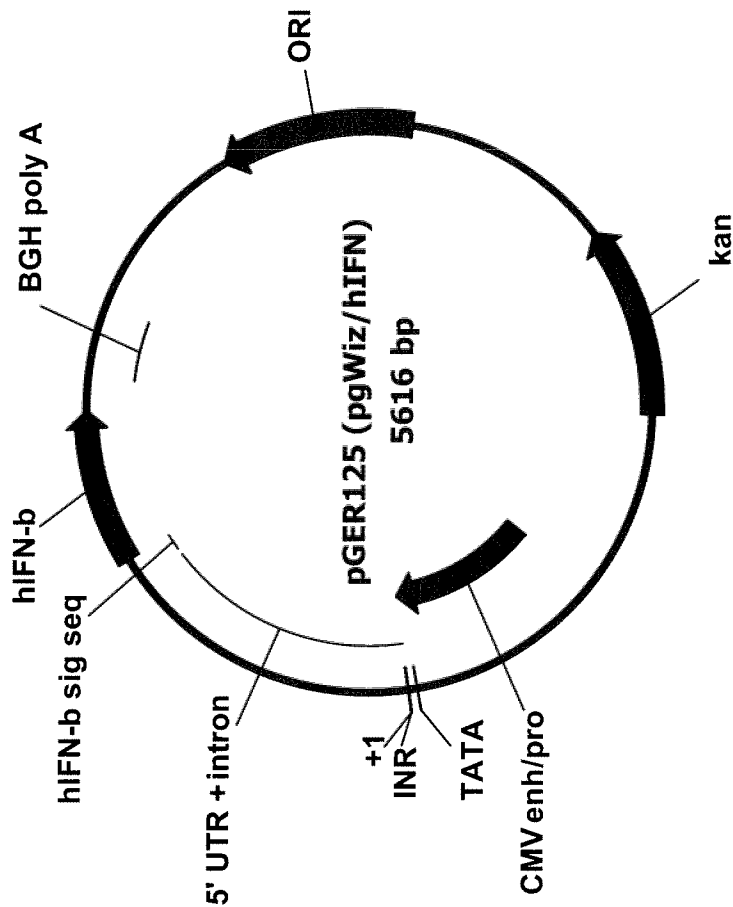


FIG. 2C

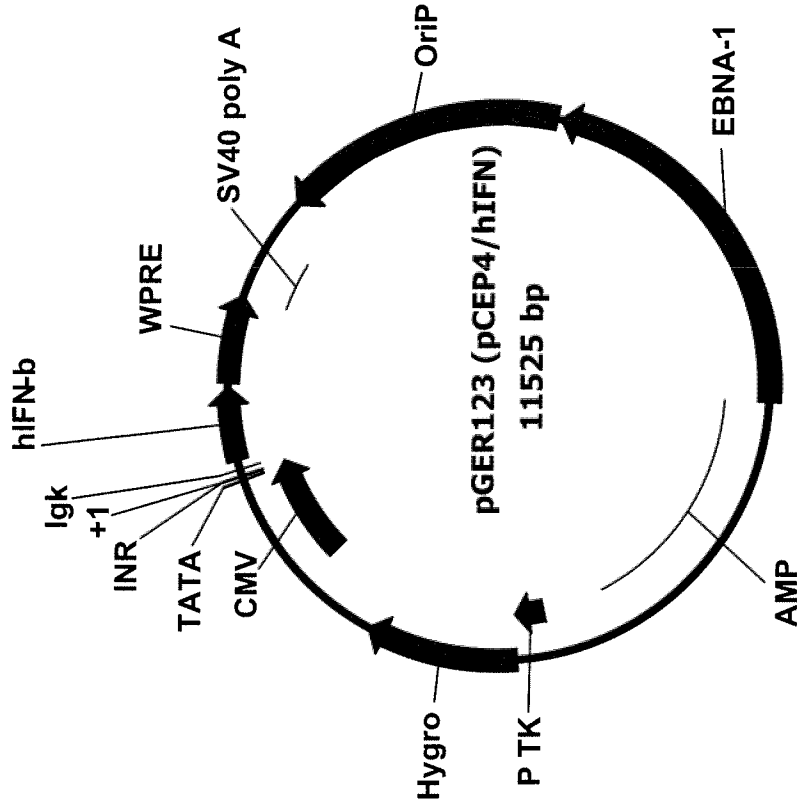


FIG. 3

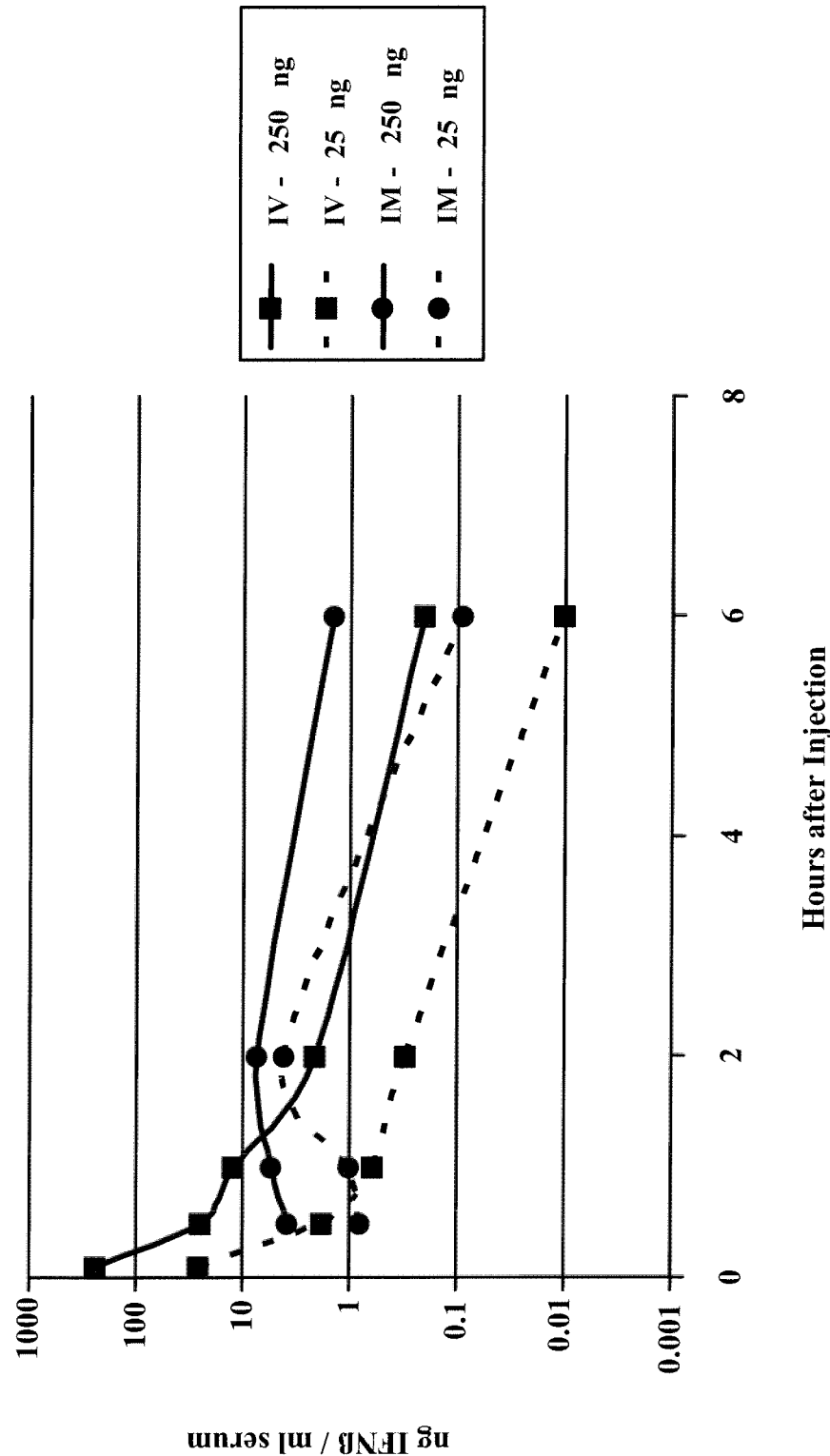


FIG. 4

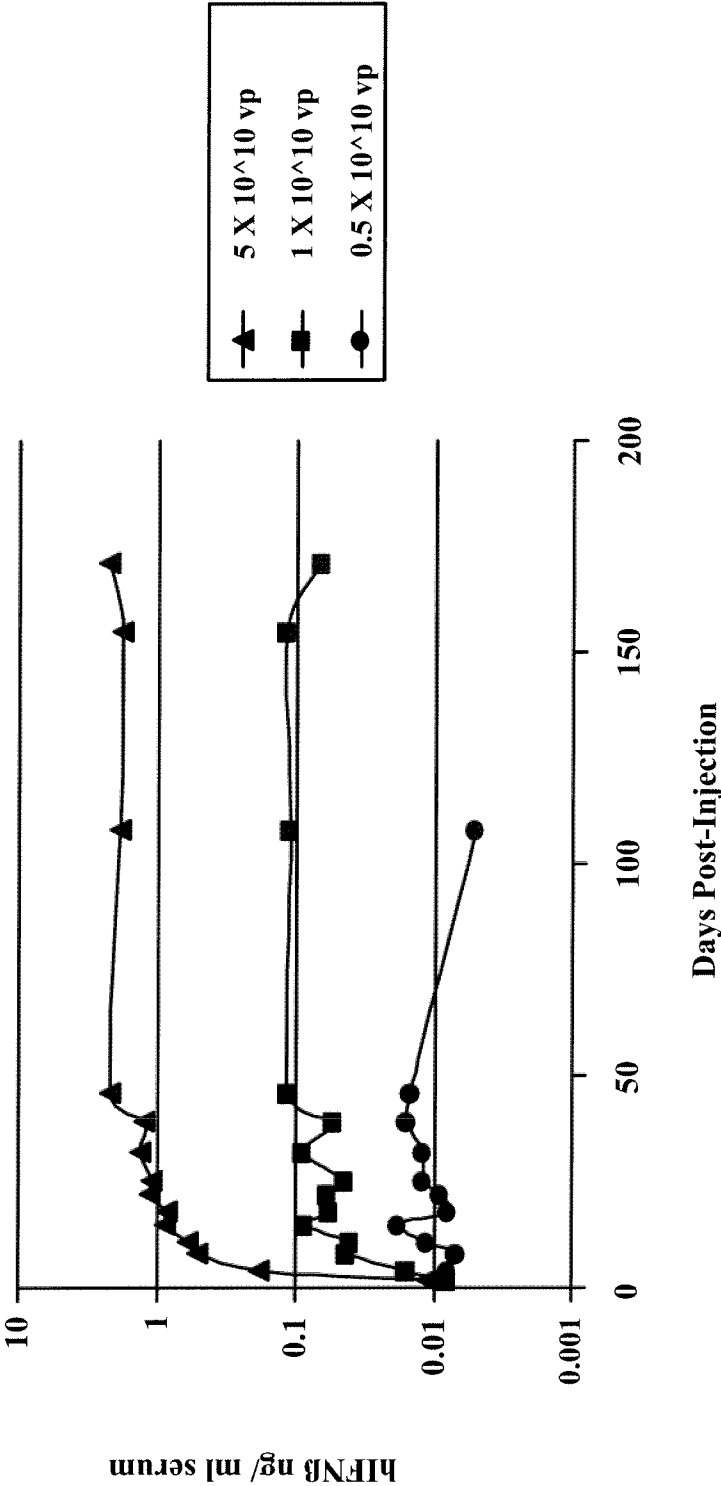


FIG. 5

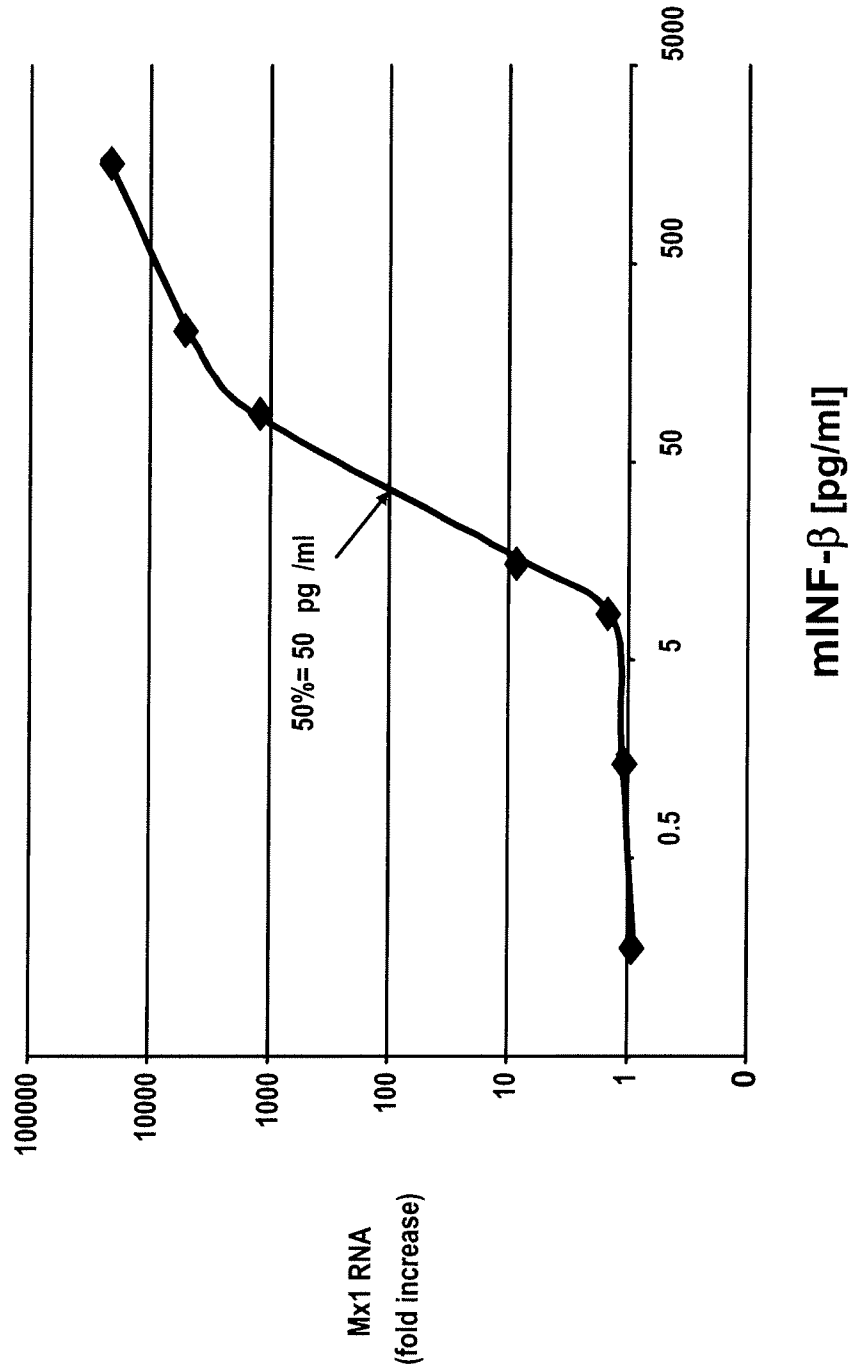


FIG. 6

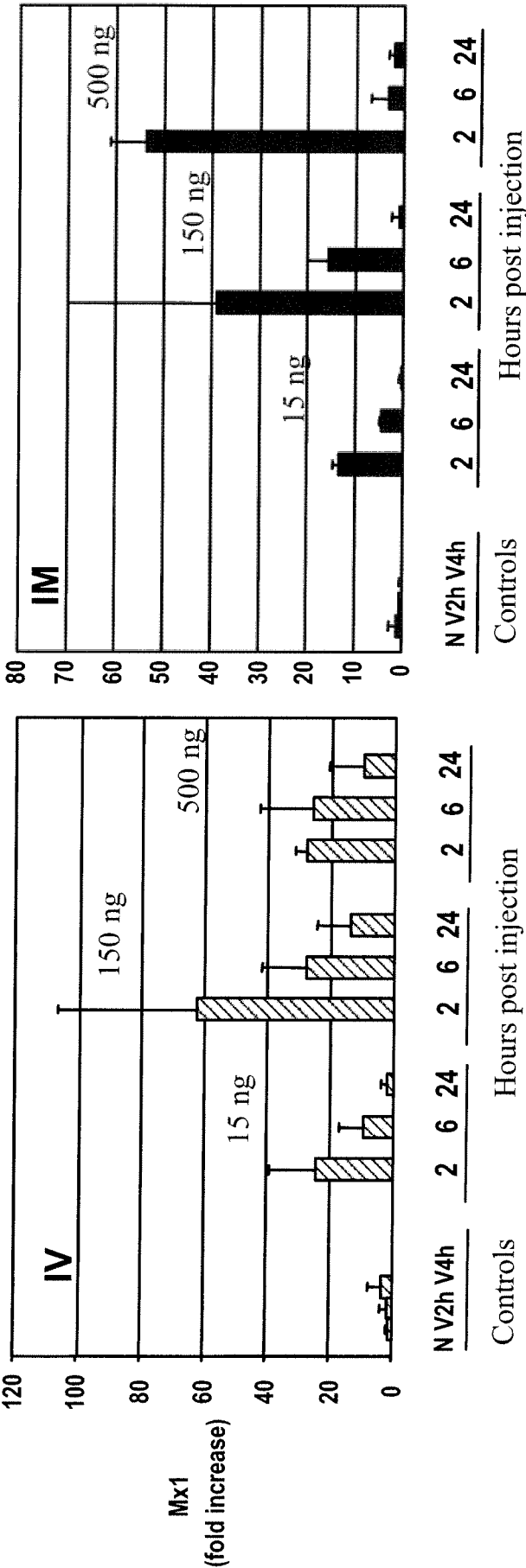


FIG. 7A

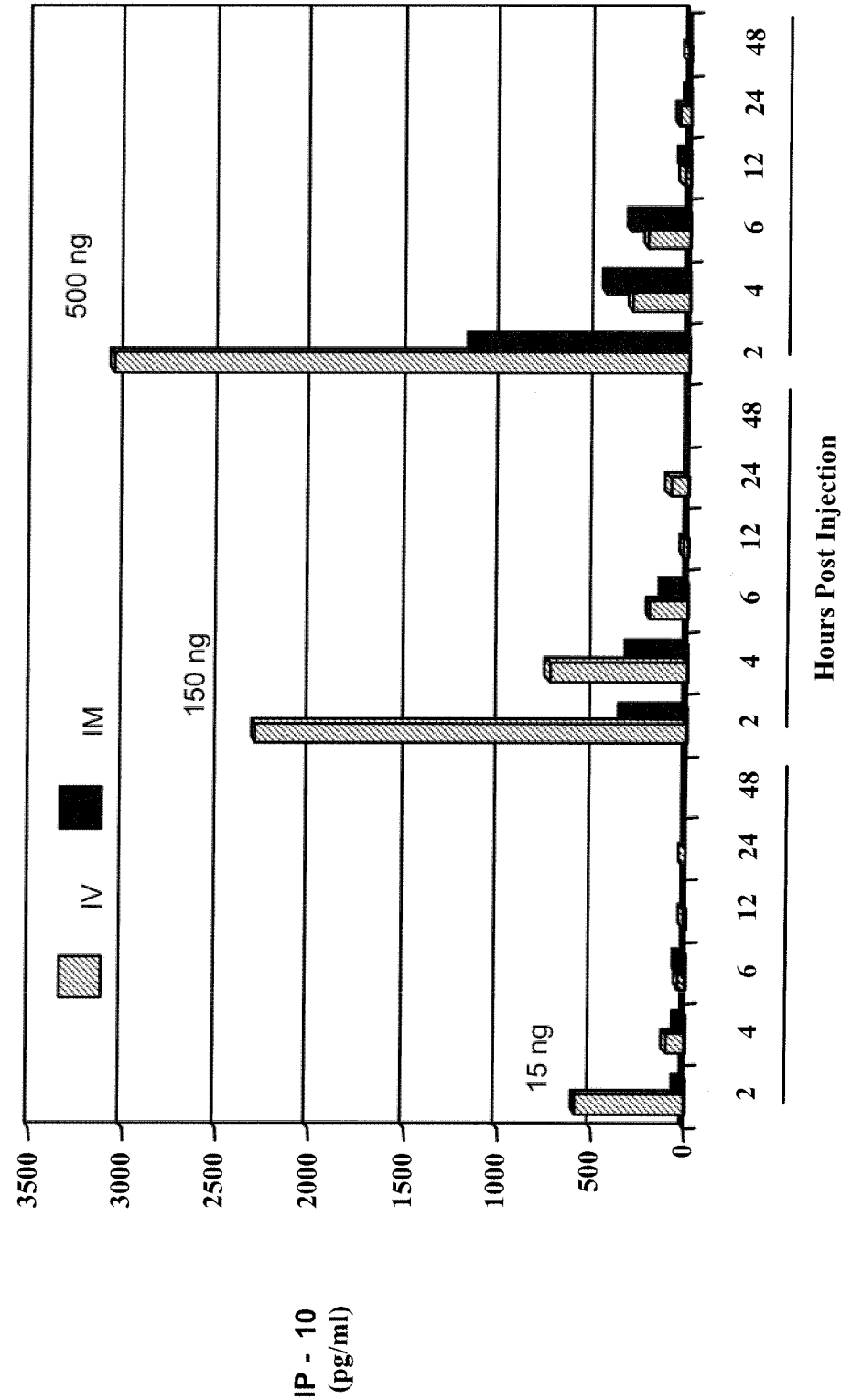


FIG. 7B

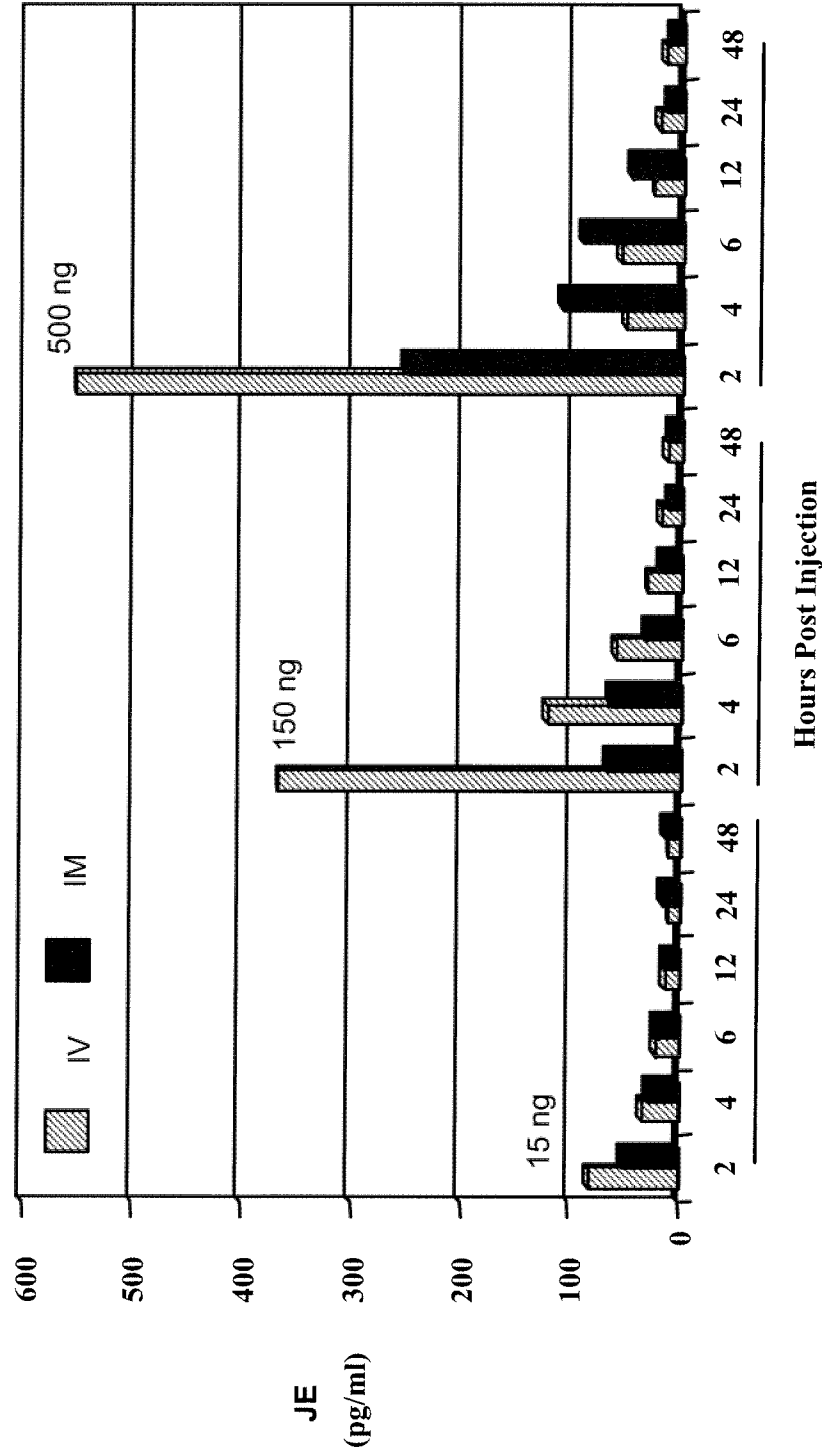


FIG. 8

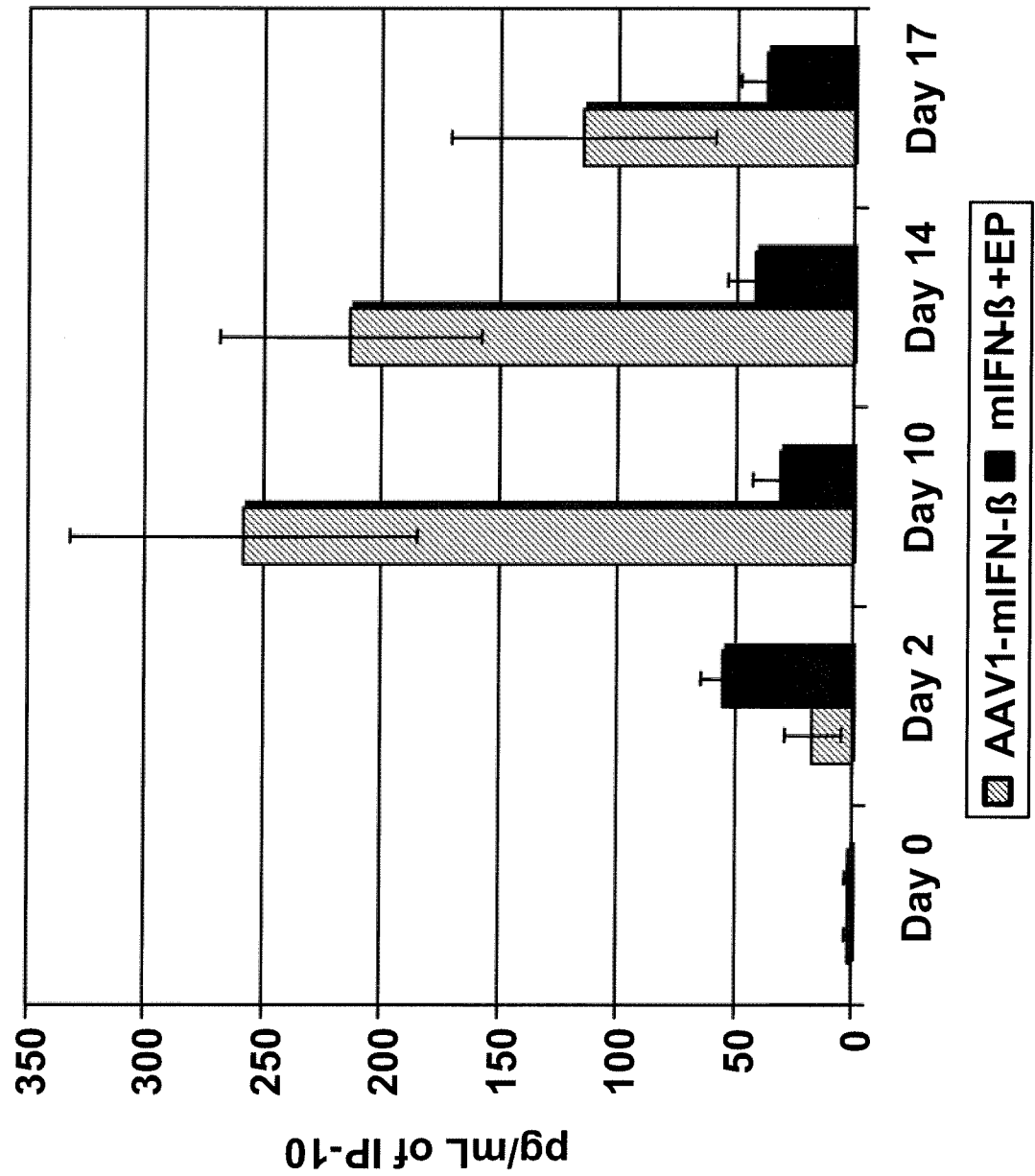


FIG. 9

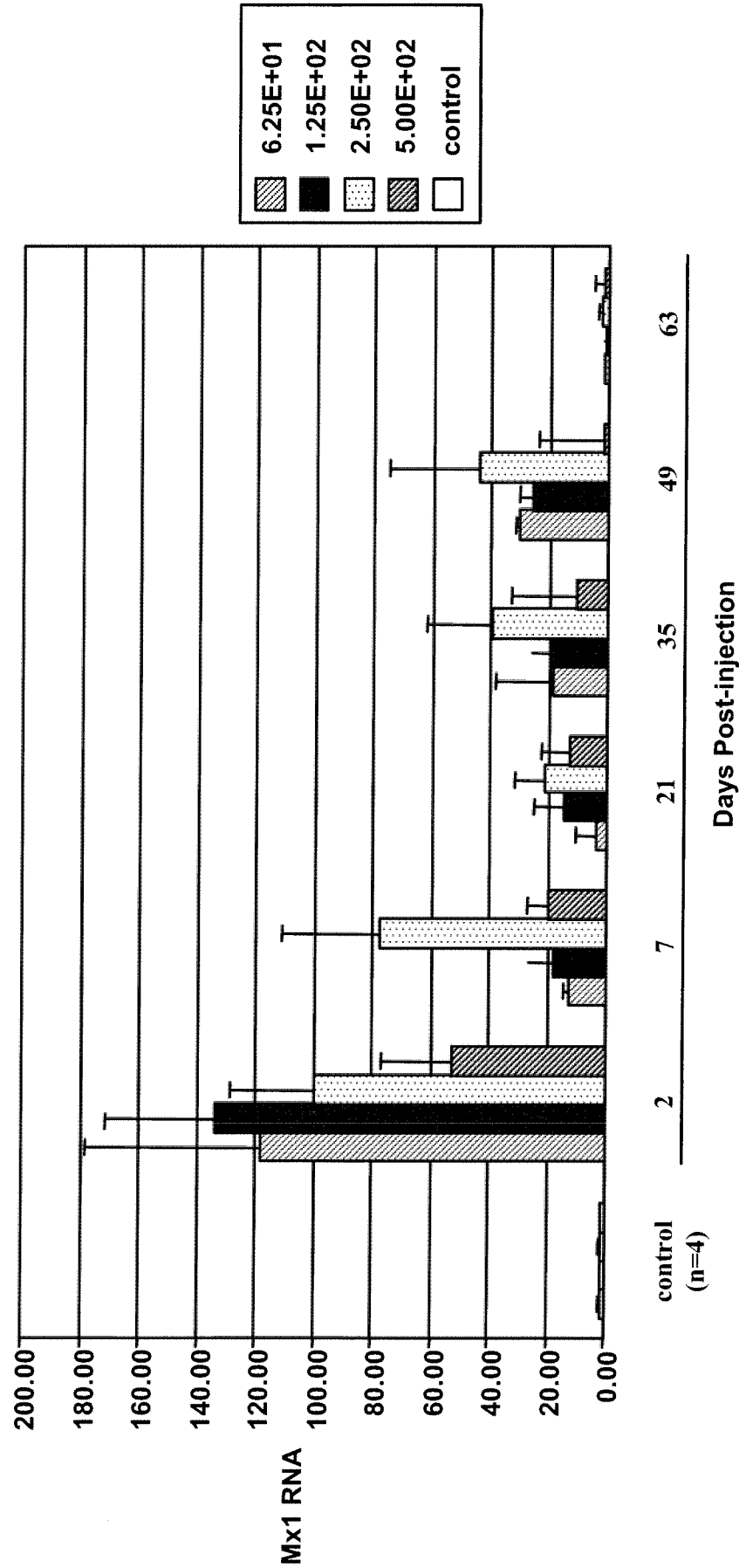
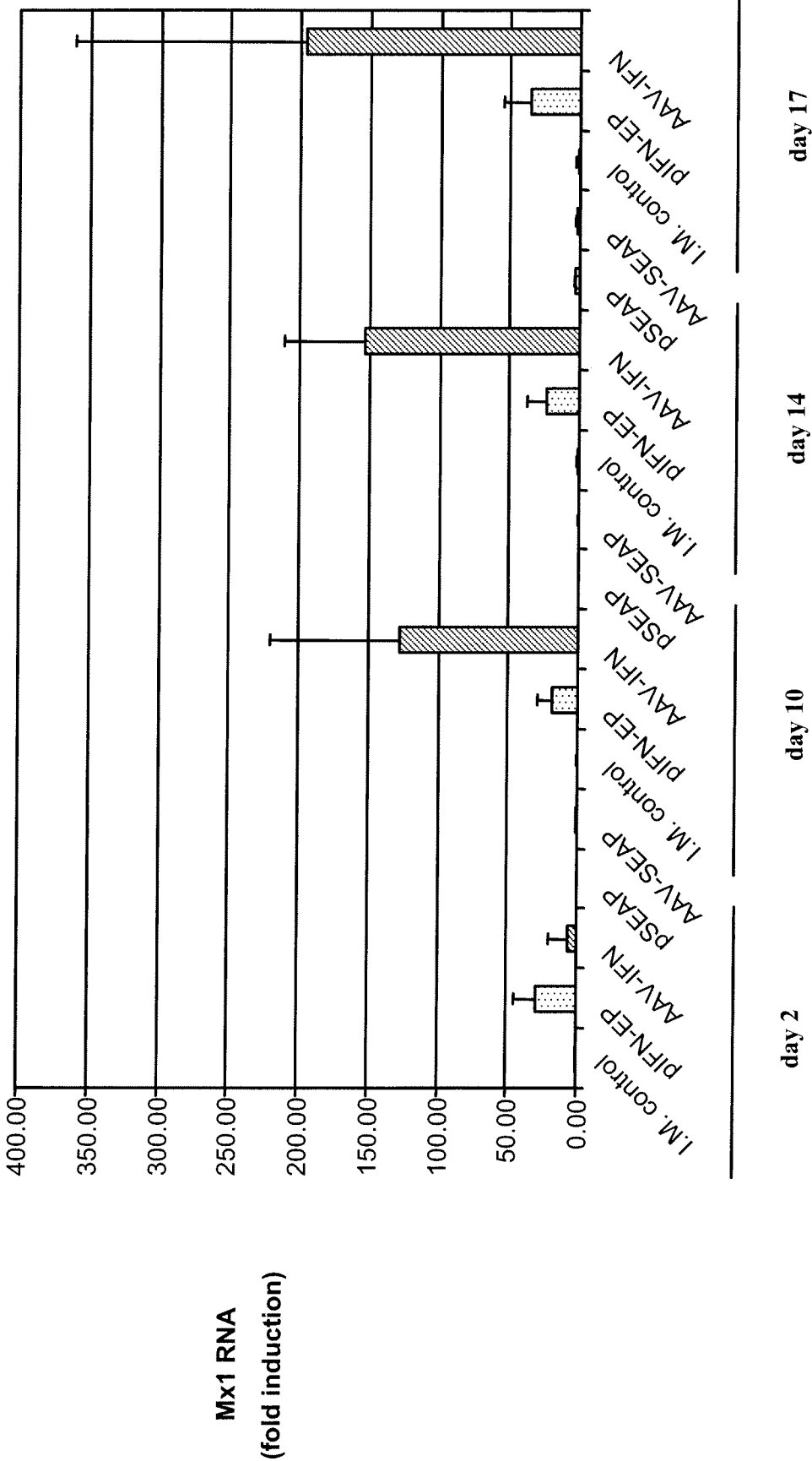


