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(57) Abstract: A hybrid promoter for recombinant expression of proteins of interest is disclosed that combines a mCMV enhancer sequence with a rat EF-1alpha intron sequence. Also disclosed are an expression cassette containing the hybrid promoter and a recombinant expression vector containing the expression cassette. A mammalian host cell, which comprises the recombinant expression vector is also disclosed, as is a method of producing a protein of interest that employs the mammalian host cell, optionally involving tetracycline-inducible expression of the protein.



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HYBRID PROMOTER AND USES THEREOF

This application claims priority from United States Provisional Patent Application Serial No. 62/388,391, filed in the United States Patent and Trademark Office on January 27, 2016.

Sequence Listing

The instant application contains a Sequence Listing which has been filed electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 25, 2017, is named JUST0051_SL.txt and is 25,408 bytes in size.

[0001] BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] This invention relates to recombinant production of proteins in mammalian cells.

[0004] 2. Discussion of the Related Art

[0005] Industrial scale production of therapeutic proteins by recombinant expression in mammalian cell culture is a relatively recent endeavor that strives for the greatest possible recombinant expression efficiency and product quality characteristics that meet or exceed regulatory guidelines.

[0006] Different mammalian cell lines have been used for recombinant protein production, including various lines of Chinese Hamster Ovary (CHO) cells. (See, e.g., Hu et al., Overexpressing Cyclin D1 in a Eukaryotic Cell line, U. S. Patent No. 6,210,924; Goepfert et al., Protein Expression from Multiple Nucleic Acids, U.S. Patent No. 8,771,988; and Wurm, F.M., CHO quasispecies- Implications for Manufacturing Processes, Processes 1:296-311; doi:10.3390/pr1030296 (2013)).

[0007] Hybrid promoters combining enhancer and promoter elements from different genetic sources have been used to enhance recombinant expression in a variety of host cells. (E.g., Harvey, Hybrid promoters, WO 2008/020960 A1; US 2008/12492 A1).

[0008] Previous studies have shown that the human CMV promoter and murine CMV promoter can drive high level expression of heterologous expression in mammalian cells. (Mizushima & Nagata, pEF-BOS, a powerful mammalian expression vector, Nucl. Acids Res., 18(17):5322 (1990); Masayuki & Tanaka, The CMV Enhancer Stimulates Expression of Foreign Genes from the Human EF-1 α Promoter, Analytical Biochemistry 247:179–181 (1997); Chattellard, P. et al., The Lupac bifunctional marker and its use in protein production, WO 2006/058900 A1; Chattellard, P. et al., Expression vectors comprising the mCMV IE2 promoter, U.S. Patent No. 7,824,907; Hjelmstrom et al., Single IFN-beta fused to a mutated IgG Fc fragment, WO 2009/053368 A1; Gaucher et al., Cell line having a high transcription activity for the production of proteins, in particular therapeutic proteins, WO 2008/096070 A2; Flannery et al., Recombinant lubricin molecules and uses thereof, U.S. Patent No. 7,642,236; Mosyak et al., Method for identifying or designing a candidate agent that interacts with LINGO-1 polypeptide using a LINGO-1 three-dimensional structure, U.S. Patent No. 7,693,698; Mosyak et al., WO 2007/092370 A1).

[0009] Addition of introns also can increase expression of heterologous proteins (Lacy-Hulbert, A. et al., Interruption of coding sequences by heterologous introns can enhance the functional expression of recombinant genes, Gene Ther. 8(8):649–653 (2001); Brinster, R.L., et al., Introns increase transcriptional efficiency in transgenic mice, Proc. Natl. Acad. Sci. USA 85: 836-40 (1988)). The human and hamster EF-1 α promoter and introns have been shown to efficiently promote gene expression (Mizushima & Nagata, pEF-BOS, a powerful mammalian expression vector, Nucl. Acids Res. 18(17):5322 (1990); Running Deer, J., & Allison, D. S., High-level expression of proteins in mammalian cells using Transcription Regulatory sequences from Chinese Hamster Ovary EF-1 α Gene, Biotechnology Progress 20:880–889 (2004); Allison, D.S., Recombinant method for making multimeric proteins, WO 2006/063292 A1; Orlova et al., Improved elongation factor-1 α -based vectors for stable high-level expression of heterologous proteins in Chinese hamster ovary cells, BMC Biotechnology 14:56 (2014)).

[00010] Combinations of human or murine CMV promoters combined with human EF-1 α introns showed a significant improvement over expression constructs with no intron (Kim, S.-Y. et al., The human elongation factor 1 α (EF-1 α) first intron highly enhances expression of foreign genes from the murine cytomegalovirus promoter, J. Biotechnol. 93(2):183–87 (2002)).

[00011] The Tet repressor (TetR), which is encoded by the bacterial transposon, Tn10, has been used to regulate gene expression in mammalian cells. TetR binds to a DNA sequence (TetO) in the absence of tetracycline. Upon binding to tetracycline, TetR undergoes a conformational change that abrogates the binding of TetR to TetO. Yao et al. had shown that the human CMV enhancer promoter could be regulated by tetracycline by incorporating a TetO sequence between the TATA box and the transcriptional start site (TSS). (See, Yao et al., *Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells*, Hum. Gene Ther. 9(13):1939-50 (1998); Yao et al. U.S. Patent No. 5,972,650; Yao et al., WO99/00510).

[00012] There is a need for enhanced recombinant expression of proteins by mammalian cells in large batch or continuous culture to support robust production of biologics in a variety of mammalian cell lines, which the present invention provides.

[00013] SUMMARY OF THE INVENTION

[00014] The present invention relates to a hybrid promoter for regulating recombinant expression of one or more proteins of interest. The hybrid promoter includes: (i) a mCMV enhancer sequence, comprising a mCMV enhancer element (mCMV-E) and a CMV promoter (CMV-P) sequence at its 3' end, operably linked 5' to a rat EF-1alpha intron sequence, operably linked 5' to a rat EF-1alpha intron sequence; (ii) an intervening first leader sequence operably linked, 3' to the CMV promoter sequence of the mCMV enhancer sequence, and 5' to the rat EF-1alpha intron sequence; and (iii) a second leader sequence operably linked 3' to the EF-1 alpha intron sequence. This inventive combination of a mCMV enhancer sequence with a rat EF-1alpha intron sequence in the hybrid promoter facilitates recombinant expression of proteins of interest, particularly by CHO cells, with high titers and high specific productivity suitable for industrial production of biologic molecules, such as, but not limited to, an antigen binding protein, an immunoglobulin, an antibody or antibody fragment, (e.g., as human therapeutics to treat disease), and was generally superior to other promoters tested for some cell lines.

[00015] Accordingly, the present invention also relates to an expression cassette containing the inventive hybrid promoter, operably linked 5' to an open reading frame encoding an exogenous protein of interest; and a polyadenylation site operably linked 3' to the open reading frame.

[00016] Another aspect of the invention is a recombinant expression vector comprising the inventive expression cassette containing the hybrid promoter.

[00017] Also included within the invention is a mammalian host cell that contains the inventive recombinant expression vector containing the expression cassette with the hybrid promoter, such as a cell derived from a Chinese Hamster Ovary (CHO) cell, e.g., a CHO-K1 cell, a DXB11 cell, or a DG44 cell.

[00018] The present invention also relates to a method of producing a protein of interest, *in vitro*, that involves culturing the mammalian host cell containing the inventive expression vector, in an aqueous medium under physiological conditions permitting expression of the protein of interest; and recovering the protein of interest from the medium. The present invention also includes a method of producing a protein of interest involving tetracycline-inducible expression of the protein of interest.

[00019] The foregoing summary is not intended to define every aspect of the invention, and additional aspects are described in other sections, such as the Detailed Description of Embodiments. The entire document is intended to be related as a unified

disclosure, and it should be understood that all combinations of features described herein are contemplated, even if the combination of features are not found together in the same sentence, or paragraph, or section of this document.

[00020] In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations defined by specific paragraphs above. For example, certain aspects of the invention that are described as a genus, and it should be understood that every member of a genus is, individually, an aspect of the invention. Also, aspects described as a genus or selecting a member of a genus, should be understood to embrace combinations of two or more members of the genus. Although the applicant(s) invented the full scope of the invention described herein, the applicants do not intend to claim subject matter described in the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

[00021] BRIEF DESCRIPTION OF THE DRAWINGS

[00022] Figure 1 shows schematic maps of some exemplary expression constructs.

[00023] Figure 2 shows a schematic map of exemplary Vector D.

[00024] Figure 3A-B shows DXB11 cell produced Fc-A protein titer (Figure 3A) and specific productivity (Figure 3B) in ProCHOTM 4 culture medium containing 150 nM methotrexate (MTX) on Day 8. Each error bar is constructed using 1 standard error of the mean.

[00025] Figure 4A-B shows DXB11 cell produced Fc-A protein titer (Figure 4A) and specific productivity (Figure 4B) in PowerCHOTM 2 medium at 500 nM MTX on Day 8. Each error bar is constructed using 1 standard error of the mean.

[00026] Figure 5A-B shows Fc-A protein titer (Figure 5A) and specific productivity (Figure 5B) for DG44 in PowerCHOTM 2 medium at 1 μ M MTX and DXB11 in Excell302 medium at 500 nM MTX, on Day 10. Each error bar is constructed using 1 standard error of the mean.

[00027] Figure 6 shows a schematic map of pJV56 with the inventive mCMV enhancer/rEF-1 α intron hybrid promoter driving LacZ expression.

[00028] Figure 7 shows a schematic map of pJV57, which was the same as pJV56, except that part of the mCMV promoter (mCMV-P) was replaced with human CMV promoter (hCMV-P) and TetO sequences.

[00029] Figure 8 shows a schematic map of pJV59, which was the same as pJV56, but with an optimized human CMV promoter ((hCMV-P); after Patwardhan et al., *High-resolution analysis of DNA regulatory elements by synthetic saturation mutagenesis*, Nature Biotechnology 27(12):1173-75 (2009)), substituted for part of the mCMV promoter (mCMV-P) sequence.

[00030] Figure 9 shows a schematic map of pJV60, which was the same as pJV57, except for changes to the TetO sequences (maintaining TetR binding) to match the optimized human CMV promoter (hCMV-P) sequence.

[00031] Figure 10 below represents a schematic representation of the human CMV promoter-Tet operator (TetO; "Tet") inserted into the mCMV promoter ("mCMVP") sequence, 3' to the mCMV enhancer element ("MCVE") sequence. This insertion replaced part of the mCMV promoter with a hCMV promoter (hCMVP) sequence. The relative position of the first leader sequence is also represented by "TPL."

[00032] Figure 11 shows a schematic comparison of the DNA sequences of a segment of pJV56 (SEQ ID NO:16) and a segment of pJV57 (SEQ ID NO:15). The segment of

pJV57 shown in SEQ ID NO:15 includes a segment (SEQ ID NO:9) that incorporates a partial hCMV promoter sequence (SEQ ID NO:24) and, 3' to SEQ ID NO:24, a TetO sequence (SEQ ID NO:23, which contains a smaller TetO sequence SEQ ID NO:29). Arrows shown in the TetO sequence (SEQ ID NO:29) indicate palindromic TetR binding sites. The mCMV promoter (mCMV-P) sequence and hCMV promoter (hCMV-P) sequence are those sequences from the TATA box through the start site of transcription. SEQ ID NO:25 is the 3' end of a mCMV-P sequence found in both pJV57 and pJV56, and in pJV57 is found 3' to SEQ ID NO:9. The transcription start is the guanine residue in the 3' subsequence taccg of SEQ ID NO:25. Note that the inserted TetO sequence (SEQ ID NO:23) most certainly impacts the transcription start site, since the transcription start site is typically ~30 bases 3' to the 5' T of the TATA box. The relative position of the first leader sequence is also represented by "TPL," and some nucleotide residues at the 5' end of the TPL are shown.

[00033] Figure 12 shows a schematic comparison of segments of the DNA sequences of pJV57 and pJV60. In the segment shown of pJV60 (SEQ ID NO:18), the TetO sequences in the corresponding segment of pJV57 (SEQ ID NO:17) were changed to match the sequences that increased expression surrounding the transcription start site in the study by Patwardhan et al., *High-resolution analysis of DNA regulatory elements by synthetic saturation mutagenesis*, Nature Biotechnology 27(12):1173-75 (2009).

[00034] Figure 13 shows a schematic comparison of segments of the DNA sequences of pJV56 and pJV59. In this segment of pJV59 (SEQ ID NO:20), optimized hCMV promoter sequences in the corresponding sequence of pJV56 (SEQ ID NO:19) replaced part of the mCMV promoter (mCMV-P) sequence in a variation of the mCMV enhancer/rat EF-1 α intron hybrid promoter of the invention.

[00035] Figure 14 shows regulation of specific β -galactosidase expression for each of the indicated vectors. T-RExTM-CHO cells expressing TetR were transiently transfected with pJV56, pJV57, pJV59, pJV60, or the control plasmid pcDNA5/TO/LacZ. Cells were incubated for 24 hours and then treated with tetracycline (Tet) or left untreated for 24 hours. Cells were lysed and LacZ was analyzed enzymatically. The fold-difference between the (+Tet) compared to (-Tet) is shown above the bars, except for the positive control (pcDNA5/TO/LacZ).

[00036] Figure 15 shows a schematic map of pJV40, including an open reading frame for TetR.

[00037] Figure 16 illustrates expression of LacZ (beta-galactosidase protein) by CHO-K1/TetR cells (clone 3E7) expressing TetR, which were transiently transfected with pJV56, pJV57, pJV59, pJV60, or the control plasmid pcDNA5/TO/LacZ. Cell were incubated for 24 hours and then treated with tetracycline (Tet) or left untreated for 24 hours. Cells were lysed and LacZ was analyzed enzymatically. The fold-difference between the (+Tet) compared to (-Tet) is shown above the bars.

[00038] Figure 17 illustrates expression of LacZ (beta-galactosidase protein) by CHO-K1/TetR cells (clone 3F9) expressing TetR, which were transiently transfected with pJV56, pJV57, pJV59, pJV60, or the control plasmid pcDNA5/TO/LacZ. Cell were incubated for 24 hours and then treated with tetracycline (Tet) or left untreated for 24 hours. Cells were lysed and LacZ was analyzed enzymatically. The fold-difference between the (+Tet) compared to (-Tet) is shown above the bars.

[00039] Figure 18 illustrates expression of LacZ (beta-galactosidase protein) by CHO-K1/TetR cells (clone 4G2) expressing TetR, which were transiently transfected with pJV56, pJV57, pJV59, pJV60, or the control plasmid pcDNA5/TO/LacZ. Cell were incubated for 24 hours and then treated with tetracycline (Tet) or left untreated for 24 hours. Cells were lysed and LacZ was analyzed enzymatically. The fold-difference between the (+Tet) compared to (-Tet) is shown above the bars.

[00040] DETAILED DESCRIPTION OF EMBODIMENTS

[00041] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[00042] Definitions

[00043] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Thus, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly indicates otherwise. For example, reference to "a protein" includes a plurality of proteins; reference to "a cell" includes populations of a plurality of cells.

[00044] The present invention relates to a hybrid promoter for regulating recombinant expression of one or more proteins of interest. The inventive promoter includes a mCMV enhancer sequence operably linked to a rat EF-1alpha intron sequence. The term "mCMV," used interchangeably with "muCMV," refers to murine cytomegalovirus, which is a herpesvirus of the subfamily betaherpesviridae. The mCMV is a double-stranded enveloped DNA virus with host specificity for mice. Similarly, the term "hCMV," or interchangeably "huCMV," refers to human cytomegalovirus.

[00045] The "mCMV enhancer sequence," with respect to the present invention, includes:

[00046] (i) a mCMV enhancer element ("mCMV-E"); *and*, at the 3' end of the mCMV enhancer sequence,

[00047] (ii) a CMV promoter sequence ("CMV-P"; i.e., a nucleotide sequence segment beginning at, and including, the TATA box through the start site of transcription); the CMV promoter sequence can be derived from mCMV, hCMV, simian CMV, rat CMV, or any other variety of CMV, or it can be an optimized version of a CMV-P, which is functional to enable transcription in mammalian cells, such as CHO cells.

[00048] The term "recombinant" indicates that the material (e.g., a nucleic acid or a polypeptide) has been artificially or synthetically (i.e., non-naturally) altered by human intervention. The alteration can be performed on the material within, or removed from, its natural environment or state. For example, a "recombinant nucleic acid" is one that is made by recombining nucleic acids, e.g., during cloning, DNA shuffling or other well known molecular biological procedures. Examples of such molecular biological

procedures are found in Maniatis et al., *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982). A "recombinant DNA molecule," is comprised of segments of DNA joined together by means of such molecular biological techniques. The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule which is expressed using a recombinant DNA molecule. A "recombinant host cell" is a cell that contains and/or expresses a recombinant nucleic acid.

[00049] The term "naturally occurring" as used throughout the specification in connection with biological materials such as polypeptides, nucleic acids, host cells, and the like, refers to materials which are found in nature.

[00050] The term "control sequence" or "control signal" refers to a polynucleotide sequence that can, in a particular host cell, affect the expression and processing of coding sequences to which it is ligated. The nature of such control sequences may depend upon the host organism. In particular embodiments, control sequences for prokaryotes may include a promoter, a ribosomal binding site, and a transcription termination sequence. Control sequences for eukaryotes may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences or elements, polyadenylation sites, and transcription termination sequences. Control sequences can include leader sequences and/or fusion partner sequences. Promoters and enhancers consist of short arrays of DNA that interact specifically with cellular proteins involved in transcription (Maniatis, et al., *Science* 236:1237 (1987)). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, i.e., promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review see Voss, et al., *Trends Biochem. Sci.*, 11:287 (1986) and Maniatis, et al., *Science* 236:1237 (1987)).

[00051] A "promoter" is a region of DNA including a site at which RNA polymerase binds to initiate transcription of messenger RNA by one or more downstream structural genes. Promoters are located near the transcription start sites of genes, on the same strand and upstream on the DNA (towards the 5' region of the sense strand). Promoters are typically about 100-1000 bp in length.

[00052] In general, an “enhancer” is a short (50-1500 bp) region of DNA that can be bound with one or more activator proteins (transcription factors) to activate transcription of a gene.

[00053] A mCMV enhancer sequence useful in the inventive hybrid promoter comprises a nucleotide sequence at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the nucleotide sequence of SEQ ID NO:2, which contains a CMV promoter (CMV-P) sequence at its 3' end (bold underlined sequence in SEQ ID NO:2) beginning at nucleotide position 588 (including a TATA-box and transcriptional start site, i.e., TATAAGAGG CGCGA C CAGCG TCGG TACCG//SEQ ID NO:28; bold underlined in SEQ ID NO:2 below; SEQ ID NO:28 also comprises SEQ ID NO:26):

[00054] GTCAACAGGA AAGTTCCATT GGAGCCAAGT ACATTGAGTC
AATAGGGACT TTCCAATGGG TTTTGCCCAG TACATAAGGT CAATGGGAGG
TAAGCCAATG GGTTTTCCC ATTACTGGCA CGTATACTGA GTCATTAGGG
ACTTTCGAAT GGGTTTGGC CAGTACATAA GGTCAATAGG GGTGAATCAA
CAGGAAAGTC CCATTGGAGC CAAGTACACT GAGTCAATAG GGACTTTCCA
TTGGGTTTTG CCCAGTACAA AAGGTCAATA GGGGGTGAGT CAATGGGTTT
TTCCCATTTAT TGGCACGTAC ATAAGGTCAA TAGGGGTGAG TCATTGGGTT
TTTCCAGCCA ATTTAATTAA AACGCCATGT ACTTCCCAC CATTGACGTC
AATGGGCTAT TGAACTAAT GCAACGTGAC CTTTAAACGG TACTTTCCCA
TAGCTGATTA ATGGGAAAGT ACCGTTCTCG AGCCAATACA CGTCAATGGG
AAGTGAAAGG GCAGCCAAAA CGTAACACCG CCCC GGTTTT CCCCTGGAAA
TTCCATATTG GCACGCATTC TATTGGCTGA GCTGCGTTCT ACGTGGG **TAT**
AAGAGGCGCG ACCAGCGTCG GTACCG//SEQ ID NO:2.

[00055] Another exemplary mCMV enhancer sequence useful in the inventive hybrid promoter comprises a nucleotide sequence at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the nucleotide sequence of SEQ ID NO:33, which contains a CMV promoter sequence at its 3' end (bold underlined sequence in SEQ ID NO:33) beginning at position 588, which includes a TATA-box and transcriptional start site, i.e.,

TATATAAGCAGAGCTCGTTTAGTGAACCGTCAGTTCGTCTCTAGACGCCAACC
G//SEQ ID NO:35; bold underlined sequence in SEQ ID NO:33 below; SEQ ID NO:35 also comprises SEQ ID NO:24):

[00056] GTCAACAGGAAAGTTCCATTGGAGCCAAGTACATTGAGTCAATAG
GGACTTTCGAATGGGTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCA

ATGGGTTTTTCCCATTACTGGCACGTATACTGAGTCATTAGGGACTTTCCAAT
 GGGTTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCC
 ATTGGAGCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCAG
 TACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTTTCCCATTATTGGCACGT
 ACATAAGGTCAATAGGGGTGAGTCATTGGGTTTTTCCAGCCAATTTAATTA
 ACGCCATGTACTTTCCACCATTGACGTCAATGGGCTATTGAACTAATGCAA
 CGTGACCTTTAAACGGTACTTTCCCATAGCTGATTAATGGGAAAGTACCGTTC
 TCGAGCCAATACACGTCAATGGGAAGTGAAAGGGCAGCCAAAACGTAACAC
 CGCCCCGGTTTTCCCCTGGAAATTCCATATTGGCACGCATTCTATTGGCTGAG
 CTGCGTTCTACGTGGGTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGTT
CGTCTCTAGACGCCAACCG//SEQ ID NO:33.

