REENGINEERING MRNA PRIMARY STRUCTURE FOR ENHANCED PROTEIN PRODUCTION

REFERENCE TO PRIORITY DOCUMENT

[0001] This application claims the benefit of priority under 35 U.S.C. §119(e) of *U.S. Provisional Application Serial No. 61/155,049, filed February 24, 2009*, entitled "Reengineering mRNA Primary Structure for Enhanced Protein Production." The subject matter of the above-noted application is incorporated by reference in its entirety by reference thereto.

BACKGROUND

[0002] Translation initiation in eukaryotes involves recruitment by mRNAs of the 40S ribosomal subunit and other components of the translation machinery at either the *5'* cap-structure or an internal ribosome entry site (IRES). Following its recruitment, the 40S subunit moves to an initiation codon. One widely held notion of translation initiation postulates that the 40S subunit moves from the site of recruitment to the initiation codon by scanning through the *5'* leader in a *5'* to 3' direction until the first AUG codon that resides in a good nucleotide context is encountered (Kozak "The Scanning Model for Translation: An Update" *J. Cell Biol.* 108:229-241 (1989)). More recently, it has been postulated that translation initiation does not involve scanning, but may involve tethering of ribosomal subunits at either the cap-structure or an IRES, or clustering of ribosomal subunits at internal sites (Chappell et al. "Ribosomal shunting mediated by a translational enhancer element that base pairs to 18S rRNA" *PNAS* USA 103(25):9488-9493 (2006); Chappell et al., "Ribosomal tethering and clustering as mechanisms for translation initiation" *PNAS* USA 103(48):18077-82 (2006)). The 40S subunit moves to an accessible AUG codon that is not necessarily the first AUG codon in the mRNA. Once the subunit reaches the initiation codon by whatever mechanism, the initiator Methionine-tRNA, which is associated with the subunit, base-pairs to the initiation codon, the large (60S) ribosomal subunit attaches, and peptide synthesis begins.

[0003] Inasmuch as translation is generally thought to initiate by a scanning mechanism, the effects on translation of AUG codons contained within *5'* leaders, termed upstream AUG codons, have been considered, and it is known that an AUG codon in the *5'* leader can have either a positive or a negative effect on protein synthesis depending on the gene, the nucleotide context, and cellular conditions. For example, an upstream AUG codon can inhibit translation initiation by diverting ribosomes from the authentic initiation codon. However, the notion that translation initiates by a scanning mechanism does not consider the effects of potential initiation codons in coding sequences on protein synthesis. In contrast, the tethering/clustering mechanisms of translation initiation suggests that putative initiation codons in coding sequences, which include both AUG codons and non-canonical codons, may be utilized, consequentially lowering the rate of protein synthesis by competing with the authentic initiation codon for ribosomes.

[0004] Micro RNA (miRNA)-mediated down-regulation can also negatively impact translation efficiency. miRNAs are generally between 21-23 nucleotides in length and are components of ribonucleoprotein complexes. It has been suggested that miRNAs can negatively impact protein levels by base-pairing to mRNAs and reducing mRNA stability, nascent peptide stability and translation efficiency (Eulalio *et al.* "Getting to the Root of miRNA-Mediated Gene Silencing" *Cell* 132:9-14 (1998)). Although miRNAs generally mediate their effects by base-pairing to binding sites in the 3' untranslated sequences (UTRs) of mRNAs, they have been shown to have similar repressive effects from binding sites contained within coding sequences and 5' leader sequences. Base-pairing occurs via the so-called "seed sequence," which includes nucleotides 2-8 of the miRNA. There may be more than 1,000 different miRNAs in humans.

[0005] The negative impact of putative initiation codons in mRNA coding sequences and miRNA-binding sites in mRNAs pose challenges to the pharmaceutical industry. For example, the industrial production of protein drugs, DNA vaccines for antigen production, general research purposes and for gene therapy applications are all affected by a sub-optimal rate of protein synthesis or sequence stability. Improving protein yields and higher protein concentration can minimize the costs associated with industrial scale cultures, reduce costs of producing drugs and can facilitate protein

purification. Poor protein expression limits the large-scale use of certain technologies, for example, problems in expressing enough antigen from a DNA vaccine to generate an immune response to conduct a phase 3 clinical trial.

SUMMARY

[0006] There is a need in the art for improving the efficiency and stability of protein translation and improving protein yield and concentration, for example, in the industrial production of protein drugs.

[0006a] In one aspect, there is provided a method of improving full-length protein expression efficiency comprising:

a) providing a polynucleotide comprising:

i) a coding sequence for the full-length protein;

ii) a primary initiation codon that is upstream of the coding sequence of the full-length protein, said primary initiation codon encoding the first amino acid of the coding sequence of the full-length protein; and

iii) one or more secondary initiation codons located within the coding sequence of the full-length protein downstream of the primary initiation codon; and

b) mutating the one or more secondary initiation codons, located within the coding sequence of the full-length protein downstream of the primary initiation codon, wherein the mutation results in a decrease in initiation of protein synthesis at the one or more secondary initiation codons resulting in a reduction of ribosomal diversion away from the primary initiation codon,

thereby increasing expression efficiency of the full-length protein initiated at the primary initiation codon,

wherein mutating the one or more secondary initiation codons located within the coding sequence of the full-length protein downstream of the primary initiation codon comprises mutating one or more nucleotides such that the amino acid sequence of the protein remains unaltered.

[0007] Disclosed is a method of improving full-length protein expression efficiency. The method includes providing a polynucleotide having a coding sequence for the protein; a primary initiation codon that is upstream of the coding sequence; and one or more secondary initiation codons located within the coding sequence. The method also includes mutating one or more secondary initiation codons resulting in a decrease in initiation of protein synthesis at the one or more secondary initiation codons resulting in a reduction of ribosomal diversion away from the primary initiation codon, thereby increasing full-length protein expression efficiency.