FIG. 10



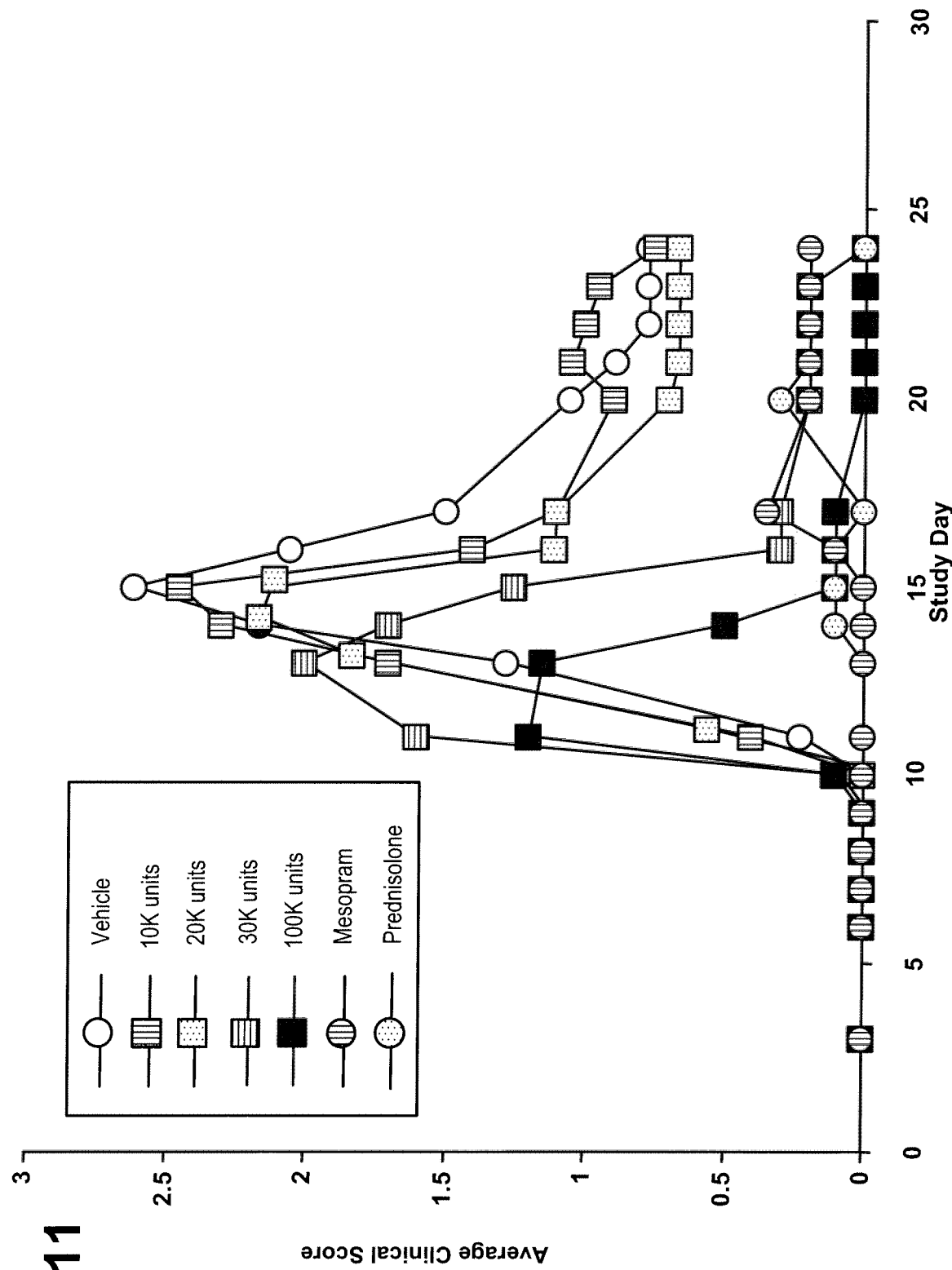


FIG. 12

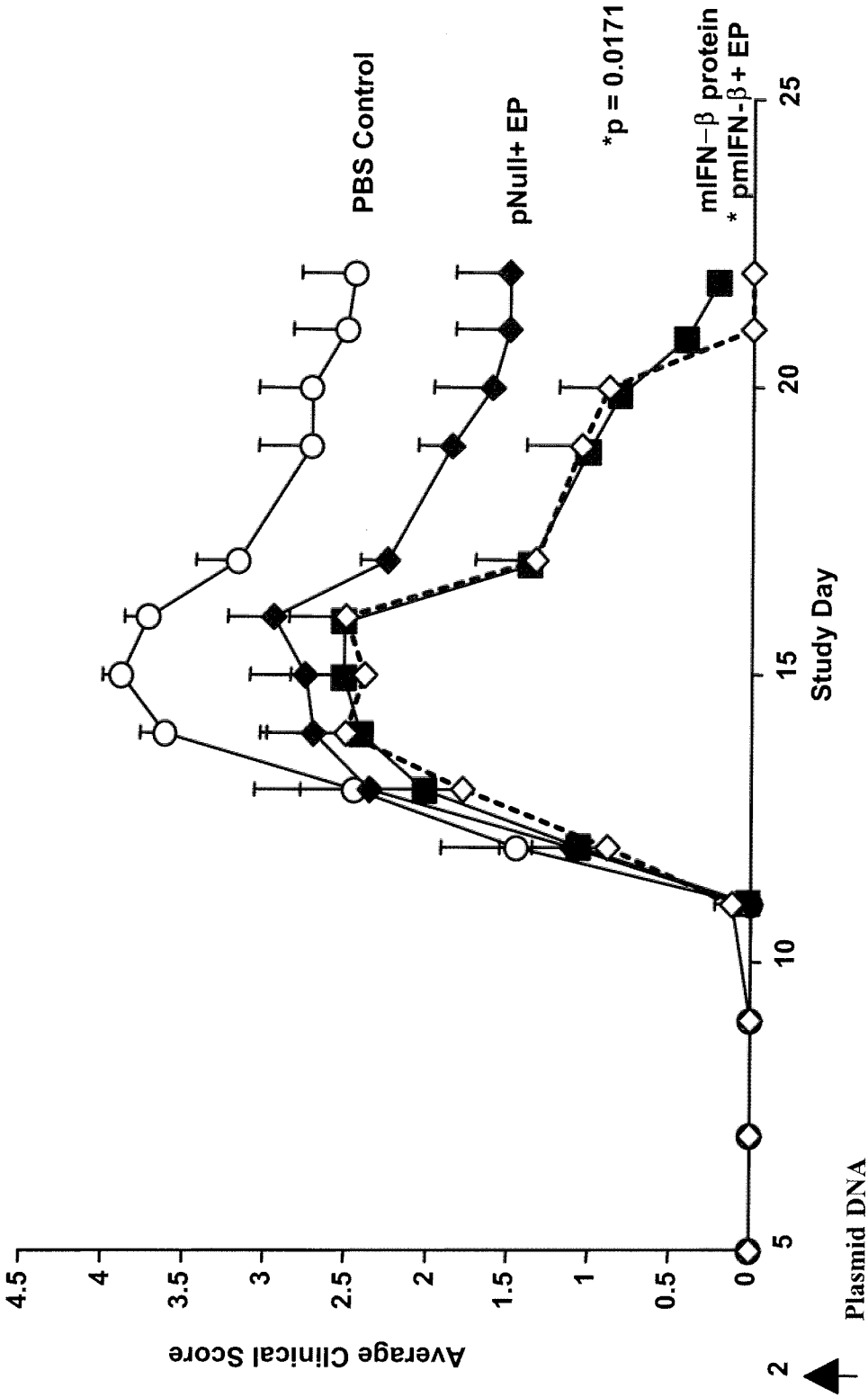


FIG. 13

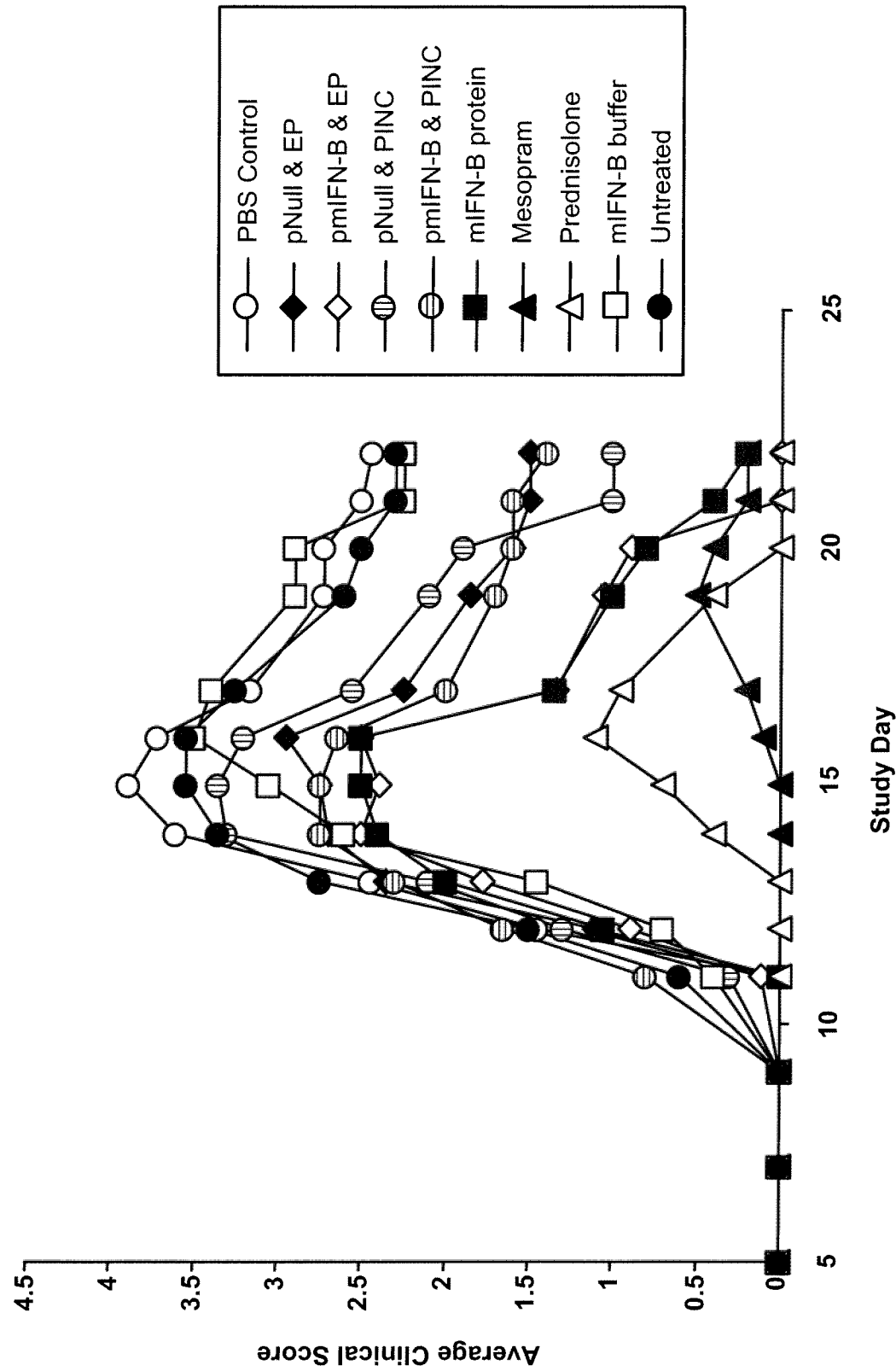


FIG. 14B

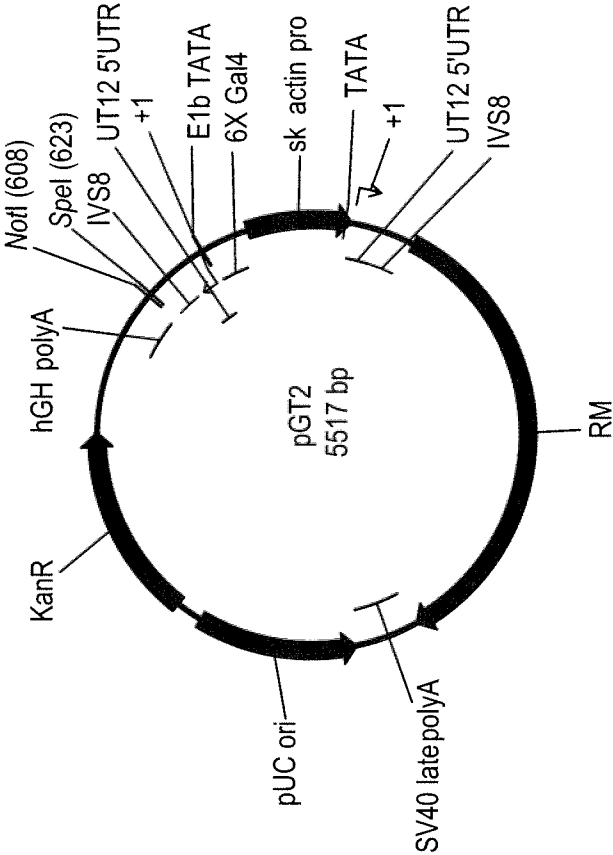


FIG. 14A

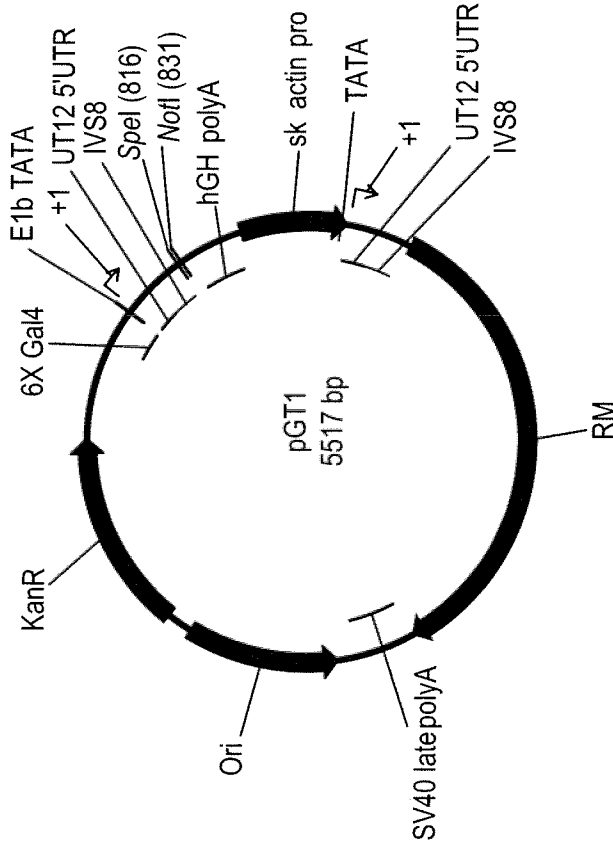


FIG. 14D

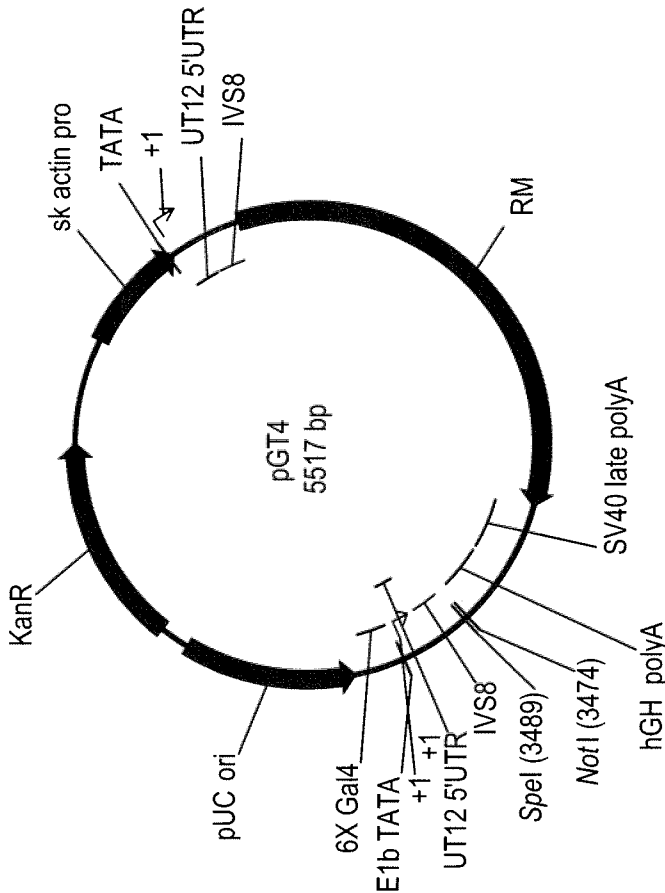
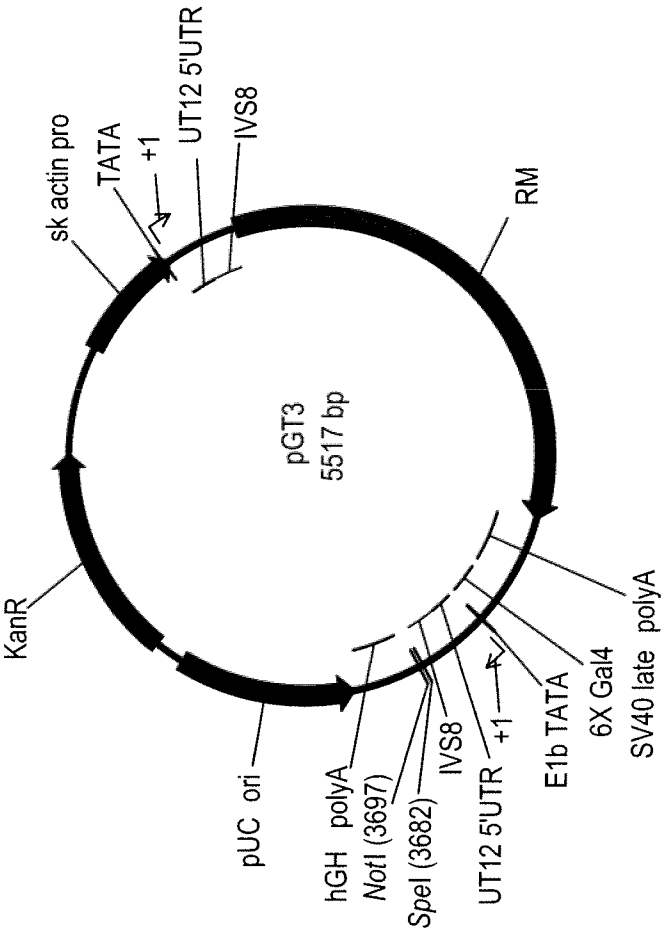