[00057] In some useful embodiments, in which tetracycline-inducible expression is desired, the inventive hybrid promoter comprises one or more TetO sequences operably linked 3' to the mCMV enhancer sequence, inserted within the CMV promoter (CMV-P) sequence at the 3' end of the mCMV enhancer sequence. A "TetO" sequence means a nucleotide sequence, which maintains the ability to bind tetracycline repressor protein (TetR). The TetO sequence is placed so that, in the presence of TetR protein, there is binding of TetR to the TetO sequence, thereby disrupting transcription to a detectable extent, compared to a control not having TetO in the promoter driving transcription of a gene of interest, or to a control not having TetR. Examples of the TetO sequence include a sequence having at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO:29

(TCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGA//SEQ ID NO:29) or having at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO:34

(CTCCCTATCAGTGATCAGTTCCTCCCTATCAGTGATAGAGA//SEQ ID NO:34).

In some useful embodiments, there can be additional nucleotide residues 5' or 3' to the TetO sequence(s), or there can be intervening nucleotide linker sequences, as long as the ability to bind TetR protein is not eliminated.

[00058] "Repression," or "repressed," within the context of the invention, refers to the interference of transcription of a gene of interest (encoding a protein of interest), occurring when TetR protein binds to a TetO binding site in the promoter that drives expression of the gene of interest, resulting in decreased expression of the protein of interest by the cell(s) (which are cell(s) that express TetR). Expression of a gene of

interest or of a protein of interest is said to be “derepressed,” when, in the presence of tetracycline in the medium, expression of the protein of interest is at least 1.5-fold over the basal levels of expression by the cell(s) in the absence of tetracycline in the medium.

[00059] “Tetracycline” means tetracycline or an analog of tetracycline, such as doxycycline, anhydrotetracycline, minocycline, oxytetracycline, methacycline, chlortetracycline, or COL-3 (Chemically modified tetracycline-3).

[00060] A rat EF-1alpha intron sequence useful in the inventive hybrid promoter comprises a nucleotide sequence at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the nucleotide sequence of SEQ ID NO:4:

[00061] GTGAGTGGCGGGTGTGGCTTCCGCGGGCCCCGGAGCTGGAGCCCTG
CTCTGAGCGGGCCGGGCTGATATGCGAGTGTCTCGTCAGGGTTTAGCTGTG
AGCATTCCCACTTCGAGTGGCGGGCGGTGCGGGGGTGAGAGTGCGAGGCCTA
GCGGCAACCCCGTAGCCTCGCCTCGTGTCCGGCTTGAGGCCTAGCGTGGTGTG
CGCCGCCGCGTGCCACTCCGGCCGCACTATGCGTTTTTTGTCCTTGCTGCCCTC
GATTGCCTTCCAGCAGCATGGGCTAACAAAGGGAGGGTGTGGGGCTCACTCT
TAAGGAGCCCATGAAGCTTACGTTGGATAGGAATGGAAGGGCAGGAGGGGC
GACTGGGGCCCCGCCCTTCGGAGCACATGTCCGACGCCACCTGGATGGGG
CGAGGCCTGTGGCTTTCCGAAGCAATCGGGCGTGAGTTTAGCCTACCTGGGCC
ATGTGGCCCTAGCACTGGGCACGGTCTGGCCTGGCGGTGCCGCGTTCCTTGC
CTCCCAACAAGGGTGAGGCCGTCCCGCCCCGGCACCAGTTGCTTGCGCGGAAA
GATGGCCGCTCCCGGGGCCCTGTTGCAAGGAGCTCAAAATGGAGGACGCGGC
AGCCCGGTGGAGCGGGCGGGTGAGTCACCCACACAAAGGAAGAGGGCCTTG
CCCCTCGCCGGCCGCTGCTTCCTGTGACCCCGTGGTCTATCGGCCGCATAGTC
ACCTCGGGCTTCTCTTGAGCACCGCTCGTCGCGGCGGGGGGAGGGGATCTAA
TGGCGTTGGAGTTTGTTACATTGGTGGGTGGAGACTAGTCAGGCCAGCCTG
GCGCTGGAAGTCATTCTTGGAATTTGCCCTTTGAGTTTGAGCGAGGCTAAT
TCTCAAGCCTCTTAGCGGTTCAAAGGTATTTTCTAAACCCGTTTCCAG//SEQ ID
NO:4.

[00062] The inventive hybrid promoter also includes an intervening first leader sequence, operably linked 3' to the mCMV enhancer and 5' to the EF-1alpha intron sequence in an operable orientation. The intervening first leader sequence is about 10 to 200 nucleotide residues in length-- more preferably about 10 to 60 nucleotide residues in length or even more preferably about 20-50 nucleotide residues in length-- and lacks any secondary structure with stability greater than 20 kcal and lacks any ATG translational

start sites. An example of a useful first leader sequence is an untranslated (5'UTR) leader sequence derived from adenovirus tripartite leader (TPL), i.e., the nucleotide sequence of SEQ ID NO:3:

[00063] TACCTCTTCCGCATCGCTGTCTGCGAGGGCCAGCTGTTGGG//SEQ ID NO:3.

[00064] The inventive hybrid promoter also includes a second leader sequence operably linked 3' to the EF-1 alpha intron sequence in an operable orientation. The second leader sequence is about 5 to 200 nucleotide residues in length-- more preferably about 10 to 200 nucleotide residues in length or even more preferably about 10-150 nucleotide residues in length-- and lacks any secondary structure with stability greater than 20 kcal and lacks any ATG translational start sites. An example of a useful second leader sequence is another untranslated (5'UTR) leader sequence region derived from adenovirus tripartite leader (TPL), i.e., the nucleotide sequence of SEQ ID NO:5:

[00065] CTCGCGGTTGAGGACAACTCTTCGCGGTCTTCCAGTACTCTTGG
ATCGGAAACCCGTCGGCCTCCGAACGGTACTCCGCCACCGAGGGACCTGAGC
GAGTCCGCATCGACCGGATCGGAAAACCTC//SEQ ID NO:5.

[00066] For other examples of useful leader sequences and design principles, see, e.g., Mignone, F. et al., Untranslated regions of mRNAs. *Genome Biology*, 3(3):reviews0004.1-0004.10 (2002)), incorporated herein by reference in its entirety. Any suitable leader sequences, with the above mentioned characteristics, can be used in practicing the present invention.

[00067] One useful embodiment of the inventive hybrid promoter is a promoter that comprises a nucleotide sequence at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the nucleotide sequence of SEQ ID NO:1, following, which contains mCMV GenBank: L06816.1, nucleotides 4067-4682, Rat EF-1alpha intron Genbank: AC158987.3, nucleotides 22137-21728; and an intervening first leader sequence underlined in lower case letters (SEQ ID NO:3); and a second leader sequence underlined in italic lower case letters (SEQ ID NO:5):

[00068] GTCAACAGGAAAGTTCCATTGGAGCCAAGTACATTGAGTCAATAG
GGACTTTCCAATGGGTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCA
ATGGGTTTTTCCCATTACTGGCACGTATACTGAGTCATTAGGGACTTTCCAAT
GGGTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCC
ATTGGAGCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCCAG
TACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTTTCCCATTATTGGCACGT

ACATAAGGTCAATAGGGGTGAGTCATTGGGTTTTTCCAGCCAATTTAATTAAA
 ACGCCATGTACTTTCCCAACATTGACGTCAATGGGCTATTGAAACTAATGCAA
 CGTGACCTTTAAACGGTACTTTCCCATAGCTGATTAATGGGAAAGTACCGTTC
 TCGAGCCAATACACGTCAATGGGAAGTGAAAGGGCAGCCAAAACGTAAACAC
 CGCCCCGGTTTTCCCCTGGAAATTCCATATTGGCACGCATTCTATTGGCTGAG
 CTGCGTTCTACGTGGGTATAAGAGGCGCGACCAGCGTCGGTACCGtacctcttcgca
tcgctgtctgcgagggccagctgttgggGTGAGTGGCGGGTGTGGCTTCCGCGGGCCCCGGAG
 CTGGAGCCCTGCTCTGAGCGGGCCGGGCTGATATGCGAGTGTCTGTCGCAGG
 GTTTAGCTGTGAGCATTCCCCTTCGAGTGGCGGGCGGTGCGGGGGTGAGAG
 TCGAGGCCTAGCGGCAACCCCGTAGCCTCGCCTCGTGTCCGGCTTGAGGCCT
 AGCGTGGTGTCCGCCGCCGCGTGCCACTCCGGCCGCACTATGCGTTTTTTGTC
 CTTGCTGCCCTCGATTGCCTTCCAGCAGCATGGGCTAACAAAGGGAGGGTGT
 GGGGCTCACTCTTAAGGAGCCCATGAAGCTTACGTTGGATAGGAATGGAAGG
 GCAGGAGGGGGCGACTGGGGCCCCGCCGCTTCGGAGCACATGTCCGACGCCA
 CCTGGATGGGGCGAGGCCTGTGGCTTTCCGAAGCAATCGGGCGTGAGTTTAG
 CCTACCTGGGCCATGTGGCCCTAGCACTGGGCACGGTCTGGCCTGGCGGTGC
 CGCGTTCCCTTGCTCCCAACAAGGGTGAGGCCGTCCCGCCCCGGCACCAGTTG
 CTTGCGCGGAAAGATGGCCGCTCCCGGGGCCCTGTTGCAAGGAGCTCAAAAT
 GGAGGACGCGGCAGCCCGGTGGAGCGGGCGGGTGAGTCACCCACACAAAGG
 AAGAGGGCCTTGCCCCCTCGCCGGCCGCTGCTTCCTGTGACCCCGTGGTCTATC
 GGCCGCATAGTCACCTCGGGCTTCTCTTGAGCACCGCTCGTCGCGGCGGGGG
 GAGGGGATCTAATGGCGTTGGAGTTTGTTCACATTTGGTGGGTGGAGACTAGT
 CAGGCCAGCCTGGCGCTGGAAGTCATTCTTGGAATTTGCCCTTTGAGTTTGG
 AGCGAGGCTAATTCTCAAGCCTCTTAGCGGTTCAAAGGTATTTTCTAAACCCG
 TTTCCAGtctgcggttgaggacaaactcttcgcggtctttccagtaactcttggatcggaaccgctcggcctcggaacgg
tactccgccaccgagggacctgagcgagtcggcatcgaccggatcggaaccctc//SEQ ID NO:1.

[00069] Examples of additional useful embodiments of the inventive hybrid promoter include (i) SEQ ID NO:30 (hybrid promoter sequence including TetO, as in pJV57), (ii) SEQ ID NO:31 (hybrid promoter including a mCMV enhancer element with optimized hCMV promoter sequence, as in pJV59), and (iii) SEQ ID NO:32 (hybrid promoter including mCMV enhancer element with optimized hCMV promoter sequence and optimized TetO sequence, as in pJV60):

[00070] GTCAACAGGAAAGTTCATTGGAGCCAAGTACATTGAGTCAATAG
 GGACTTTC AATGGGTTTTGCCAGTACATAAGGTCAATGGGAGGTAAGCCA

ATGGGTTTTTCCCATTACTGGCACGTATACTGAGTCATTAGGGACTTTCCAAT
GGGTTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCC
ATTGGAGCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTGCCAG
TACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTTTCCCATTATTGGCACGT
ACATAAGGTCAATAGGGGTGAGTCATTGGGTTTTTCCAGCCAATTTAATTA
ACGCCATGTACTTTCCCACCATTGACGTCAATGGGCTATTGAAACTAATGCAA
CGTGACCTTTAAACGGTACTTTCCCATAGCTGATTAATGGGAAAGTACCGTTC
TCGAGCCAATACACGTCAATGGGAAGTGAAAGGGCAGCCAAAACGTAACAC
CGCCCCGGTTTTCCCCTGGAAATTCATATTGGCACGCATTCTATTGGCTGAG
CTGCGTTCTACGTGGGTATATAAGCAGAGCTCTCCCTATCAGTGATAGAGATC
TCCCTATCAGTGATAGAGATCGTCGACGAGCTCAGCGTCGGTACCGTACCTCT
TCCGCATCGCTGTCTGCGAGGGCCAGCTGTTGGGGTGAGTGGCGGGGTGTGGC
TTCCGCGGGCCCCGGAGCTGGAGCCCTGCTCTGAGCGGGCCGGGCTGATATG
CGAGTGTCGTCCGCAGGGTTTAGCTGTGAGCATTCCCACCTCGAGTGGCGGGC
GGTGCGGGGGTGAGAGTGCGAGGCCTAGCGGCAACCCCGTAGCCTCGCCTCG
TGTCGGGCTTGAGGCCTAGCGTGGTGTCCGCCGCCGCGTGCCACTCCGGCCGC
ACTATGCGTTTTTTGTCCTTGCTGCCCTCGATTGCCTTCCAGCAGCATGGGCTA
ACAAAGGGAGGGTGTGGGGCTCACTCTTAAGGAGCCCATGAAGCTTACGTTG
GATAGGAATGGAAGGGCAGGAGGGGCGACTGGGGCCCCGCCCGCCTTCGGAG
CACATGTCCGACGCCACCTGGATGGGGCGAGGCCTGTGGCTTTCCGAAGCAA
TCGGGCGTGAGTTTAGCCTACCTGGGCCATGTGGCCCTAGCACTGGGCACGGT
CTGGCCTGGCGGTGCCGCGTTCCCTTGCCCTCCCAACAAGGGTGAGGCCGTCCC
GCCCCGGCACCAGTTGCTTGCGCGGAAAGATGGCCGCTCCCGGGGCCCTGTTG
CAAGGAGCTCAAAATGGAGGACGCGGCAGCCCGGTGGAGCGGGCGGGTGAG
TCACCCACACAAAGGAAGAGGGCCTTGCCCCTCGCCGGCCGCTGCTTCCTGT
GACCCCGTGGTCTATCGGCCGCATAGTCACCTCGGGCTTCTCTTGAGCACCGC
TCGTGCGGGCGGGGGGAGGGGATCTAATGGCGTTGGAGTTTGTTCACATTTG
GTGGGTGGAGACTAGTCAGGCCAGCCTGGCGCTGGAAGTCATTCTTGGAATT
TGCCCCCTTGAGTTTGGAGCGAGGCTAATTCTCAAGCCTCTTAGCGGTTCAAA
GGTATTTTCTAAACCCGTTTCCAGCTCGCGGTTGAGGACAACTCTTCGCGGT
CTTTCAGTACTCTTGATCGGAAACCCGTCGGCCTCCGAACGGTACTCCGCC
ACCGAGGGACCTGAGCGAGTCCGCATCGACCGGATCGGAAAACCTC//SEQ ID
NO:30;

[00071] GTCAACAGGAAAGTTCCATTGGAGCCAAGTACATTGAGTCAATAG
GGACTTTCCAATGGGTTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCA
ATGGGTTTTTCCCATTACTGGCACGTATACTGAGTCATTAGGGACTTTCCAAT
GGGTTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCC
ATTGGAGCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTTGCCCAG
TACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTTTCCCATTATTGGCACGT
ACATAAGGTCAATAGGGGGTGAGTCATTGGGTTTTTCCAGCCAATTTAATTAAA
ACGCCATGTACTTTCCCACCATTGACGTCAATGGGCTATTGAACTAATGCAA
CGTGACCTTTAAACGGTACTTTCCCATAGCTGATTAATGGGAAAGTACCGTTC
TCGAGCCAATACACGTCAATGGGAAGTGAAAGGGCAGCCAAAACGTAACAC
CGCCCCGGTTTTCCCCTGGAAATTCATATTGGCACGCATTCTATTGGCTGAG
CTGCGTTCTACGTGGGTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGTTCG
TCTCTAGACGCCAACCGCCTCTTCCGCATCGCTGTCTGCGAGGGCCAGCTGTT
GGGGTGAGTGGCGGGTGTGGCTTCCGCGGGCCCCGGAGCTGGAGCCCTGCTC
TGAGCGGGCCGGGCTGATATGCGAGTGTCGTCCGCAGGGTTTAGCTGTGAGC
ATTCCCACTTCGAGTGGCGGGCGGTGCGGGGGTGAGAGTGCGAGGCCTAGCG
GCAACCCCGTAGCCTCGCCTCGTGTCCGGCTTGAGGCCTAGCGTGGTGTCCGC
CGCCGCGTGCCACTCCGGCCGCACTATGCGTTTTTTGTCTTGCTGCCCTCGAT
TGCCTTCCAGCAGCATGGGCTAACAAAGGGAGGGTGTGGGGCTCACTCTTAA
GGAGCCCATGAAGCTTACGTTGGATAGGAATGGAAGGGCAGGAGGGGCGAC
TGGGGCCCCGCCCTTCGGAGCACATGTCCGACGCCACCTGGATGGGGCGA
GGCCTGTGGCTTTCCGAAGCAATCGGGCGTGAGTTTAGCCTACCTGGGCCATG
TGGCCCTAGCACTGGGCACGGTCTGGCCTGGCGGTGCCGCGTTCCCTTGCCTC
CCAACAAGGGTGAGGCCGTCCCGCCCGGCACCAGTTGCTTGCGCGGAAAGAT
GGCCGCTCCCGGGGCCCTGTTGCAAGGAGCTCAAAATGGAGGACGCGGCAGC
CCGGTGGAGCGGGCGGGTGAGTACCCACACAAAGGAAGAGGGCCTTGCCC
CTCGCCGGCCGCTGCTTCCTGTGACCCCGTGGTCTATCGGCCGCATAGTCACC
TCGGGCTTCTCTTGAGCACCGCTCGTCGCGGCGGGGGGAGGGGATCTAATGG
CGTTGGAGTTTGTTACATTTGGTGGGTGGAGACTAGTCAGGCCAGCCTGGCG
CTGGAAGTCATTCTTGGAATTTGCCCTTTGAGTTTGGAGCGAGGCTAATTCT
CAAGCCTCTTAGCGGTTCAAAGGTATTTTCTAAACCCGTTTCCAGCTCGCGGT
TGAGGACAAACTCTTCGCGGTCTTTCCAGTACTCTTGGATCGGAAACCCGTCG
GCCTCCGAACGGTACTCCGCCACCGAGGGACCTGAGCGAGTCCGCATCGACC
GGATCGGAAAACCTC//SEQ ID NO:31; and

[00072] GTCAACAGGAAAGTTCCATTGGAGCCAAGTACATTGAGTCAATAG
GGACTTTCCAATGGGTTTTTGCCCAGTACATAAGGTCAATGGGAGGTAAGCCA
ATGGGTTTTTCCCATTACTGGCACGTATACTGAGTCATTAGGGACTTTCCAAT
GGGTTTTTGCCCAGTACATAAGGTCAATAGGGGTGAATCAACAGGAAAGTCCC
ATTGGAGCCAAGTACACTGAGTCAATAGGGACTTTCCATTGGGTTTTTGCCCAG
TACAAAAGGTCAATAGGGGGTGAGTCAATGGGTTTTTCCCATTATTGGCACGT
ACATAAGGTCAATAGGGGGTGAGTCATTGGGTTTTTCCAGCCAATTTAATTAAA
ACGCCATGTACTTTCCCACCATTGACGTCAATGGGCTATTGAACTAATGCAA
CGTGACCTTTAAACGGTACTTTCCCATAGCTGATTAATGGGAAAGTACCGTTC
TCGAGCCAATACACGTCAATGGGAAGTGAAAGGGCAGCCAAAACGTAACAC
CGCCCCGGTTTTCCCCTGGAAATTCATATTGGCACGCATTCTATTGGCTGAG
CTGCGTTCTACGTGGGTATATAAGCAGAGCTCTCCCTATCAGTGATCAGTTCC
TCCCTATCAGTGATAGAGATCGTCGACGAGCTCAGCGTCGGTACCGTACCTCT
TCCGCATCGCTGTCTGCGAGGGCCAGCTGTTGGGGTGAGTGGCGGGTGTTGGC
TTCCGCGGGCCCCGGAGCTGGAGCCCTGCTCTGAGCGGGCCGGGCTGATATG
CGAGTGTCTGTCGCGAGGGTTTAGCTGTGAGCATTCCCACCTTCGAGTGGCGGGC
GGTGCGGGGGTGAGAGTGCGAGGCCTAGCGGCAACCCCGTAGCCTCGCCTCG
TGTCGGGCTTGAGGCCTAGCGTGGTGTCCGCCGCCGCGTGCCACTCCGGCCGC
ACTATGCGTTTTTTGTCTTGTCTGCCCTCGATTGCCTTCCAGCAGCATGGGCTA
ACAAAGGGAGGGTGTGGGGCTCACTCTTAAGGAGCCCATGAAGCTTACGTTG
GATAGGAATGGAAGGGCAGGAGGGGGCGACTGGGGCCCCGCCCTTCGGAG
CACATGTCCGACGCCACCTGGATGGGGCGAGGCCTGTGGCTTTCCGAAGCAA
TCGGGCGTGAGTTTAGCCTACCTGGGCCATGTGGCCCTAGCACTGGGCACGGT
CTGGCCTGGCGGTGCCGCGTTCCCTTGCCCTCCCAACAAGGGTGAGGCCGTCCC
GCCCCGCACCAAGTTGCTTGCGCGGAAAGATGGCCGCTCCCGGGGCCCTGTTG
CAAGGAGCTCAAAATGGAGGACGCGGCAGCCCGGTGGAGCGGGCGGGTGAG
TCACCCACACAAAGGAAGAGGGCCTTGCCCCCTCGCCGGCCGCTGCTTCCTGT
GACCCCGTGGTCTATCGGCCGCATAGTCACCTCGGGCTTCTCTTGAGCACCGC
TCGTGCGCGCGGGGGGAGGGGATCTAATGGCGTTGGAGTTTGTTCACATTTG
GTGGGTGGAGACTAGTCAGGCCAGCCTGGCGCTGGAAGTCATTCTTGGAATT
TGCCCCTTTGAGTTTGGAGCGAGGCTAATTCTCAAGCCTCTTAGCGGTTCAAA
GGTATTTTCTAAACCCGTTTCCAGCTCGCGGTTGAGGACAACTCTTCGCGGT
CTTCCAGTACTCTTGATCGGAAACCCGTCGGCCTCCGAACGGTACTCCGCC

ACCGAGGGACCTGAGCGAGTCCGCATCGACCGGATCGGAAAACCTC// SEQ ID NO:32.