[0008] The method can also include mutating one or more nucleotides such that the amino acid sequence remains unaltered. The one or more secondary initiation codons can be in the same reading frame as the coding sequence or out-of-frame with the coding sequence. The one or more secondary initiation codons can be located one or more nucleotides upstream or downstream from a ribosomal recruitment site. The ribosomal recruitment site can include a cap or an IRES. The one or more secondary initiation codons can be selected from AUG, ACG, GUG, UUG, CUG, AUA, AUC, and AUU. The method can include mutating more than one secondary initiation codon within the coding sequence. The method can include mutating all the secondary initiation codons within the coding sequence. A flanking nucleotide can be mutated to a less favorable nucleotide context. The mutation of the one or more secondary initiation codons can avoid introducing new initiation codons. The mutation of the one or more secondary initiation codons can avoid introducing miRNA seed sequences. The mutation of the one or more secondary initiation codons can avoid altering usage bias of mutated codons. The generation of truncated proteins, polypeptide, or peptides other than the full-

length encoded protein can be reduced. Mutating one or more secondary initiation codons can avoid introducing miRNA seed sequences, splice donor or acceptor sites, or mRNA destabilization elements.

[0009] Also disclosed is a method of improving full-length protein expression efficiency. The method includes providing a polynucleotide sequence having a coding sequence for the protein and one or more miRNA binding sites located within the coding sequence; and mutating the one or more miRNA binding sites. The mutation results in a decrease in miRNA binding at the one or more miRNA binding sites resulting in a reduction of miRNA-mediated down regulation of protein translation, thereby increasing full-length protein expression efficiency.

[0010] The method can also include mutating one or more nucleotides such that the amino acid sequence remains unaltered. The method can include mutating one or more nucleotides in an miRNA seed sequence. The method can include mutating one or more nucleotides such that initiation codons are not introduced into the polynucleotide sequence. The method can include mutating one or more nucleotides such that rare codons are not introduced into the polynucleotide sequence. The method can include mutating one or more nucleotides such that additional miRNA seed sequences are not introduced into the polynucleotide sequence. The one or more miRNA binding sites can be located within the coding sequence. The one or more miRNA binding sites can be located within the 3' untranslated region. The one or more miRNA binding sites can be located within the 5' leader sequence.

[0011] A further understanding of the nature and advantages of the present disclosure may be realized by reference to the remaining portions of the specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A-1B show growth curves of *E. coli* DH5αcell cultures transformed with CAT (diamonds) or mCAT expression constructs (squares);

[0013] Figure 2 shows a Western blot analysis of lysates collected from *E. coli* DH5α cells transformed with CAT (C) or mCAT (mC) expression constructs;

[0014] Figure 3 shows a Western blot analysis of extracts from DG44 cells transformed with wild type CAT or modified CAT expression constructs;

[0015] Figure 4 shows a Western blot analysis of supernatants from DG44 cells transformed with the wild type CD5 (cd5-1) or modified CD5 signal peptide α-thyroglobulin light chain expression constructs (cd5-2 to cd5-5).

DETAILED DESCRIPTION

I. Overview

[0016] Described herein are methods to modify natural mRNAs or to engineer synthetic mRNAs to increase levels of the encoded protein. These rules describe modifications to mRNA coding and 3' UTR sequences intended to enhance protein synthesis by: 1) decreasing ribosomal diversion via AUG or non-canonical initiation codons in coding sequences, and/or 2) by evading miRNA-mediated down-regulation by eliminating miRNA binding sites in coding sequences.

[0017] Described are methods of reengineering mRNA primary structure that can be used to increase the yield of specific proteins in eukaryotic and bacterial cells. The methods described herein can be applied to the industrial production of protein drugs as well as for research purposes, gene therapy applications, and DNA vaccines for increasing antigen production. Greater protein yields minimize the costs associated with industrial scale cultures and reduce drug costs. In addition, higher protein concentrations can facilitate protein purification. Moreover, processes that may otherwise not be possible due to poor protein expression, e.g. in the conduct of phase 3 clinical trials, or in expressing enough antigen from a DNA vaccine to generate an immune response can be possible using the methods described herein.

II. Definitions

[0018] This specification is not limited to the particular methodology, protocols, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only,

and is not intended to limit the scope of the present methods which will be described by the appended claims.

[0019] As used herein, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells, reference to "a protein" includes one or more proteins and equivalents thereof known to those skilled in the art, and so forth.

[0020] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this disclosure pertains. The following references provide one of skill with a general definition of many of the terms used in this disclosure: *Academic Press Dictionary of Science and Technology*, Morris (Ed.), Academic Press (1st ed., 1992); *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith *et al.* (Eds.), Oxford University Press (revised ed., 2000); *Encyclopaedic Dictionary of Chemistry*, Kumar (Ed.), Anmol Publications Pvt. Ltd. (2002); *Dictionary of Microbiology and Molecular Biology*, Singleton *et al.* (Eds.), John Wiley & Sons (3rd ed., 2002); *Dictionary of Chemistry*, Hunt (Ed.), Routledge (1st ed., 1999); *Dictionary of Pharmaceutical Medicine*, Nahler (Ed.), Springer-Verlag Telos (1994); *Dictionary of Organic Chemistry*, Kumar and Anandand (Eds.), Anmol Publications Pvt. Ltd. (2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). Further clarifications of some of these terms as they apply specifically to this disclosure are provided herein.

[0021] The term "agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, *e.g.*, protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" are used interchangeably herein.