FIG. 14C



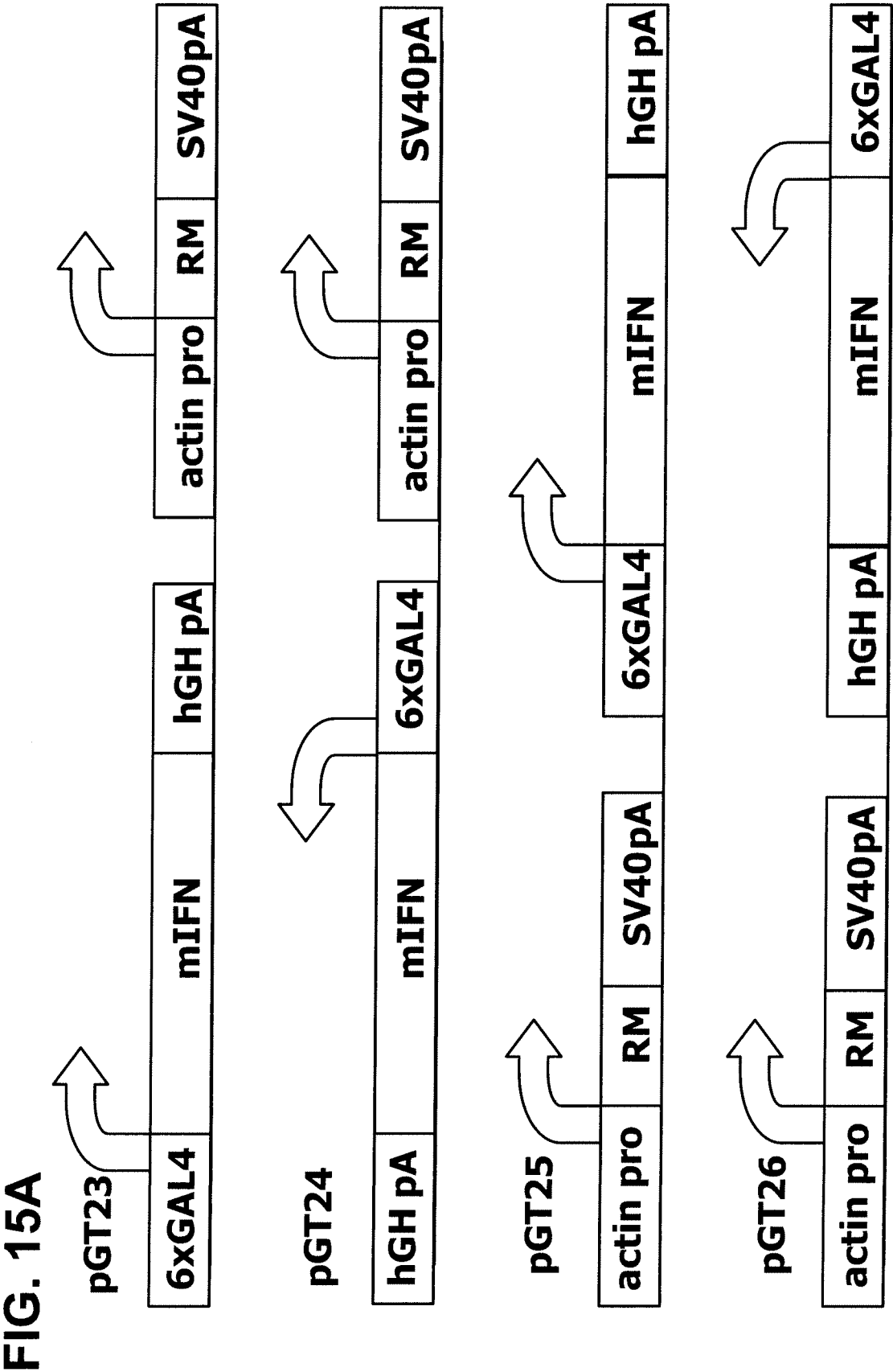


FIG. 15B

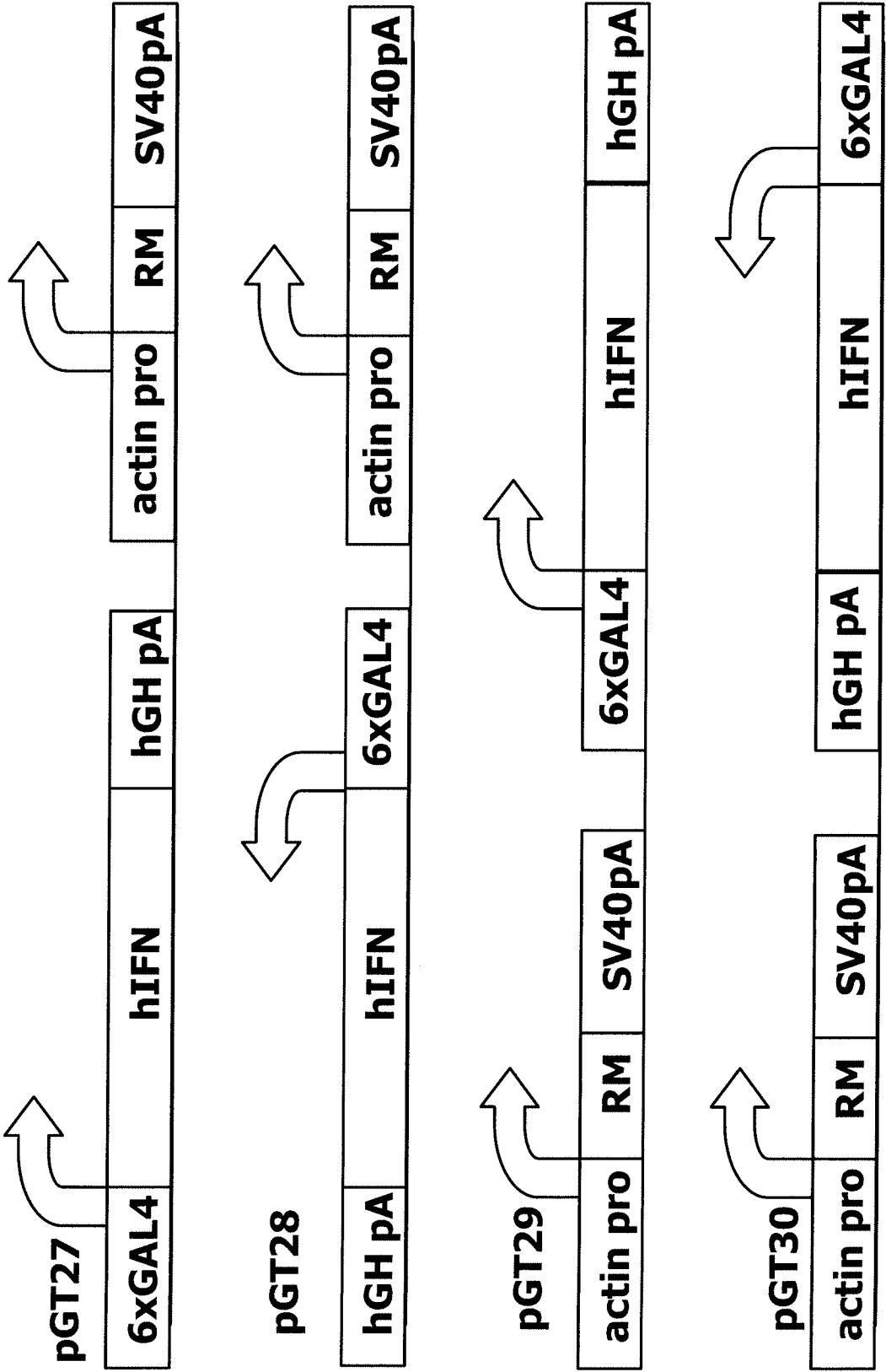


FIG. 16

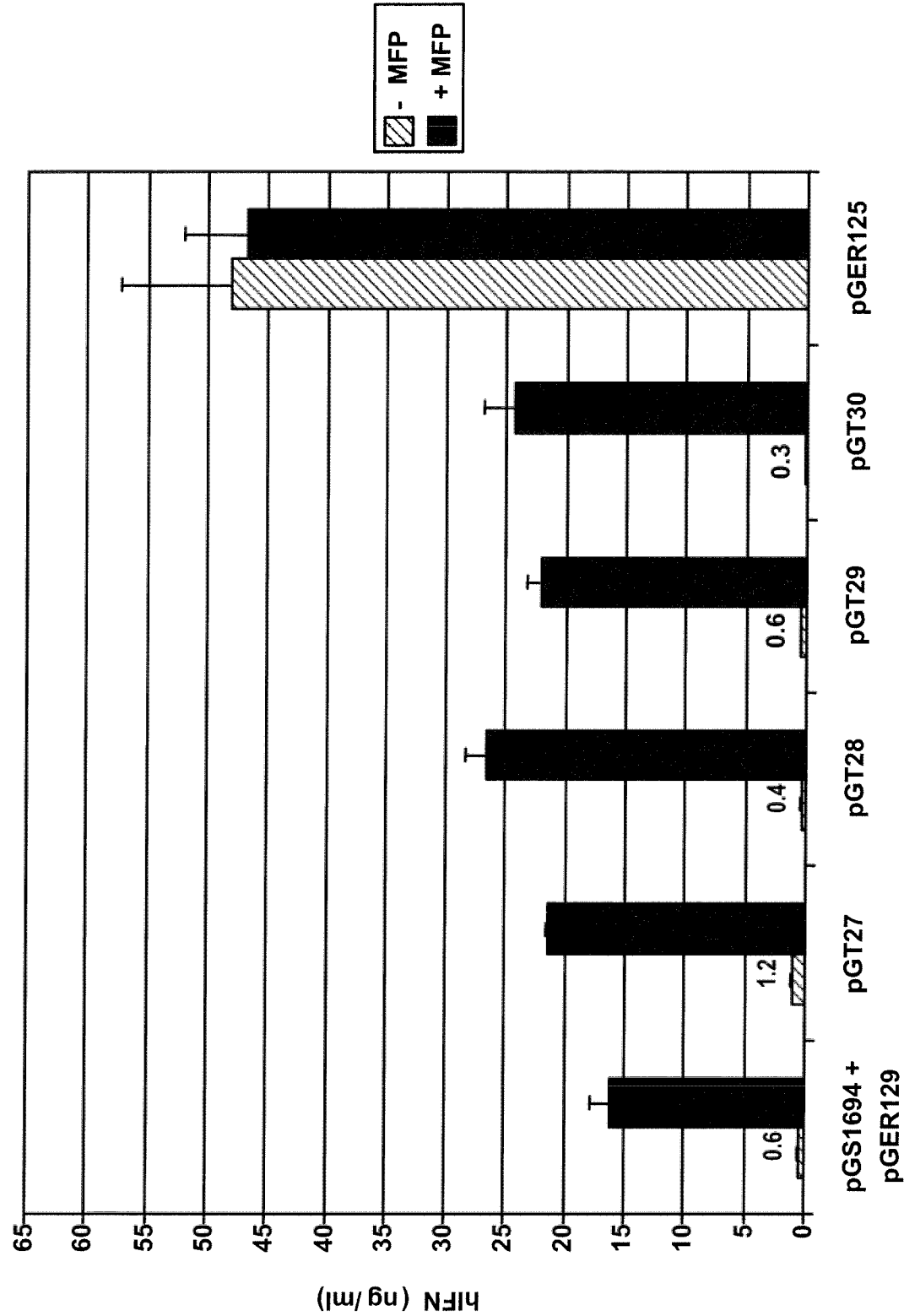


FIG. 17

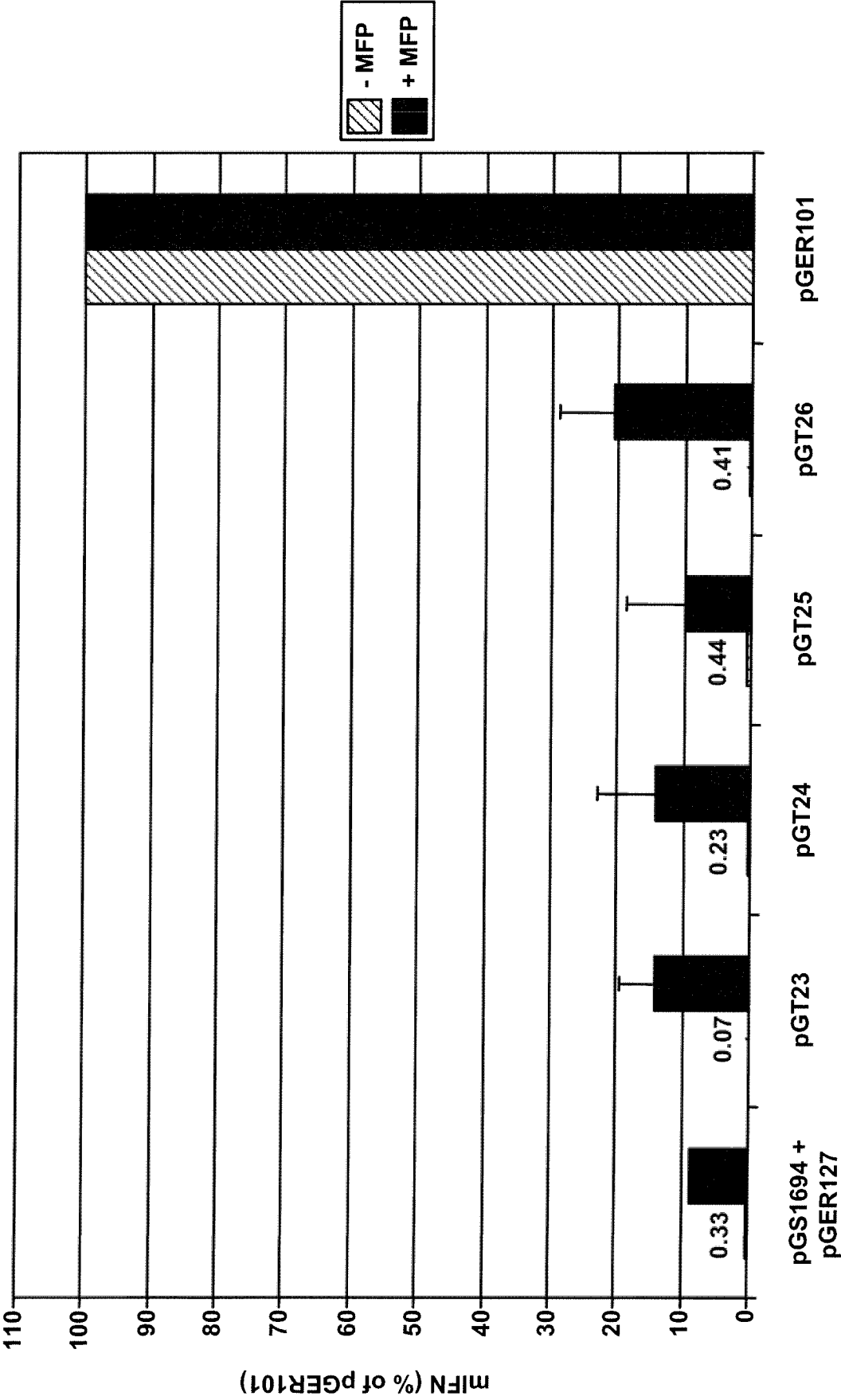


FIG. 18

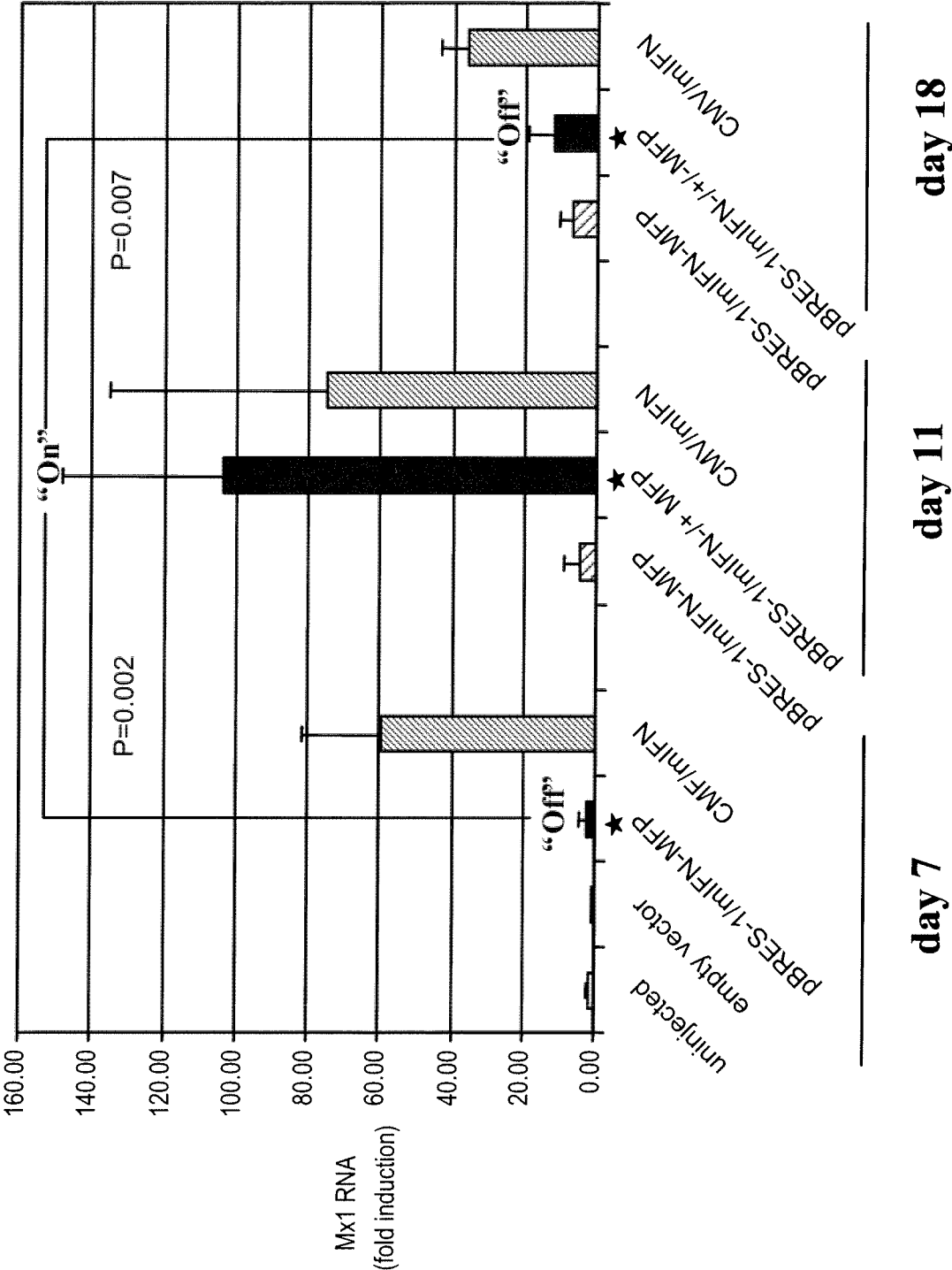


FIG. 19

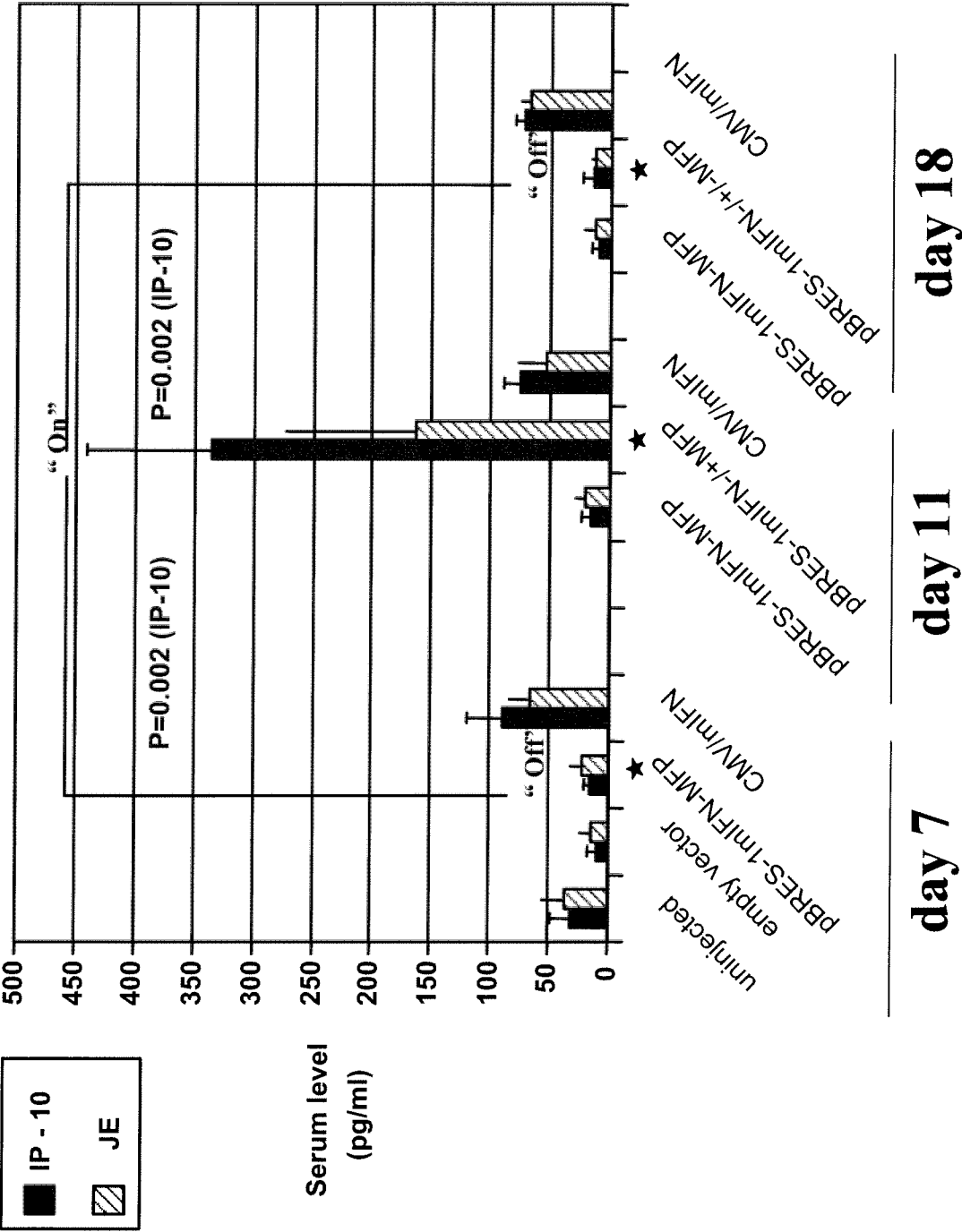


FIG. 20A