[00073] The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced. For example, a control sequence in a vector that is "operably linked" to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

[00074] "Polypeptide" and "protein" are used interchangeably herein and include a molecular chain of two or more amino acids linked covalently through peptide bonds. The terms do not refer to a specific length of the product. Thus, "peptides," and "oligopeptides," are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. In addition, protein fragments, analogs, mutated or variant proteins, fusion proteins and the like are included within the meaning of polypeptide. The terms also include molecules in which one or more amino acid analogs or non-canonical or unnatural amino acids are included as can be expressed recombinantly using known protein engineering techniques. In addition, fusion proteins can be derivatized as described herein by well-known organic chemistry techniques.

[00075] A "variant" of a polypeptide (e.g., an immunoglobulin, or an antibody) comprises an amino acid sequence wherein one or more amino acid residues are inserted into, deleted from and/or substituted into the amino acid sequence relative to another polypeptide sequence. Variants include fusion proteins.

[00076] The term "fusion protein" indicates that the protein includes polypeptide components derived from more than one parental protein or polypeptide. Typically, a fusion protein is expressed from a "fusion gene" in which a nucleotide sequence encoding a polypeptide sequence from one protein is appended in frame with, and optionally separated by a linker from, a nucleotide sequence encoding a polypeptide sequence from a different protein. The fusion gene can then be expressed by a recombinant host cell as a single protein.

[00077] A "secreted" protein refers to those proteins capable of being directed to the ER, secretory vesicles, or the extracellular space as a result of a secretory signal peptide

sequence, as well as those proteins released into the extracellular space without necessarily containing a signal sequence. If the secreted protein is released into the extracellular space, the secreted protein can undergo extracellular processing to produce a "mature" protein. Release into the extracellular space can occur by many mechanisms, including exocytosis and proteolytic cleavage. In some other embodiments of the inventive method, the protein of interest can be synthesized by the host cell as a secreted protein, which can then be further purified from the extracellular space and/or medium.

[00078] As used herein "soluble" when in reference to a protein produced by recombinant DNA technology in a host cell is a protein that exists in aqueous solution; if the protein contains a twin-arginine signal amino acid sequence the soluble protein is exported to the periplasmic space in gram negative bacterial hosts, or is secreted into the culture medium by eukaryotic host cells capable of secretion, or by bacterial host possessing the appropriate genes (e.g., the *kil* gene). Thus, a soluble protein is a protein which is not found in an inclusion body inside the host cell. Alternatively, depending on the context, a soluble protein is a protein which is not found integrated in cellular membranes, or, in vitro, is dissolved, or is capable of being dissolved in an aqueous buffer under physiological conditions without forming significant amounts of insoluble aggregates (i.e., forms aggregates less than 10%, and typically less than about 5%, of total protein) when it is suspended without other proteins in an aqueous buffer of interest under physiological conditions, such buffer not containing a detergent or chaotropic agent, such as urea, guanidinium hydrochloride, or lithium perchlorate. In contrast, an insoluble protein is one which exists in denatured form inside cytoplasmic granules (called an inclusion body) in the host cell, or again depending on the context, an insoluble protein is one which is present in cell membranes, including but not limited to, cytoplasmic membranes, mitochondrial membranes, chloroplast membranes, endoplasmic reticulum membranes, etc., or in an in vitro aqueous buffer under physiological conditions forms significant amounts of insoluble aggregates (i.e., forms aggregates equal to or more than about 10% of total protein) when it is suspended without other proteins (at physiologically compatible temperature) in an aqueous buffer of interest under physiological conditions, such buffer not containing a detergent or chaotropic agent, such as urea, guanidinium hydrochloride, or lithium perchlorate.

[00079] The term "polynucleotide" or "nucleic acid" includes both single-stranded and double-stranded nucleotide polymers containing two or more nucleotide residues. The nucleotide residues comprising the polynucleotide can be ribonucleotides or

deoxyribonucleotides or a modified form of either type of nucleotide. Said modifications include base modifications such as bromouridine and inosine derivatives, ribose modifications such as 2',3'-dideoxyribose, and internucleotide linkage modifications such as phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoraniladate and phosphoroamidate.

[00080] The term "oligonucleotide" means a polynucleotide comprising 200 or fewer nucleotide residues. In some embodiments, oligonucleotides are 10 to 60 bases in length. In other embodiments, oligonucleotides are 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 nucleotides in length. Oligonucleotides may be single stranded or double stranded, e.g., for use in the construction of a mutant gene. Oligonucleotides may be sense or antisense oligonucleotides. An oligonucleotide can include a label, including a radiolabel, a fluorescent label, a hapten or an antigenic label, for detection assays. Oligonucleotides may be used, for example, as PCR primers, cloning primers or hybridization probes.

[00081] A "polynucleotide sequence" or "nucleotide sequence" or "nucleic acid sequence," as used interchangeably herein, is the primary sequence of nucleotide residues in a polynucleotide, including of an oligonucleotide, a DNA, and RNA, a nucleic acid, or a character string representing the primary sequence of nucleotide residues, depending on context. From any specified polynucleotide sequence, either the given nucleic acid or the complementary polynucleotide sequence can be determined. Included are DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Unless specified otherwise, the left-hand end of any single-stranded polynucleotide sequence discussed herein is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences;" sequence regions on the DNA strand having the same sequence as the RNA transcript that are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences."

[00082] As used herein, an "isolated nucleic acid molecule" or "isolated nucleic acid sequence" is a nucleic acid molecule that is either (1) identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid or (2) cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the sequence of the nucleic acid of interest can be determined. An isolated nucleic acid molecule is other than in the form or

setting in which it is found in nature. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the immunoglobulin (e.g., antibody) where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

[00083] As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of ribonucleotides along the mRNA chain, and also determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the RNA sequence and for the amino acid sequence.

[00084] The term "gene" is used broadly to refer to any nucleic acid associated with a biological function. Genes typically include coding sequences and/or the regulatory sequences required for expression of such coding sequences. The term "gene" applies to a specific genomic or recombinant sequence, as well as to a cDNA or mRNA encoded by that sequence. Genes also include non-expressed nucleic acid segments that, for example, form recognition sequences for other proteins. Non-expressed regulatory sequences including transcriptional control elements to which regulatory proteins, such as transcription factors, bind, resulting in transcription of adjacent or nearby sequences.

[00085] "Expression of a gene" or "expression of a nucleic acid" means transcription of DNA into RNA (optionally including modification of the RNA, e.g., splicing), translation of RNA into a polypeptide (possibly including subsequent post-translational modification of the polypeptide), or both transcription and translation, as indicated by the context.

[00086] The present invention relates to an expression cassette that includes the inventive hybrid promoter. A eukaryotic "expression cassette" refers to the part of an expression vector that enables production of protein in a eukaryotic cell, such as a mammalian cell. It includes a promoter, operable in a eukaryotic cell, for mRNA transcription, one or more gene(s) encoding protein(s) of interest and a mRNA termination and processing signal. An expression cassette can usefully include among the coding sequences, a gene useful as a selective marker. In the expression cassette of the present invention, the hybrid promoter is operably linked 5' to an open reading frame encoding an exogenous protein of interest; and a polyadenylation site is operably linked 3' to the open reading frame. One embodiment of a useful polyadenylation site sequence is a SV40 late polyadenylation site SEQ ID NO:14:

[00087] CACACATCATAAGATACATTGATGAGTTTGGACAAACCACAACCTAG
 AATGCAGTGAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTAT
 TTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCAT
 TTTATGTTTCAGGTTTCAAGGGGAGATGTGGGAGGTTTTTTAAAGCAAGTAAAA
 CCTCTACAAATGTGGTA//SEQ ID NO:14.

[00088] Other suitable control sequences can also be included as long as the expression cassette remains operable. The open reading frame can optionally include a coding sequence for more than one protein of interest.

[00089] As used herein the term "coding region" or "coding sequence" when used in reference to a structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of an mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (i.e., TAA, TAG, TGA).

[00090] The present invention also encompasses a recombinant expression vector comprising the inventive expression cassette.

[00091] The term "vector" means any molecule or entity (e.g., nucleic acid, plasmid, bacteriophage or virus) used to transfer protein coding information into a host cell.

[00092] The term "expression vector" or "expression construct" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid control sequences necessary for the expression of the operably linked coding sequence in a particular host cell. An expression vector can include, but is not limited to, sequences that affect or control transcription, translation, and, if introns are present, affect RNA splicing of a coding region operably linked thereto. Nucleic acid sequences necessary for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. A secretory signal peptide sequence can also, optionally, be encoded by the expression vector, operably linked to the coding sequence of interest, so that the expressed polypeptide can be secreted by the recombinant host cell, for more facile isolation of the polypeptide of interest from the cell, if desired. Such techniques are well known in the art. (E.g., Goodey, Andrew R.; et al., Peptide and DNA sequences, U.S. Pat. No. 5,302,697; Weiner et al., Compositions and methods for protein secretion, U.S. Pat. No. 6,022,952 and U.S. Pat. No. 6,335,178; Uemura et al., Protein expression vector and utilization

thereof, U.S. Pat. No. 7,029,909; Ruben et al., 27 human secreted proteins, US 2003/0104400 A1). For expression of multi-subunit proteins of interest, separate expression vectors in suitable numbers and proportions, each containing a coding sequence for each of the different monomers, can be used to transform a host cell. In other embodiments a single expression vector can be used to express the different subunits of the protein of interest.

[00093] The present invention also relates to a mammalian host cell comprising the inventive recombinant expression vector.

[00094] The term "host cell" means a cell that has been transformed, or is capable of being transformed, with a nucleic acid and thereby expresses a gene of interest. The term includes the progeny of the parent cell, whether or not the progeny is identical in morphology or in genetic make-up to the original parent cell, so long as the gene of interest is present. Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells in culture include bacteria (such as *Escherichia coli* sp.), yeast (such as *Saccharomyces* sp.) and other fungal cells, insect cells, plant cells, mammalian (including human) cells, e.g., CHO cells and HEK-293 cells. Modifications can be made at the DNA level, as well. The peptide-encoding DNA sequence may be changed to codons more compatible with the chosen host cell. For *E. coli*, optimized codons are known in the art. Codons can be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed. Such fermentation conditions are well known in the art.

[00095] Examples of useful mammalian host cell lines are Chinese hamster ovary cells, including CHO-K1 cells (e.g., ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al, Proc. Natl. Acad. Sci. USA 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension

culture, [Graham et al, J. Gen Virol. 36: 59 (1977)]; baby hamster kidney cells (BHK, ATCC CCL 10); mouse Sertoli cells (TM4, Mather, Biol. Reprod. 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human hepatoma cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al, Annals N.Y Acad. Sci. 383: 44-68 (1982)); MRC 5 cells or FS4 cells; or mammalian myeloma cells.

[00096] "Cell," "cell line," and "cell culture" are often used interchangeably and all such designations herein include cellular progeny. For example, a cell "derived" from a CHO cell is a cellular progeny of a Chinese Hamster Ovary cell, which may be removed from the original primary cell parent by any number of generations, and which can also include a transformant progeny cell. Transformants and transformed cells include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

[00097] Host cells are transformed or transfected with the above-described nucleic acids or vectors for production of polypeptides (including antigen binding proteins, such as antibodies) and are cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In addition, novel vectors and transfected cell lines with multiple copies of transcription units separated by a selective marker are particularly useful for the expression of polypeptides, such as antibodies.

[00098] The term "transfection" means the uptake of foreign or exogenous DNA by a cell, and a cell has been "transfected" when the exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are well known in the art and are disclosed herein. See, e.g., Graham et al., 1973, Virology 52:456; Sambrook et al., 2001, Molecular Cloning: A Laboratory Manual, supra; Davis et al., 1986, Basic Methods in Molecular Biology, Elsevier; Chu et al., 1981, Gene 13:197. Such techniques can be used to introduce one or more exogenous DNA moieties into suitable host cells.

[00099] The term "transformation" refers to a change in a cell's genetic characteristics, and a cell has been transformed when it has been modified to contain new DNA or RNA. For example, a cell is transformed where it is genetically modified from its native state by introducing new genetic material via transfection, transduction, or other techniques. Following transfection or transduction, the transforming DNA may recombine with that of the cell by physically integrating into a chromosome of the cell, or may be maintained transiently as an episomal element without being replicated, or may replicate independently as a plasmid. A cell is considered to have been "stably transformed" when the transforming DNA is replicated with the division of the cell.

[000100] The present invention also relates to a method of producing a protein of interest involving culturing the mammalian host cell, in aqueous medium under physiological conditions permitting expression of the protein of interest; and recovering the protein of interest from the medium.

[000101] The host cells used to produce the polypeptides useful in the invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58: 44 (1979), Barnes et al., Anal. Biochem. 102: 255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO90103430; WO 87/00195; or U.S. Patent Re. No. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source, such that the physiological conditions of the cell in, or on, the medium promote expression of the protein of interest by the host cell; any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature (typically, but not necessarily, about 37°C), pH (typically, but not necessarily, about pH 6.5-7.5), oxygenation, and the like, are those previously used with the host cell selected for expression of the protein of interest, and will be apparent to the ordinarily skilled artisan. The culture medium can include a suitable amount of serum such as fetal bovine serum

(FBS), or preferably, the host cells can be adapted for culture in serum-free medium. In some embodiments, the aqueous medium is liquid, such that the host cells are cultured in a cell suspension within the liquid medium. The host cells can be usefully grown in batch culture or in continuous culture systems.

[000102] In other embodiments, the mammalian host cells can be cultured on solid or semi-solid aqueous medium, for example, containing agar or agarose, to form a medium or substrate surface to which the cells adhere and form an adhesion layer.

[000103] Upon culturing the host cells, the recombinant polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide, such as an antigen binding protein (e.g., an antibody), is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration.

[000104] A protein of interest, such as an antibody or antibody fragment can be purified using, for example, hydroxylapatite chromatography, cation or anion exchange chromatography, or preferably affinity chromatography, using the antigen of interest or protein A or protein G as an affinity ligand. Protein A can be used to purify proteins that include polypeptides are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark et al., J. Immunol. Meth. 62: 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss et al, EMBO J. 5: 15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the protein comprises a CH 3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as ethanol precipitation, Reverse Phase HPLC, chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also possible depending on the antibody to be recovered.

[000105] "Under physiological conditions" with respect to incubating buffers and immunoglobulins, or other binding assay reagents means incubation under conditions of temperature, pH, and ionic strength, that permit a biochemical reaction, such as a non-covalent binding reaction, to occur. Typically, the temperature is at room or ambient temperature up to about 37°C and at pH 6.5-7.5.

[000106] "Physiologically acceptable salt" of a composition of matter, for example a salt of an immunoglobulin, such as an antibody, or other protein of interest, means any

salt, or salts, that are known or later discovered to be pharmaceutically acceptable. Some non-limiting examples of pharmaceutically acceptable salts are: acetate; trifluoroacetate; hydrohalides, such as hydrochloride and hydrobromide; sulfate; citrate; maleate; tartrate; glycolate; gluconate; succinate; mesylate; besylate; salts of gallic acid esters (gallic acid is also known as 3,4, 5 trihydroxybenzoic acid) such as PentaGalloylGlucose (PGG) and epigallocatechin gallate (EGCG), salts of cholesteryl sulfate, pamoate, tannate and oxalate salts.

[000107] A "reaction mixture" is an aqueous mixture containing all the reagents and factors necessary, which under physiological conditions of incubation, permit an in vitro biochemical reaction of interest to occur, such as a covalent or non-covalent binding reaction.

[000108] A "domain" or "region" (used interchangeably herein) of a polynucleotide is any portion of the entire polynucleotide, up to and including the complete polynucleotide, but typically comprising less than the complete polynucleotide. A domain can, but need not, fold independently (e.g., DNA hairpin folding) of the rest of the polynucleotide chain and/or be correlated with a particular biological, biochemical, or structural function or location, such as a coding region or a regulatory region.

[000109] A "domain" or "region" (used interchangeably herein) of a protein is any portion of the entire protein, up to and including the complete protein, but typically comprising less than the complete protein. A domain can, but need not, fold independently of the rest of the protein chain and/or be correlated with a particular biological, biochemical, or structural function or location (e.g., a ligand binding domain, or a cytosolic, transmembrane or extracellular domain).

[000110] The term "antibody", or interchangeably "Ab", is used in the broadest sense and includes fully assembled antibodies, monoclonal antibodies (including human, humanized or chimeric antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments that can bind antigen (e.g., Fab, Fab', F(ab')₂, Fv, single chain antibodies, diabodies), comprising complementarity determining regions (CDRs) of the foregoing as long as they exhibit the desired biological activity. Multimers or aggregates of intact molecules and/or fragments, including chemically derivatized antibodies, are contemplated. Antibodies of any isotype class or subclass, including IgG, IgM, IgD, IgA, and IgE, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2, or any allotype, are contemplated. Different isotypes have different effector functions; for

example, IgG1 and IgG3 isotypes have antibody-dependent cellular cytotoxicity (ADCC) activity.

[000111] An "isolated" protein, e.g., an immunoglobulin, such as an antibody or antibody fragment, is one that has been identified and separated from one or more components of its natural environment or of a culture medium in which it has been secreted by a producing cell. In some embodiments, the isolated protein is substantially free from proteins or polypeptides or other contaminants that are found in its natural or culture medium environment that would interfere with its therapeutic, diagnostic, prophylactic, research or other use. "Contaminant" components of its natural environment or medium are materials that would interfere with diagnostic or therapeutic uses for the protein, e.g., an antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous (e.g., polynucleotides, lipids, carbohydrates) solutes. Typically, an "isolated protein" constitutes at least about 5%, at least about 10%, at least about 25%, or at least about 50% of a given sample. In some embodiments, the protein of interest, e.g., an antibody will be purified (1) to greater than 95% by weight of protein, and most preferably more than 99% by weight, or (2) to homogeneity by SDS-PAGE under reducing or nonreducing conditions, optionally using a stain, e.g., Coomassie blue or silver stain. Isolated naturally occurring antibody includes the antibody in situ within recombinant cells since at least one component of the protein's natural environment will not be present. Typically, however, the isolated protein of interest (e.g., an antibody) will be prepared by at least one purification step.

[000112] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies that are antigen binding proteins are highly specific binders, being directed against an individual antigenic site or epitope, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different epitopes. Nonlimiting examples of monoclonal antibodies include murine, rabbit, rat, chicken, chimeric, humanized, or human antibodies, fully assembled antibodies, multispecific antibodies (including bispecific antibodies), antibody fragments that can bind an antigen (including, Fab, Fab', F(ab)₂, Fv, single chain antibodies, diabodies), maxibodies, nanobodies, and recombinant peptides comprising CDRs of the foregoing as long as they exhibit the desired biological activity, or variants or derivatives thereof.

[000113] The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256:495 [1975], or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature*, 352:624-628[1991] and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

[000114] A "multispecific" binding agent or antigen binding protein or antibody is one that targets more than one antigen or epitope.

[000115] A "bispecific," "dual-specific" or "bifunctional" binding agent or antigen binding protein or antibody is a hybrid having two different antigen binding sites. Biantigen binding proteins, antigen binding proteins and antibodies are a species of multiantigen binding protein, antigen binding protein or multispecific antibody and may be produced by a variety of methods including, but not limited to, fusion of hybridomas or linking of Fab' fragments. (See, e.g., Songsivilai and Lachmann, 1990, *Clin. Exp. Immunol.* 79:315-321; Kostelny et al., 1992, *J. Immunol.* 148:1547-1553; Spiess et al., Alternative molecular formats and therapeutic applications for bispecific antibodies, *Mol. Immunol.* 67:95-106 (2015)). The two binding sites of a bispecific antigen binding protein or antibody will bind to two different epitopes, which may reside on the same or different protein targets.

[000116] The term "immunoglobulin" encompasses full antibodies comprising two dimerized heavy chains (HC), each covalently linked to a light chain (LC); a single undimerized immunoglobulin heavy chain and covalently linked light chain (HC+LC), or a chimeric immunoglobulin (light chain+heavy chain)-Fc heterotrimer (a so-called "hemibody"). An "immunoglobulin" is a protein, but is not necessarily an antigen binding protein.

[000117] In an "antibody", each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" chain of about 220 amino acids (about 25 kDa) and one "heavy" chain of about 440 amino acids (about 50-70 kDa). The amino-terminal portion of each chain includes a "variable" ("V") region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The carboxy-terminal portion of each chain defines a constant region primarily responsible for effector function.