[0022] The term "cistron" means a unit of DNA that encodes a single polypeptide or protein. The term "transcriptional unit" refers to the segment of DNA within which the synthesis of RNA occurs.

[0023] The term "DNA vaccines" refers to a DNA that can be introduced into a host cell or a tissue and therein expressed by cells to produce a messenger ribonucleic acid (mRNA) molecule, which is then translated to produce a vaccine antigen encoded by the DNA.

[0024] The language "gene of interest" is intended to include a cistron, an open reading frame (ORF), or a polynucleotide sequence which codes for a protein product (protein of interest) whose production is to be modulated. Examples of genes of interest include genes encoding therapeutic proteins, nutritional proteins and industrial useful proteins. Genes of interest can also include reporter genes or selectable marker genes such as enhanced green fluorescent protein (EGFP), luciferase genes (*Renilla* or *Photinus*).

[0025] Expression is the process by which a polypeptide is produced from DNA. The process involves the transcription of the gene into mRNA and the subsequent translation of the mRNA into a polypeptide.

[0026] The term "endogenous" as used herein refers to a gene normally found in the wild-type host, while the term "exogenous" refers to a gene not normally found in the wild-type host.

[0027] A "host cell" refers to a living cell into which a heterologous polynucleotide sequence is to be or has been introduced. The living cell includes both a cultured cell and a cell within a living organism. Means for introducing the heterologous polynucleotide sequence into the cell are well known, *e.g.*, transfection, electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like. Often, the heterologous polynucleotide sequence to be introduced into the cell is a replicable expression vector or cloning vector. In some embodiments, host cells can be engineered to incorporate a desired gene on its chromosome or in its genome. Many host cells that can be employed in the practice of the present methods (*e.g.*, CHO cells) serve as hosts are well known in the art. See, *e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press (3rd ed., 2001); and Brent *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (Ringbou ed., 2003). In some embodiments, the host cell is a eukaryotic cell.

[0028] The term "inducing agent" is used to refer to a chemical, biological or physical agent that effects translation from an inducible translational regulatory element. In response to exposure to an inducing agent, translation from the element generally is initiated *de novo* or is increased above a basal or constitutive level of expression. An inducing agent can be, for example, a stress condition to which a cell is exposed, for example, a heat or cold shock, a toxic agent such as a heavy metal ion, or a lack of a nutrient, hormone, growth factor, or the like; or can be a compound that affects the growth or differentiation state of a cell such as a hormone or a growth factor.

[0029] The phrase "isolated or purified polynucleotide" is intended to include a piece of polynucleotide sequence (*e.g.*, DNA) which has been isolated at both ends from the sequences with which it is immediately contiguous in the naturally occurring genome of the organism. The purified polynucleotide can be an oligonucleotide which is either double or single stranded; a polynucleotide fragment *incorporated into a vector; a fragment inserted into the genome of a eukaryotic or* prokaryotic organism; or a fragment used as a probe. The phrase "substantially pure," when referring to a polynucleotide, means that the molecule has been separated from other accompanying biological components so that, typically, it has at least 85 percent of a sample or greater percentage.

[0030] The term "nucleotide sequence," "nucleic acid sequence," "nucleic acid," or "polynucleotide sequence," refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogs of natural nucleotides that hybridize to nucleic acids in a manner similar to naturally-occurring nucleotides. Nucleic acid sequences can be, *e.g.*, prokaryotic sequences, eukaryotic mRNA sequences, cDNA sequences from eukaryotic mRNA, genomic DNA sequences from eukaryotic DNA (*e.g.*, mammalian DNA), and synthetic DNA or RNA sequences, but are not limited thereto.

[0031] The term "promoter" means a nucleic acid sequence capable of directing transcription and at which transcription is initiated. A variety of promoter sequences are known in the art. For example, such elements can include, but are not limited to, TATA-boxes, CCAAT-boxes, bacteriophage RNA polymerase specific

promoters (*e.g.*, T7, SP6, and T3 promoters), an SP1 site, and a cyclic AMP response element. If the promoter is of the inducible type, then its activity increases in response to an inducing agent.

[0032] The five prime leader or untranslated region (5' leader, 5' leader sequence or 5' UTR) is a particular section of messenger RNA (mRNA) and the DNA that codes for it. It starts at the +1 position (where transcription begins) and ends just before the start codon (typically AUG) of the coding region. In bacteria, it may contain a ribosome binding site (RBS) known as the Shine-Delgarno sequence. 5' leader sequences range in length from no nucleotides (in rare leaderless messages) up to >1,000-nucleotides. 3' UTRs tend to be even longer (up to several kilobases in length).

[0033] The term "operably linked" or "operably associated" refers to functional linkage between genetic elements that are joined in a manner that enables them to carry out their normal functions. For example, a gene is operably linked to a promoter when its transcription is under the control of the promoter and the transcript produced is correctly translated into the protein normally encoded by the gene. Similarly, a translational enhancer element is operably associated with a gene of interest if it allows up-regulated translation of a mRNA transcribed from the gene.

[0034] A sequence of nucleotides adapted for directional ligation, *e.g.*, a polylinker, is a region of an expression vector that provides a site or means for directional ligation of a polynucleotide sequence into the vector. Typically, a directional polylinker is a sequence of nucleotides that defines two or more restriction endonuclease recognition sequences, or restriction sites. Upon restriction cleavage, the two sites yield cohesive termini to which a polynucleotide sequence can be ligated to the expression vector. In an embodiment, the two restriction sites provide, upon restriction cleavage, cohesive termini that are non-complementary and thereby permit directional insertion of a polynucleotide sequence into the cassette. For example, the sequence of nucleotides adapted for directional ligation can contain a sequence of nucleotides that defines multiple directional cloning means. Where the sequence of nucleotides adapted for directional ligation defines numerous restriction sites, it is referred to as a multiple cloning site.