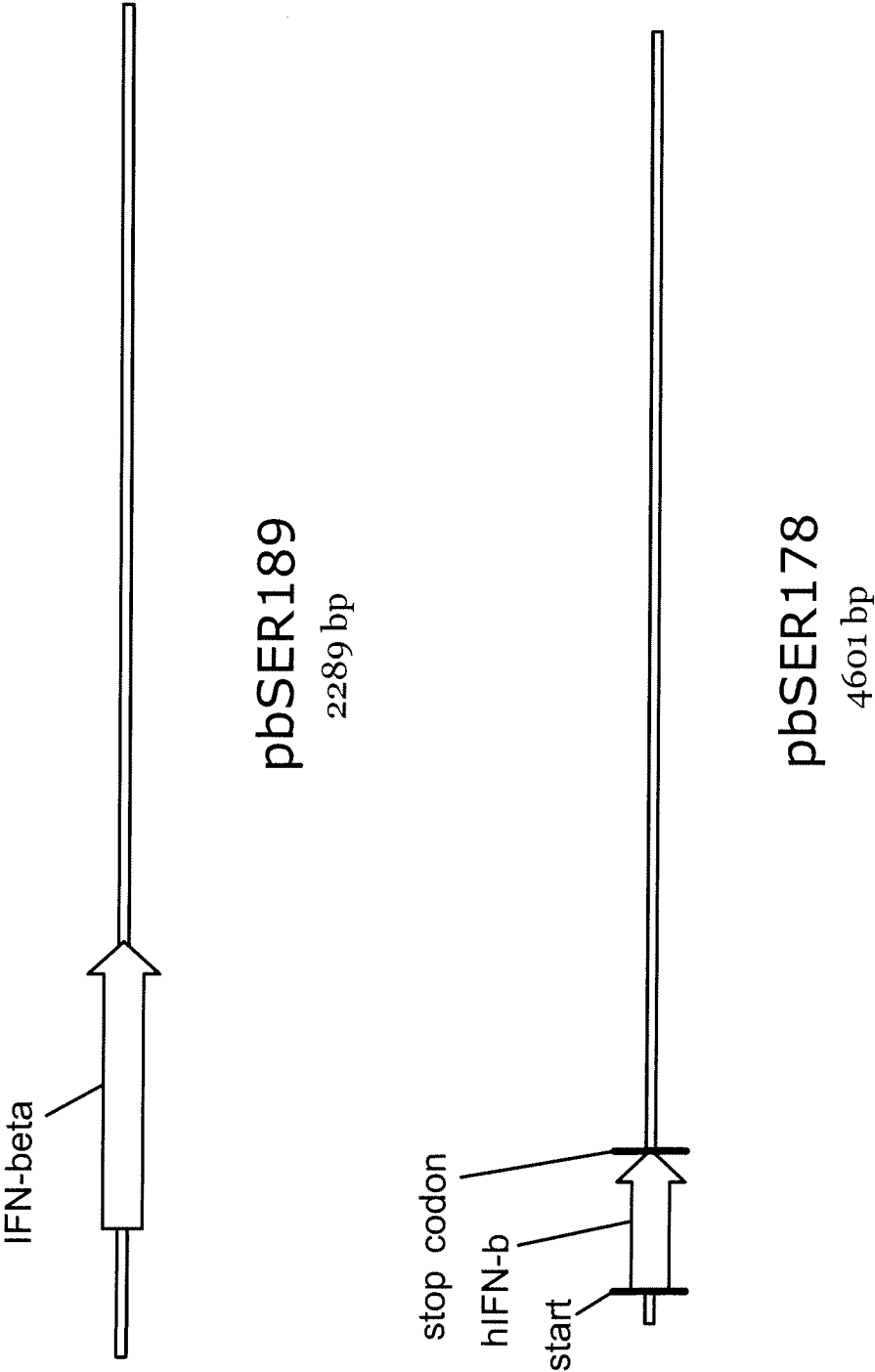
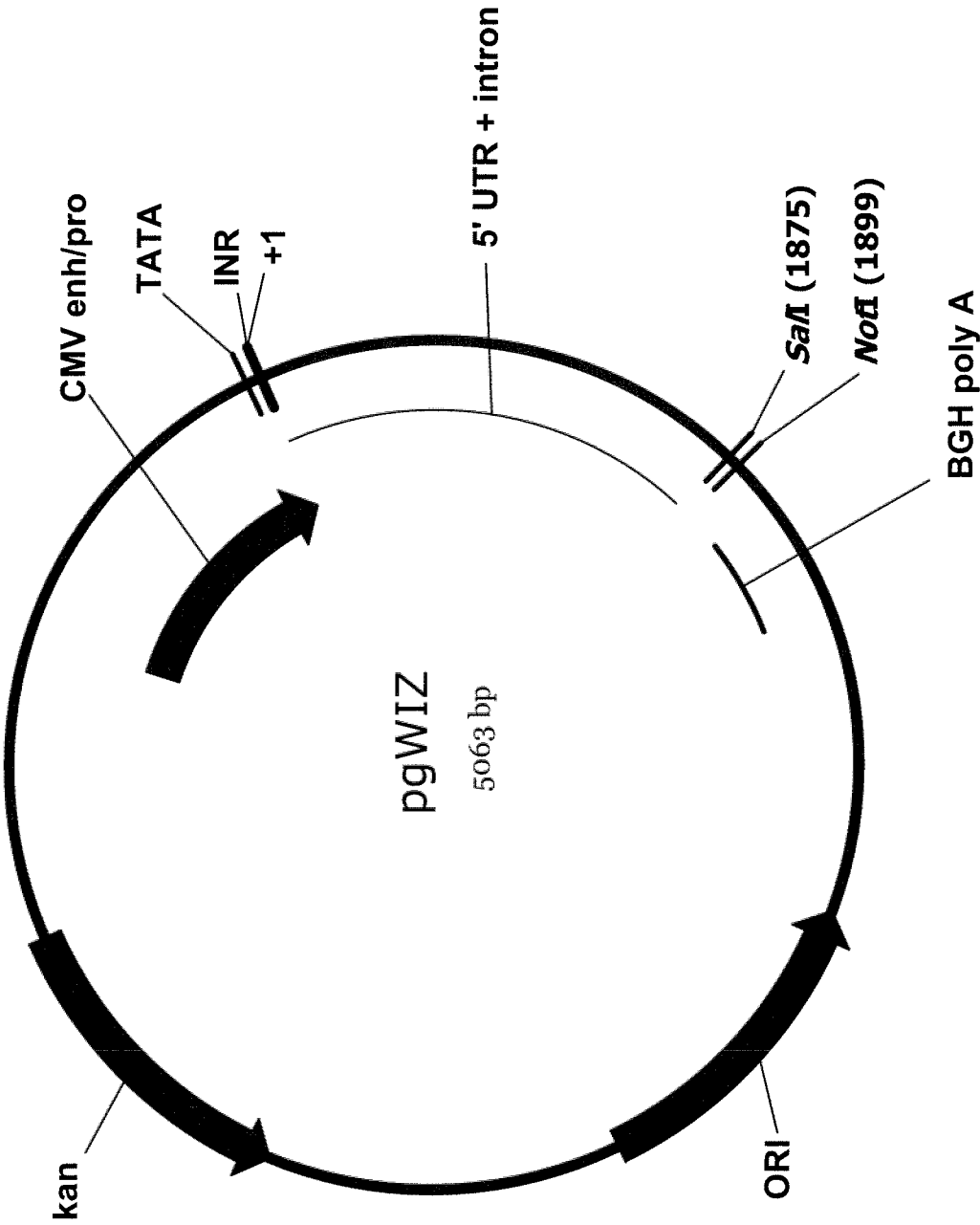


FIG. 20B



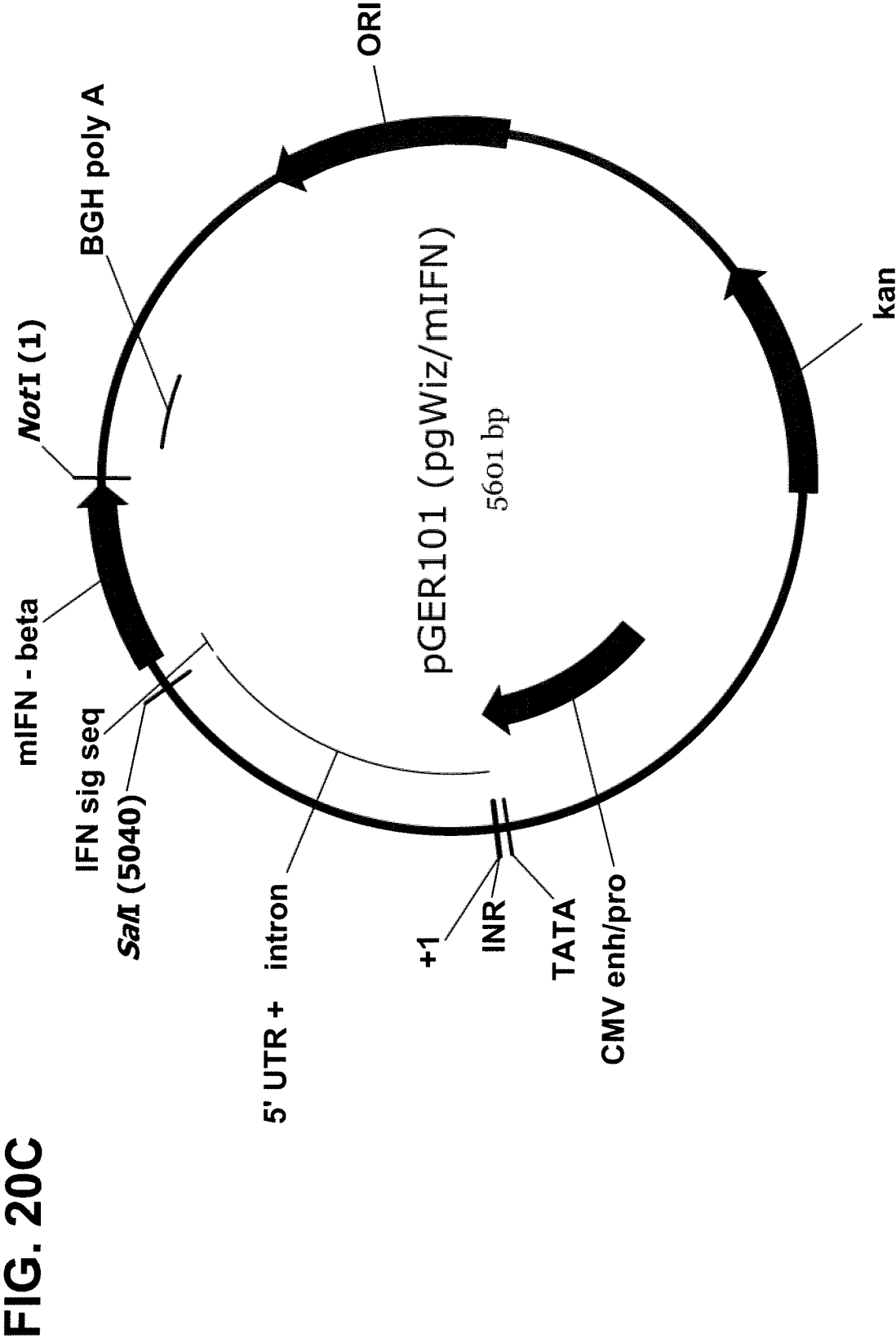
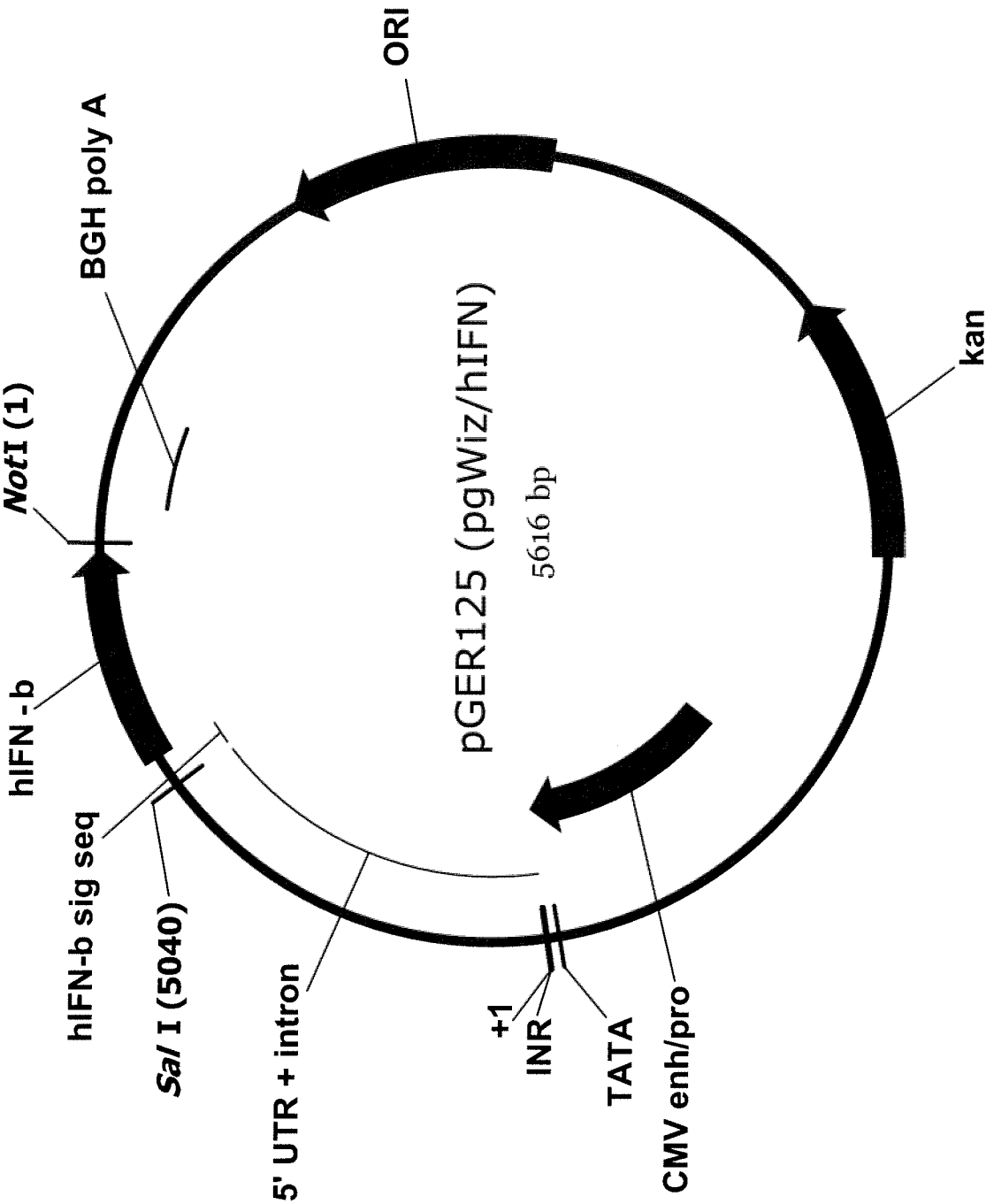


FIG. 21



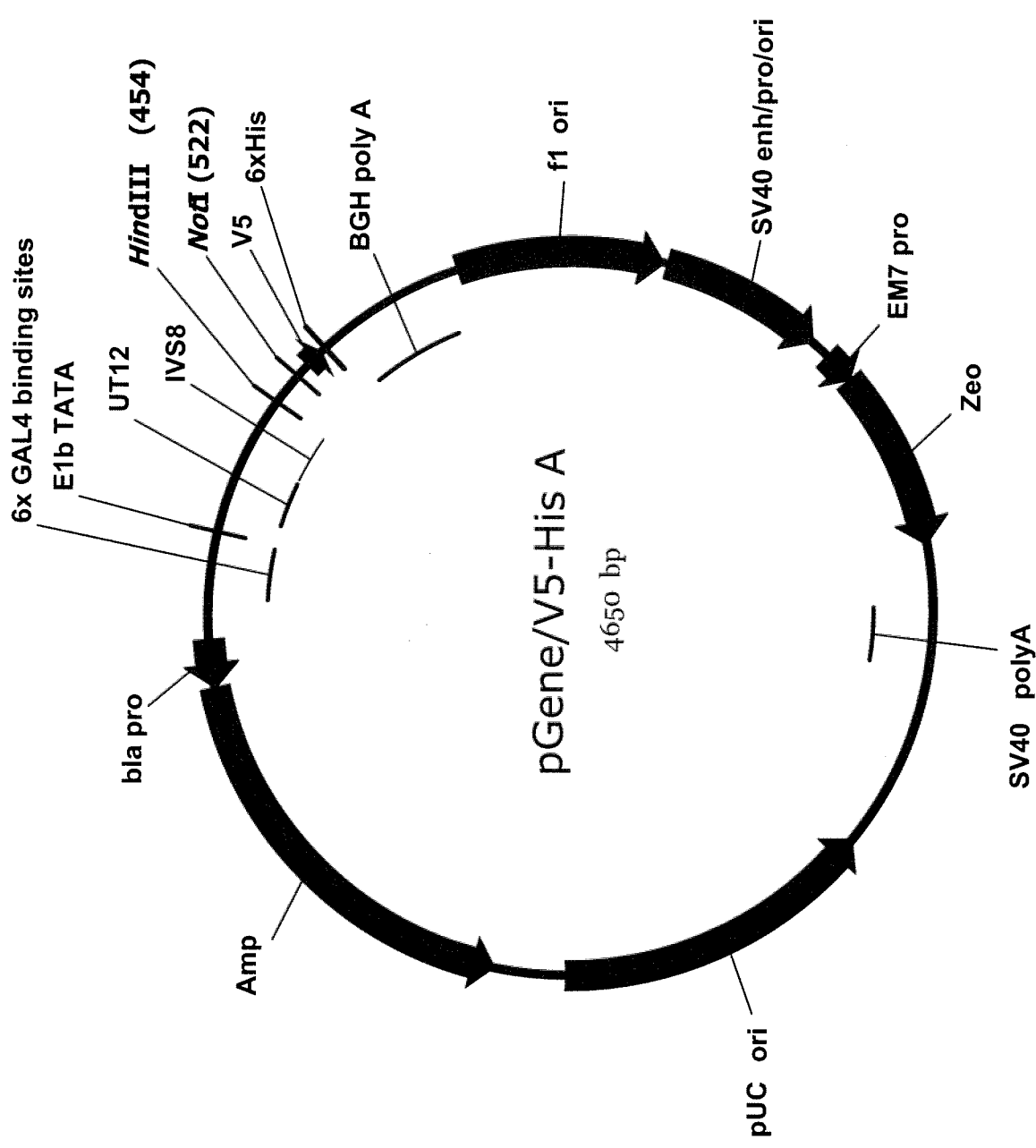
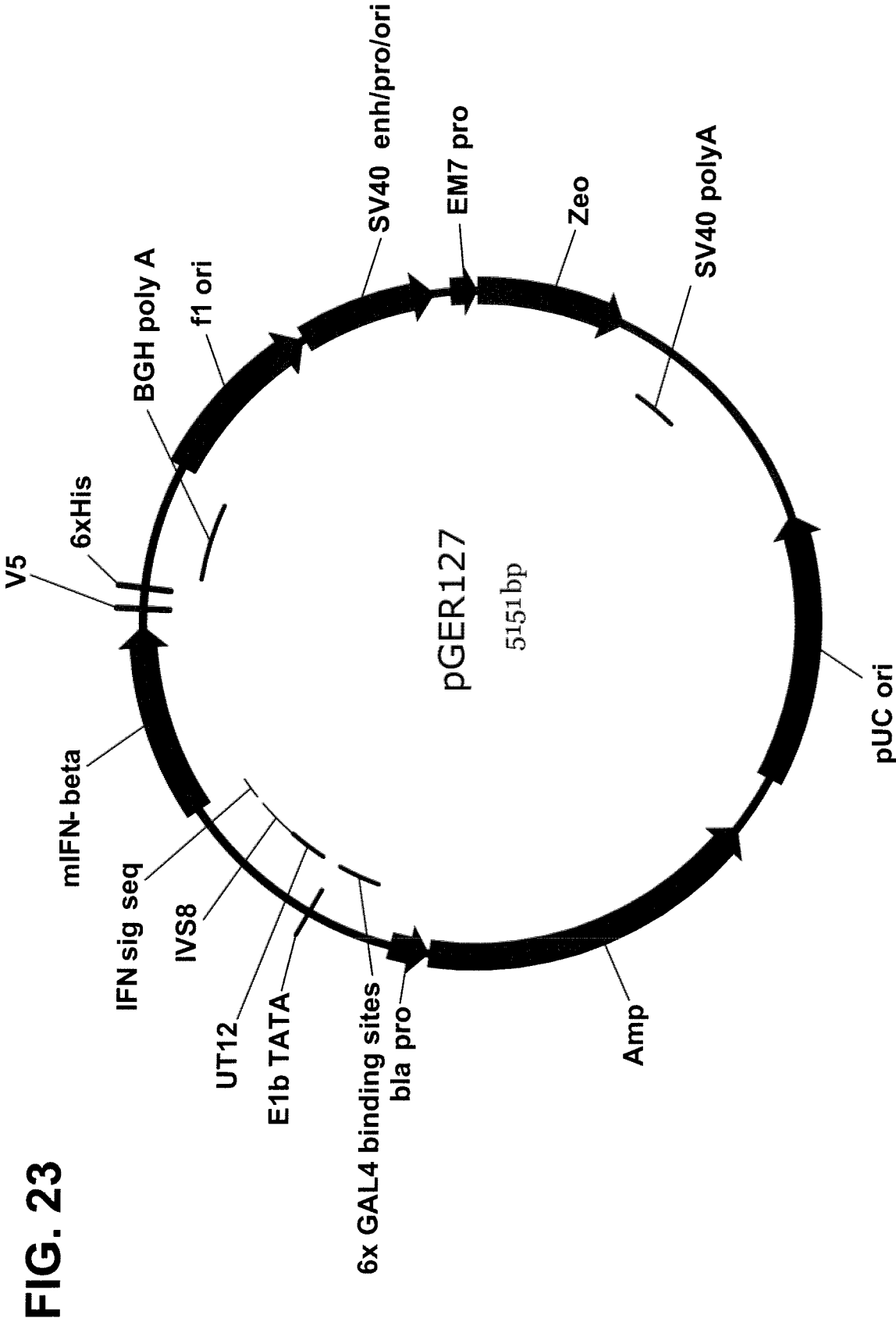
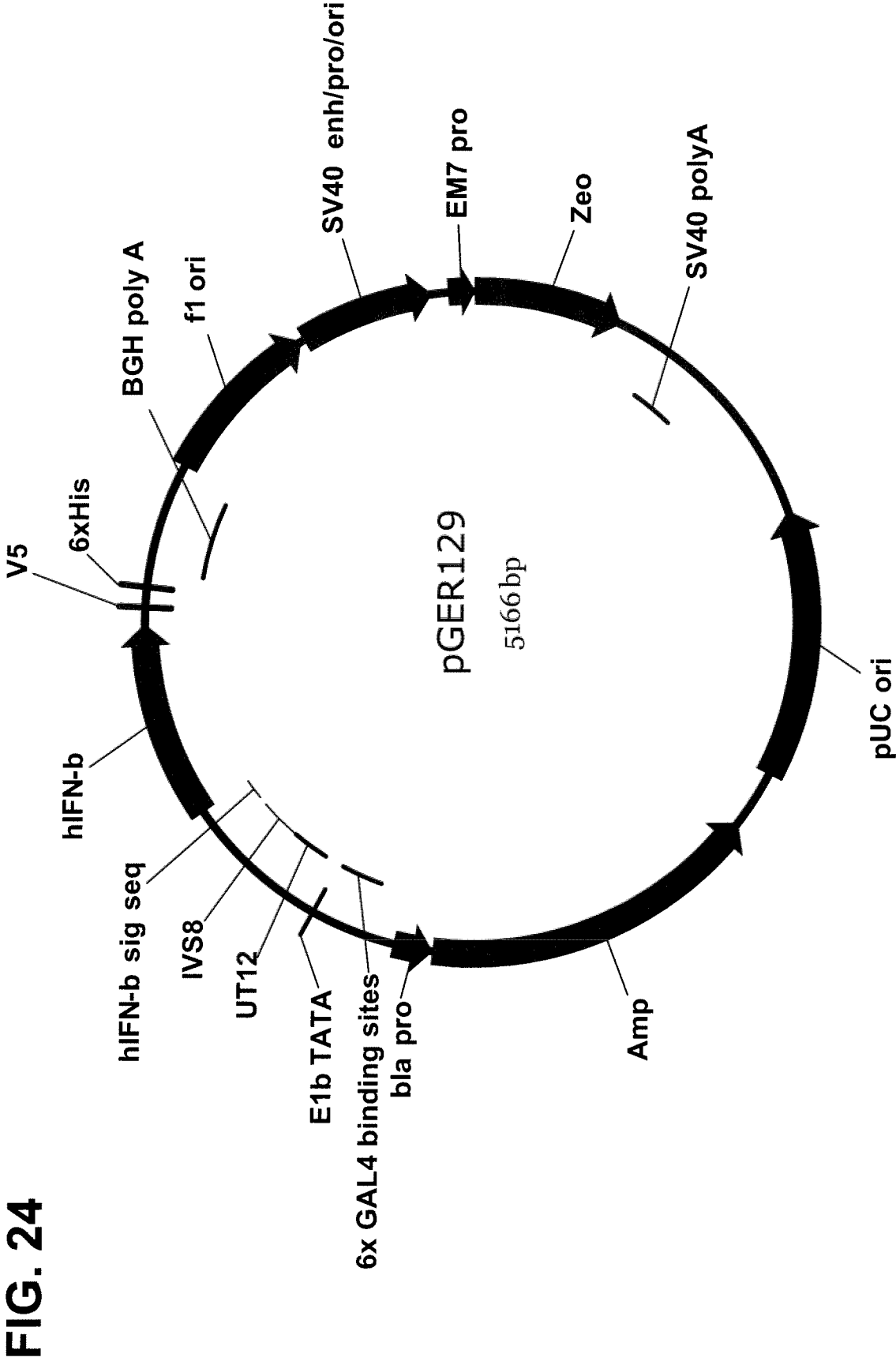
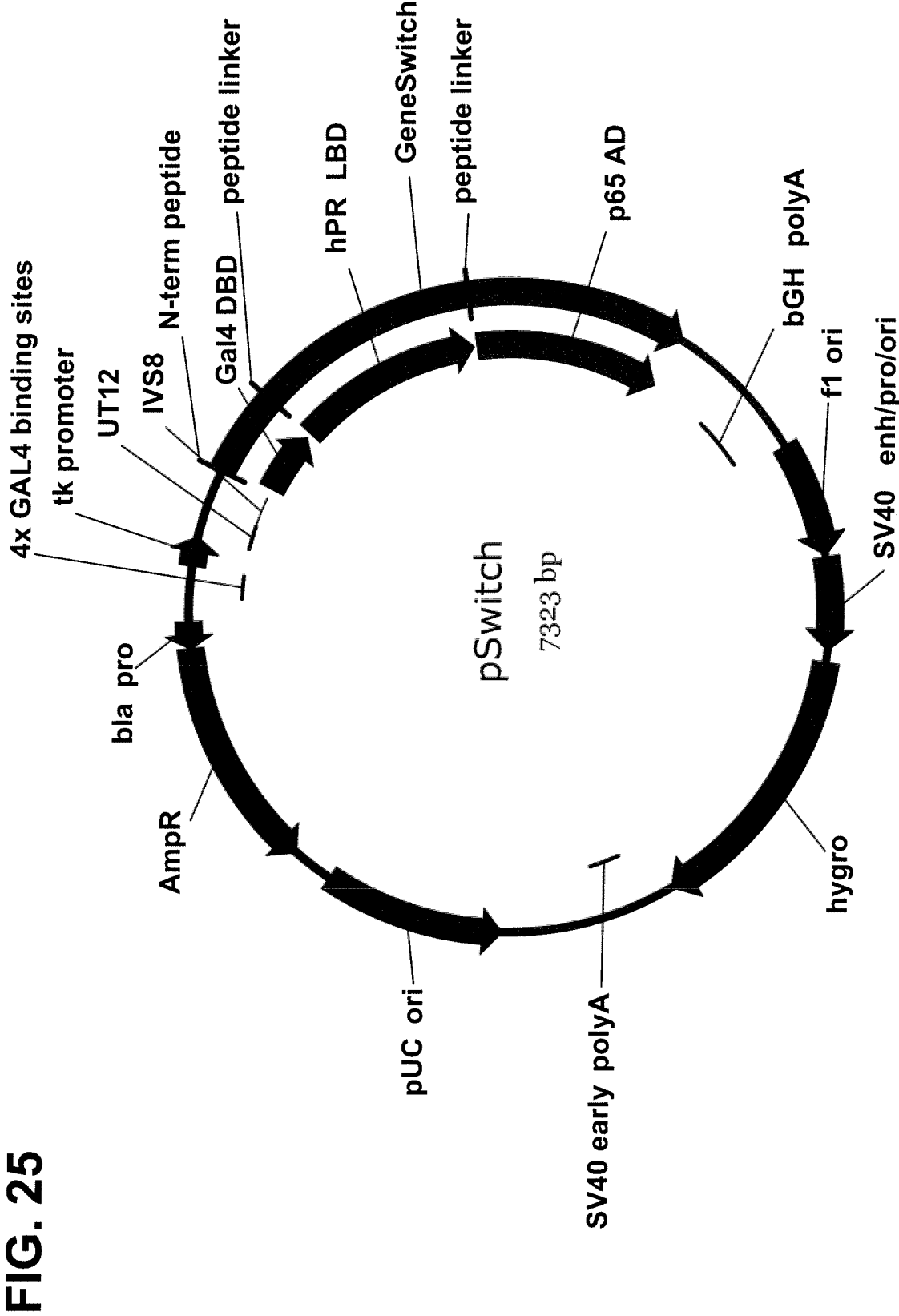