The variable region differs among different antibodies. The constant region is the same among different antibodies. Within the variable region of each heavy or light chain, there are three hypervariable subregions that help determine the antibody's specificity for antigen in the case of an antibody that is an antigen binding protein. However, within the scope of the present invention, an embodiment of the immunoglobulin, e.g., an antibody, need not be an antigen binding protein, or need not be known to specifically bind to an antigen. The variable domain residues between the hypervariable regions are called the framework residues and generally are somewhat homologous among different antibodies. Immunoglobulins can be assigned to different classes depending on the amino acid sequence of the constant domain of their heavy chains. Human light chains are classified as kappa (.kappa.) and lambda (.lamda.) light chains. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See generally, *Fundamental Immunology*, Ch. 7 (Paul, W., ed., 2nd ed. Raven Press, N.Y. (1989)). Within the scope of the invention, an "antibody" also encompasses a recombinantly made antibody, and antibodies that are glycosylated or lacking glycosylation.

[000118] The term "light chain" or "immunoglobulin light chain" includes a full-length light chain and fragments thereof having sufficient variable region sequence to confer binding specificity. A full-length light chain includes a variable region domain, V_L , and a constant region domain, C_L . The variable region domain of the light chain is at the amino-terminus of the polypeptide. Light chains include kappa chains and lambda chains.

[000119] The term "heavy chain" or "immunoglobulin heavy chain" includes a full-length heavy chain and fragments thereof having sufficient variable region sequence to confer binding specificity. A full-length heavy chain includes a variable region domain, V_H , and three constant region domains, C_{H1} , C_{H2} , and C_{H3} . The V_H domain is at the amino-terminus of the polypeptide, and the C_H domains are at the carboxyl-terminus, with the C_{H3} being closest to the carboxy-terminus of the polypeptide. Heavy chains are classified as mu (μ), delta (δ), gamma (γ), alpha (α), and epsilon (ϵ), and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. In separate embodiments of the invention, heavy chains may be of any isotype, including IgG (including IgG1, IgG2, IgG3 and IgG4 subtypes), IgA (including IgA1 and IgA2 subtypes), IgM and IgE. Several of these may be further divided into subclasses or isotypes, e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. Different IgG isotypes may have different effector functions

(mediated by the Fc region), such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, the Fc region of an antibody binds to Fc receptors (Fc γ Rs) on the surface of immune effector cells such as natural killers and macrophages, leading to the phagocytosis or lysis of the targeted cells. In CDC, the antibodies kill the targeted cells by triggering the complement cascade at the cell surface.

[000120] An "Fc region", or used interchangeably herein, "Fc domain" or "immunoglobulin Fc domain", contains two heavy chain fragments, which in a full antibody comprise the C_{H1} and C_{H2} domains of the antibody. The two heavy chain fragments are held together by two or more disulfide bonds and by hydrophobic interactions of the C_{H3} domains.

[000121] The term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

[000122] In many embodiments of the invention, the protein of interest is an antigen binding protein, such as but not limited to, an antibody, antibody subunit, or antibody fragment. For a detailed description of the structure and generation of antibodies, see Roth, D. B., and Craig, N. L., Cell, 94:411-414 (1998), herein incorporated by reference in its entirety. Briefly, the process for generating DNA encoding the heavy and light chain immunoglobulin sequences occurs primarily in developing B-cells. Prior to the rearranging and joining of various immunoglobulin gene segments, the V, D, J and constant (C) gene segments are found generally in relatively close proximity on a single chromosome. During B-cell-differentiation, one of each of the appropriate family members of the V, D, J (or only V and J in the case of light chain genes) gene segments are recombined to form functionally rearranged variable regions of the heavy and light immunoglobulin genes. This gene segment rearrangement process appears to be sequential. First, heavy chain D-to-J joints are made, followed by heavy chain V-to-DJ joints and light chain V-to-J joints. In addition to the rearrangement of V, D and J segments, further diversity is generated in the primary repertoire of immunoglobulin heavy and light chains by way of variable recombination at the locations where the V and J segments in the light chain are joined and where the D and J segments of the heavy chain are joined. Such variation in the light chain typically occurs within the last codon of the V gene segment and the first codon of the J segment. Similar imprecision in joining occurs on the heavy chain chromosome between the D and J_H segments and may extend

over as many as 10 nucleotides. Furthermore, several nucleotides may be inserted between the D and J_H and between the V_H and D gene segments which are not encoded by genomic DNA. The addition of these nucleotides is known as N-region diversity. The net effect of such rearrangements in the variable region gene segments and the variable recombination which may occur during such joining is the production of a primary antibody repertoire.

[000123] The term "hypervariable" region refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a complementarity determining region or CDR [i.e., residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain as described by Kabat et al., *Sequences of Proteins of Immunological Interest*, 3rd Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)]. Even a single CDR may recognize and bind antigen, although with a lower affinity than the entire antigen binding site containing all of the CDRs.

[000124] An alternative definition of residues from a hypervariable "loop" is described by Chothia et al., *J. Mol. Biol.* 196: 901-917 (1987) as residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain.

[000125] "Framework" or "FR" residues are those variable region residues other than the hypervariable region residues.

[000126] "Antibody fragments" comprise a portion of an intact full length antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies (Zapata et al., *Protein Eng.*, 8(10):1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[000127] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment which contains the constant region. The Fab fragment contains all of the variable domain, as well as the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. The Fc fragment displays carbohydrates and is responsible for many antibody effector functions (such as binding complement and cell receptors), that distinguish one class of antibody from another.

[000128] Pepsin treatment yields an $F(ab')_2$ fragment that has two "Single-chain Fv" or "scFv" antibody fragments comprising the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Fab fragments differ from Fab' fragments by the inclusion of a few additional residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Preferably, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the Fv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 1 13, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[000129] A "Fab fragment" is comprised of one light chain and the C_{H1} and variable regions of one heavy chain. The heavy chain of a Fab molecule cannot form a disulfide bond with another heavy chain molecule.

[000130] A "Fab' fragment" contains one light chain and a portion of one heavy chain that contains the V_H domain and the C_{H1} domain and also the region between the C_{H1} and C_{H2} domains, such that an interchain disulfide bond can be formed between the two heavy chains of two Fab' fragments to form an $F(ab')_2$ molecule.

[000131] A " $F(ab')_2$ fragment" contains two light chains and two heavy chains containing a portion of the constant region between the C_{H1} and C_{H2} domains, such that an interchain disulfide bond is formed between the two heavy chains. A $F(ab')_2$ fragment thus is composed of two Fab' fragments that are held together by a disulfide bond between the two heavy chains.

[000132] "Fv" is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the $V_H V_L$ dimer. A single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[000133] "Single-chain antibodies" are Fv molecules in which the heavy and light chain variable regions have been connected by a flexible linker to form a single polypeptide chain, which forms an antigen-binding region. Single chain antibodies are discussed in detail in International Patent Application Publication No. WO 88/01649 and U.S. Pat. No. 4,946,778 and No. 5,260,203, the disclosures of which are incorporated by reference in their entireties.

[000134] "Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain, and optionally comprising a polypeptide linker between the V_H and V_L domains that enables the Fv to form the desired structure for antigen binding (Bird et al., Science 242:423-426, 1988, and Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883, 1988). An "Fd" fragment consists of the V_H and C_{H1} domains.

[000135] The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain ($V_H V_L$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

[000136] A "domain antibody" is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. In some instances, two or more V_H regions are covalently joined with a peptide linker to create a bivalent domain antibody. The two V_H regions of a bivalent domain antibody may target the same or different antigens.

[000137] The term "antigen binding protein" (ABP) includes antibodies or antibody fragments, as defined above, and recombinant peptides or other compounds that contain sequences derived from CDRs having the desired antigen-binding properties such that they specifically bind a target antigen of interest.

[000138] In general, an antigen binding protein, e.g., an antibody or antibody fragment, "specifically binds" to an antigen of interest when it has a significantly higher binding affinity for, and consequently is capable of distinguishing, that antigen, compared to its affinity for other unrelated proteins, under similar binding assay conditions. Typically, an antigen binding protein is said to "specifically bind" its target antigen when the dissociation constant (K_D) is 10^{-8} M or lower. The antigen binding protein specifically binds antigen with "high affinity" when the K_D is 10^{-9} M or lower, and with "very high affinity" when the K_D is 10^{-10} M or lower.

[000139] "Antigen binding region" or "antigen binding site" means a portion of a protein that specifically binds a specified antigen. For example, that portion of an antigen binding protein that contains the amino acid residues that interact with an antigen and

confer on the antigen binding protein its specificity and affinity for the antigen is referred to as "antigen binding region." An antigen binding region typically includes one or more "complementary binding regions" ("CDRs"). Certain antigen binding regions also include one or more "framework" regions ("FRs"). A "CDR" is an amino acid sequence that contributes to antigen binding specificity and affinity. "Framework" regions can aid in maintaining the proper conformation of the CDRs to promote binding between the antigen binding region and an antigen. In a traditional antibody, the CDRs are embedded within a framework in the heavy and light chain variable region where they constitute the regions responsible for antigen binding and recognition. A variable region of an immunoglobulin antigen binding protein comprises at least three heavy or light chain CDRs, see, *supra* (Kabat et al., 1991, Sequences of Proteins of Immunological Interest, Public Health Service N.I.H., Bethesda, Md.; see also Chothia and Lesk, 1987, J. Mol. Biol. 196:901-917; Chothia et al., 1989, Nature 342: 877-883), within a framework region (designated framework regions 1-4, FR1, FR2, FR3, and FR4, by Kabat et al., 1991, *supra*; see also Chothia and Lesk, 1987, *supra*).

[000140] The term "antigen" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antigen binding protein (including, e.g., an antibody or immunological functional fragment thereof), and additionally capable of being used in an animal to produce antibodies capable of binding to that antigen. An antigen may possess one or more epitopes that are capable of interacting with different antigen binding proteins, e.g., antibodies.

[000141] The term "epitope" is the portion of a molecule that is bound by an antigen binding protein (for example, an antibody). The term includes any determinant capable of specifically binding to an antigen binding protein, such as an antibody or to a T-cell receptor. An epitope can be contiguous or non-contiguous (e.g., in a single-chain polypeptide, amino acid residues that are not contiguous to one another in the polypeptide sequence but that within the context of the molecule are bound by the antigen binding protein). In certain embodiments, epitopes may be mimetic in that they comprise a three dimensional structure that is similar to an epitope used to generate the antigen binding protein, yet comprise none or only some of the amino acid residues found in that epitope used to generate the antigen binding protein. Most often, epitopes reside on proteins, but in some instances may reside on other kinds of molecules, such as nucleic acids. Epitope determinants may include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl or sulfonyl groups, and may have specific

three dimensional structural characteristics, and/or specific charge characteristics. Generally, antibodies specific for a particular target antigen will preferentially recognize an epitope on the target antigen in a complex mixture of proteins and/or macromolecules.

[000142] The term "identity" refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by aligning and comparing the sequences. "Percent identity" means the percent of identical residues between the amino acids or nucleotides in the compared molecules and is calculated based on the size of the smallest of the molecules being compared. For these calculations, gaps in alignments (if any) must be addressed by a particular mathematical model or computer program (i.e., an "algorithm"). Methods that can be used to calculate the identity of the aligned nucleic acids or polypeptides include those described in Computational Molecular Biology, (Lesk, A. M., ed.), 1988, New York: Oxford University Press; Biocomputing Informatics and Genome Projects, (Smith, D. W., ed.), 1993, New York: Academic Press; Computer Analysis of Sequence Data, Part I, (Griffin, A. M., and Griffin, H. G., eds.), 1994, New Jersey: Humana Press; von Heinje, G., 1987, Sequence Analysis in Molecular Biology, New York: Academic Press; Sequence Analysis Primer, (Gribskov, M. and Devereux, J., eds.), 1991, New York: M. Stockton Press; and Carillo et al., 1988, SIAM J. Applied Math. 48:1073. For example, sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. Using a computer program such as BLAST or FASTA, two polypeptide or two polynucleotide sequences are aligned for optimal matching of their respective residues (either along the full length of one or both sequences, or along a pre-determined portion of one or both sequences). The programs provide a default opening penalty and a default gap penalty, and a scoring matrix such as PAM 250 [a standard scoring matrix; see Dayhoff et al., in Atlas of Protein Sequence and Structure, vol. 5, supp. 3 (1978)] can be used in conjunction with the computer program. For example, the percent identity can then be calculated as: the total number of identical matches multiplied by 100 and then divided by the sum of the length of the longer sequence within the matched span and the number of gaps introduced into the longer sequences in order to align the two sequences. In calculating percent identity, the sequences being compared are aligned in a way that gives the largest match between the sequences.

[000143] The GCG program package is a computer program that can be used to determine percent identity, which package includes GAP (Devereux et al., 1984, Nucl.

Acid Res. 12:387; Genetics Computer Group, University of Wisconsin, Madison, Wis.). The computer algorithm GAP is used to align the two polypeptides or two polynucleotides for which the percent sequence identity is to be determined. The sequences are aligned for optimal matching of their respective amino acid or nucleotide (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3.times. the average diagonal, wherein the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. In certain embodiments, a standard comparison matrix (see, Dayhoff et al., 1978, Atlas of Protein Sequence and Structure 5:345-352 for the PAM 250 comparison matrix; Henikoff et al., 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10915-10919 for the BLOSUM 62 comparison matrix) is also used by the algorithm.

[000144] Recommended parameters for determining percent identity for polypeptides or nucleotide sequences using the GAP program include the following:

[000145] Algorithm: Needleman et al., 1970, J. Mol. Biol. 48:443-453;

[000146] Comparison matrix: BLOSUM 62 from Henikoff et al., 1992, supra;

[000147] Gap Penalty: 12 (but with no penalty for end gaps)

[000148] Gap Length Penalty: 4

[000149] Threshold of Similarity: 0

[000150] Certain alignment schemes for aligning two amino acid sequences may result in matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full-length sequences. Accordingly, the selected alignment method (GAP program) can be adjusted if so desired to result in an alignment that spans at least 50 contiguous amino acids of the target polypeptide.

[000151] The term "modification" when used in connection with proteins of interest (e.g., immunoglobulins, including antibodies and antibody fragments), include, but are not limited to, one or more amino acid changes (including substitutions, insertions or deletions); chemical modifications; covalent modification by conjugation to therapeutic or diagnostic agents; labeling (e.g., with radionuclides or various enzymes); covalent polymer attachment such as PEGylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of non-natural amino acids. Modified

immunoglobulins of the invention will retain the binding (or non-binding) properties of unmodified molecules of the invention. By methods known to the skilled artisan, immunoglobulins, such as antibodies, or other proteins, can be "engineered" or modified for improved target affinity, selectivity, stability, and/or manufacturability before the coding sequence of the "engineered" protein is included in the inventive expression cassette.

[000152] The term "derivative" when used in connection with proteins of interest, such as immunoglobulins (including antibodies and antibody fragments) refers to proteins that are covalently modified by conjugation to therapeutic or diagnostic agents, labeling (e.g., with radionuclides or various enzymes), covalent polymer attachment such as PEGylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of non-natural amino acids. Derivatives of the invention will retain the binding properties of un-derivatized molecules.

[000153] Within the scope of the invention, proteins of interest can be therapeutic proteins, or "biologics," for the treatment of disease, including but not limited to human disease or disorder. "Treatment" or "treating" is an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" includes any indication(s) of success in the amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the injury, pathology or condition more tolerable to the patient; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; improving a patient's physical or mental well-being. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of a physical examination by a physician or other health care provider, self-reporting by a patient, neuropsychiatric exams, and/or a psychiatric evaluation.

[000154] An "effective amount" of a therapeutic is generally an amount sufficient to reduce the severity and/or frequency of symptoms, eliminate the symptoms and/or underlying cause, prevent the occurrence of symptoms and/or their underlying cause, and/or improve or remediate the damage that results from or is associated with migraine headache. In some embodiments, the effective amount is a therapeutically effective amount or a prophylactically effective amount. A "therapeutically effective amount" is an

amount sufficient to remedy a disease state (e.g., transplant rejection or GVHD, inflammation, multiple sclerosis, cancer, cardiovascular disease, diabetes, neuropathy, pain) or symptom(s), particularly a state or symptom(s) associated with the disease state, or otherwise prevent, hinder, retard or reverse the progression of the disease state or any other undesirable symptom associated with the disease in any way whatsoever (i.e. that provides "therapeutic efficacy"). A "prophylactically effective amount" is an amount of a pharmaceutical composition that, when administered to a subject, will have the intended prophylactic effect, e.g., preventing or delaying the onset (or reoccurrence) of migraine headache or multiple sclerosis symptoms, or reducing the likelihood of the onset (or reoccurrence) of migraine headache, migraine headache symptoms, or multiple sclerosis symptoms. The full therapeutic or prophylactic effect does not necessarily occur by administration of one dose, and may occur only after administration of a series of doses. Thus, a therapeutically or prophylactically effective amount may be administered in one or more administrations.

[000155] The protein of interest can be any protein, but in many embodiments the protein is a pharmacologically active protein or peptide.

[000156] In some embodiments of the invention, the protein of interest can be a mimetic or agonist peptide. The terms "-mimetic peptide," "peptide mimetic," and "-agonist peptide" refer to a peptide or protein having biological activity comparable to a naturally occurring protein of interest. These terms further include peptides that indirectly mimic the activity of a naturally occurring peptide molecule, such as by potentiating the effects of the naturally occurring molecule.

[000157] In some embodiments of the invention, the protein of interest can be an antagonist peptide or inhibitor peptide. The term "-antagonist peptide," "peptide antagonist," and "inhibitor peptide" refer to a peptide that blocks or in some way interferes with the biological activity of a receptor of interest, or has biological activity comparable to a known antagonist or inhibitor of a receptor of interest (such as, but not limited to, an ion channel or a G-Protein Coupled Receptor (GPCR)).

[000158] Examples of pharmacologically active proteins that can be used within the present invention include, but are not limited to, a toxin peptide, an IL-6 binding peptide, a CD3 binding protein, a CD19 binding protein, a CD20 binding protein, a CD22 binding protein, a HER2 binding protein, a HER3 binding protein, a VEGF-A binding protein, a TNF- α binding protein, an EGFR binding protein, a RANK ligand binding protein, an IL-1 α binding protein, an IL-1 β binding protein, an IL-17A binding protein, an EPCAM

(CD326) binding protein, a CGRP peptide antagonist, a bradykinin B1 receptor peptide antagonist, a parathyroid hormone (PTH) agonist peptide, a parathyroid hormone (PTH) antagonist peptide, an ang-1 binding peptide, an ang-2 binding peptide, a myostatin binding peptide, an erythropoietin-mimetic (EPO-mimetic) peptide, a FGF21 peptide, a thrombopoietin-mimetic (TPO-mimetic) peptide (e.g., AMP2 or AMPS), a nerve growth factor (NGF) binding peptide, a B cell activating factor (BAFF) binding peptide, and a glucagon-like peptide (GLP)-1 or a peptide mimetic thereof or GLP-2 or a peptide mimetic thereof.

[000159] The term peptide or protein "analog" refers to a polypeptide having a sequence that differs from a peptide sequence existing in nature by at least one amino acid residue substitution, internal addition, or internal deletion of at least one amino acid, and/or amino- or carboxy-terminal end truncations, or additions). An "internal deletion" refers to absence of an amino acid from a sequence existing in nature at a position other than the N- or C-terminus. Likewise, an "internal addition" refers to presence of an amino acid in a sequence existing in nature at a position other than the N- or C-terminus.

[000160] Cloning DNA

[000161] Cloning of DNA is carried out using standard techniques (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1-3, Cold Spring Harbor Press, which is incorporated herein by reference). For example, a cDNA library may be constructed by reverse transcription of polyA⁺ mRNA, preferably membrane-associated mRNA, and the library screened using probes specific for human immunoglobulin polypeptide gene sequences. In one embodiment, however, the polymerase chain reaction (PCR) is used to amplify cDNAs (or portions of full-length cDNAs) encoding an immunoglobulin gene segment of interest (e.g., a light or heavy chain variable segment). The amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used is not critical, so long as it is possible to determine the sequence of some portion of the immunoglobulin polypeptide of interest.

[000162] One source for antibody nucleic acids is a hybridoma produced by obtaining a B cell from an animal immunized with the antigen of interest and fusing it to an immortal cell. Alternatively, nucleic acid can be isolated from B cells (or whole spleen) of the immunized animal. Yet another source of nucleic acids encoding antibodies is a library of such nucleic acids generated, for example, through phage display technology.

Polynucleotides encoding peptides of interest, e.g., variable region peptides with desired binding characteristics, can be identified by standard techniques such as panning.

[000163] The sequence encoding an entire variable region of the immunoglobulin polypeptide may be determined; however, it will sometimes be adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Sequencing is carried out using standard techniques (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) *Proc. Natl. Acad. Sci. USA* 74: 5463-5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoma immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

[000164] Isolated DNA can be operably linked to control sequences or placed into expression vectors, which are then transfected into host cells that do not otherwise produce immunoglobulin protein, to direct the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies is well known in the art.

[000165] Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

[000166] Many vectors are known in the art. Vector components may include one or more of the following: a signal sequence (that may, for example, direct secretion of the

antibody; e.g.,

ATGGACATGAGGGTGCCCGCTCAGCTCCTGGGGCTCCTGCTGCTGTGGCTGAG
AGGT GCGCGCTGT//SEQ ID NO:7, which encodes the VK-1 signal peptide sequence

MDMRVPAQLLGLLLWLRGARC//SEQ ID NO:8), an origin of replication, one or more selective marker genes (that may, for example, confer antibiotic or other drug resistance, complement auxotrophic deficiencies, or supply critical nutrients not available in the media), an enhancer element, a promoter, and a transcription termination sequence, all of which are well known in the art.