[0035] The term "subject" for purposes of treatment refers to any animal classified as a mammal, *e.g.*, human and non-human mammals. Examples of non-human animals include dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, and etc. Except when noted, the terms "patient" or "subject" are used herein interchangeably. In an embodiment, the subject is human.

[0036] Transcription factor refers to any polypeptide that is required to initiate or regulate transcription. For example, such factors include, but are not limited to, c-Myc, c-Fos, c-Jun, CREB, cEts, GATA, GAL4, GAL4/Vp16, c-Myb, MyoD, NF-κB, bacteriophage-specific RNA polymerases, Hif-1, and TRE. Example of sequences encoding such factors include, but are not limited to, GenBank accession numbers K02276 (c-Myc), K00650 (c-fos), BC002981 (c-jun), M27691 (CREB), X14798 (cEts), M77810 (GATA), K01486 (GAL4), AY136632 (GAL4/Vp16), M95584 (c-Myb), M84918 (MyoD), 2006293A (NF-κB), NP 853568 (SP6 RNA polymerase), AAB28111 (T7 RNA polymerase), NP 523301 (T3 RNA polymerase), AF364604 (HIF-1), and X63547 (TRE).

[0037] A "substantially identical" nucleic acid or amino acid sequence refers to a nucleic acid or amino acid sequence which includes a sequence that has at least 90% sequence identity to a reference sequence as measured by one of the well known programs described herein (*e.g.*, BLAST) using standard parameters. The sequence identity can be at least 95%, at least 98%, and at least 99%. In some embodiments, the subject sequence is of about the same length as compared to the reference sequence, *i.e.*, consisting of about the same number of contiguous amino acid residues (for polypeptide sequences) or nucleotide residues (for polynucleotide sequences).

[0038] Sequence identity can be readily determined with various methods known in the art. For example, the BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)). Percentage of sequence identity is determined by comparing two optimally aligned sequences over a

comparison window, wherein the portion of the polynucleotide sequence in the comparison window may include additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which does not include additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0039] The term "treating" or "alleviating" includes the administration of compounds or agents to a subject to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease (*e.g.*, a cardiac dysfunction), alleviating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder. Subjects in need of treatment include patients already suffering from the disease or disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

[0040] Treatment may be prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease. In the treatment of cardiac remodeling and/or heart failure, a therapeutic agent may directly decrease the pathology of the disease, or render the disease more susceptible to treatment by other therapeutic agents.

[0041] The term "vector" or "construct" refers to polynucleotide sequence elements arranged in a definite pattern of organization such that the expression of genes/gene products that are operably linked to these elements can be predictably controlled. Typically, they are transmissible polynucleotide sequences (*e.g.*, plasmid or virus) into which a segment of foreign DNA can be spliced in order to introduce the foreign DNA into host cells to promote its replication and/or transcription.

[0042] A cloning vector is a DNA sequence (typically a plasmid or phage) which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites. A foreign DNA fragment

may be spliced into the vector at these sites in order to bring about the replication and cloning of the fragment. The vector may contain one or more markers suitable for use in the identification of transformed cells. For example, markers may provide tetracycline or ampicillin resistance.

[0043] An expression vector is similar to a cloning vector but is capable of inducing the expression of the DNA that has been cloned into it, after transformation into a host. The cloned DNA is usually placed under the control of (*i.e.*, operably linked to) certain regulatory sequences such as promoters or enhancers. Promoter sequences may be constitutive, inducible or repressible.

[0044] An "initiation codon" or "initiation triplet" is the position within a cistron where protein synthesis starts. It is generally located at the 5' end of the coding sequence. In eukaryotic mRNAs, the initiation codon typically consists of the three nucleotides (the Adenine, Uracil, and Guanine (AUG) nucleotides) which encode the amino acid Methionine (Met). In bacteria, the initiation codon is also typically AUG, but this codon encodes a modified Methionine (N-Formylmethionine (fMet)). Nucleotide triplets other than AUG are sometimes used as initiation codons, both in eukaryotes and in bacteria.

[0045] A "downstream initiation codon" refers to an initiation codon that is located downstream of the authentic initiation codon, typically in the coding region of the gene. An "upstream initiation codon" refers to an initiation codon that is located upstream of the authentic initiation codon in the 5' leader region.

[0046] As used herein, reference to "downstream" and "upstream" refers to a location with respect to the authentic initiation codon. For example, an upstream codon on an mRNA sequence is a codon that is towards the 5'-end of the mRNA sequence relative to another location within the sequence (such as the authentic initiation codon) and a downstream codon refers to a codon that is towards the 3'-end of the mRNA sequence relative to anther location within the sequence.

[0047] As used herein, "authentic initiation codon" or "primary initiation codon" refers to the initiation codon of a cistron that encodes the first amino acid of the coding sequence of the encoded protein of interest whose production is to be modulated.

A "secondary initiation codon" refers to an initiation codon that is other than the primary or authentic initiation codon for the encoded protein of interest. The secondary initiation codon is generally downstream of the primary or authentic initiation codon and located within the coding sequence.

[0048] As used herein, "increased protein expression" refers to translation of a modified mRNA where one or more secondary initiation codons are mutated that generates polypeptide concentration that is at least about 5%, 10%, 20%, 30%, 40%, 50% or greater over the polypeptide concentration obtained from the wild type mRNA where the one or more secondary initiation codons have not been mutated. Increased protein expression can also refer to protein expression of a mutated mRNA that is 1.5-fold, 2-fold, 3-fold, 5-fold, 10-fold or more over the wild type mRNA.