FIG. 22







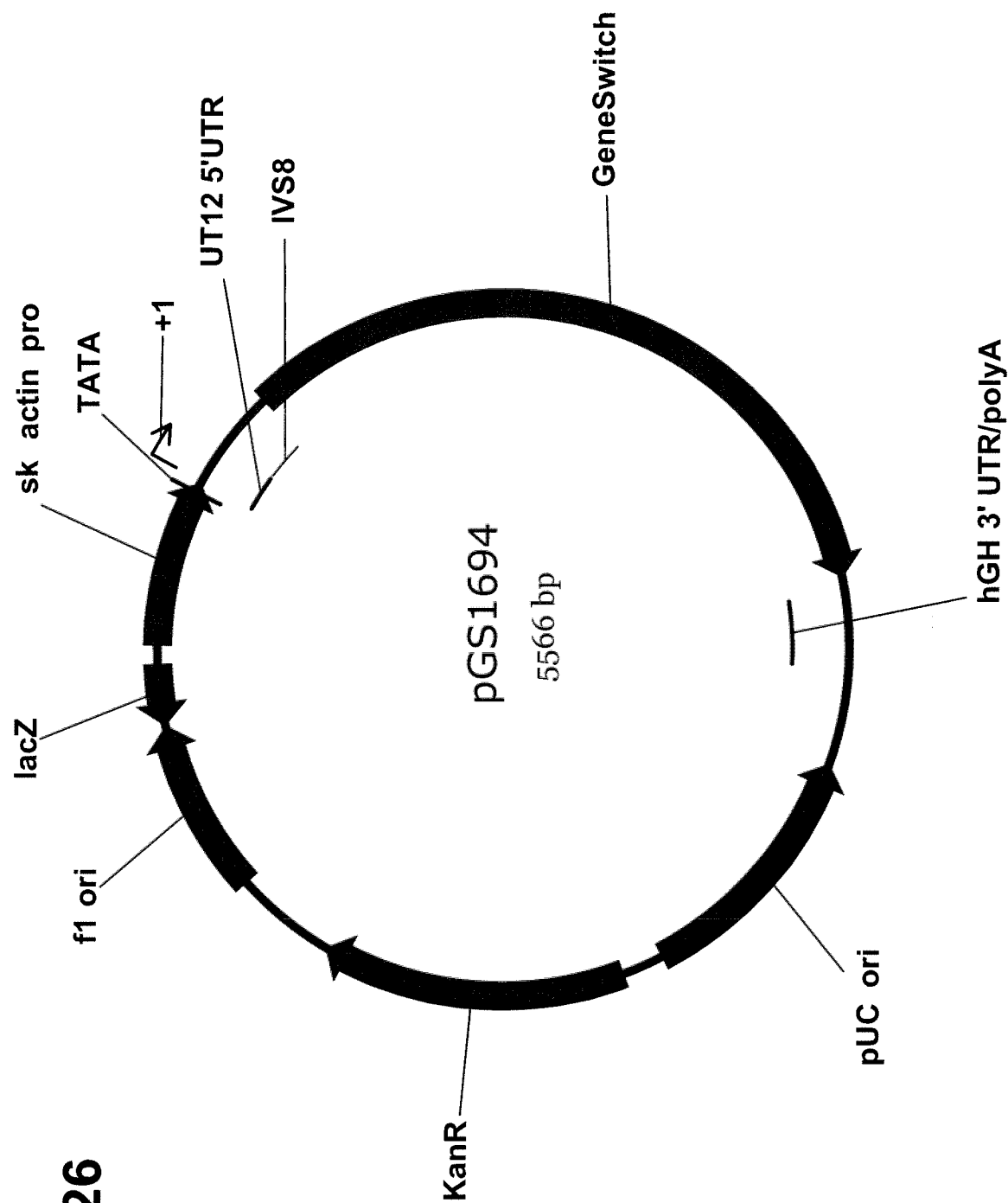
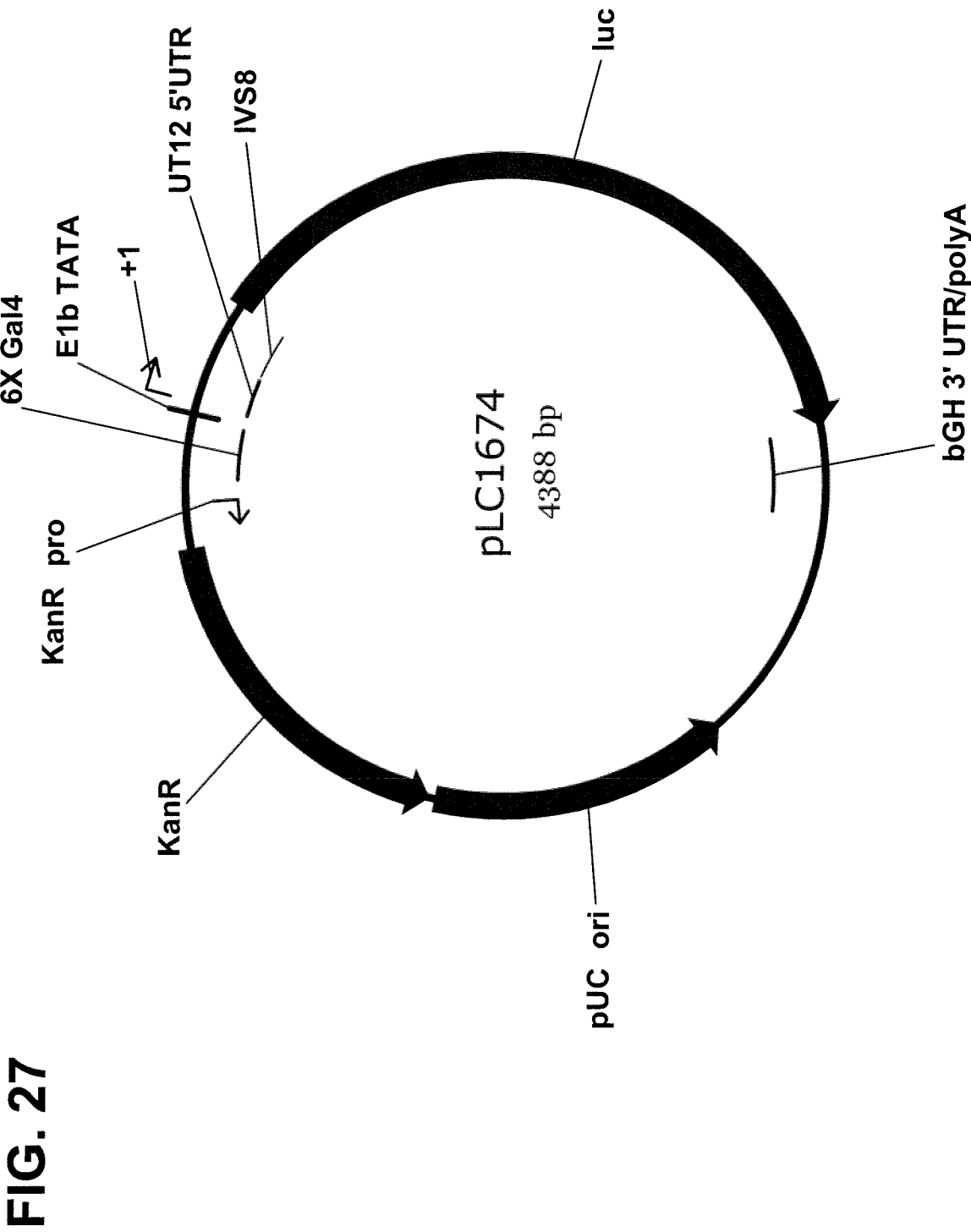
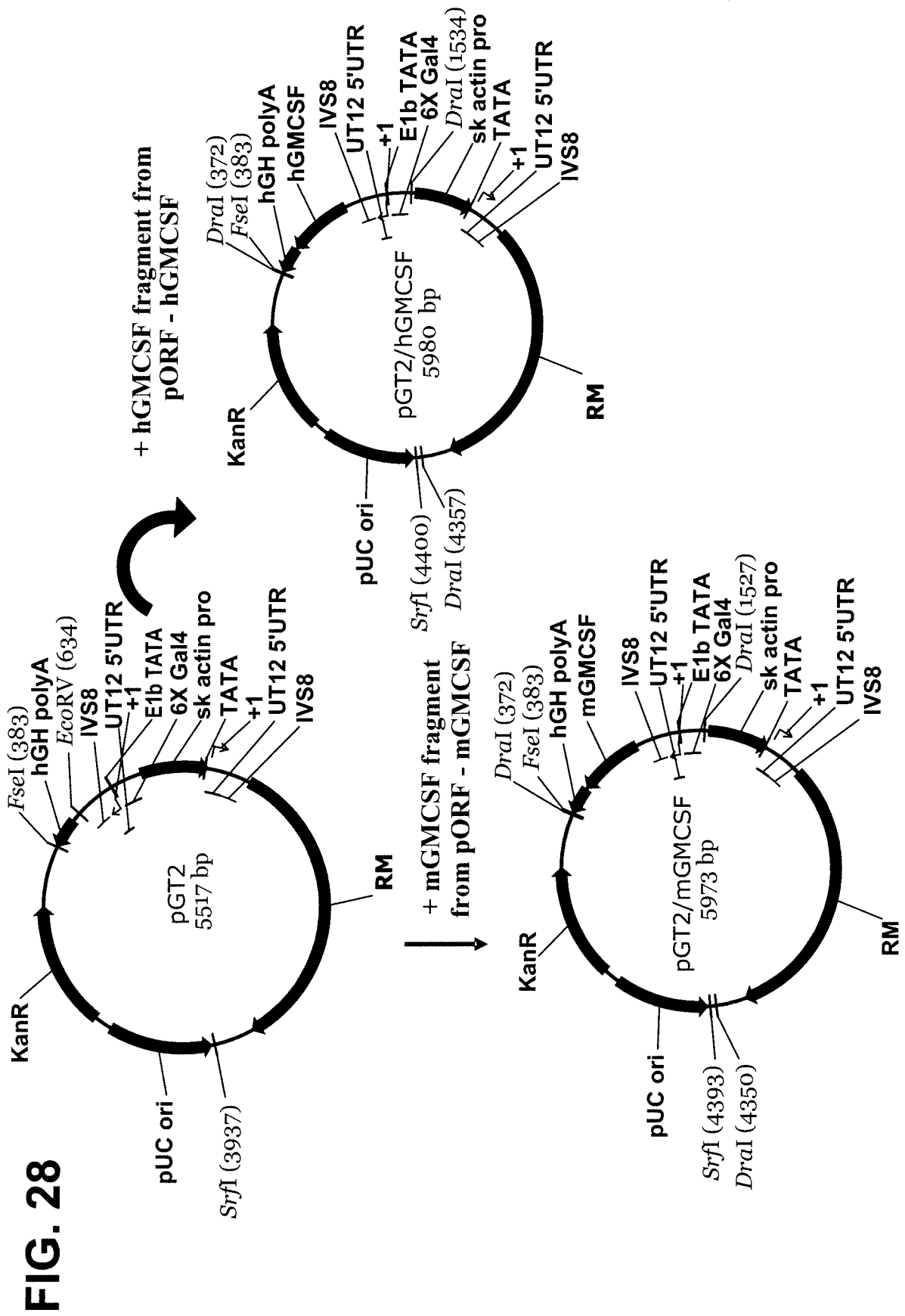


FIG. 26





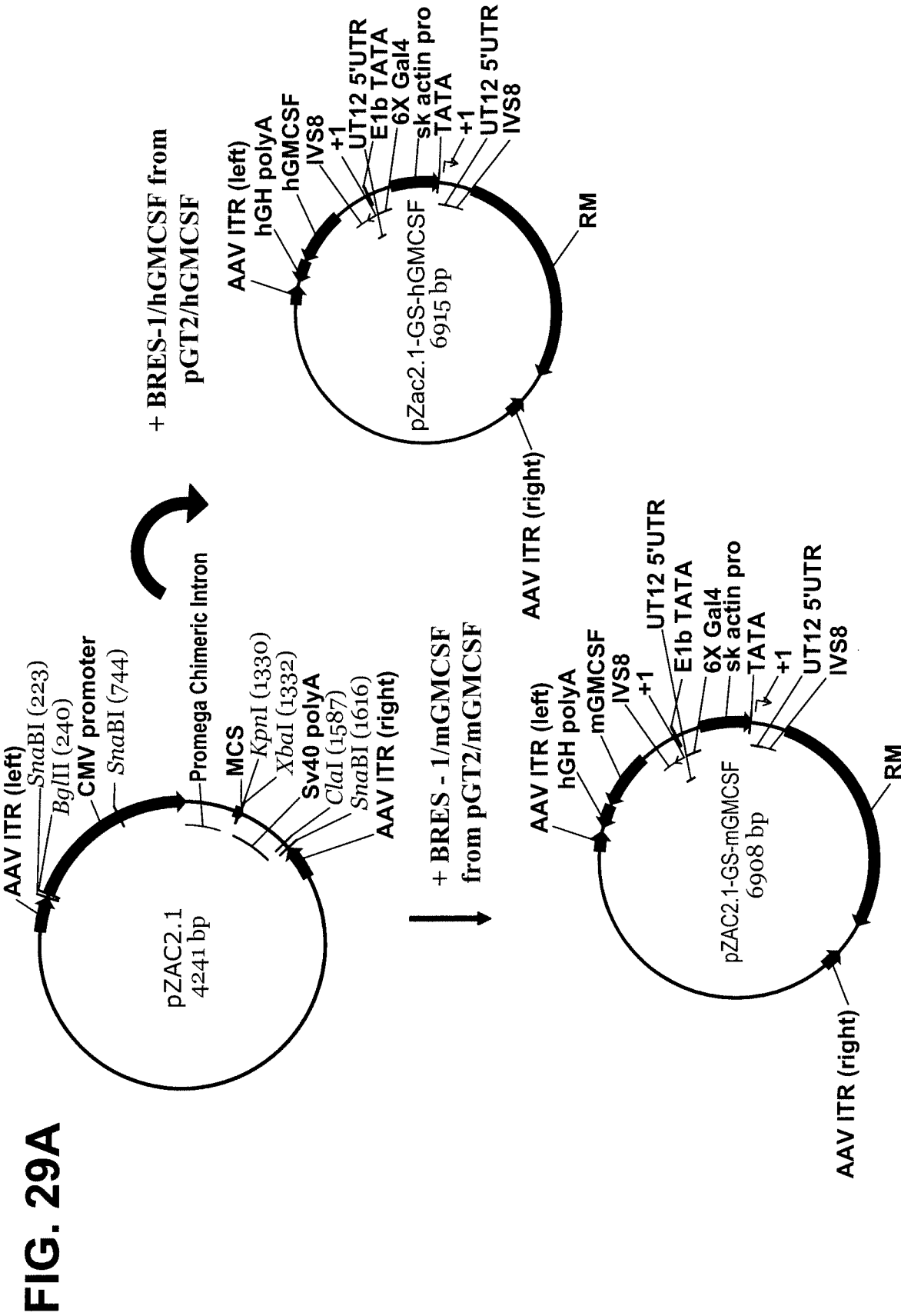
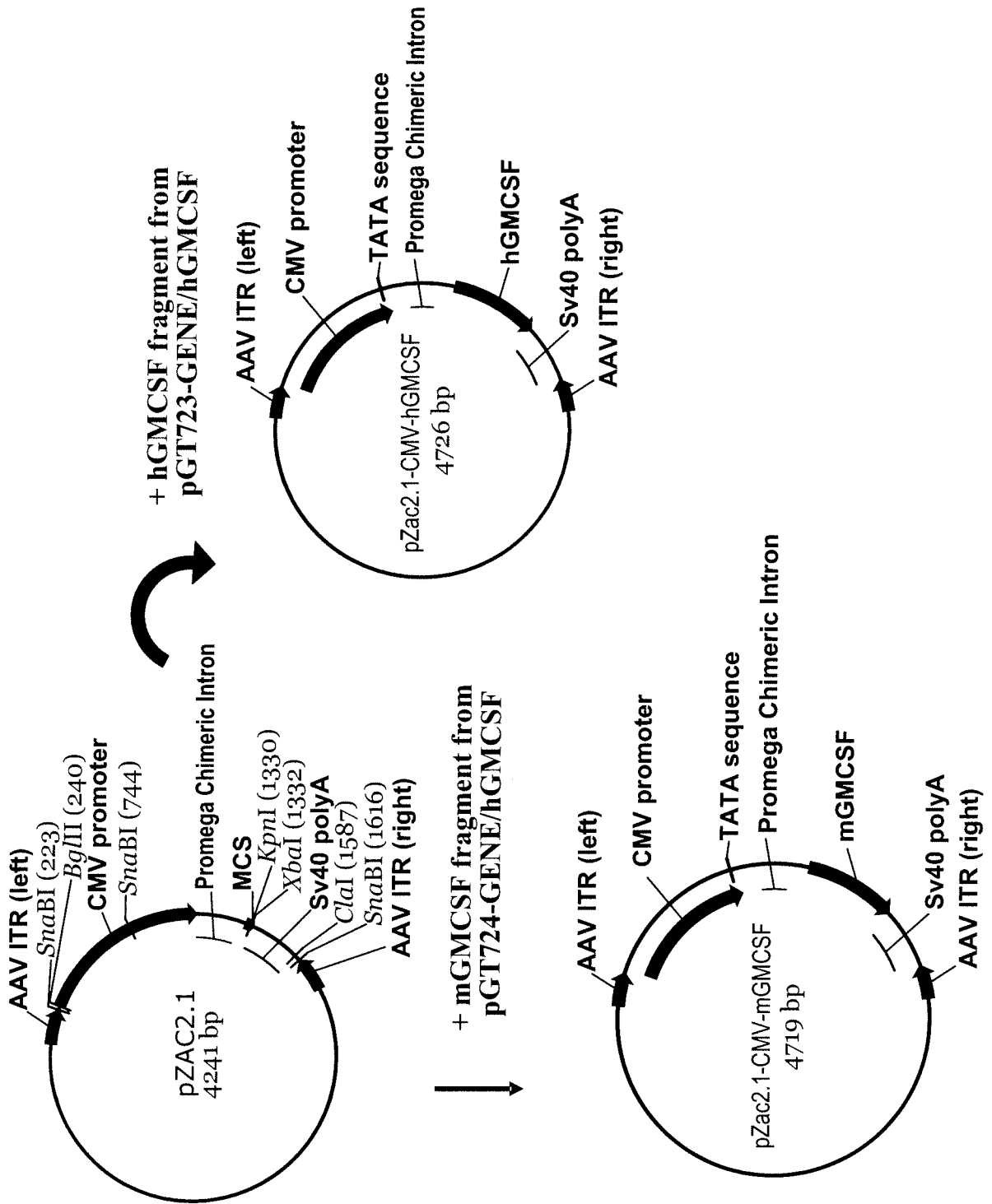
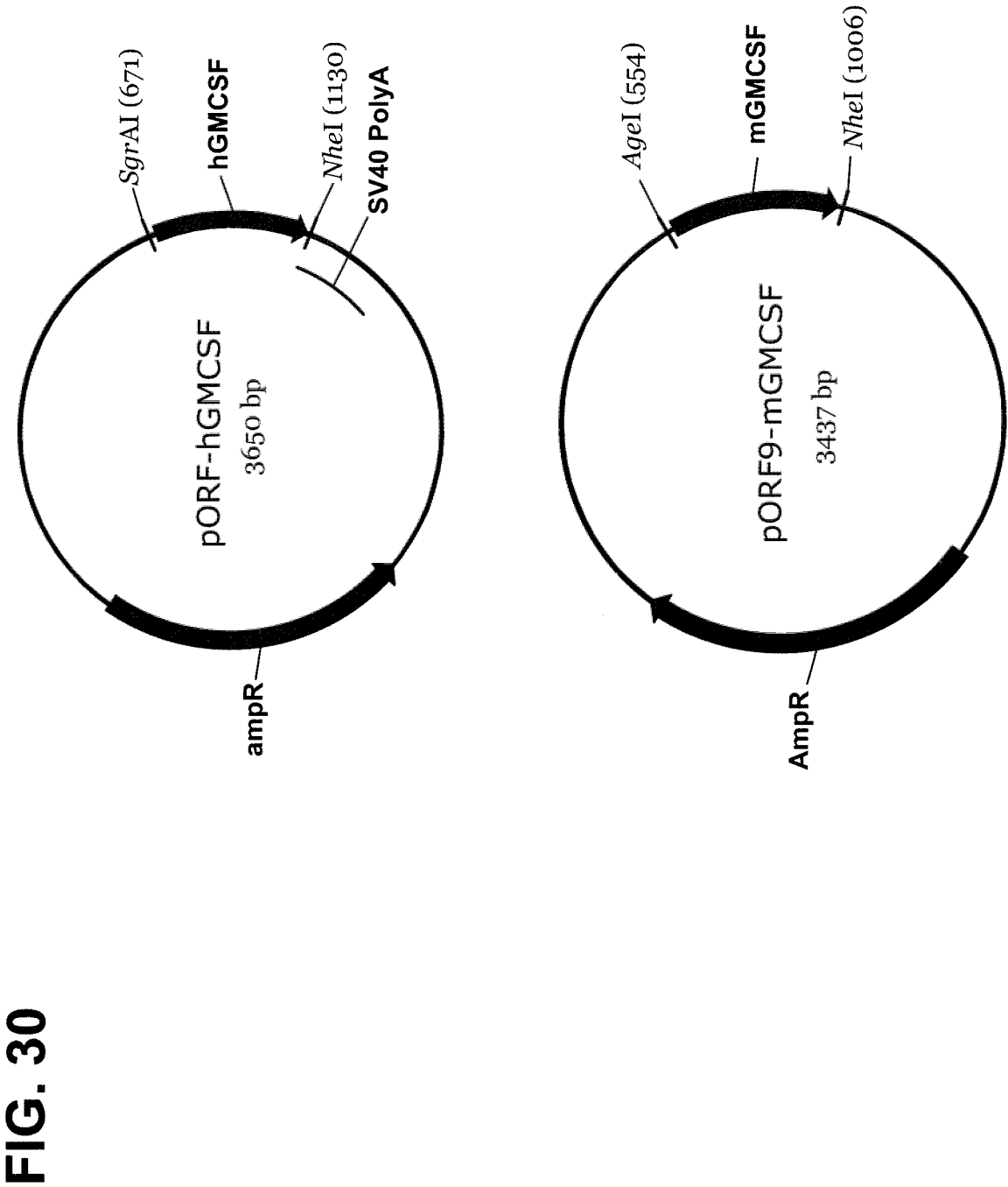