[000167] By way of further illustration, the following numbered embodiments are encompassed by the present invention:

[000168] Embodiment 1: A hybrid promoter comprising:

- (i) a mCMV enhancer sequence, comprising a mCMV enhancer element (mCMV-E) and a CMV promoter (CMV-P) sequence at its 3' end, operably linked 5' to a rat EF-1alpha intron sequence;
- (ii) an intervening first leader sequence operably linked, 3' to the CMV promoter sequence of the mCMV enhancer sequence, and 5' to the rat EF-1alpha intron sequence; and
- (iii) a second leader sequence operably linked 3' to the rat EF-1 alpha intron sequence.

[000169] Embodiment 2: The hybrid promoter of Embodiment 1, wherein the CMV promoter sequence at the 3' end of the mCMV enhancer sequence comprises a segment having the nucleotide sequence of SEQ ID NO:24 or SEQ ID NO:26.

[000170] Embodiment 3: The hybrid promoter of any of Embodiments 1-2, wherein the rat EF-1alpha intron sequence comprises the nucleotide sequence of SEQ ID NO:4.

[000171] Embodiment 4: The hybrid promoter of any of Embodiments 1-3, wherein the mCMV enhancer sequence comprises the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:33; and the rat EF-1alpha intron sequence comprises the nucleotide sequence of SEQ ID NO:4.

[000172] Embodiment 5: The hybrid promoter of any of Embodiments 1-4, wherein the first leader sequence comprises the nucleotide sequence of SEQ ID NO:3.

[000173] Embodiment 6: The hybrid promoter of any of Embodiments 1-5, wherein the second leader sequence comprises the nucleotide sequence of SEQ ID NO:5.

- [000174] Embodiment 7: The hybrid promoter of any of Embodiments 1-6, further comprising one or more TetO sequences inserted within the CMV promoter sequence.
- [000175] Embodiment 8: The hybrid promoter of any of Embodiments 1-7, comprising a nucleotide sequence having at least 95% sequence identity to the nucleotide sequence of any of SEQ ID NO:1, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32.
- [000176] Embodiment 9: An expression cassette, comprising:
- [000177] the hybrid promoter of any of Embodiments 1-8, operably linked 5' to an open reading frame encoding an exogenous protein of interest; and a polyadenylation site operably linked 3' to the open reading frame.
- [000178] Embodiment 10: A recombinant expression vector comprising the expression cassette of Embodiment 9.
- [000179] Embodiment 11: A mammalian host cell comprising the recombinant expression vector of Embodiment 10.
- [000180] Embodiment 12: The mammalian host cell of Embodiment 11, further being capable of expressing TetR.
- [000181] Embodiment 13: The mammalian host cell of any of Embodiments 11-12, being derived from a Chinese Hamster Ovary (CHO) cell.
- [000182] Embodiment 14: The mammalian host cell of any of Embodiments 11-13, selected from the group consisting of a CHO-K1 cell, a DXB11 cell, and a DG44 cell.
- [000183] Embodiment 15: A method of producing a protein of interest, comprising:
- (a) culturing the mammalian host cell of any of Embodiments 11-14, in an aqueous medium under physiological conditions permitting expression of the protein of interest; and
 - (b) recovering the protein of interest from the medium.
- [000184] Embodiment 16: A method of producing a protein of interest, comprising:
- (a) culturing a mammalian host cell that comprises an expression vector comprising the hybrid promoter of any of Embodiments 7-8, in an aqueous medium under physiological conditions, wherein the mammalian host cell is capable of expressing TetR, whereby, in the absence of tetracycline in the medium, the expression of the protein of interest is repressed;

(b) adding tetracycline to the aqueous medium in an amount sufficient to bind TetR in the host cell, whereby expression of the protein of interest by the host cell is derepressed; and

(c) recovering the protein of interest from the medium.

[000185] Embodiment 17: The method of any of Embodiments 15-16, wherein the aqueous medium is serum-free.

[000186] Embodiment 18: The method of any of Embodiments 15-16, wherein culturing the mammalian host cell is in a suspension in liquid aqueous medium.

[000187] Embodiment 19: The method of any of Embodiments 15-16, wherein culturing the mammalian host cell is in an adhesion layer on a solid or semisolid substrate.

[000188] The following working examples are illustrative and not to be construed in any way as limiting the scope of the invention.

[000189] EXAMPLES

[000190] Example 1. Materials and Methods.

[000191] Vector construction. All vectors C, D, E, F, and G included a DNA sequence encoding an exogenous protein of interest “Fc-A” (or “FC-A”; an exemplary Fc fusion with a therapeutic anti-inflammatory antibody), an IRES derived from EMCV virus, the murine dihydrofolate reductase gene, and a 221-bp late polyadenylation derived from SV40 virus as described previously (Kaufman, R.J. et al., Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus, *Nucl. Acids Res.* 19(16): 4485–4490 (1991); Schek, et al., Definition of the upstream efficiency element of the simian virus 40 late polyadenylation signal by using in vitro analyses, *Mol. Cell. Biol.* 12(12):5386-93 (1992)). The junction between the IRES and DHFR was *aaaaacacgatTGCTCGAGAGTTGCCACCCATC**atg*//SEQ ID NO:6, where the lower case letters represent the endogenous IRES sequence and the ATG transcription start site of DHFR is italicized lower case. A single transcript is initiated from these various constructs, which then includes the Fc-A gene, the EMCV IRES, the murine DHFR gene and terminates at the SV40 polyadenylation site.

[000192] Various transcription promoting sequences were included either 5’ or both 5’ and 3’ to the sequence described above. This allowed all plasmids to be selected using the DHFR gene and Fc-A as a marker for relative expression. The plasmid backbone for all plasmids was pUC57-simple Genscript (Piscataway, NJ).

[000193] Several exemplary plasmids are represented schematically in Figure 1. Vector C contained the human CMV and human EF-1alpha intron sequences described previously (Kim, Lee, Shin, Kang, & Kim, 2002). Vector F (Figure 1) contained sequences flanking the endogenous Chinese hamster ovary (CHO) EF-1alpha gene as described (Running Deer, J., & Allison, D. S., High-level expression of proteins in mammalian cells using Transcription Regulatory sequences from Chinese Hamster Ovary EF-1alpha Gene, *Biotechnology Progress* 20:880–889 (2004)) with 4083 bp 5' sequences flanking the ATG and 4174 bp 3' flanking sequences following the polyadenylation site. Vector G (Figure 1) was constructed by deleting a 240bp sequence from the transcription start site of the CHO EF-1alpha gene to the FSEI site and replacing it with a DNA fragment encompassing the murine CMV fragment from position -615 to the transcriptional start site. Vector D (Figure 1) was constructed by synthesizing the fragment shown in SEQ ID NO:1 (containing murine CMV GenBank: L06816.1, nucleotides 4067-4682, Rat EF-1alpha intron Genbank: AC158987.3, nucleotides 22137-21728) and placing these sequences 5' of the Fc-A coding sequence. Vector C and Vector D also contained the adenovirus tripartite leader that replaces the endogenous 3' untranslated leader sequence (Kaufman et al., 1991). The pUC57-simple vector is not shown in Figure 1. The complete map of Vector D is shown in Figure 2.

[000194] Electroporation and culture of cells. Long duration electroporation were performed as described previously (Bodwell, et al., Long Duration electroporation for achieving high level expression of glucocorticoid receptors in mammalian cell lines. *J. Steroid Biochem. and Mol. Biol.*, 68(e 8), 77–82 (1999)). Briefly, 2×10^7 cells were resuspended in 0.3mL of HBS buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Polysorbate20), 25mg of DNA was added, and electroporated in a 4mm gap cuvette using a BTX EM 600 with a capacitance of 3175 μ F, 720 ohms, and 200 volts.

[000195] DXB11 and DG44 cells were cultured in either PowerCHO2 (Lonza), ProCHO4 (Lonza) or Ex-cell 302 (Sigma) according to the manufacturer's instructions. Cells were cultured every 3-4 days and seeded at 4×10^5 cells/ml for a 3-day culture or 3×10^5 cells/ml for a 4-day culture.

[000196] Protein titer was determined by affinity High Performance Liquid Chromatography (HPLC) using POROS A/20 Protein A column.

[000197] Example 2. Transfection of CHO cells

[000198] CHO cells were transfected with various constructs expressing the Fc-A fusion protein. These constructs are shown in Figure 1 and Figure 2. Transfected pools of cells were compared for their expression of Fc-A fusion protein.

[000199] Transfection of DXB11 cells. The various Fc-A expression plasmids were used to transfect DXB11, a DHFR mutant CHO cell line, using electroporation. These cells were grown in ProCHO4 and were first selected for growth in media lacking hypoxanthine and thymidine and then selected at 150 nM methotrexate (MTX). Pools of cells selected with 150 nM MTX were seeded at 1×10^6 cells/ml and fed Ex-cell Advanced CHO Feed 1 (SAFC) and glucose on days 2, 4 and 7. Supernatant fluid was harvested on day 8 and analyzed by porous protein A as described above.

[000200] The Fc-A protein titer and specific productivity (picograms per cell per day) are shown in Figure 3A and Figure 3B, respectively. The results demonstrated that the hybrid promoter containing murine CMV enhancer combined with the rat EF-1alpha intron and suitable leader sequences (e.g., here the adenovirus tripartite leader; vector D), enhanced expression of the Fc-A recombinant protein by pools of transfected cells. The protein titers achieved were superior to other constructs and specific productivity was as good or better than that obtained using other constructs.

[000201] Transfection of DXB-11 with Vectors D, F, and G. Vectors D, F, and G (Figure 1) were used to transfect DXB11 cells growing in PowerCHO2. Cells were first selected for the growth in media lacking hypoxanthine and thymidine, amplified to 150nM MTX and then re-amplified to 500nM MTX to further enhance expression. These pools were seeded at 1×10^6 cells/ml and fed with Ex-cell Advanced CHO Feed 1 and glucose on days 2, 3 and 6. Supernatant fluid was harvested on day 8. As shown in Figure 4A and Figure 4B, respectively, the titer of those pools transfected with Vector D were significantly higher compared to Vectors F and G. The specific productivities of Vector D were generally higher than Vector F and G.

[000202] Transfection of DG44 and DXB-11 with Vectors D and F. CHO cells were transfected with Vectors D and F expressing Fc-A fusion protein. DXB11 cells growing in Ex-cell 302 were transfected as described above. DG44, another DHFR mutant cell line, was grown in PowerCHO2 and transfected identically to the DXB11 cells. Transfected pools of cells were compared for their expression of Fc-A. Cells were transfected using electroporation, selected for growth in media lacking hypoxanthine and thymidine, and then amplified to 150nM MTX. The DXB11 were further amplified to 500nM MTX and the DG44 cells were further amplified to 1uM MTX. Pools of cells

were then seeded by a 1:5 dilution into growth medium supplemented with Ex-cell Advanced CHO Feed 1, fed on days 3, 6 and 8 with Ex-cell Advanced CHO Feed 1 and glucose. Supernatant fluid was harvested on day 10 and analyzed by porous protein A as described above. The titer and specific productivity are shown in Figure 5A and Figure 5B, respectively.

[000203] DXB11 Pools transfected with Vector D expressed nearly twice as much Fc-A fusion protein as those pools transfected with Vector F. Pools of DG44 cells transfected with Vectors D and F expressed at similar levels. However, the pools transfected with the Vector D had slightly higher specific productivity. The lower specific productivity of DG44 cells compared to DXB11 cells implies that DG44 cells are less sensitive to the MTX than DXB11 cells.

[000204] Vector D also included a 5' untranslated (5'UTR) leader sequence derived from adenovirus tripartite leader (TPL). The TPL has been shown to enhance expression of proteins under stress conditions (Logan & Shenk, Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA 81(12): 3655-59 (1984)). Whether the TPL is enhancing expression in these studies is unclear, but other studies have shown that the exact nucleotide sequences of the 5'UTR that one may use in this position varies as long, as they do not contain extensive secondary structures or initiator codons prior to the authentic initiator codon (Mignone, F. et al., Untranslated regions of mRNAs. Genome Biology, 3(3):reviews0004.1-0004.10 (2002)).

[000205] Example 3. Tetracycline-inducible expression from the inventive hybrid promoter

[000206] In cells expressing the Tet repressor (TetR), tetracycline can be used to regulate expression from promoters containing the Tet operator sequence (Yao et al.). Introduction of the Tet operator (TetO) sequence just 3' of the TATA box prevents transcription from this promoter in the presence of the TetR. Presumably, TetR binds to TetO and prevents transcription factors from interacting with the transcription start site or interfere with the transcription initiation complex formation (Yao et al). Notably, positioning of the TetO sequences is critical in determining if TetR can effectively modulate transcription. (See, Yao et al.,

Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells, Hum. Gene Ther. 9(13):1939-50 (1998)).

[000207] Yao et al. hypothesized that positioning the TetO sequence 10bp downstream of the TATATAA sequence allows binding of the TetR to the same surface as the TATA binding protein. Consistent with this hypothesis, Kim et al. found that insertion of TetO sequences at position 0 or 15 downstream of the hCMV US11 TATAAG sequence failed to preserve repression in the presence of TetR. (See, Kim et al., *Tetracycline repressor-regulated gene repression in recombinant human cytomegalovirus*, J. Virol. 69: 2565-257 (1995)).

[000208] Tetracycline (Tet), when added to the culture, will bind TetR and inhibit binding to the TetO sequence, thus allowing transcription. To create a powerful Tet-regulated promoter based on the inventive mCMV enhancer sequence/rat EF-1 α intron hybrid promoter sequences, we first generated a plasmid driving LacZ (beta-galactosidase protein) expression, JV56_pJV39_pJV10_pUC57S (herein abbreviated as "pJV56"; Figure 6). The LacZ open reading frame is SEQ ID NO:12:

[000209] ATGGGGGGTTCTCATCATCATCATCATGGTATGGCTAGCATGA
CTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGTACC
TAAGGATCAGCTTGGAGTTGATCCCGTCGTTTTACAACGTCGTGACTGGGAAA
ACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGC
TGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCA
GCCTGAATGGCGAATGGCGCTTTGCCTGGTTTTCCGGCACCAGAAGCGGTGCC
GGAAAGCTGGCTGGAGTGCATCTTCCTGAGGCCGATACTGTCGTCGTCCCCCT
CAAACCTGGCAGATGCACGGTTACGATGCGCCCATCTACACCAACGTAACCTA
TCCCATTACGGTCAATCCGCCGTTTGTTCACGGAGAATCCGACGGGTGTGTT
ACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCG
AATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCT
GGGTCGGTTACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCA
TTTTTACGCGCCGGAGAAAACCGCCTCGCGGTGATGGTGCTGCGTTGGAGTG
ACGGCAGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGCATTTTCCG
TGACGTCTCGTTGCTGCATAAACCGACTACACAAATCAGCGATTTCCATGTTG
CCACTCGCTTTAATGATGATTTTACGCCGCGCTGTACTGGAGGCTGAAGTTCAG
ATGTGCGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCTTTATGGCAGGG
TGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGAT
GAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAAACC

CGAAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCGGTGGTTGAACT
GCACACCGCCGACGGCACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTC
CGCGAGGTGCGGATTGAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGC
TGATTCGAGGGCGTTAACCGTCACGAGCATCATCCTCTGCATGGTCAGGTCATG
GATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAGCAGAACAACCTTTA
ACGCCGTGCGCTGTTTCGATTATCCGAACCATCCGCTGTGGTACACGCTGTGC
GACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCA
TGGTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAG
CGAACGCGTAACGCGAATGGTGCAGCGCGATCGTAATACCCGAGTGTGATC
ATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAATCACGACGCGCTGT
ATCGCTGGATCAAATCTGTCGATCCTTCCCGCCCGGTGCAGTATGAAGGCGGC
GGAGCCGACACCACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTGG
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TTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCACGCG
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TCCCGGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTA
AATATGATGAAAACGGCAACCCGTGGTTCGGCTTACGGCGGTGATTTTGGCGA
TACGCCGAACGATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGACCGCA
CGCCGCATCCAGCGCTGACGGAAGCAAAACACCAGCAGCAGTTTTTCCAGTT
CCGTTTATCCGGGCAAACCATCGAAGTGACCAGCGAATACCTGTTCCGTCATA
GCGATAACGAGCTCCTGCACTGGATGGTGGCGCTGGATGGTAAGCCGCTGGC
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CTGCCTGAACTACCGCAGCCGGAGAGCGCCGGGCAACTCTGGCTCACAGTAC
GCGTAGTGCAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATCAGCGC
CTGGCAGCAGTGGCGTCTGGCGGAAAACCTCAGTGTGACGCTCCCCGCCGCG
TCCCACGCCATCCCGCATCTGACCACCAGCGAAATGGATTTTTGCATCGAGCT
GGGTAATAAGCGTTGGCAATTAAACCGCCAGTCAGGCTTTCTTTCACAGATGT
GGATTGGCGATAAAAAACAACCTGCTGACGCCGCTGCGCGATCAGTTCACCCG
TGCACCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGCATTGACCTT
AACGCCTGGGTGGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCA
GCGTTGTTGCAGTGCACGGCAGATACACTTGCTGATGCGGTGCTGATTACGAC
CGCTCACGCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACC
TACCGGATTGATGGTAGTGGTCAAATGGCGATTACCGTTGATGTTGAAGTGGC
GAGCGATACACCGCATCCGGCGCGGATTGGCCTGAACTGCCAGCTGGCGCAG

GTAGCAGAGCGGGTAAACTGGCTCGGATTAGGGCCGCAAGAAAACCTATCCCG
 ACCGCCTTACTGCCGCCTGTTTTGACCGCTGGGATCTGCCATTGTCAGACATG
 TATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGGACGCGCG
 AATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAG
 CCGCTACAGTCAACAGCAACTGATGGAAACCAGCCATCGCCATCTGCTGCAC
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 GCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAGTTCCAGCTGAGCGCCGG
 TCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAA//SEQ ID NO:12.

[000210] The basic idea was to exchange part of the mCMV promoter (mCMV-P) element sequence with the human CMV promoter (hCMV-P) element that includes the TetO binding sites responsible for regulation by tetracycline (Tet). This construct, JV57_JV56_pJV39_pJV10 (herein abbreviated as “pJV57”; Figure 7), includes the tetracycline-sensitive TetO regulatory element for expression of LacZ. Figure 10 shows schematically the hCMV-P/TetO inserted into the mCMV promoter sequence. This insertion replaces part of the mCMV-P promoter sequence with a part of the hCMV-P promoter sequence. Another construct was made to incorporate expression-increasing changes in a hCMV promoter described by Patwardhan et al. in the context of an unregulated promoter (hybrid mCMV enhancer/(partial) hCMV promoter/rat EF-1alpha intron). (Patwardhan et al., *High-resolution analysis of DNA regulatory elements by synthetic saturation mutagenesis*, Nature Biotechnology 27(12):1173-75 (2009)). This construct was JV59_JV56_pJV39_pJV10 (abbreviated herein as “pJV59”; Figure 8). In another construct, JV60_JV56_pJV39_pJV10 (herein abbreviated as “pJV60”; Figure 9), the TetO sequences of pJV57 were changed to match the sequences that increased expression surrounding the transcription start site in the study by Patwardhan et al. (see, Figure 9 and Figure 12).

[000211] Figure 11 shows the DNA sequence of hCMV-P/TetO that we used to replace the mCMV promoter (mCMV-P) sequences. Arrows indicate palindromic TetR binding sites. The mCMV-P and hCMV-P promoter sequences shown are those sequences from the TATA box through the start site of transcription. The transcription start is the g residue in the 3' sequence taccg. Note that the TetO sequence most certainly impacts the transcription start site, since the transcription start site is typically ~30 bases 3' to the 5' T of the TATA box.

[000212] We expressed beta-galactosidase from various constructs to monitor the level of protein expression. This inserted substitute sequence, which includes muCMV

enhancer element (mCMV-E)/(partial) huCMV promoter/TetO/ratEF-1 α intron, was indeed regulated by tetracycline in the medium (See, e.g., Figure 14, pJV57 +/- Tet). Furthermore, the construct pJV57 yielded approximately 10-fold as much recombinant protein as a human CMV/TetO control (pcDNA5/TO/LacZ; Thermo Fisher Scientific), when Tet was present in the medium, but about 3.5-fold less beta-galactosidase in the absence of tetracycline, as shown in Figure 14. T-Rex CHO cells bearing the muCMV enhancer/ratEF-1 α intron hybrid promoter in pJV56, absent a TetO sequence within the CMV-P segment of the mCMV enhancer sequence, also expressed approximately 10 times more protein than the human CMV/TetO control (pcDNA5/TO/LacZ), regardless of whether tetracycline was in the medium.

[000213] Materials and Methods.

[000214] T-RExTM-CHO cells. T-REx-CHO cell line (Thermo Fisher Scientific, Product No. R71807) were cultured in Ham's F12 media + glutamine + 10 μ g/ml Blast per manufacturer's protocol. T-REx-CHO cells were electroporated using the long duration electroporation (LDE) method as described by Bodwell et al. (Bodwell et al., *Long Duration Electroporation for Achieving High Level Expression of Glucocorticoid Receptors in Mammalian Cell Lines*, J. Steroid Biochem. Molec. Biol. 68:77–82 (1999)), with various constructs pJV56, pJV57, pJV59, and pJV60, expressing the beta-galactosidase protein, as shown in Figure 6, Figure 7, Figure 8, and Figure 9, respectively. Transfected cells were allowed to recover for 24 hours and then were treated with or without 1 μ g/ml tetracycline for 24 hours. Cells were lysed and beta-galactosidase activity was assayed using a β -Gal Assay Kit per manufacturer's protocol (Thermo Fisher Scientific, Product No. K1455-01). Briefly, lysates were diluted 10-fold and 10- μ l aliquots were assayed using a BCA protein assay kit (Thermo Fisher Scientific, Product No. 23227) to determine protein concentration of the lysate. Lysates were diluted 100-fold and 10- μ l aliquots were assayed for beta-galactosidase activity. All samples were transfected at least in duplicates. Specific beta-galactosidase activity was normalized to the amount of protein assayed and background activity of mock-transfected cells was subtracted from transfected samples. Results were graphed with standard deviation shown (Figure 14).