[0049] As used herein, "ribosomal recruitment site" refers to a site within an mRNA to which a ribosome subunit associates prior to initiation of translation of the encoded protein. Ribosomal recruitment sites can include the cap structure, a modified nucleotide (m^7G cap-structure) found at the 5' ends of mRNAs, and sequences termed internal ribosome entry sites (IRES), which are contained within mRNAs. Other ribosomal recruitment sites can include a 9-nucleotide sequence from the *Gtx* homeodomain mRNA. The ribosomal recruitment site is often upstream of the authentic initiation codon, but can also be downstream of the authentic initiation codon.

[0050] As used herein, "usage bias" refers to the particular preference an organism shows for one of the several codons that encode the same amino acid. Altering usage bias refers to mutations that lead to use of a different codon for the same amino acid with a higher or lower preference than the original codon.

[0051] As used herein, "full-length protein" refers to a protein which encompasses essentially every amino acid encoded by the gene encoding the protein. Those of skill in the art know there are subtle modifications of some proteins in living cells so that the protein is actually a group of closely related proteins with slight alterations. For example, some but not all proteins a) have amino acids removed from the amino-terminus, and/or b) have chemical groups added which could increase molecular weight. Most bacterial proteins as encoded contain a methionine and an alanine residue at

the amino-terminus of the protein; one or both of these residues are frequently removed from active forms of the protein in the bacterial cell. These types of modifications are typically heterogenous so not all modifications happen to every molecule. Thus, the natural "full-length" molecule is actually a family of molecules that start from the same amino acid sequence but have small differences in how they are modified. The term "full-length protein" encompasses such a family of molecules.

[0052] As used herein, "rescued" or "modified" refer to nucleotide alterations that remove most to all secondary initiation codons from the coding region. "Partially modified" refers to nucleotide alterations that remove a subset of all possible mutations of secondary initiation codons from the coding region.

III. Reduction of Ribosomal diversion via downstream initiation codons

[0053] As mentioned above, it is well-known that features contained within *5'* leaders can affect translation efficiency. For example, an AUG codon in the *5'* leader, termed an upstream AUG codon, can have either a positive or a negative effect on protein synthesis depending on the gene, the nucleotide context, and cellular conditions. An upstream AUG codon can inhibit translation initiation by diverting ribosomes from the authentic initiation codon (Meijer *et al.*, "Translational Control of the *Xenopus* laevis Connexin-41 5'-Untranslated Region by Three Upstream Open Reading Frames" *J. Biol. Chem.* 275(40):30787-30793 (2000)). For example, Figures 6 and 8 in Meijer et al. show the ribosomal diversion effect of upstream AUG codon in the 5' leader sequence.

[0054] Although AUG/ATG is the usual translation initiation codon in many species, it is known that translation can sometimes also initiate at other upstream codons, including ACG, GUG/GTG, UUG/TTG, CUG/CTG, AUA/ATA, AUC/ATC, and AUU/ATT *in vivo*. For example, it has been shown that mammalian ribosomes can initiate translation at a non-AUG triplet when the initiation codon of mouse dihydrofolate reductase (*dhfr*) was mutated to ACG (Peabody, D.S. (1987) *J. Biol. Chem.* 262, 11847-11851). A further study by Peabody showed that mutant initiation codons AUG of *dhfr* (GUG, UUG, CUG, AUA, AUC and AUU) all were able to direct the synthesis of apparently normal *dhfr* (Peabody, D. S. (1989) *J. Biol. Chem.* 264, 5031-5035).

[0055] The tethering and clustering models of translation initiation postulate that translation can initiate at an accessible initiation codon and studies have shown that an initiation codon can be used in a distance-dependent manner downstream of the ribosomal recruitment site (cap or IRES) (Chappell *et al.* "Ribosomal tethering and clustering as mechanisms for translation initiation" *PNAS* USA 103(48):18077-82 2006). This suggests that putative initiation codons in coding sequences may also be utilized. Translation initiation at downstream initiation codons, or secondary initiation sites, can compete with the authentic initiation codon, or primary initiation site, for ribosomes and lower the expression of the encoded protein. Decreasing the availability of these secondary initiation sites, such as by mutating them into a non-initiation codon, increases the availability of the primary initiation sites to the ribosome and a more efficient encoded protein expression.

[0056] The present method allows for improved and more efficient protein expression and reduces the competition between various initiation codons for the translation machinery. By eliminating downstream initiation codons in *coding sequences* that are in the same reading frame as the encoded protein, the generation of truncated proteins, with potential altered function, will be eliminated. In addition, by eliminating downstream initiation codons that are out-of-frame with the coding sequence, the generation of various peptides, some of which may have negative effects on cell physiology or protein production, will also be eliminated. This advantage can be particularly important for applications in DNA vaccines or gene therapy.

[0057] Direct mutation of downstream initiation codons can take place such that the encoded amino acid sequence remains unaltered. This is possible in many cases because the genetic code is degenerate and most amino acids are encoded by two or more codons. The only exceptions are Methionine and Tryptophan, which are only encoded by one codon, AUG, and UGG, respectively. Mutation of a downstream initiation codon that also alters the amino acid sequence can also be considered. In such cases, the effects of altering the amino acid sequence can be evaluated. Alternatively, if the amino acid sequence is to remain unaltered, the nucleotides flanking the putative initiation codon can sometimes be mutated to diminish the efficiency of the initiation codon. For AUG codons, this can be done according to the nucleotide context rules established by Marilyn

Kozak (Kozak, M. (1984) *Nature* 308, 241-246), which state that an AUG in excellent context contains a purine at position -3 and a G at +4, where AUG is numbered +1, +2, +3.