FIG. 29B





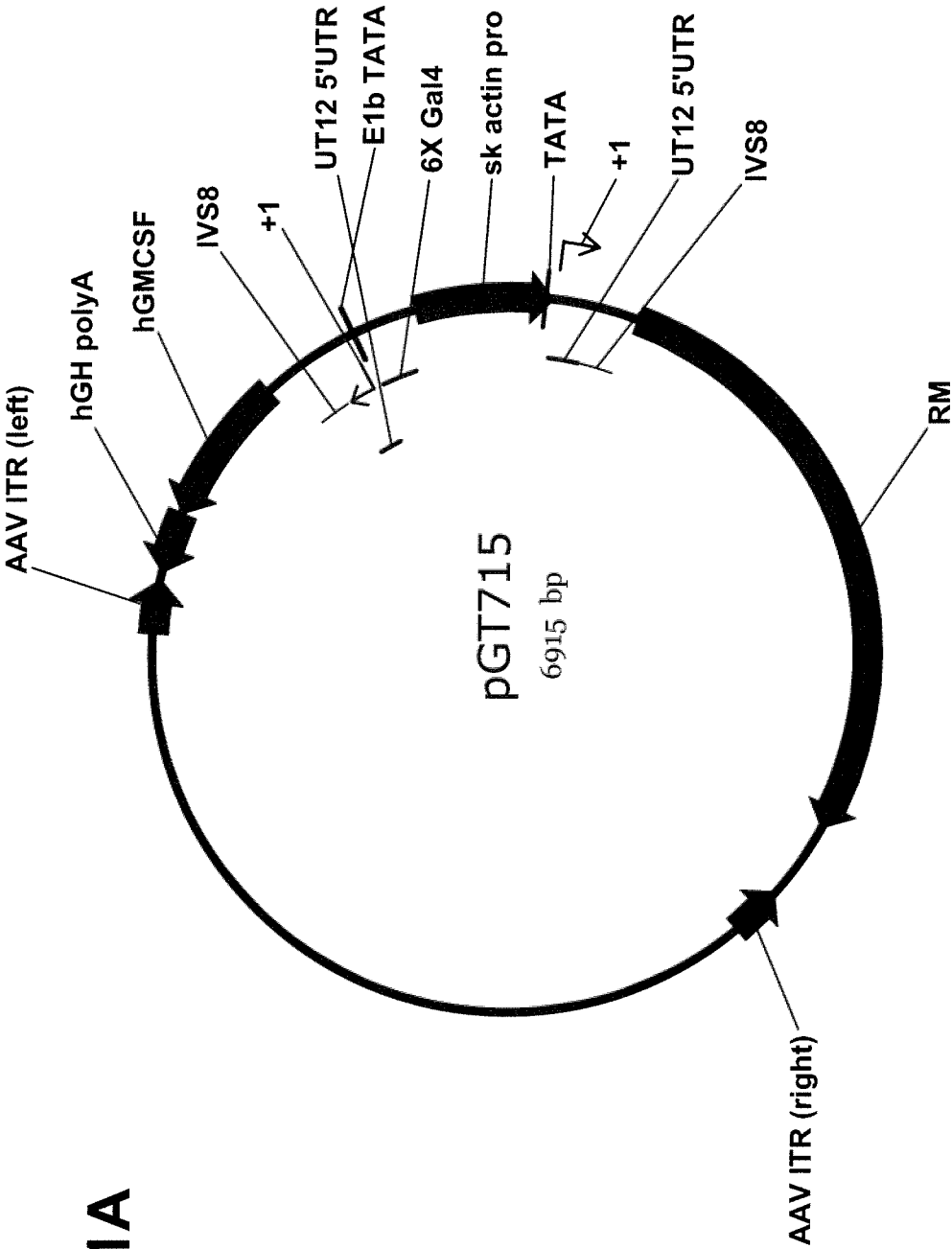


FIG. 31A

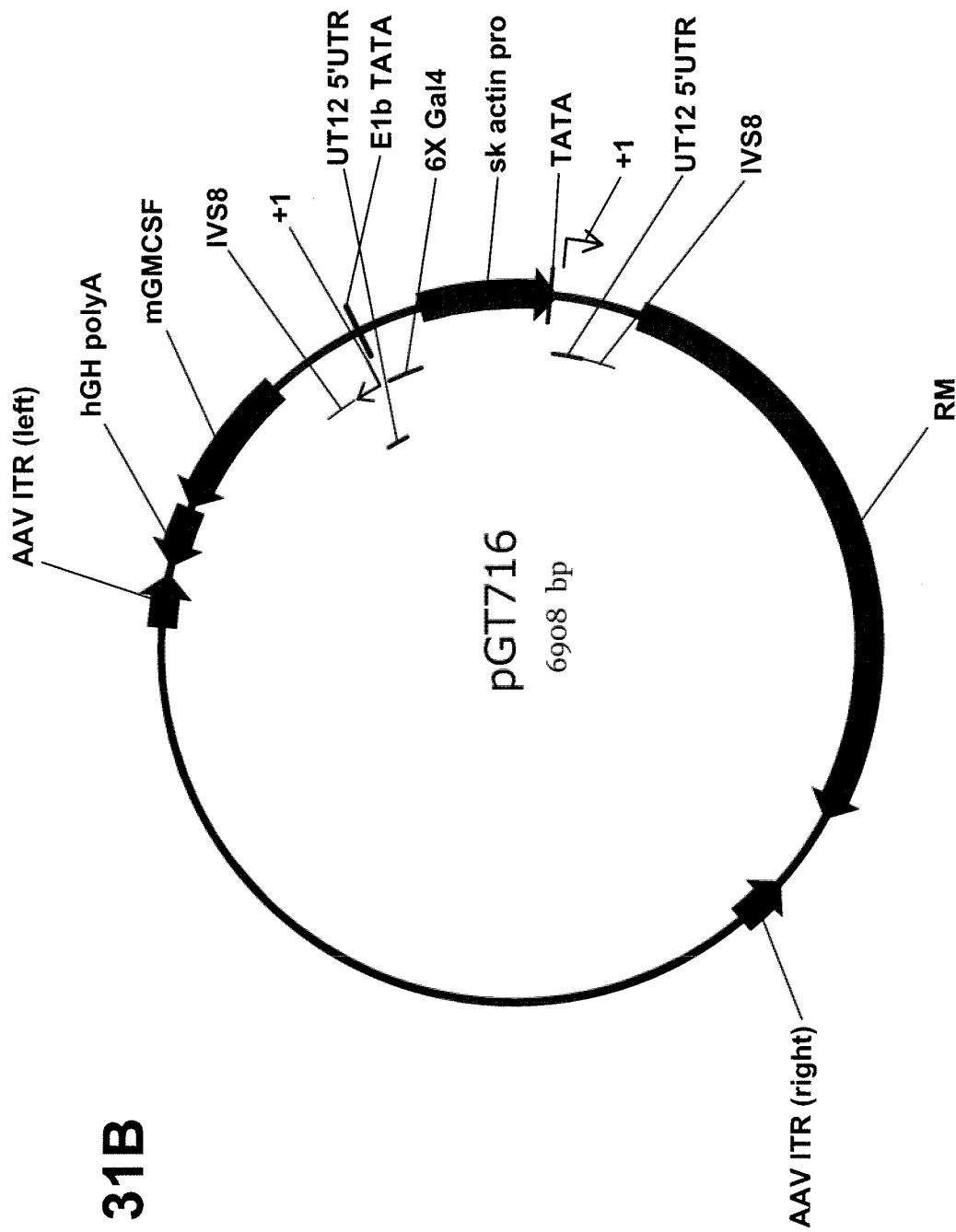


FIG. 31B

FIG. 32

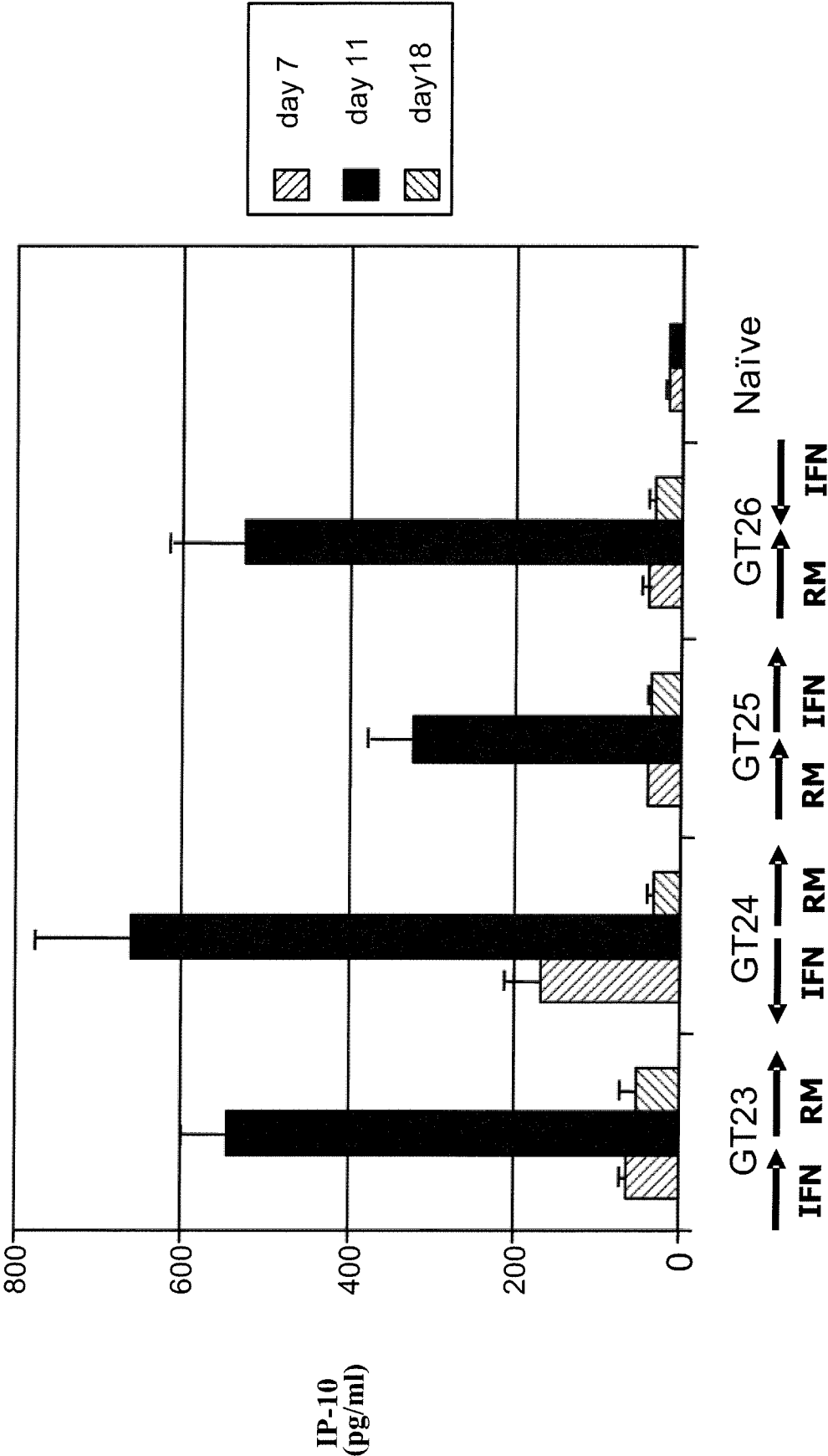


FIG. 33

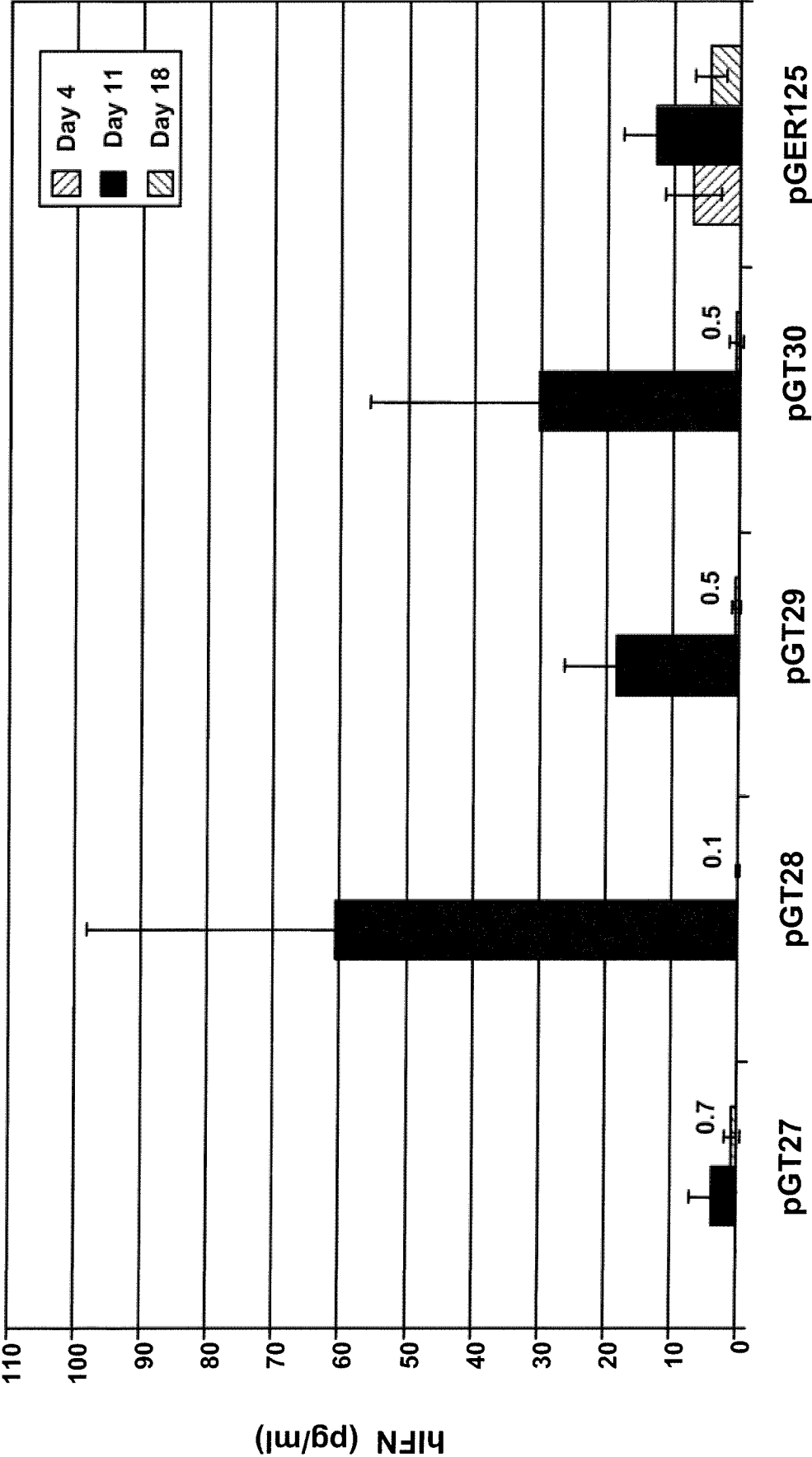


FIG. 34A

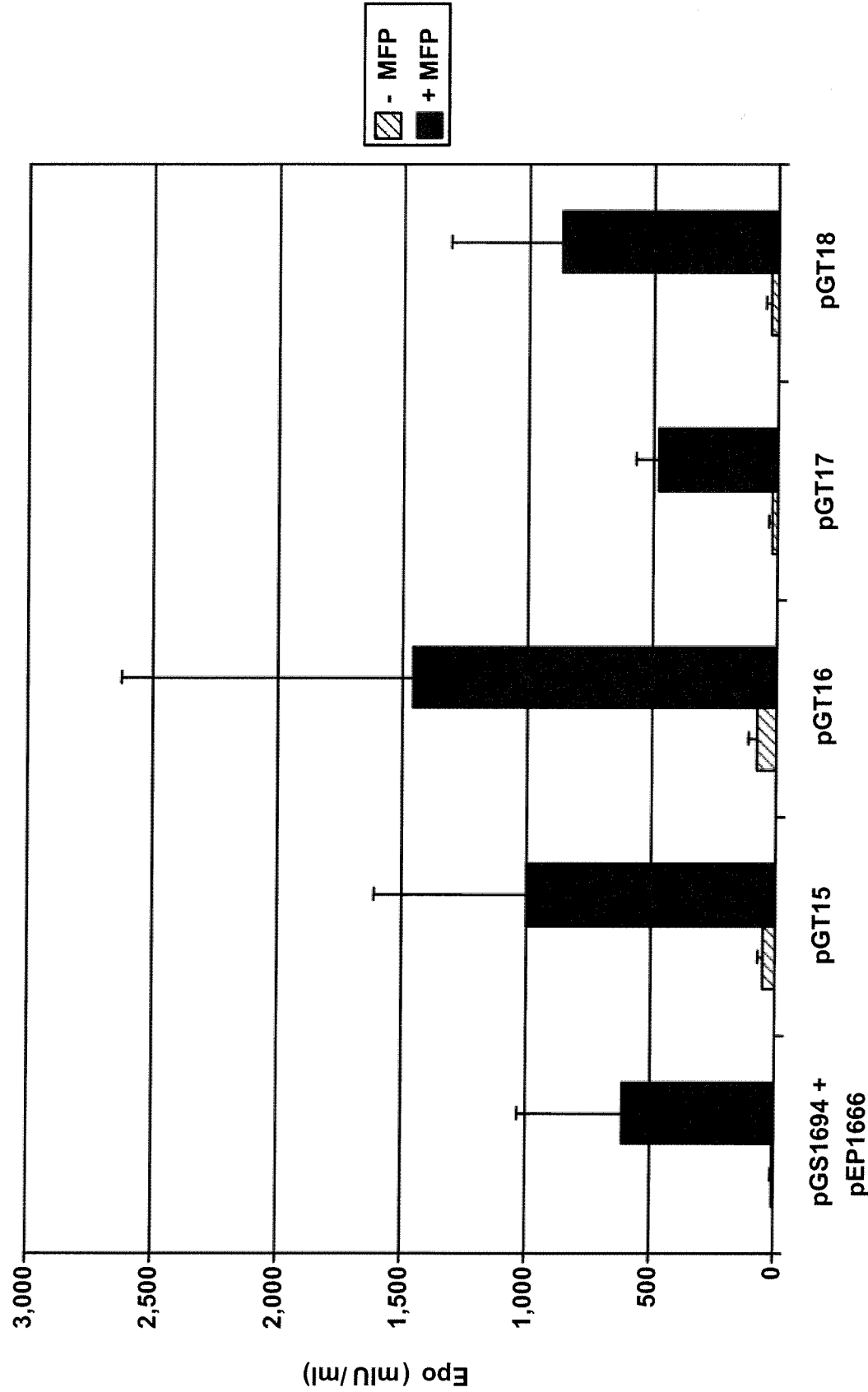