[000215] CHO-K1/TetR cells. In separate experiments, tetracycline repressor (TetR) was subcloned by Genscript[®] (Piscataway, NJ) into pcDNA3.1 + expression vector to yield vector pJV40_pcDNA3.1+tetR (abbreviated herein, as "pJV40"; Figure 15). CHO-K1 cells were routinely cultured in PowerCHOTM 2 Chemically Defined, Serum-free

CHO Medium (Lonza, Product No. BE12-771Q), and cells were electroporated using the LDE method, as described above for pJV40. Transfected pools were selected for 10 days in PowerCHO™ 2 +600 µg/ml G418 (Gibco, Product No. 10131027). Cells were then serially diluted and plated in 96-well microplates (Corning® CellBIND® 96 Well, Product #3340) at 0.75 cell per well in cloning media (EX-CELL® 302 Serum-Free Medium for CHO Cells; Sigma-Aldrich, Product No. 14326C SIGMA) +15% conditioned media in the presence of G418). Clonal populations were identified after 11-12 days and were transferred to 12-well plates in 50/50 cloning media/ PowerCHO™ 2 medium for an additional 3-4 days. Finally, clones were moved and grown in a 24 deep-well plate (VWR, Product No. P-DW-10ML-24-C-S) with constant shaking (220 rpm) in PowerCHO™ 2 medium + 600 µg/ml G418.

[000216] To screen for clones expressing high levels of TetR, CHO-K1/TetR clones were transfected with pJV57 using the ExpiFectamine™ CHO transfection kit (Thermo Fisher Scientific, Product No. A29130) per manufacturer's protocol. From those highly expressing TetR⁺ clones, some clones were subsequently chosen to be transfected with vector pJV56, pJV57, pJV59, or pJV60, using the ExpiFectamine™ CHO transfection kit (Thermo Fisher Scientific, Product No. A29130), again transfected per manufacturer's protocol. Transfected clones were allowed to recover for 24 hours and then were treated with 1 µg/ml tetracycline for 24 hours. To measure expression of the LacZ protein product, cells were lysed, and beta-galactosidase activity was assayed using a β-Gal Assay Kit per manufacturer's protocol (Thermo Fisher Scientific, Product No. K1455-01).

[000217] Briefly, lysates were diluted 10-fold and 10-µl aliquots were assayed using a BCA protein assay kit (Thermo Fisher Scientific, Product No. 23227) to determine protein concentration of the lysate. Ten (10) µl of undiluted lysate was assayed for beta-galactosidase activity. All samples were transfected at least in duplicates. Specific beta-galactosidase activity was normalized to the amount of protein assayed and background activity of mock-transfected cells was subtracted from transfected samples. Results were graphed with standard deviation shown (e.g., Figure 16, Figure 17, Figure 18).

[000218] Figure 16 (clone 3E7), Figure 17 (clone 3F9), and Figure 18 (clone 4G2) shows representative results from CHO-K1/TetR clones transfected with various constructs (pJV56, pJV57, pJV59, and pJV60) expressing the beta-galactosidase protein (or transfected with positive control: pcDNA5/TO/LacZ; Thermo Fisher Scientific, Product No. V1033-20). As above, cells were transfected with pJV57 using the

ExpiFectamine™ CHO transfection kit (Thermo Fisher Scientific, Product No. A29130) per manufacturer's protocol. Transfected clones were allowed to recover for 24 hours and then were treated with 1 µg/ml tetracycline for 24 hours. Cells were lysed, and beta-galactosidase activity was assayed using a β-Gal Assay Kit per manufacturer's protocol (Thermo Fisher Scientific, Product No. K1455-01). Briefly, lysates were diluted 10-fold and 10-µl aliquots were assayed using a BCA protein assay kit (Thermo Fisher Scientific, Product No. 23227) to determine protein concentration of the lysate. Ten (10) µl of undiluted lysate was assayed for beta-galactosidase activity. All samples were transfected at least in duplicates. Specific beta-galactosidase activity was normalized to the amount of protein assayed and background activity of mock-transfected cells was subtracted from transfected samples. Specific beta-galactosidase activity was normalized to the amount of protein assayed and background activity of mock-transfected cells was subtracted from transfected samples. Results were graphed with standard deviation shown.

[000219] Results.

[000220] T-REx™ CHO cells. In experiments using T-RExCHO cells (Thermo Fisher Scientific), a commercial cell line that stably expresses TetR, we measured beta-galactosidase (beta-gal) activity as a readout to test the ability of pJV57 to be regulated by tetracycline (Tet). As shown in Figure 14, pJV57 had equivalent expression to pJV56 when induced by the presence of tetracycline (+Tet). In addition, pJV57 had approximately 10-fold higher expression of LacZ in the presence of tetracycline (+Tet) when compared with pcDNA5/TO/LacZ, a vector shown to exhibit robust tetracycline-regulated expression. pJV57 also showed 3.5-fold regulated expression by tetracycline, a key observation demonstrating a powerfully regulated promoter. Fold-difference in Tet regulation for the positive control pcDNA5/TO/LacZ was not included in Figure 14, because of low signal-to-noise ratio between uninduced pcDNA5/TO/LacZ and background, resulting in a negative specific beta-gal activity in the control.

[000221] In an attempt to further improve upon pJV57, we hypothesized that the TetO sequences could reduce expression from pJV57 as previous studies showed that changes around the transcription start site reduced expression from the hCMV promoter (Patwardhan et al., *High-resolution analysis of DNA regulatory elements by synthetic saturation mutagenesis*, Nat Biotechnol. 27(12): 1173–1175 (2009)). The TetO sequences in pJV57 were changed to match the sequences that increased expression surrounding the transcription start site in the study by Patwardhan et al. (pJV60, Figure 9 and Figure 12). Furthermore, these changes were designed to maintain high affinity TetR

binding (the affinity of different TetO mutations is described in Sizemore et al. and reviewed in Hillen and Berens). (See, Sizemore et al., *Quantitative analysis of Tn10 Tet repressor binding to a complete set of tet operator mutants*, Nucleic Acids Research 18 (10) 2875-2880 (1990); Hillen and Berens, *Mechanisms underlying expression of Tn10 encoded tetracycline resistance*, Ann. Rev. Micro. 48:345-369 (1994)). However, this optimized construct, pJV60, did not appear to increase expression compared to pJV57 in T-REx-CHO cells (Figure 14). Tetracycline-regulated expression was maintained, but potentially slightly less regulation (2.68-fold compared to 3.50-fold (Figure 14).

[000222] The optimized hCMV promoter (hCMV-P) sequences replaced the mCMV promoter (mCMV-P) sequences in the mCMV enhancer sequence/rat EF-1 α intron hybrid promoter of the invention (see, sequence comparison in Figure 13). The changes incorporated into pJV59 did not appear to increase expression relative to pJV56 (Figure 14). Thus, we conclude that in the T-REx-CHO cells, the context of the powerful mCMV enhancer/promoter-rat EF-1 α intron, that changes around the transcription start site have little or no impact on expression.

[000223] CHO-K1/TetR cells. In separate experiments, a CHO-K1 cell line stably expressing TetR was generated by transfection of pJV40 (see, Figure 15) that has TetR gene subcloned into pcDNA3.1+ vector. Geneticin-resistant CHO-K1 pools were serially diluted to obtain single-cell colonies and screened to have high expression of TetR, i.e. more than two-fold repression of LacZ in the absence of Tet (data not shown). Three clones were selected for further analysis, as shown respectively in Figure 16, Figure 17, and Figure 18, referred to herein as CHO-K1/TetR clones. The clones were transiently transfected with pcDNA5/TO/LacZ as a control, and expression vectors of the present invention, i.e., vectors pJV56, pJV57, pJV59 and pJV60, as described above, to determine the effectiveness of a Tet-regulated promoter element based on the mCMV enhancer sequence/rat EF-1 α intron hybrid promoter sequences using TetR-expressing cells that were generated in-house.

[000224] As shown in Figure 16, Figure 17, and Figure 18, pJV56 in two of the 3 CHO-K1/TetR clones exhibited 2- to 3-fold higher expression of LacZ compared to pcDNA5/TO/LacZ in the presence of tetracycline (+Tet). Addition of hCMV-P/TetO sequences in pJV57 enabled protein expression to be regulated by tetracycline to the extent of 3.24-, 9.30- and 3.15-fold enhancement of LacZ expression in the presence of tetracycline (+Tet), compared to (-Tet) (see, pJV57, in Figure 16, Figure 17, and Figure 18 respectively), although the level of expression was variable depending on the clone

studied. This is comparison to pJV56, which did not include the hCMV-P/TetO sequences (1.27-, 0.95-, 1.19-fold differences between +Tet and minus-Tet treatments, as shown in Figure 16, Figure 17, and Figure 18, respectively).

[000225] Interestingly, total expression of LacZ was increased in all three pJV59-transfected CHO-K1/TetR clones, although as expected there was essentially no tetracycline-sensitive regulation of expression (\pm Tet comparison: 0.99-, 1.06-, 1.00-fold difference), as shown in Figure 16, Figure 17, and Figure 18, consistent with our hypothesis that protein expression can be increased by optimizing sequences surrounding the transcription start site (see, Patwardhan et al.). However, addition of TetO sites that were mutated to maintain TetR binding (pJV60) – in the context of the optimized promoter – showed decreased (Figure 16, Figure 17) or unchanged (Figure 18) LacZ expression compared to pJV59. Nevertheless, LacZ expression was augmented in all three pJV60-bearing clones compared to pJV57, in the presence of tetracyclin (2.31-, 2.82- and 1.56-fold higher expression compared to its non-Tet-treated control; Figure 16, Figure 17, Figure 18, respectively). This suggests that mutation of TetO sequences adversely affected TetR binding as observed by higher basal LacZ expression in all clones in the absence of Tet and lower fold-induction upon +Tet addition.

[000226] Overall, all of the pJV56, pJV57, pJV59, and pJV60 constructs in CHO/TetR cells demonstrated increased LacZ expression compared to CHO cells expressing from pcDNA5/TO/LacZ (i.e., positive controls) in the presence of tetracycline, and CHO cells bearing the inventive hybrid promoter with hCMV-P/TetO sequences had beta-galactosidase expression up-regulated by tetracycline (1.56- to 9.30-fold differences), compared to (-Tet), as shown in Figure 16, Figure 17, and Figure 18. It should be noted that the method of transfection is different between T-REx-CHO cells (adherent) to CHO-K1/TetR cells (cell suspension in liquid aqueous medium), and that transfection efficiency may account for differences observed in the overall levels of specific beta-galactosidase activity.

We claim:

1. A hybrid promoter, comprising:
 - (i) a mCMV enhancer sequence, comprising a mCMV enhancer element (mCMV-E) and a CMV promoter (CMV-P) sequence at its 3' end, operably linked 5' to a rat EF-1alpha intron sequence;
 - (ii) an intervening first leader sequence operably linked, 3' to the CMV promoter sequence of the mCMV enhancer sequence, and 5' to the rat EF-1alpha intron sequence; and
 - (iii) a second leader sequence operably linked 3' to the rat EF-1 alpha intron sequence.
2. The hybrid promoter of Claim 1, wherein the CMV promoter sequence at the 3' end of the mCMV enhancer sequence comprises a segment having the nucleotide sequence of SEQ ID NO:24 or SEQ ID NO:26.
3. The hybrid promoter of Claim 1, wherein the rat EF-1alpha intron sequence comprises the nucleotide sequence of SEQ ID NO:4.
4. The hybrid promoter of Claim 1, wherein the mCMV enhancer sequence comprises the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:33; and the rat EF-1alpha intron sequence comprises the nucleotide sequence of SEQ ID NO:4.
5. The hybrid promoter of Claim 4, wherein the mCMV enhancer sequence comprises the nucleotide sequence of SEQ ID NO:2.
6. The hybrid promoter of Claim 1, wherein the first leader sequence comprises the nucleotide sequence of SEQ ID NO:3.
7. The hybrid promoter of Claim 1, wherein the second leader sequence comprises the nucleotide sequence of SEQ ID NO:5.
8. The hybrid promoter of Claim 1, further comprising one or more TetO sequences inserted within the CMV promoter sequence.
9. The hybrid promoter of Claim 1, comprising a nucleotide sequence having at least 95% sequence identity to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32.
10. The hybrid promoter of Claim 1, comprising the nucleotide sequence of SEQ ID NO:1.
11. The hybrid promoter of Claim 1, comprising the nucleotide sequence of SEQ ID NO:30.

12. The hybrid promoter of Claim 1, comprising the nucleotide sequence of SEQ ID NO:31.
13. The hybrid promoter of Claim 1, comprising the nucleotide sequence of SEQ ID NO:32.
14. An expression cassette, comprising:
 - (a) the hybrid promoter of Claim 1 or Claim 8, operably linked 5' to an open reading frame encoding an exogenous protein of interest; and
 - (b) a polyadenylation site operably linked 3' to the open reading frame.
15. A recombinant expression vector comprising the expression cassette of Claim 14.
16. A mammalian host cell comprising the recombinant expression vector of Claim 15.
17. The mammalian host cell of Claim 16, further being capable of expressing TetR.
18. The mammalian host cell of Claim 16 or Claim 17, being derived from a Chinese Hamster Ovary (CHO) cell.
19. The mammalian host cell of Claim 18, selected from the group consisting of a CHO-K1 cell, a DXB11 cell, and a DG44 cell.
20. A method of producing a protein of interest, comprising:
 - (a) culturing the mammalian host cell of Claim 16, in an aqueous medium under physiological conditions permitting expression of the protein of interest; and
 - (b) recovering the protein of interest from the medium.
21. A method of producing a protein of interest, comprising:
 - (a) culturing a mammalian host cell that comprises an expression vector comprising the hybrid promoter of Claim 8, in an aqueous medium under physiological conditions, wherein the mammalian host cell is capable of expressing TetR, whereby, in the absence of tetracycline in the medium, the expression of the protein of interest is repressed;
 - (b) adding tetracycline to the aqueous medium in an amount sufficient to bind TetR in the host cell, whereby expression of the protein of interest by the host cell is derepressed; and
 - (c) recovering the protein of interest from the medium.
22. The method of producing a protein of interest of Claim 20 or Claim 21, wherein the aqueous medium is serum-free.
23. The method of producing a protein of interest of Claim 20 or Claim 21, wherein culturing the mammalian host cell is in a suspension in liquid aqueous medium.

24. The method of producing a protein of interest of Claim 20 or Claim 21, wherein culturing the mammalian host cell is in an adhesion layer on a solid or semisolid substrate.

FIG. 1

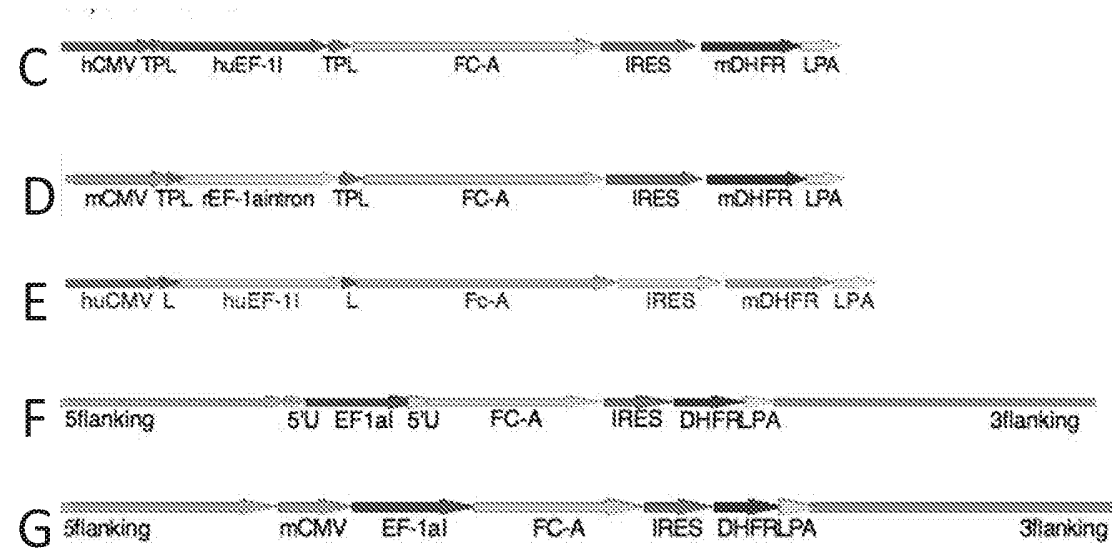
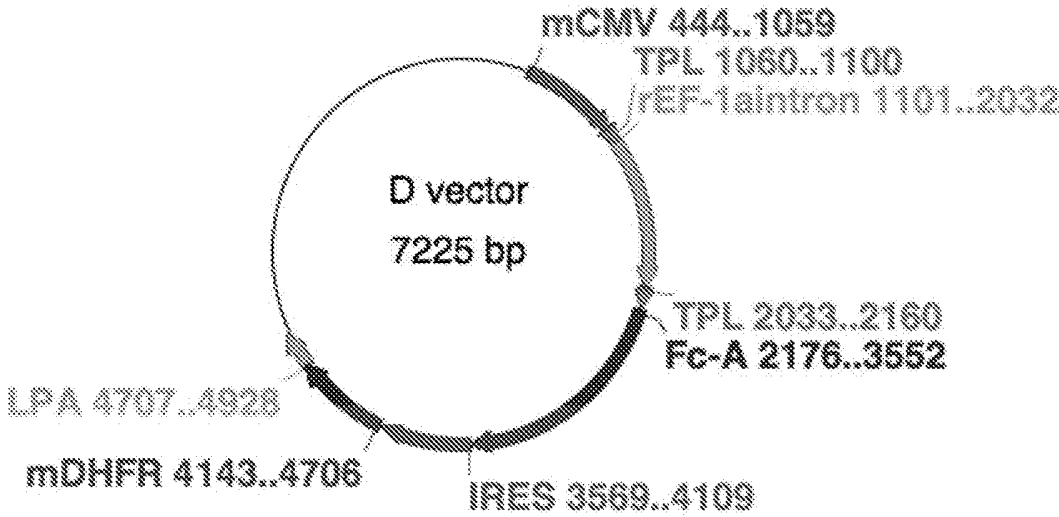
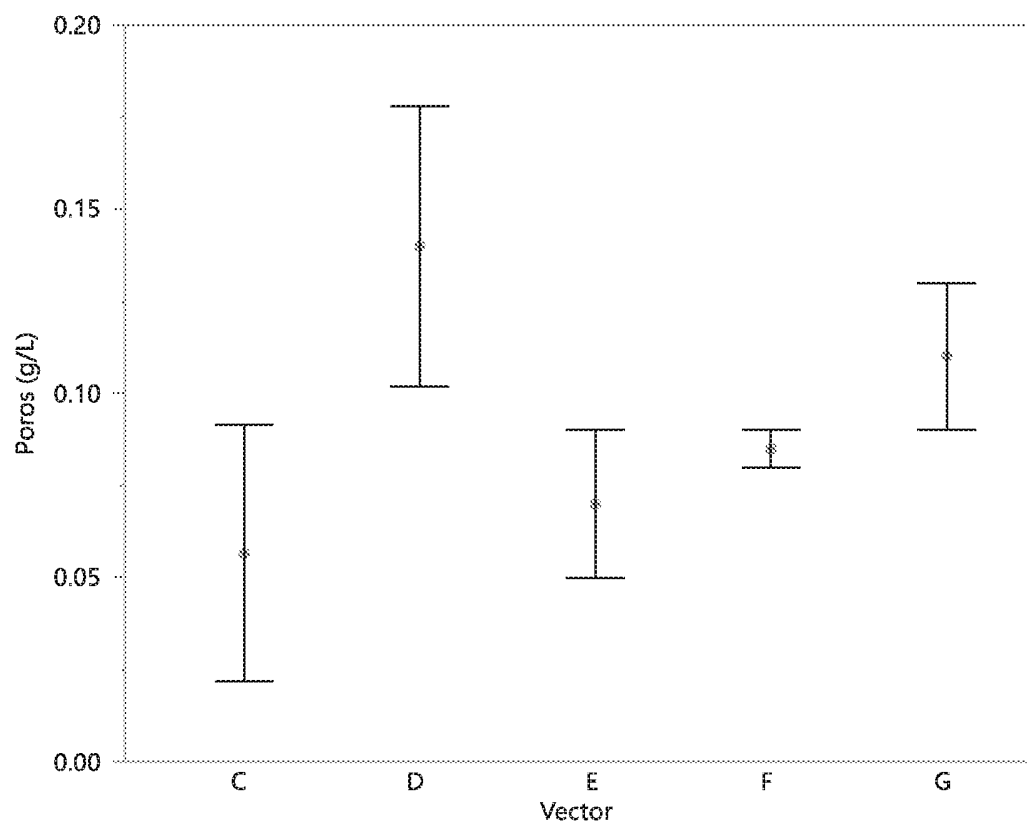