[0058] For non-AUG codons, similar rules seem to apply with additional determinants from nucleotides at positions +5 and +6. In designing mutations, the codon usage bias can, in many cases, remain relatively unaltered, *e.g.* by introducing mutated codons with similar codon bias as the wild type codon. Inasmuch as different organisms have different codon usage frequencies, the specific mutations for expression in cells from different organisms will vary accordingly.

[0059] It should be appreciated that the methods disclosed herein are not limited to eukaryotic cells, but also apply to bacteria. Although bacterial translation initiation is thought to differ from eukaryotes, ribosomal recruitment still occurs via *cis*-elements in mRNAs, which include the so-called Shine-Delgarno sequence. Non-AUG initiation codons in bacteria include ACG, GUG, UUG, CUG, AUA, AUC, and AUU.

[0060] In an embodiment, disclosed are modifications to coding sequences that enhance protein synthesis by decreasing ribosomal diversion via downstream initiation codons. These codons can include AUG/ATG and other nucleotide triplet codons known to function as initiation codons in cells, including but not limited to ACG, GUG/GTG, UUG/TTG, CUG/CTG, AUA/ATA, AUC/ATC, and AUU/ATT. In one embodiment the downstream initiation codon is mutated. Reengineering of mRNA coding sequences to increase protein production can involve mutating all downstream initiation codons or can involve mutating just some of the downstream initiation codons. In another embodiment, the flanking nucleotides are mutated to a less favorable nucleotide context. In an embodiment, ATG codons in the signal peptide can be mutated to ATC codons resulting in a Methionine to Isoleucine substitution. In another embodiment, CTG codons in the signal peptide can be mutated to CTC. In another embodiment, ATG codons can be mutated to ATC codons resulting in a Methionine (M) to Isoleucine (I) amino acid substitution, and CTG codons can be mutated to CTCs. In another embodiment, ATG codons can be mutated to ATC codons, CTG codons can be mutated to CTC codons, and the context of initiator AUG can be improved by changing

the codon 3' of the initiator from CCC to GCT resulting in a Proline (P) to Argenine (R) amino acid substitution. In other embodiments, modifications can be made to the signal peptide in which one or more AUG and CUG codons can be removed. Modifications can be made including a modified signal peptide by removal of most of the potential initiation codons, removal of ATG and CTGs of the signal peptide, removal of ATG, CTG and ACG codons resulting in a Glutamic acid (E) to Glutamine (Q) amino acid substitution or a Histidine (H) to Argenine (R) amino acid substitution.

[0061] Standard techniques in molecular biology can be used to generate the mutated nucleic acid sequences. Such techniques include various nucleic acid manipulation techniques, nucleic acid transfer protocols, nucleic acid amplification protocols and other molecular biology techniques known in the art. For example, point mutations can be introduced into a gene of interest through the use of oligonucleotide mediated site-directed mutagenesis. Modified sequences also can be generated synthetically by using oligonucleotides synthesized with the desired mutations. These approaches can be used to introduce mutations at one site or throughout the coding region. Alternatively, homologous recombination can be used to introduce a mutation or exogenous sequence into a target sequence of interest. Nucleic acid transfer protocols include calcium chloride transformation/transfection, electroporation, liposome mediated nucleic acid transfer, N-[1-(2,3-Dioloyloxy)propyl]-N,N,N-trimethylammonium methylsulfate meditated transformation, and others. In an alternative mutagenesis protocol, point mutations in a particular gene can also be selected for using a positive selection pressure. See, e.g., Current Techniques in Molecular Biology, (Ed. Ausubel, et al.). Nucleic acid amplification protocols include but are not limited to the polymerase chain reaction (PCR). Use of nucleic acid tools such as plasmids, vectors, promoters and other regulating sequences, are well known in the art for a large variety of viruses and cellular organisms. Further a large variety of nucleic acid tools are available from many different sources including ATCC, and various commercial sources. One skilled in the art will be readily able to select the appropriate tools and methods for genetic modifications of any particular virus or cellular organism according to the knowledge in the art and design choice. Protein expression can be measured also using various standard methods.

These include, but are not limited to, Western blot analysis, ELISA, metabolic labeling, and enzymatic activity measurements.

IV. Evasion of miRNA-mediated down-regulation

[0062] MicroRNAs are an abundant class of small noncoding RNAs that generally function as negative gene regulators. In an embodiment, modifications can be made to mRNA sequences, including 5' leader, coding sequence, and 3' UTR, to evade miRNA-mediated down-regulation. Such modification can thereby alter mRNA or nascent peptide stability, and enhance protein synthesis and translation efficiency.

[0063] MiRNAs can be generally between 21-23 nucleotide RNAs that are components of ribonucleoprotein complexes. miRNAs can affect mRNA stability or protein synthesis by base-pairing to mRNAs. miRNAs generally mediate their effects by base-pairing to binding sites in the 3' UTRs of mRNAs. However, they have been shown to have similar repressive effects from binding sites contained within coding sequences and 5' leader sequences. Base-pairing occurs via the so-called "seed sequence," which consists of nucleotides 2-8 of the miRNA. There may be more than 1,000 different miRNAs in humans.

[0064] Reengineering mRNAs to circumvent miRNA-mediated repression can involve mutating all seed sequences within an mRNA. As with the initiation codon mutations described above, these mutations can ensure that the encoded amino acid sequence remains unaltered, and act not to introduce initiation codons, rare codons, or other miRNA seed sequences.