FIG. 34B

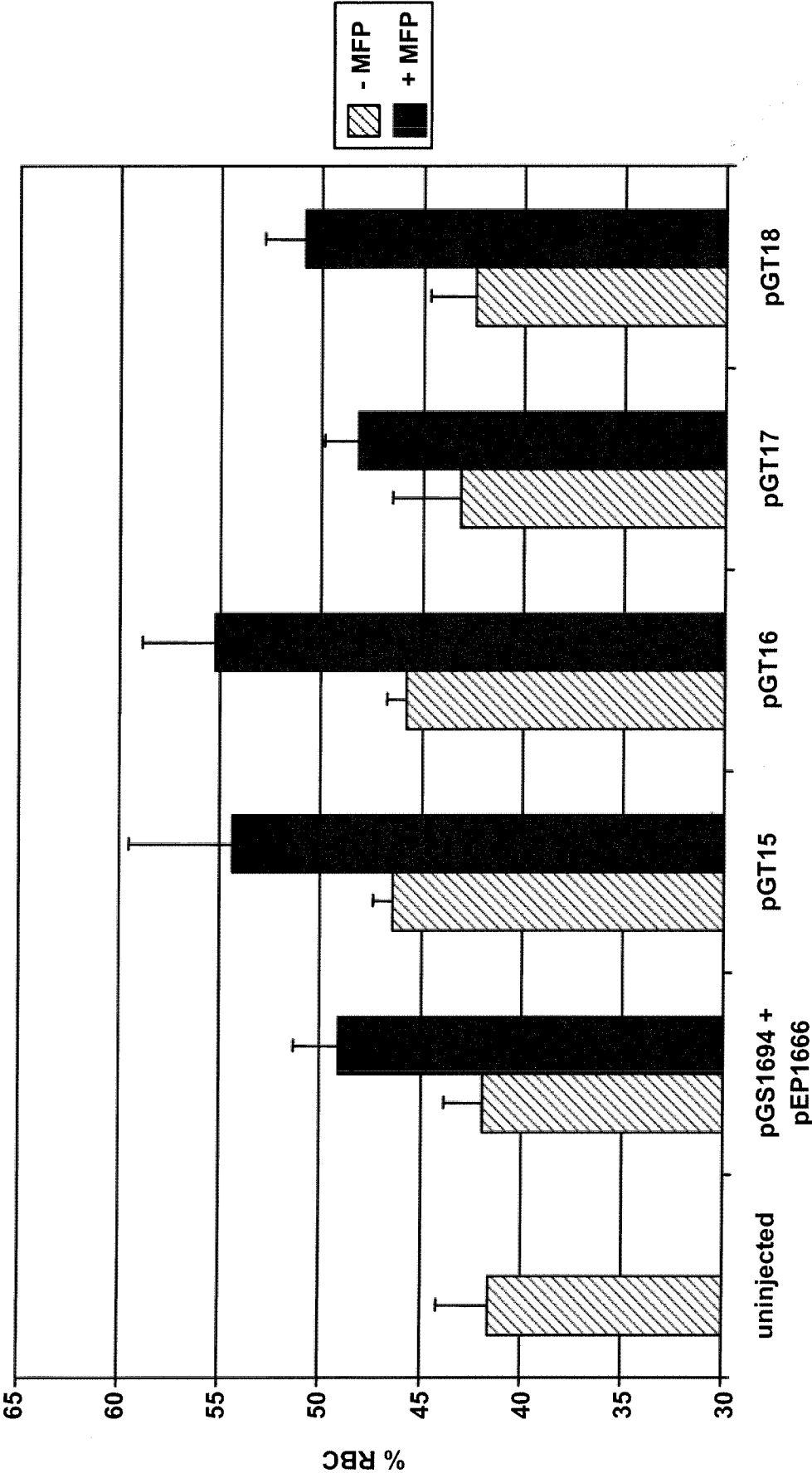


FIG. 35

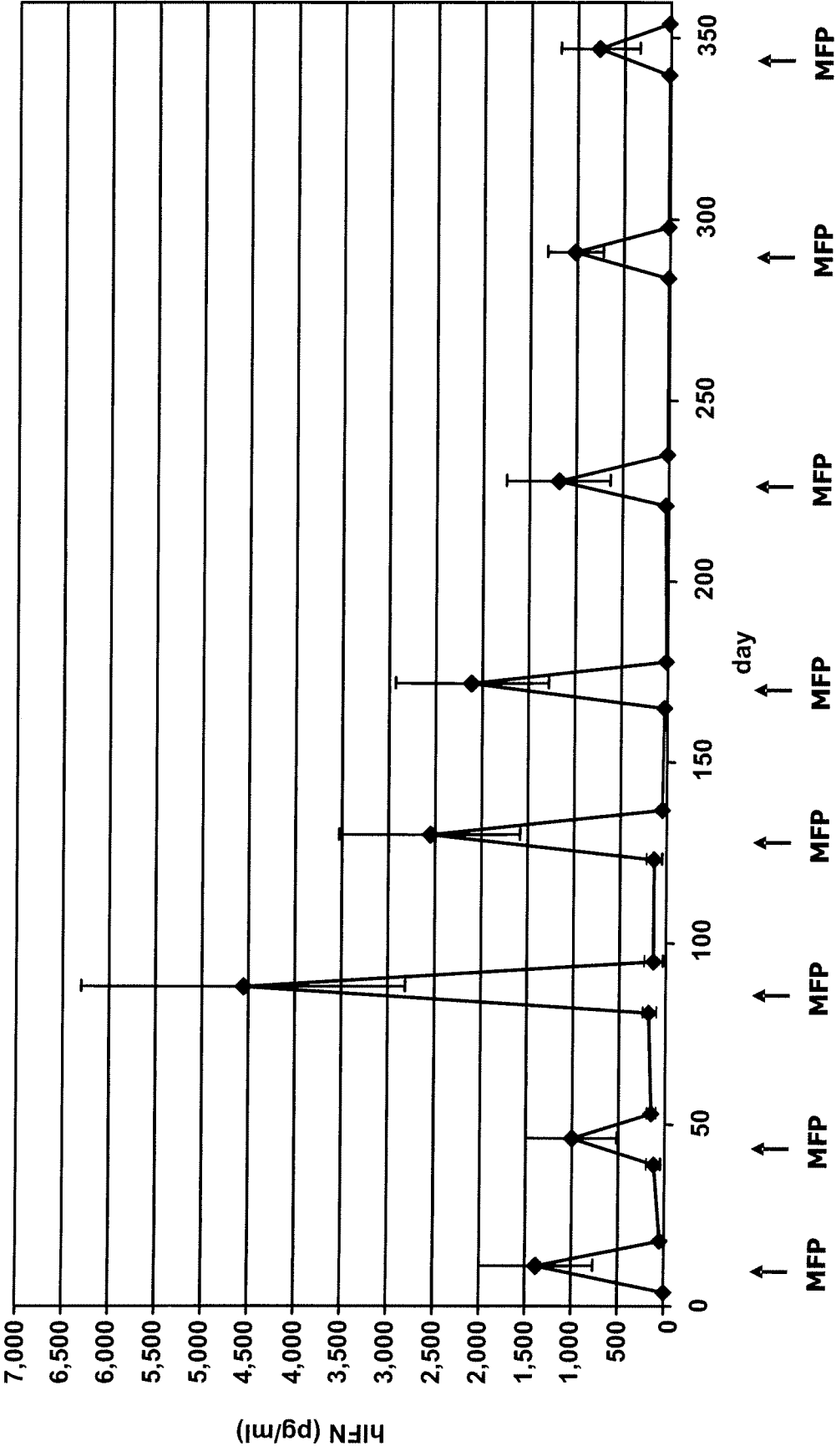


FIG. 36

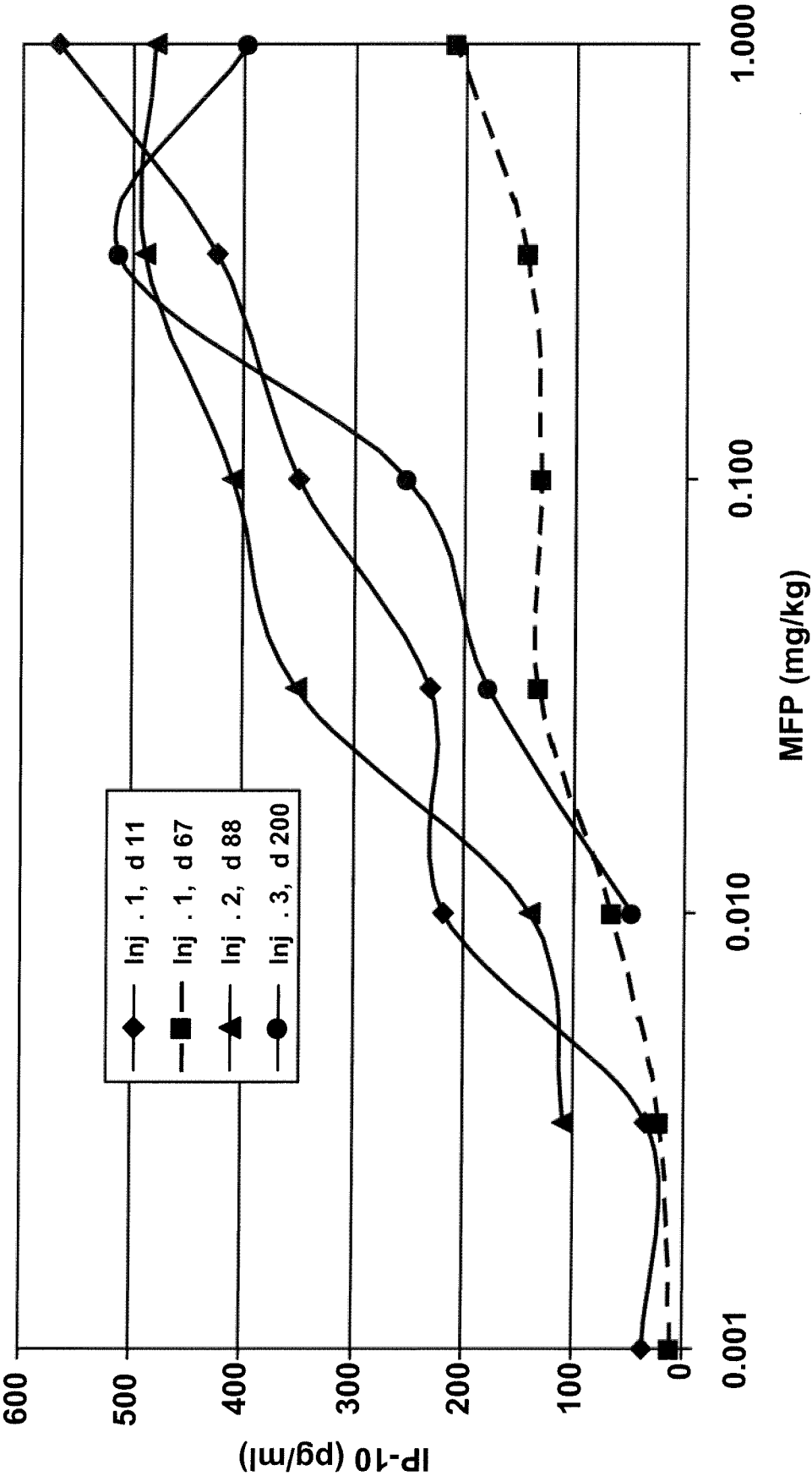


FIG. 37A

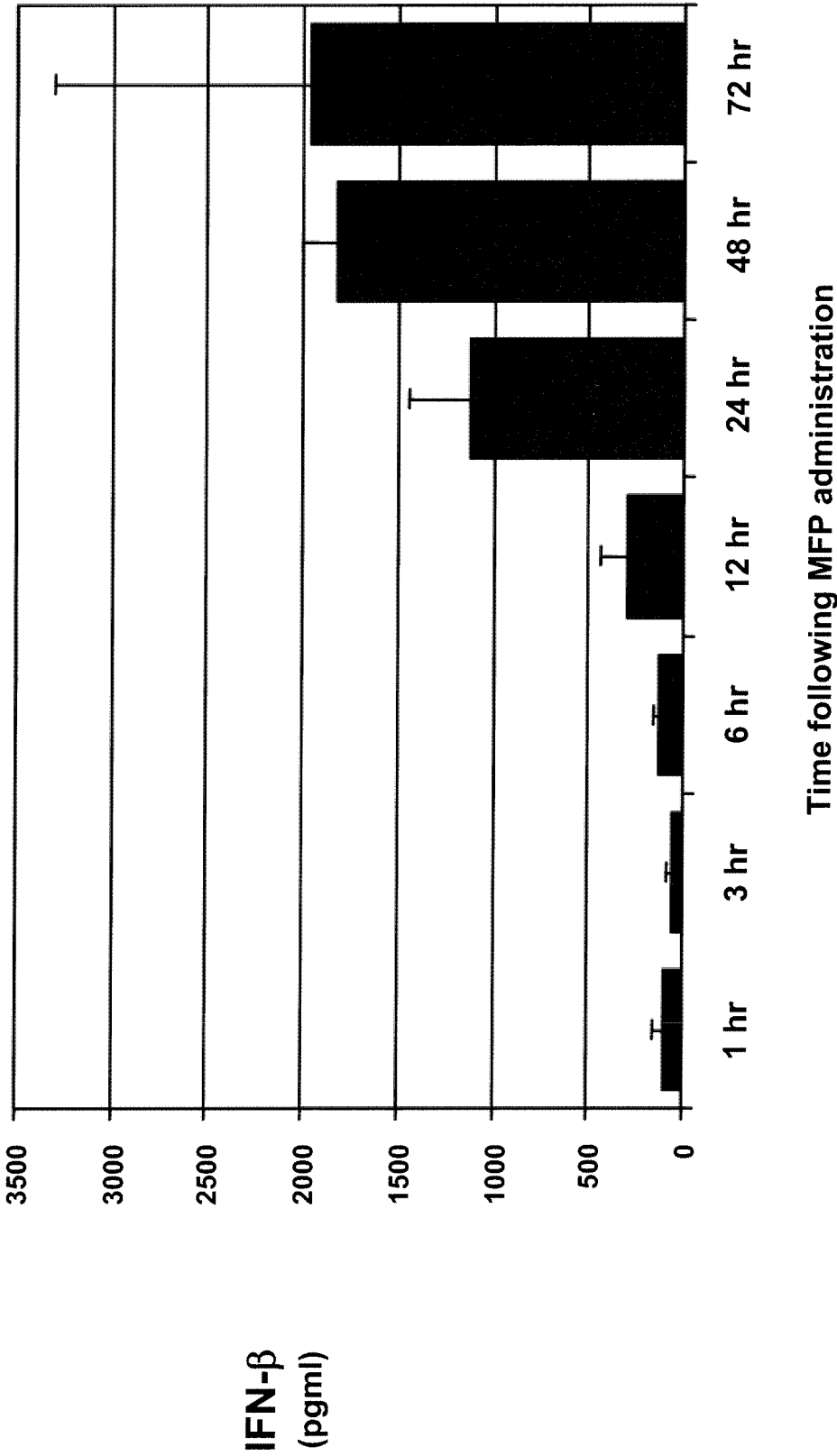
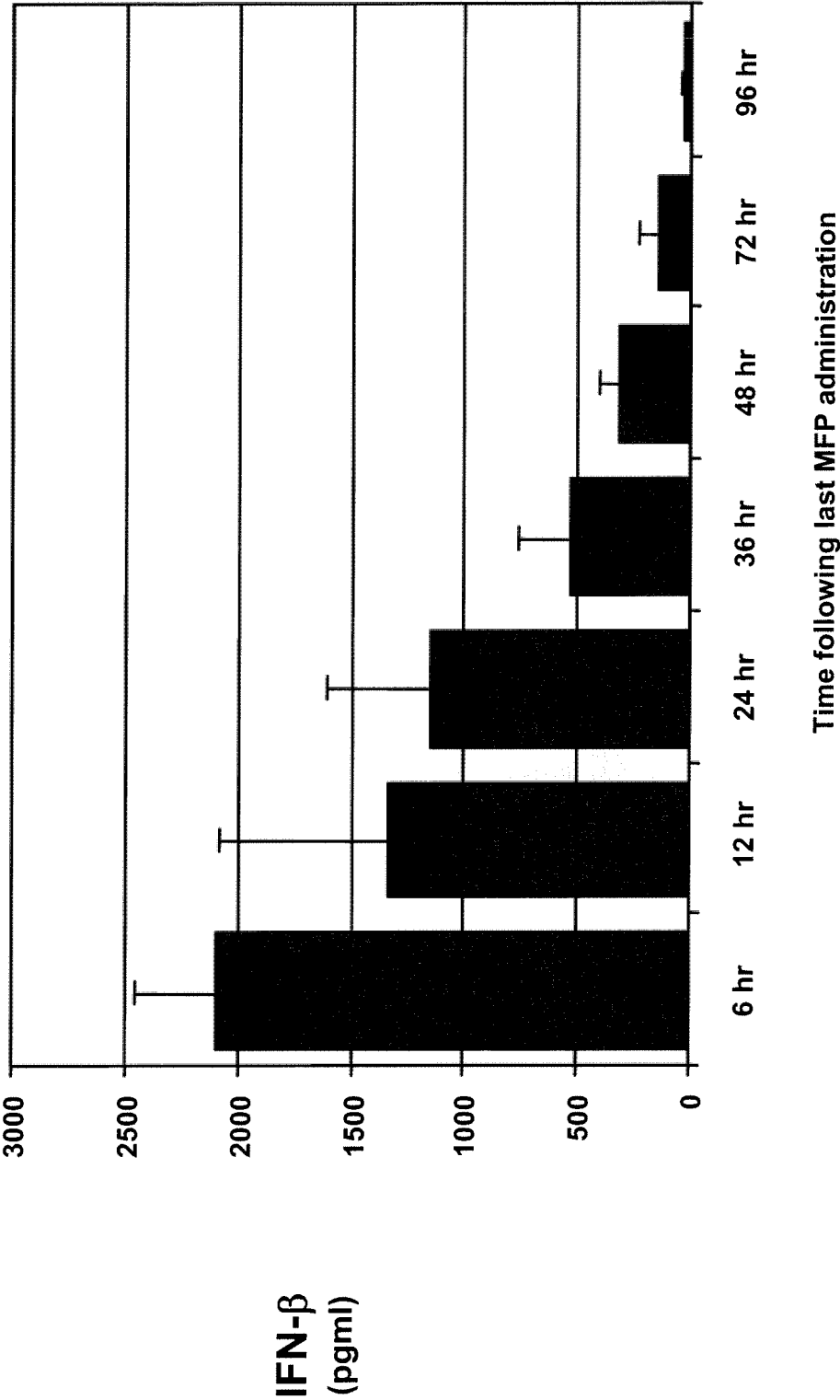