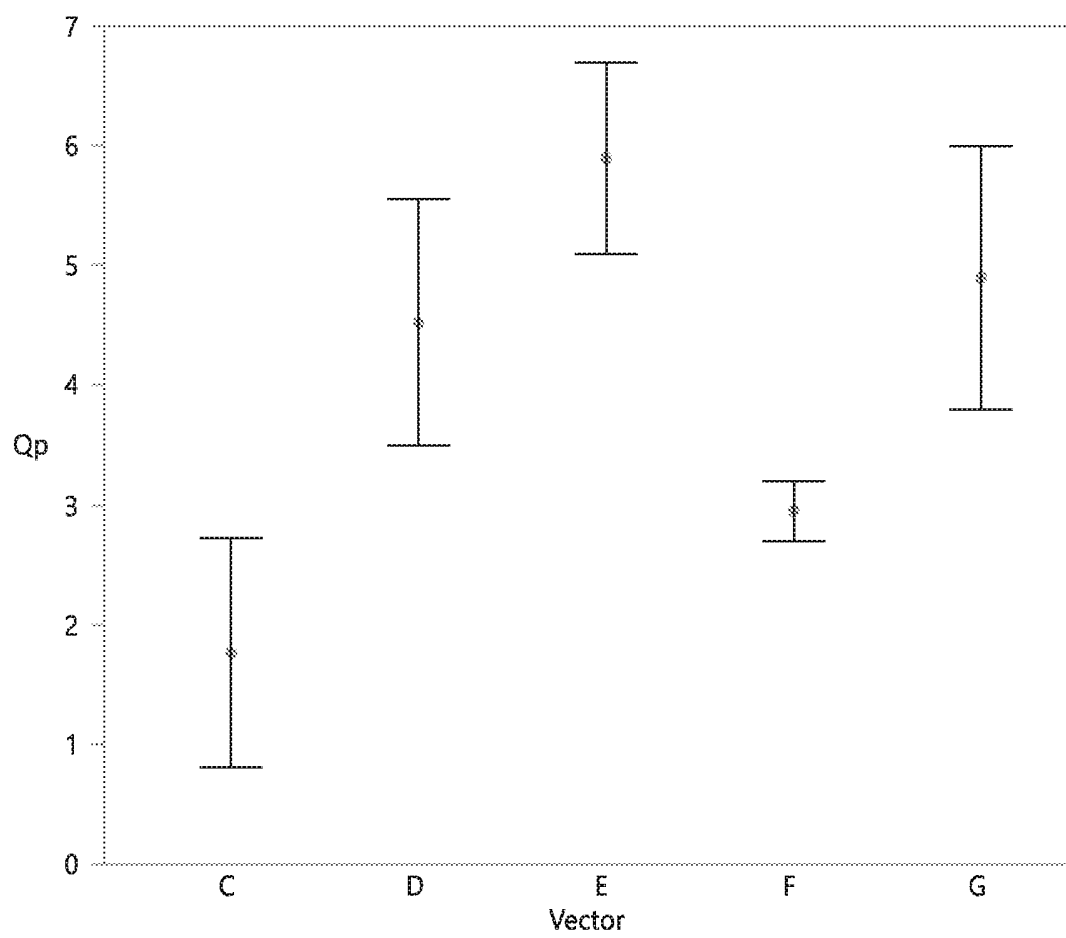
FIG.2



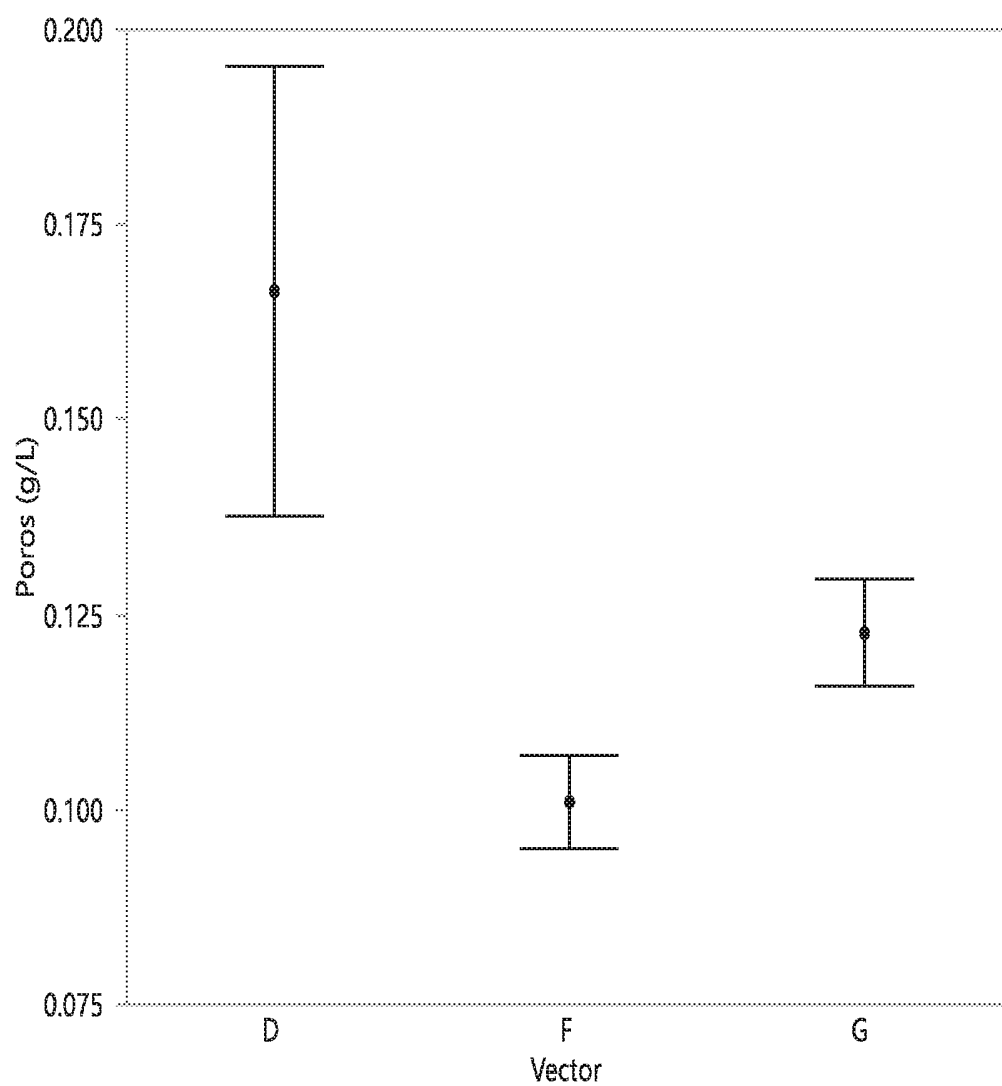
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FIG. 3A

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FIG. 3B

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FIG. 4A

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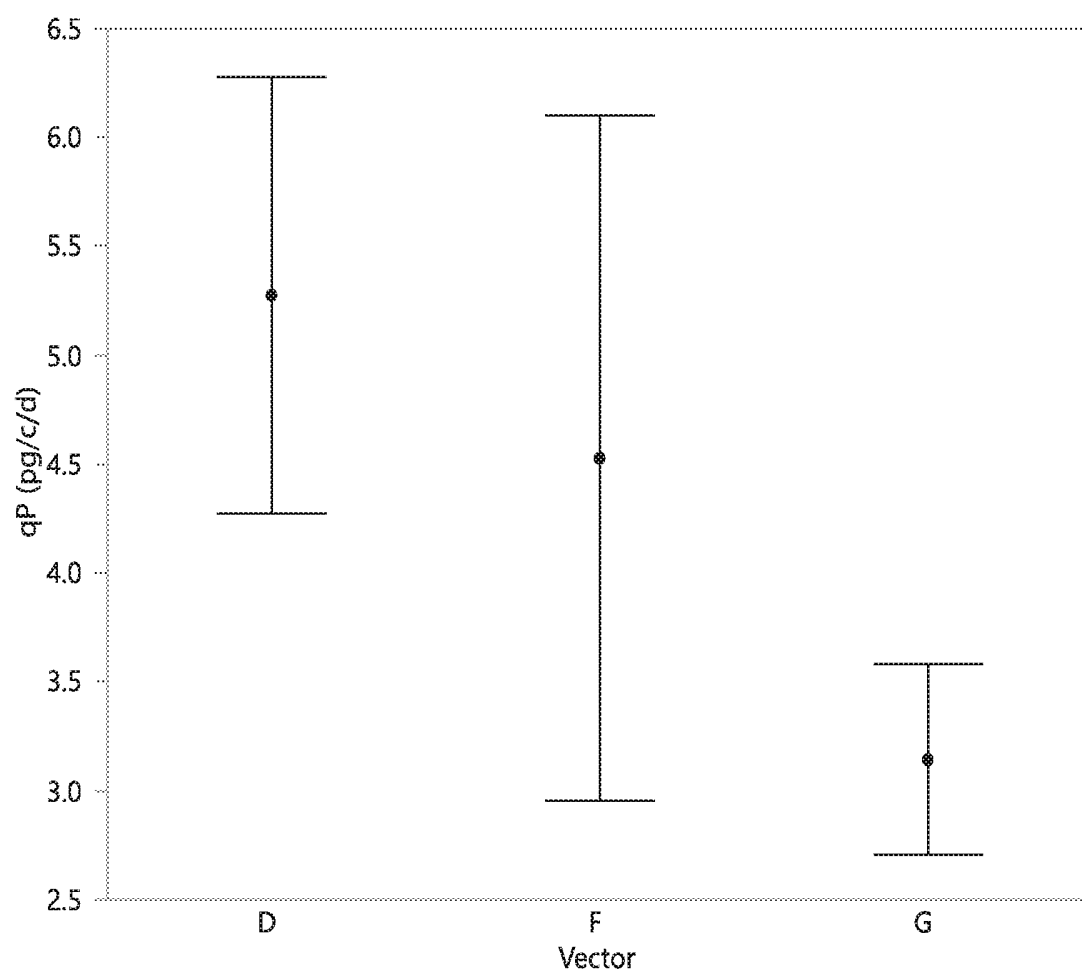
FIG. 4B