[0065] A computer program can be used to reengineer mRNA sequences according to a cell type of interest, *e.g.* rodent cells for expression in Chinese hamster ovary cells, or human cells for expression in human cell lines or for application in DNA vaccines. This program can recode an mRNA to eliminate potential initiation codons except for the initiation codon. In the case of in-frame AUG codons in the coding sequence, the context of these downstream initiation codons can be weakened if possible. Mutations can be performed according to the codon bias for the cell line of interest, e.g. human codon bias information can be used for human cell lines, *Saccharomyces*

cerevisiae codon bias information can be used for this yeast, and *E.coli* codon bias information can be used for this bacteria. In higher eukaryotic mRNAs, the recoded mRNA can then be searched for all known seed sequences in the organism of interest, e.g. human seed sequences for human cell lines. Seed sequences can be mutated with the following considerations: 1) without disrupting the amino acid sequence, 2) without dramatically altering the usage bias of mutated codons, 3) without introducing new putative initiation codons.

[0066] While this specification contains many specifics and described with references to preferred embodiments thereof, these should not be construed as limitations on the scope of a method that is claimed or of what may be claimed, but rather as descriptions of features specific to particular embodiments. It will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the meaning of the subject matter described. Certain features that are described in this specification in the context of separate embodiments can also be implemented in combination in a single embodiment. Conversely, various features that are described in the context of a single embodiment can also be implemented in multiple embodiments separately or in any suitable sub-combination. Moreover, although features may be described above as acting in certain combinations and even initially claimed as such, one or more features from a claimed combination can in some cases be excised from the combination, and the claimed combination may be directed to a sub-combination or a variation of a sub-combination. The scope of the subject matter is defined by the claims that follow.

[0067] All publications, databases, GenBank sequences, patents, and patent applications cited in this specification are herein incorporated by reference as if each was specifically and individually indicated to be incorporated by reference.

EXAMPLES

[0068] The following examples are provided as further illustration, but not to limit the scope. Other variants will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims.

and mCAT expression constructs and plated onto LB/ampicillin plates. Cultures were obtained from single colonies and cultured in LB/ampicillin (~50μg/ml) at 37°C with shaking at 220 rpm until logarithmic growth was reached as determined by measuring the A_{600} of the culture. The cultures were then diluted with LB/ampicillin to comparable A_{600}'s. The A_{600} of the culture derived from DH5α cells transformed with the CAT expression construct was 0.3, while that from the cells transformed with the mCAT expression construct was 0.25. Chloramphenicol acetyltransferase expression was induced via the *lac* operon contained within the CAT and mCAT plasmids by the introduction of Isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration of 0.4mM). Three milliliters of each culture was transferred to a fresh tube containing chloramphenicol resulting in a final concentration of 20, 40, 80, 160, 320, 640, 1280, and 2560μg/ml. Cultures were incubated at 37°C with shaking at 220 rpm and the A_{600} of each culture measured at 1 hour intervals.

[0073] Figures 1A-1B show growth curves of cultures of DH5α cells transformed with CAT (diamonds) and mCAT (squares) expression constructs. Chloramphenicol acetyltransferase expression was induced by the addition of IPTG, (0.4mM final concentration) 3 milliliters of IPTG containing culture was added to fresh tubes containing Chloramphenicol resulting in final concentrations of 0, 40, 80, 160, 320, 640, 1280, and 2560μg/ml. Cultures were incubated at 37°C with shaking at 220 rpm and the A_{600} of each culture measured over time. The results for cultures grown in the presence of 320 and 640μg/ml Chloramphenicol are shown. The X-axis represents time in hours, the Y-axis represents normalized A_{600} (relative to starting A_{600}).

[0074] The results showed that bacteria transformed with the mCAT expression construct grew better than the bacteria transformed with the CAT expression construct at all concentrations. As shown in Figures 1A-1B, in high concentrations of Chloramphenicol (320 and 640 μg/ml), cells with the modified CAT still grew, but cells with the wild type CAT did not. These results indicate that more functional Chloramphenicol acetyltransferase enzyme was expressed from the mCAT construct thus allowing the bacteria transformed with this expression construct to grow better in the presence of this antibiotic.

[0075] To determine the relative amounts of Chloramphenicol acetyltransferase enzyme synthesized from DH5α cells transformed with the CAT and mCAT expression constructs, Western blot analysis was performed on cell extracts at 5, 30, 60 and 90 minutes after induction by IPTG. 50µl of culture at each time point was centrifuged, and bacterial pellets resuspended in 30µl of TE buffer and 10µl of a 4 x SDS gel loading buffer. The sample was heated at 95°C for 3 minutes and loaded onto a 10% Bis-Tris/SDS polyacylamide gel. Proteins were transferred to a PVDF membrane and probed with an anti-CAT antibody. Figure 2 is a Western blot analysis of lysates from DH5α cells transformed with the CAT (C) and mCAT (mCAT) expression constructs at various times after IPTG induction. The results showed that the amount of Chloramphenicol acetyltransferase protein (above the 19kDa marker) is substantially increased in DH5α cells transformed with the mCAT expression construct (mC) at all time points tested.

[0076] Analysis of the Chloramphenicol acetyltransferase ORF was also performed in mammalian cells. The CAT ORF and the partially modified CAT ORF were cloned into mammalian expression constructs containing a CMV promoter and tested by transient transfection into Chinese Hamster Ovary (DG44) cells. In brief, 0.5µg of each expression construct along with 20ng of a co-transfection control plasmid that expresses the β-galactosidase reporter protein (pCMVβ,.Clontech) was transfected into 100,000 DG44 cells using the Fugene 6 (Roche) transfection reagent according to the manufacturer's instructions. Twenty-four hours post transfection, cells were lysed using 250µl of lysis buffer. Lac Z reporter assay was performed to ensure equal transfection efficiencies between samples. 30µl of lysate was added to 10µl of a 4 x SDS gel loading buffer. The sample was heated at 72°C for 10 minutes and loaded onto a 10% Bis-Tris/SDS polyacylamide gel. Proteins were transferred to a PVDF membrane and probed with an α-CAT antibody.