FIG. 37B



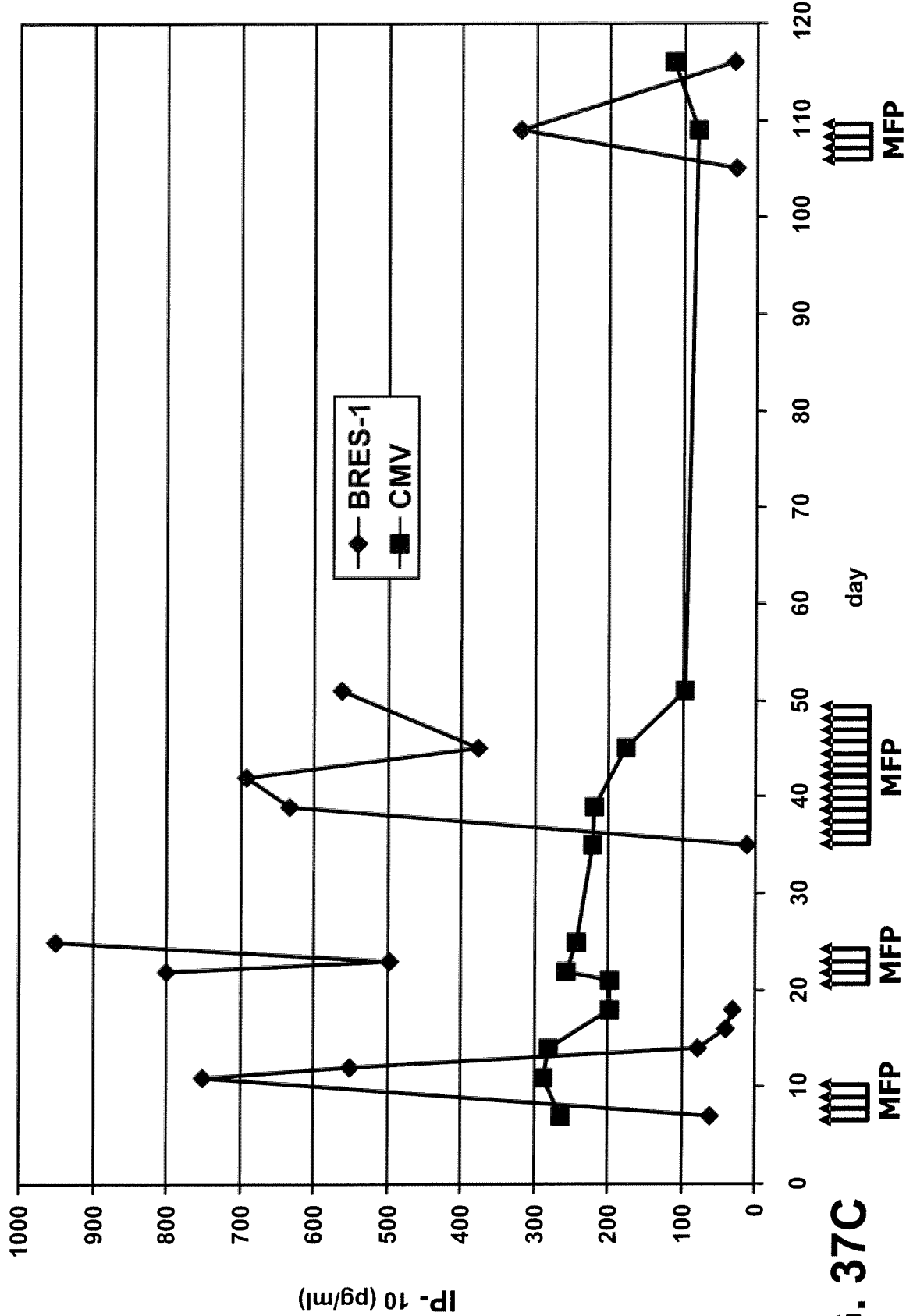


FIG. 37C

FIG. 38

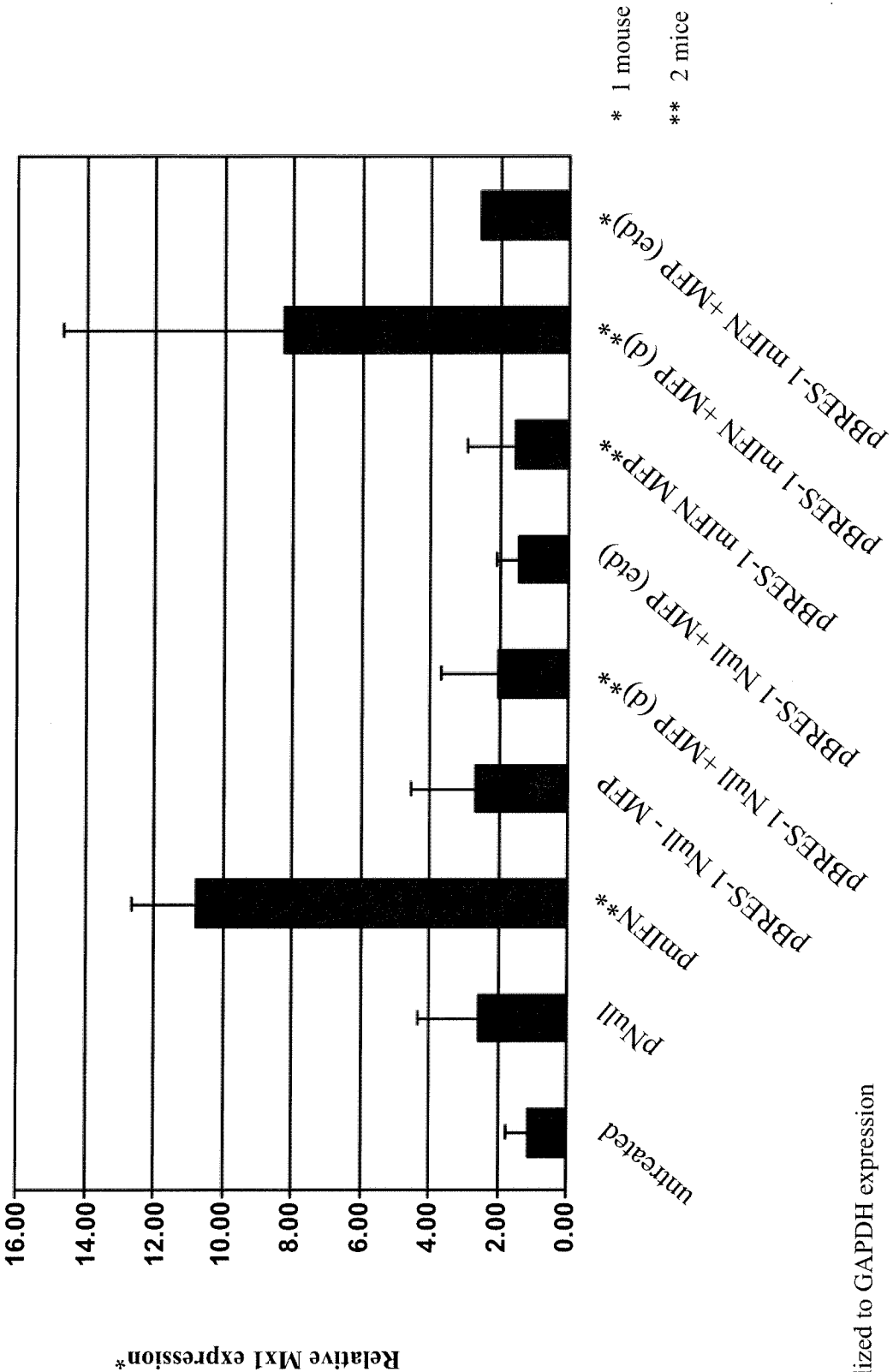


Fig. 39A

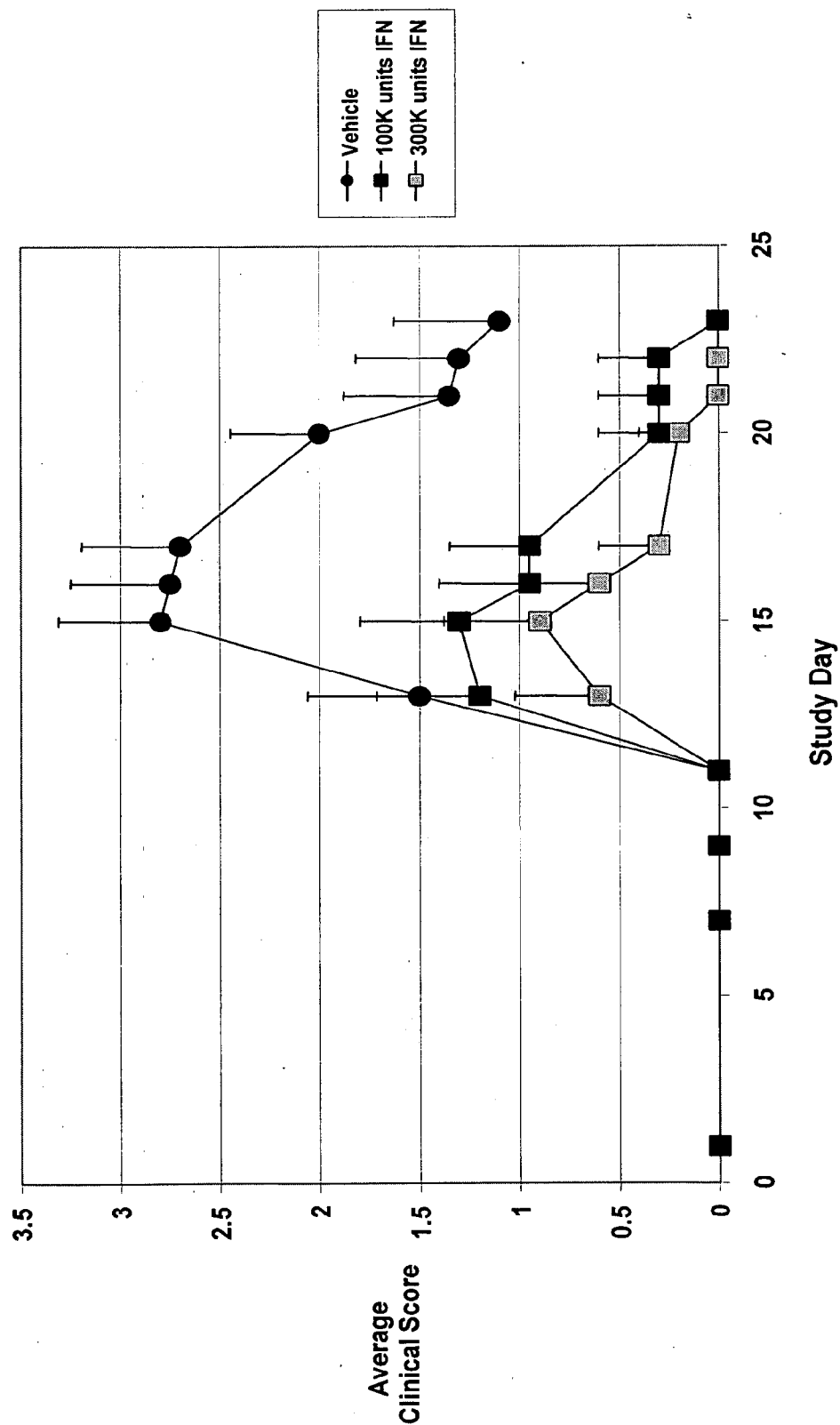


Fig. 39B

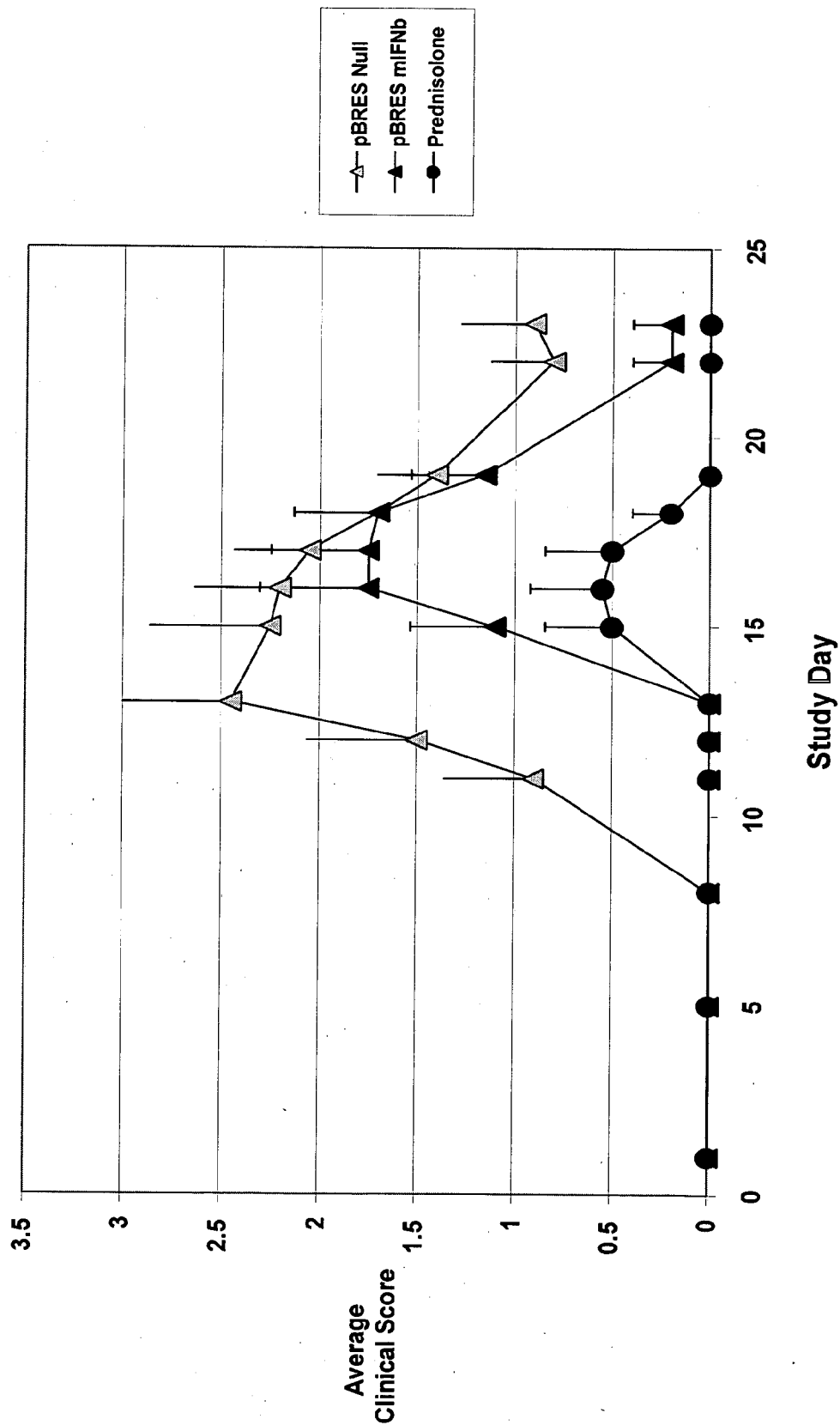


Fig. 40

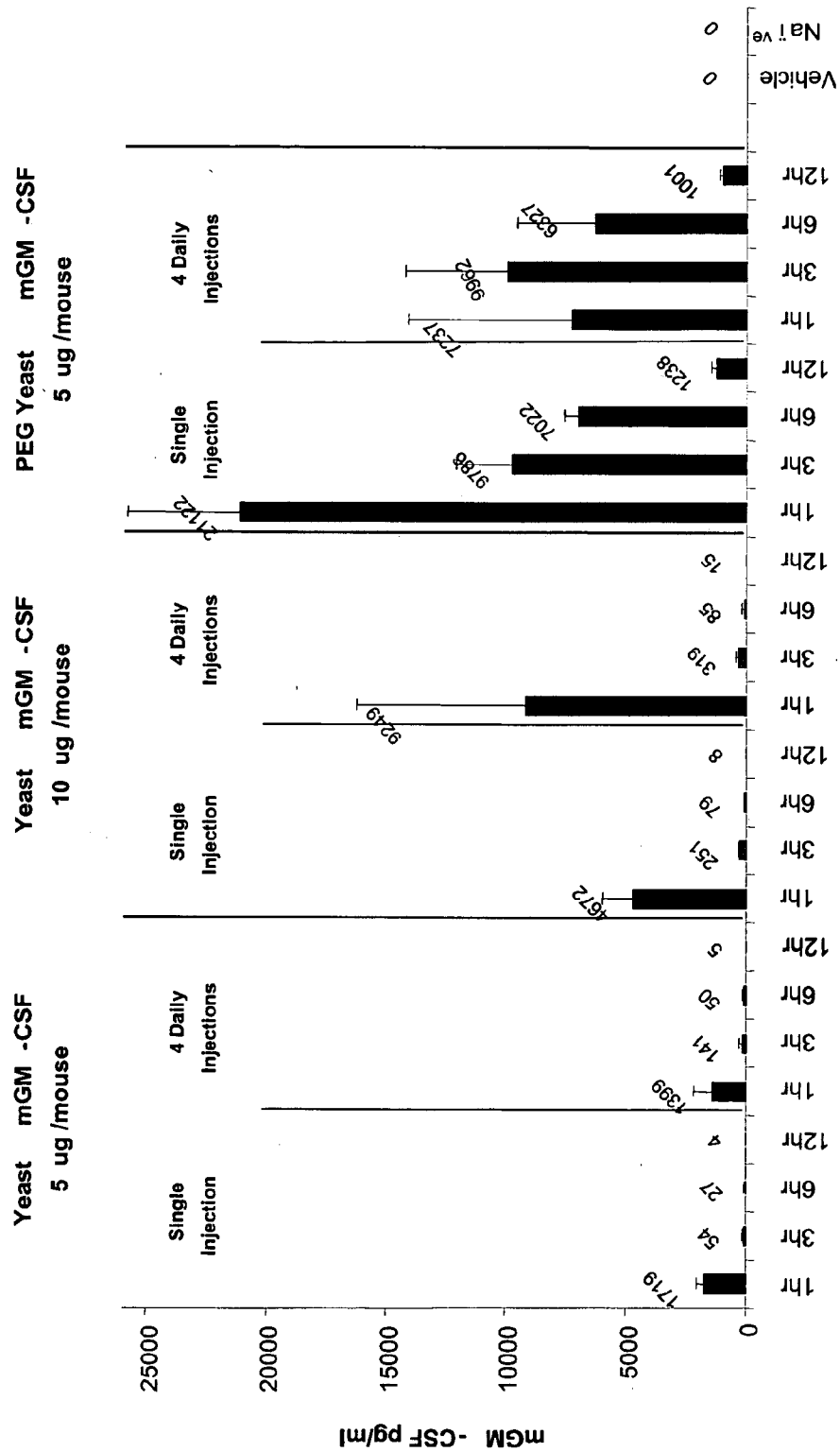


Fig. 41

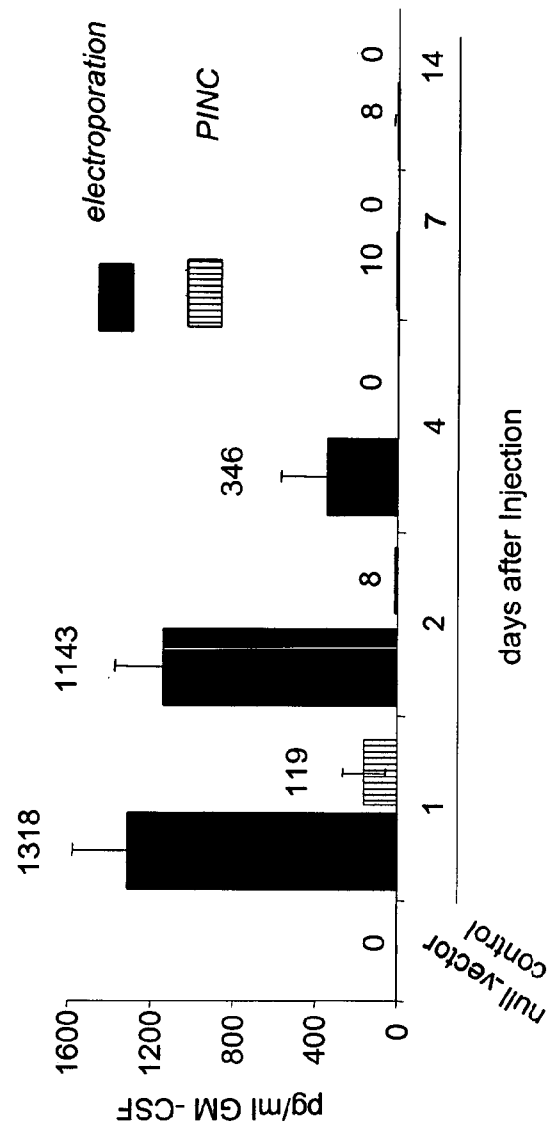


Fig. 42

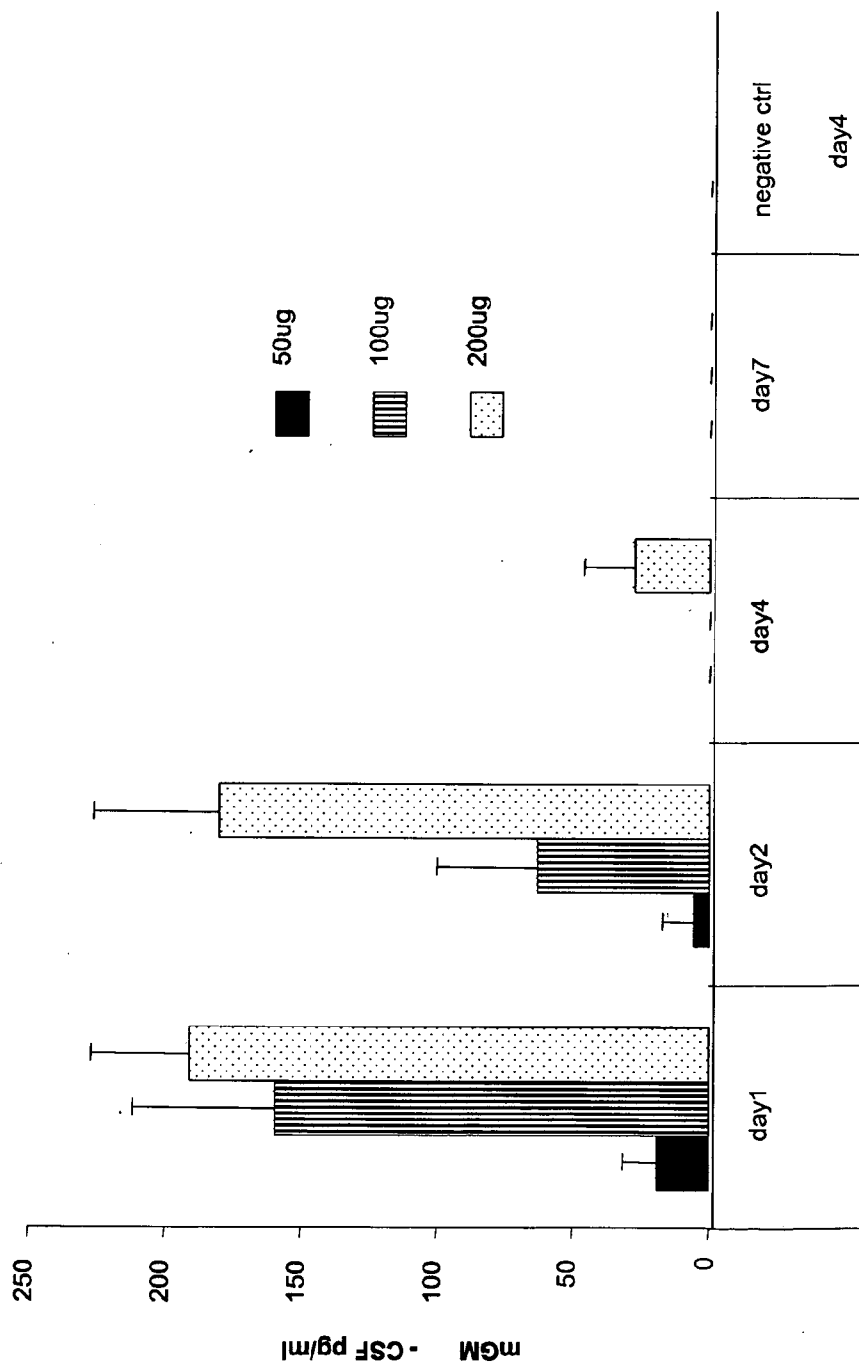


Fig. 43A

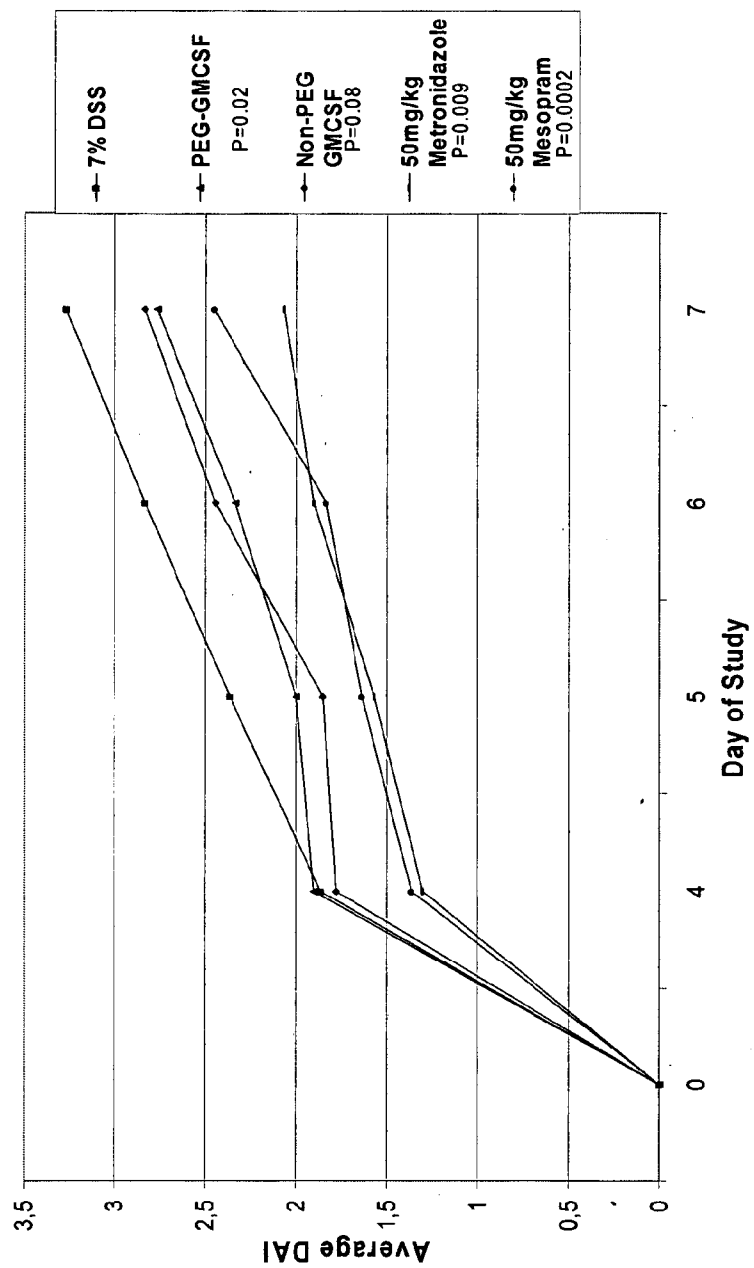


FIG. 43B

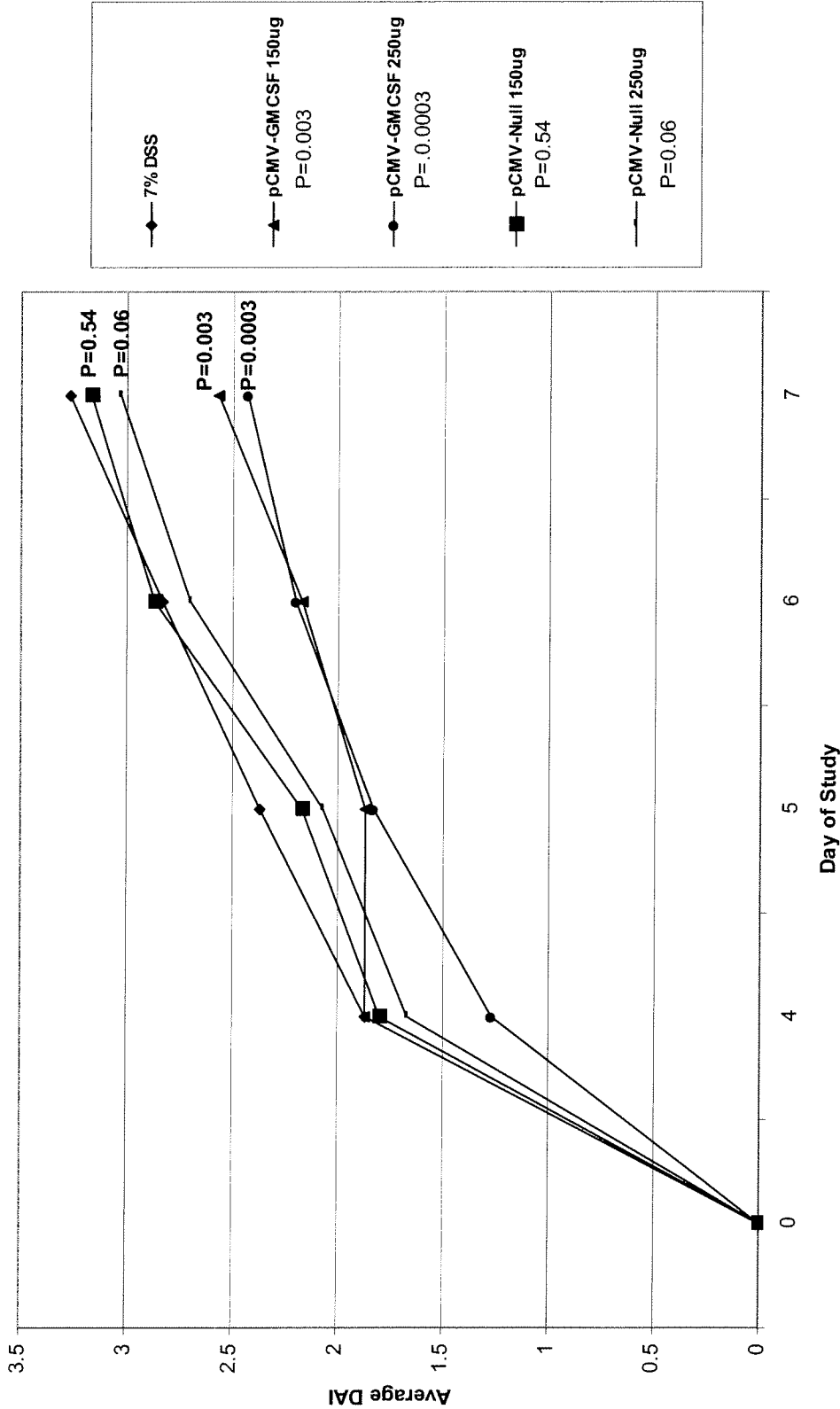


FIG. 44A

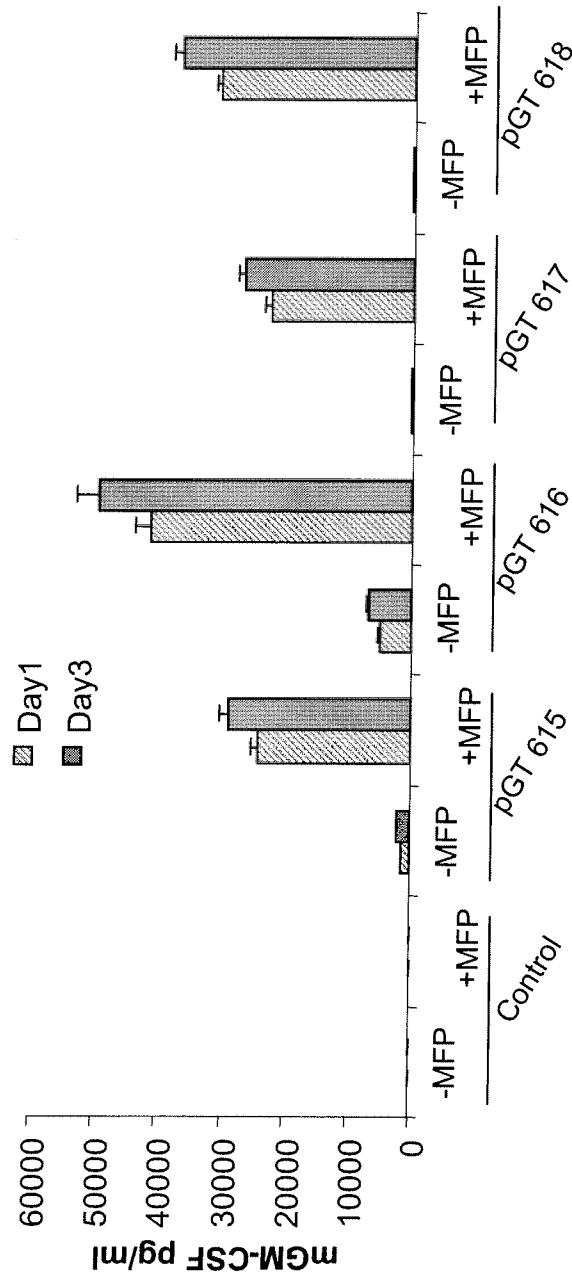


FIG. 44B

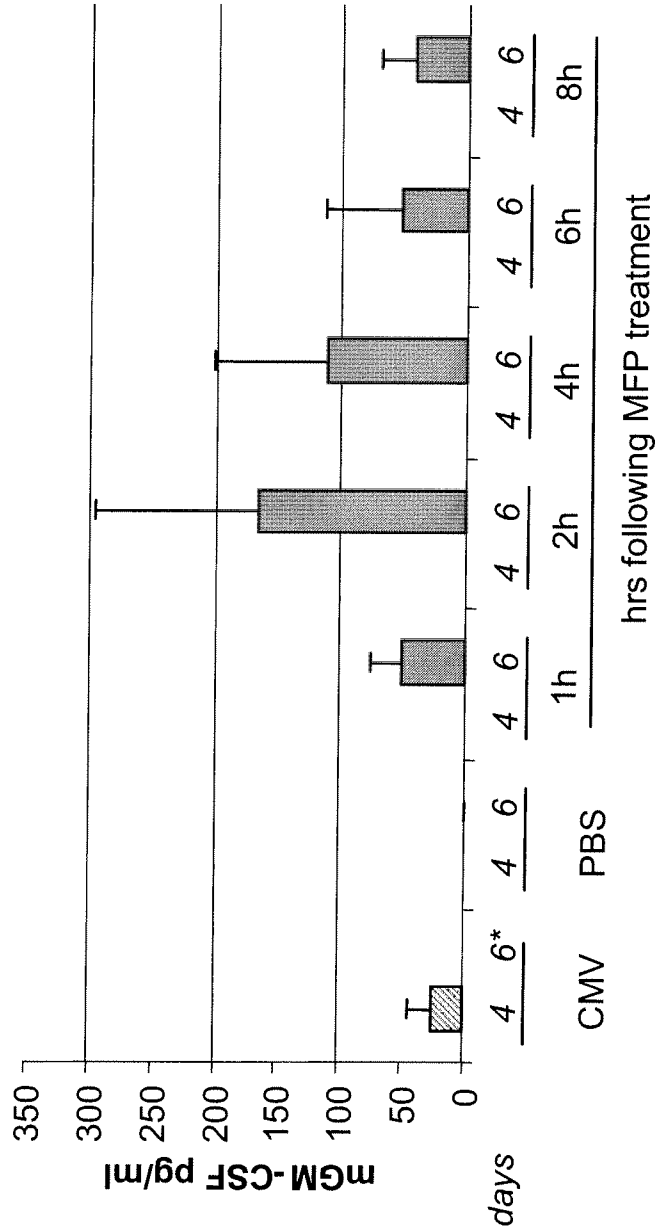


FIG. 45