FIG. 5A

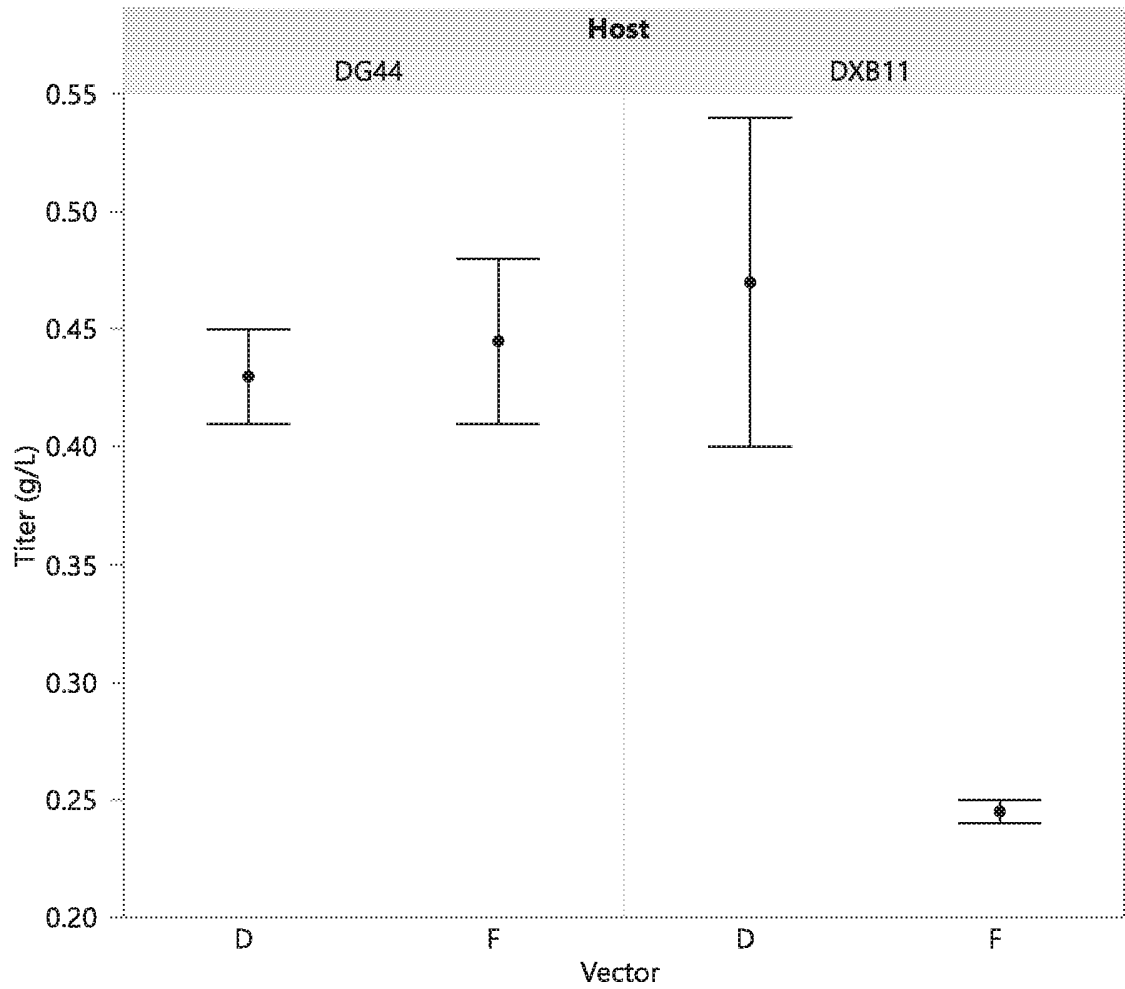


FIG. 5B

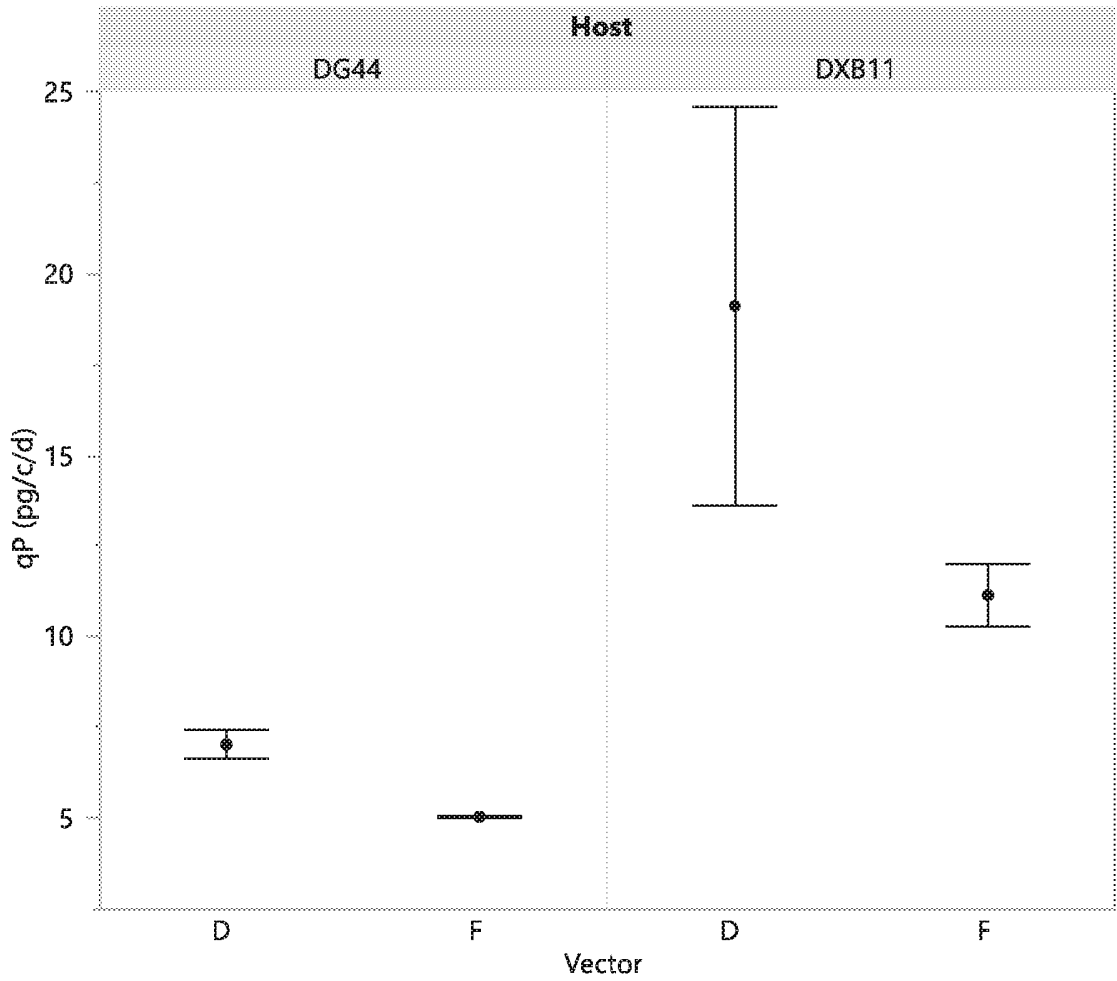


FIG. 6

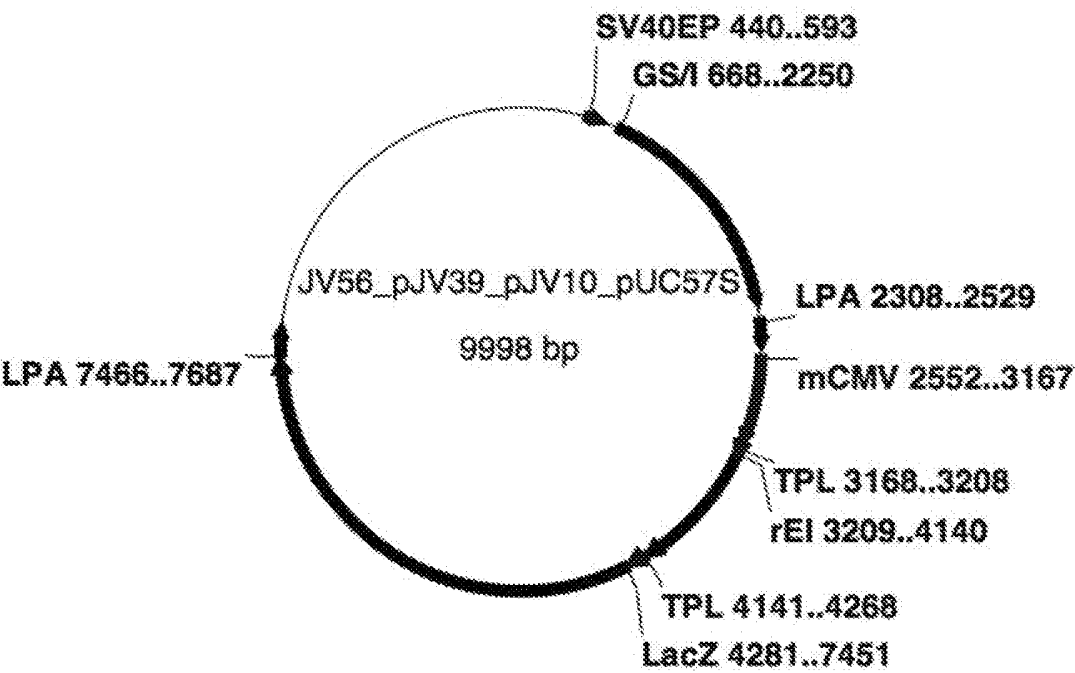


FIG. 7

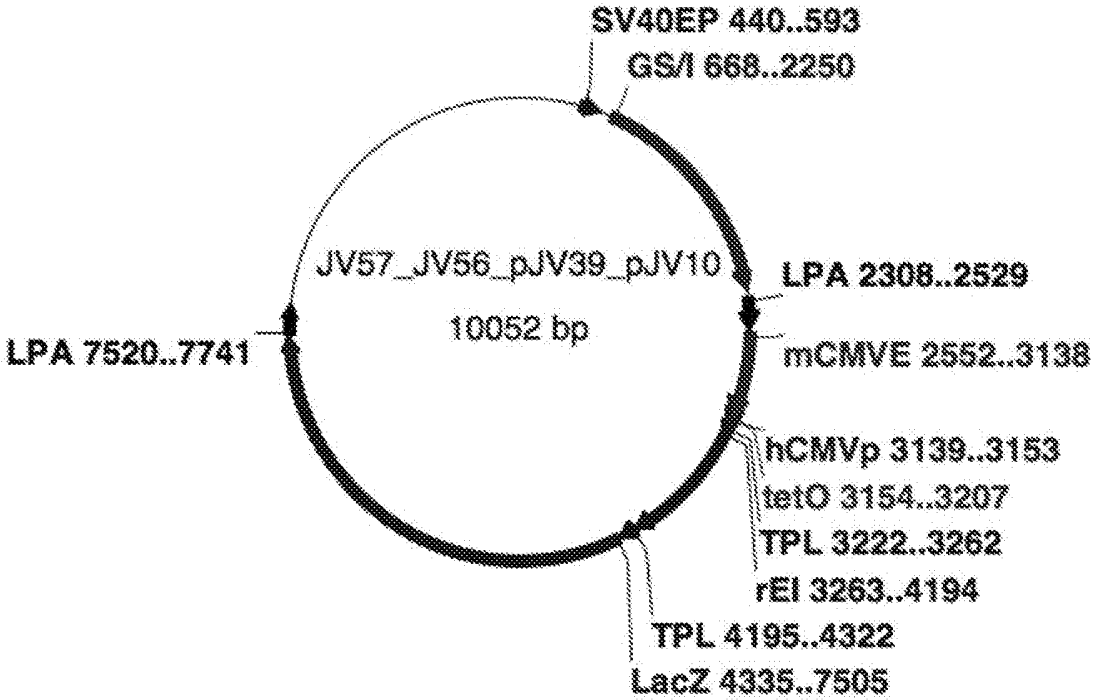


FIG. 8

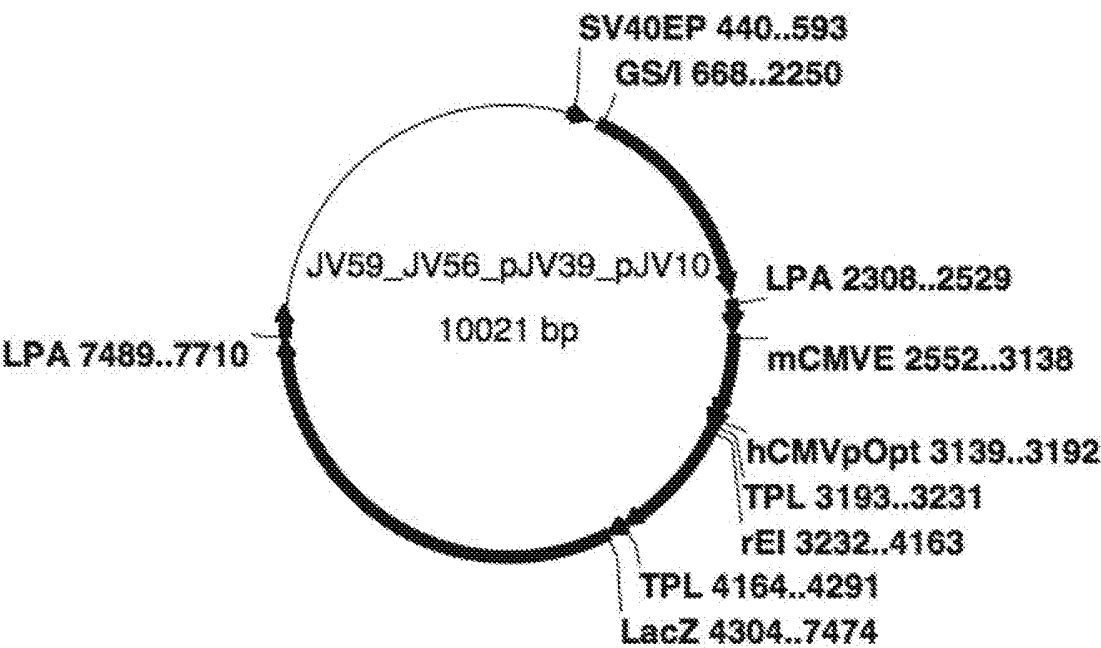


FIG. 9

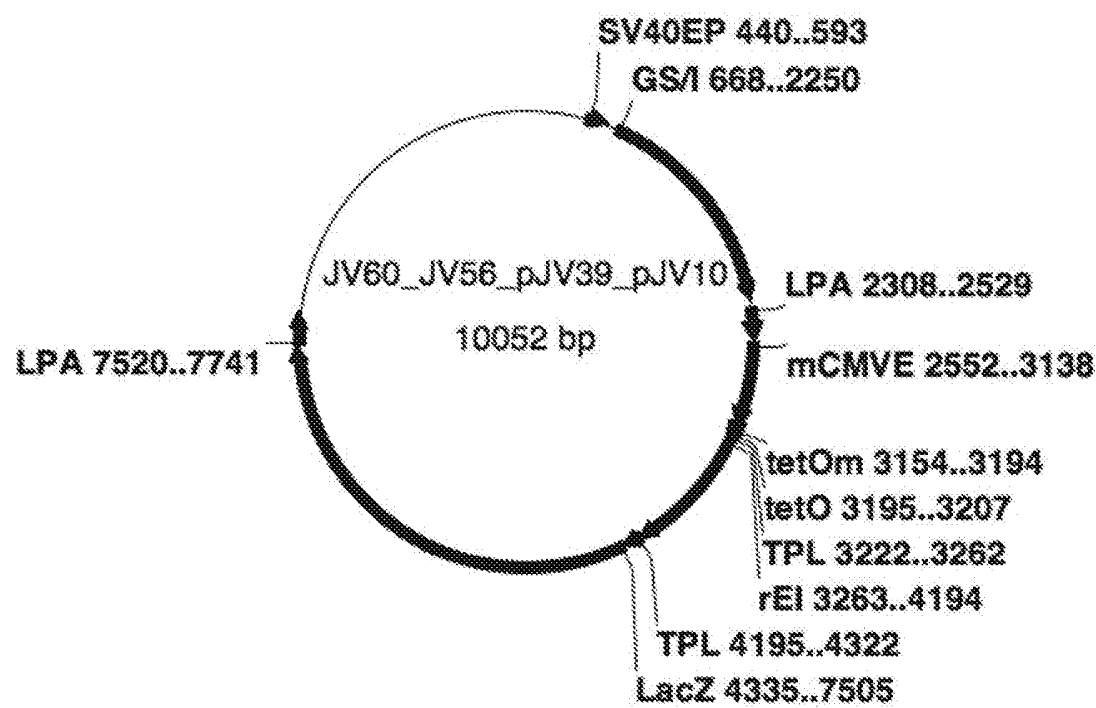


FIG. 10

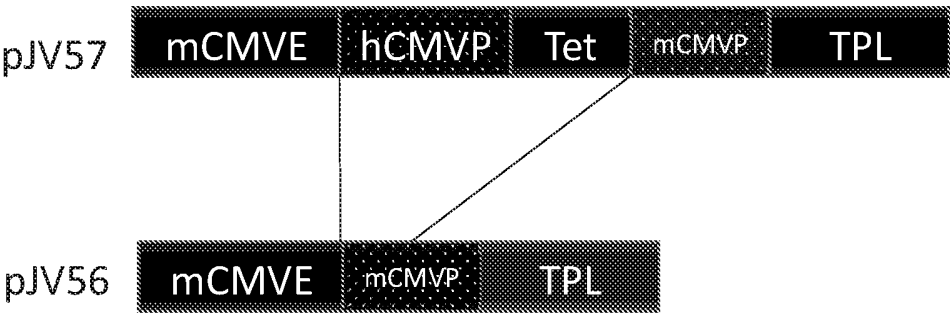


FIG. 12

pJV57 TATATAAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGA
|||||
pJV60 TATATAAGCAGAGCTCTCCCTATCAGTGATCAGTTCCTCCCTATCAGTGATAGAGA

FIG. 11

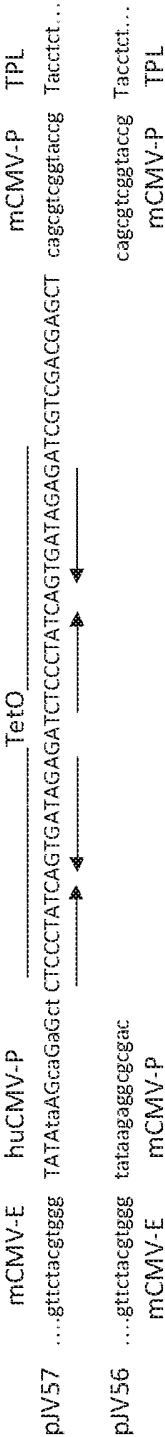
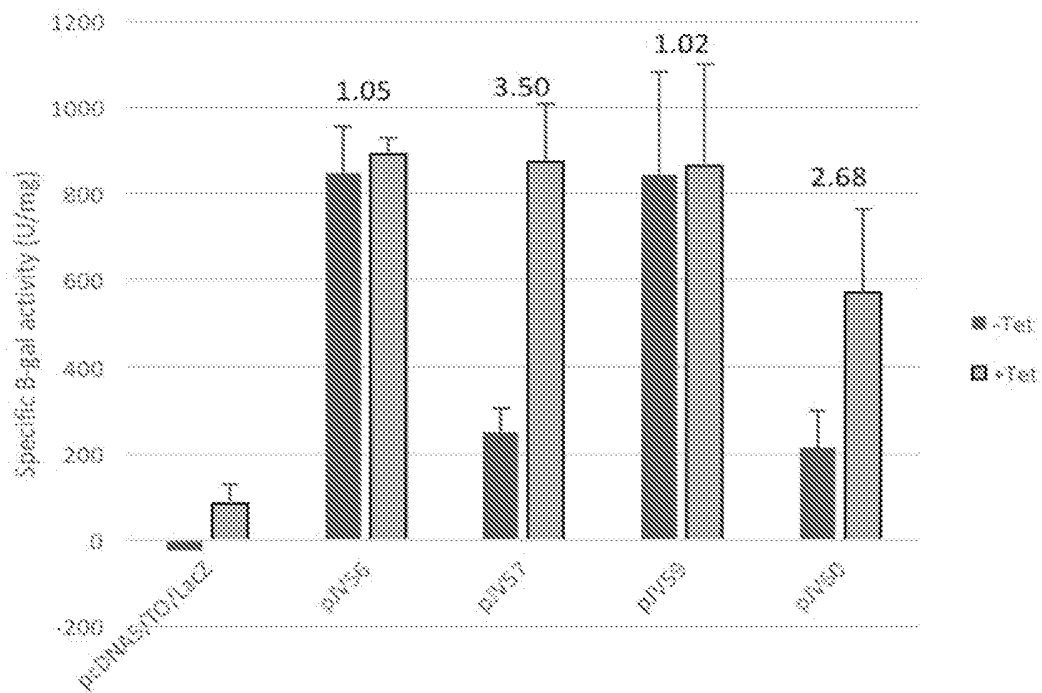


FIG. 13



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FIG. 14

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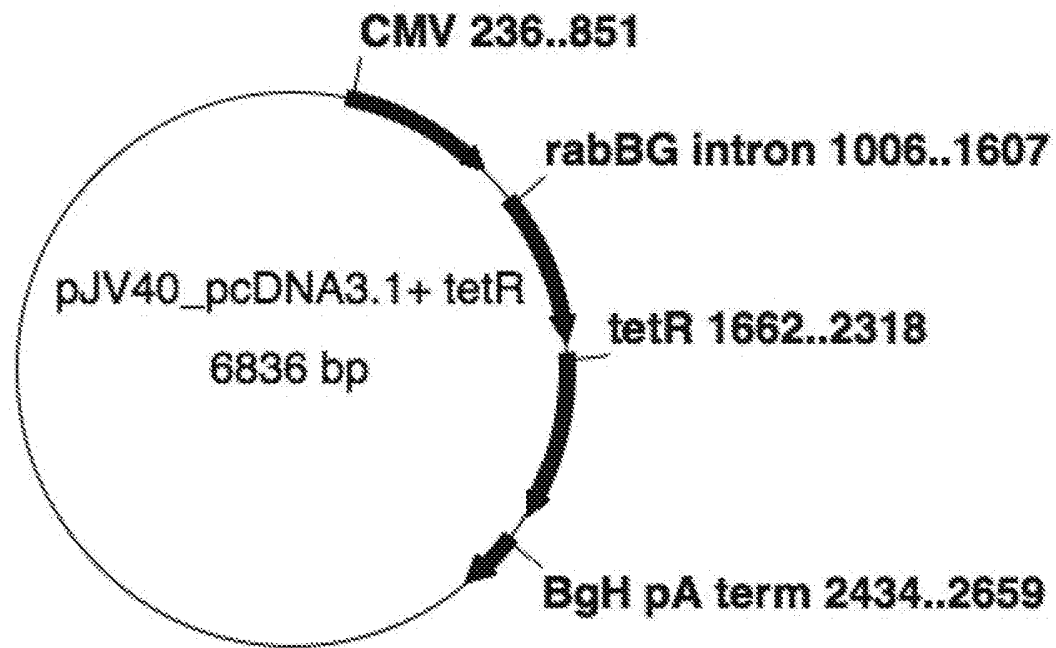
FIG. 15

FIG. 16

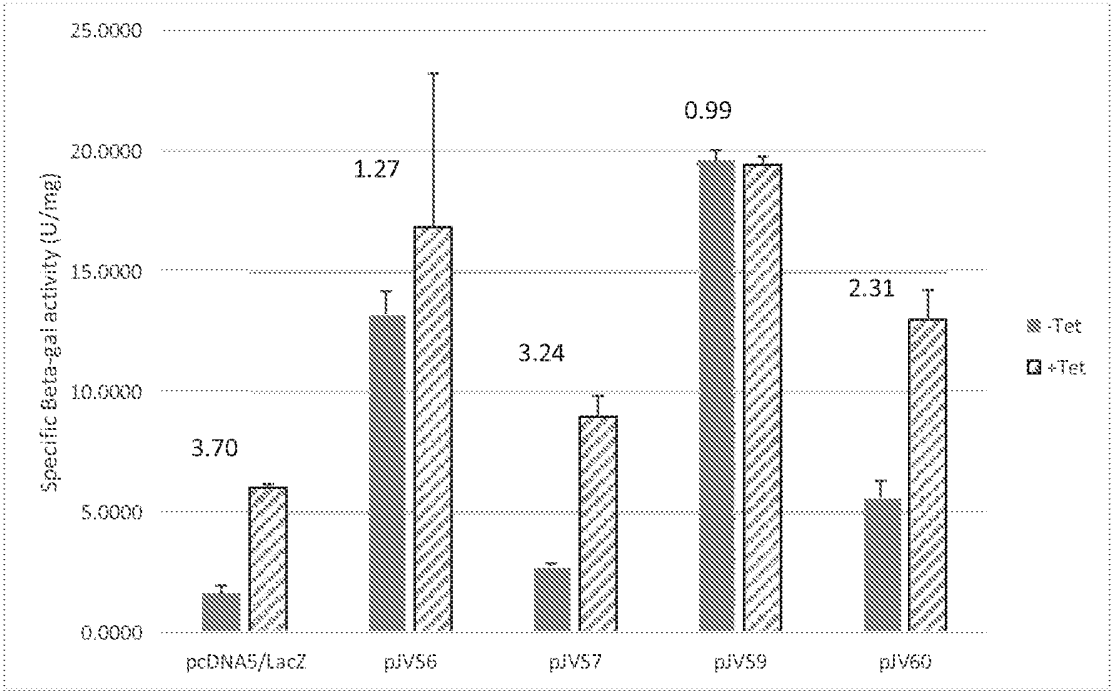


FIG. 17

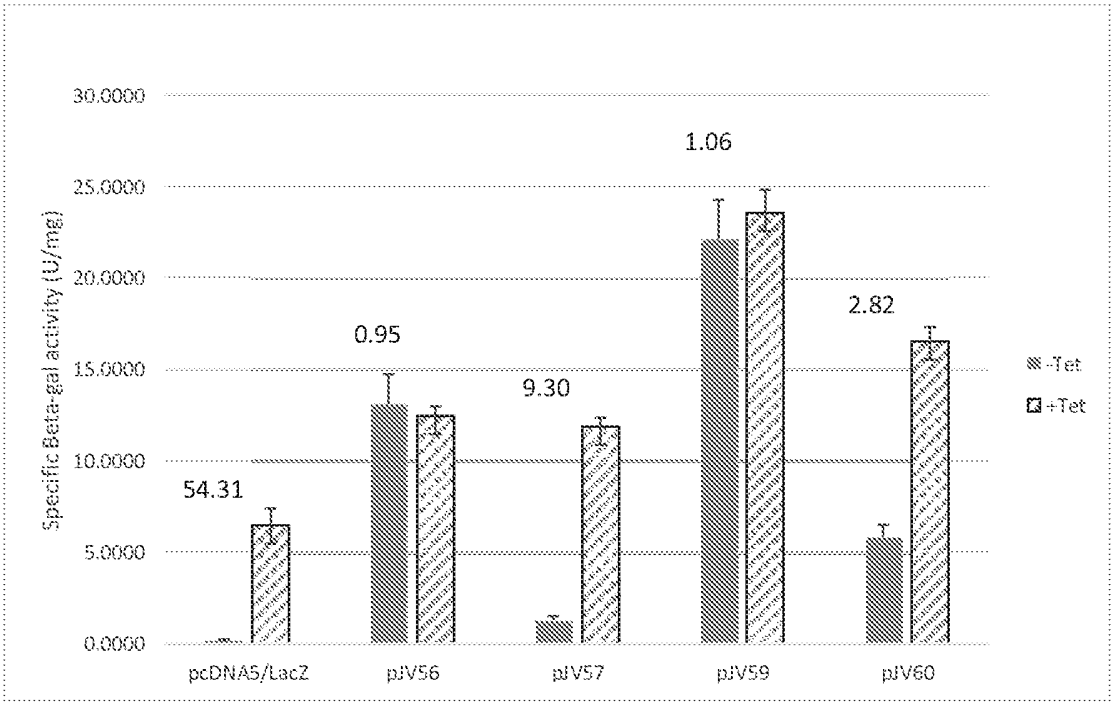
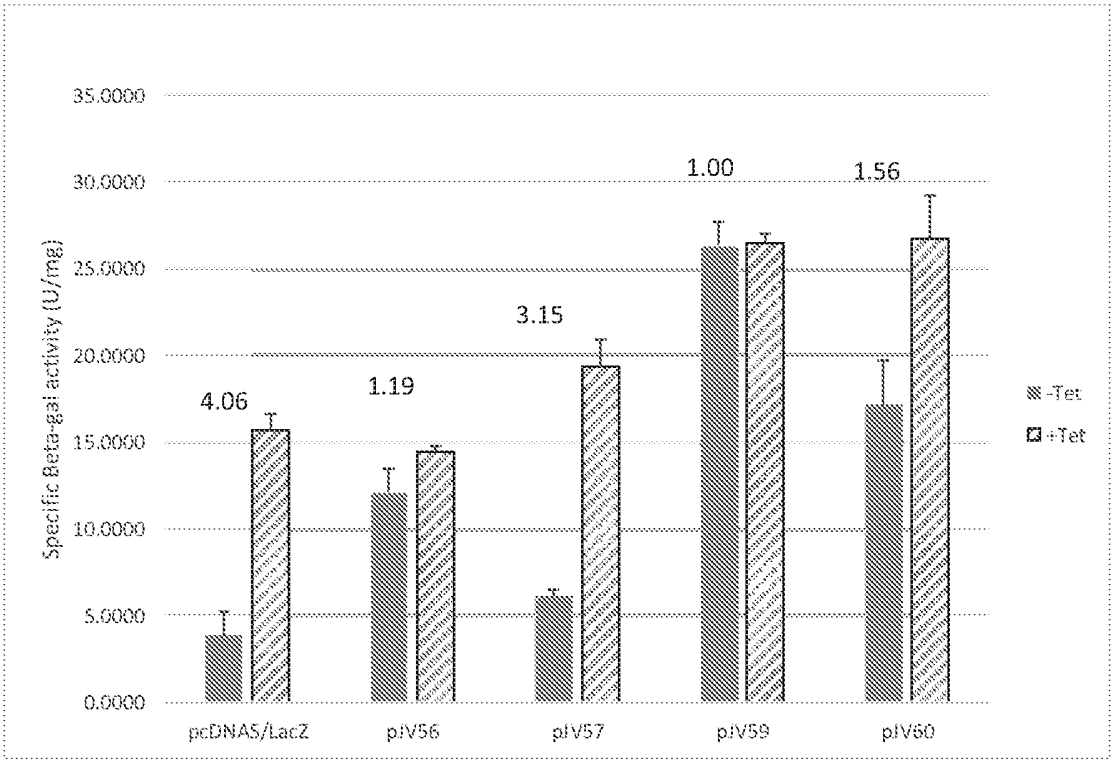


FIG. 18



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/015130

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/86
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/110864 A1 (ISIS INNOVATION [GB]; WYLLIE DAVID HAMILTON [GB]; COTTINGHAM MATTHEW G) 15 September 2011 (2011-09-15) the whole document -----	1-24
Y	SEOW THENG ONG ET AL: "Hybrid Cytomegalovirus Enhancer-H1 Promoter-Based Plasmid and Baculovirus Vectors Mediate Effective RNA Interference", HUMAN GENE THERAPY, vol. 16, no. 12, 1 December 2005 (2005-12-01), pages 1404-1412, XP55354977, US ISSN: 1043-0342, DOI: 10.1089/hum.2005.16.1404 the whole document ----- -/-	1-24



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

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

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

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

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

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Date of the actual completion of the international search

15 March 2017

Date of mailing of the international search report

05/04/2017

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Young, Craig

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/015130

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>JIANGUO SU ET AL: "Hybrid Cytomegalovirus-U6 Promoter-based Plasmid Vectors Improve Efficiency of RNA Interference in Zebrafish", MARINE BIOTECHNOLOGY, SPRINGER-VERLAG, NE, vol. 10, no. 5, 6 March 2008 (2008-03-06), pages 511-517, XP019595180, ISSN: 1436-2236 the whole document</p> <p>-----</p>	1-24
Y	<p>WANG C Y ET AL: "Improved neuronal transgene expression from an AAV-2 vector with a hybrid CMV enhancer/PDGF-.beta. promoter", JOURNAL OF GENE MEDICINE, JOHN WILEY & SONS, INC, US, vol. 7, no. 7, 1 July 2005 (2005-07-01), pages 945-955, XP008121243, ISSN: 1099-498X, DOI: 10.1002/JGM.742 [retrieved on 2005-03-09] the whole document</p> <p>-----</p>	1-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/015130

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2017/015130

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011110864	A1	15-09-2011	NONE