[0077] Figure 3 shows a Western blot analysis of extracts from the DG44 cells transformed with wild type (CAT) and modified CAT expression constructs. Cell extracts were fractionated on 10% Bis-Tris gels in 1 x MOPS/SDS, transferred to PVDF

membrane and probed with an anti-CAT antibody. Experiments were performed in triplicate with extracts from cells in which transfection efficiency was the same.

[0078] Comparisons were made between three transfections with the wild type (CAT) and three with the modified CAT. The amount of CAT protein (above the 19kDa marker) is substantially increased in cells transfected with the modified construct. The results showed that the amount of CAT protein (above the 19kDa marker) is substantially increased in DG44 cells transfected with the mCAT construct. Modification of the CAT ORF by eliminating multiple translation initiation sites within the resulting mRNA transcripts demonstrated that this technology may be of practical use in numerous organisms besides just mammalian and bacterial cells.

Secreted Proteins

[0079] The usefulness of this technology was also investigated with secreted proteins. Mammalian expression constructs were generated for a signal peptide that is encoded within the Homo sapiens CD5 molecule (CD5), mRNA. Mammalian expression constructs were generated in which transcription was driven by a CMV promoter and where the cd5 signal peptide was placed at the 5' end of the ORF that encodes a light chain of an antibody against the thyroglobulin protein (cd5-1, SEQ ID NO: 123). The CD5 signal peptide sequence contains 7 potential initiation codons including 3 ATG, 1 TTG and 3 CTG codons. A series of expression constructs was generated. In one variation, ATG codons in the cd5 signal peptide were changed to ATC codons resulting in a Methionine to Isoleucine substitution (cd5-2, SEQ ID NO: 124). In another variation, CTG codons in the cd5 signal peptide were changed to CTC (cd5-3, SEQ ID NO: 125). In another variation, ATG codons were mutated to ATC codons resulting in a Methionine (M) to Isoleucine (I) amino acid substitution, and CTG codons were changed to CTCs (cd5-4, SEQ ID NO: 126). In another variation, ATG codons were changed to ATC codons resulting in a Methionine (M) to Isoleucine (I) amino acid substitution, CTG codons were changed to CTC codons, and the context of initiator AUG was improved by changing the codon 3' of it from CCC to GCT resulting in a Proline (P) to Argenine (R) amino acid substitution (cd5-5, SEQ ID NO: 127).

[0080] These constructs were then tested by transient transfection into Chinese Hamster Ovary (DG44) cells. In brief, 0.5μg of each expression construct along with 20ng of a co-transfection control plasmid that expresses the β-galactosidase reporter protein (pCMVβ, Clontech) was transfected into 100,000 DG44 cells using the Fugene 6 (Roche) transfection reagent according to the manufacturer's instructions. Twenty-four hours post transfection cells were lysed using 250μl of lysis buffer. Lac Z reporter assay were performed to ensure equal transfection efficiencies between samples. 30μl of supernatant was added to 10μl of a 4 x SDS gel loading buffer. The sample was heated at 72°C for 10 minutes and loaded onto a 10% Bis-Tris/SDS polyacylamide gel. Proteins were transferred to a PVDF membrane and probed with an α-kappa light chain antibody.

[0081] Figure 4 shows a Western blot analysis of supernatant from DG44 cells transformed with the wild type (cd5-1) and modified cd5 signal peptide α-thyroglobulin light chain expression constructs (cd5-2 to cd5-5). Cell extracts were fractionated on 10% Bis-Tris gels in 1 x MOPS/SDS, transferred to PVDF membrane and probed with an α-kappa light chain antibody. Experiments were performed with supernatant from cells in which transfection efficiency was the same. The results show that the levels of the secreted antibody light chain product (above 28 kDa) in the supernatant of cells was substantially increased for the expression construct lacking CTG codons in the signal peptide (cd5-3). The expression construct lacking CTG, ATG codons and with improved nucleotide context around the authentic initiation codon in the signal peptide (fully rescued) also had levels of protein product in the supernatant that were substantially increased.

[0082] Thy-1 Variable Light chain ORF containing light chain signal peptide 1 (SEQ ID NO: 128) contains 104 potential initiation codons including 8 ATG, including the authentic initiation codon, 15 ATC, 6 ACG, 14 GTG, 4 TTG, 26 CTG, 16 AGG, 10 AAG, 3 ATA, and 2 ATT codons. Modifications were made in the signal peptide in which an AUG and CUG codons were removed (SEQ ID NO: 129). Thy-1 Variable Light chain ORF containing light chain signal peptide 2 (SEQ ID NOS: 130) contains 104 potential initiation codons including 7 ATG, including the authentic initiation codon, 16 ATC, 6 ACG, 13 GTG, 4 TTG, 27 CTG, 15 AGG, 10 AAG, 4 ATA,

and 2 ATT codons. Thy-1 Variable Heavy chain ORF containing heavy chain signal peptide 1 contains 225 potential initiation codons including 18 ATG, including the authentic initiation codon, 14 ATC, 18 ACG, 42 GTG, 7 TTG, 43 CTG, 43 AGG, 33 AAG, 5 ATA, and 2 ATT codons (SEQ ID NO: 131). Modifications were made in the signal peptide by removing an AUG and CUG codon (SEQ ID NO: 132). Thy-1 Variable Heavy chain ORF containing heavy chain signal peptide 2 contains 227 potential initiation codons including 18 ATG, including the authentic initiation codon, 14 ATC, 18 ACG, 43 GTG, 9 TTG, 41 CTG, 43 AGG, 33 AAG, 5 ATA, and 3 ATT codons (SEQ ID NO: 133).

[0083] Thy-1 Variable Light chain ORF in which the signal peptide is replaced with the CD5 signal peptide (SEQ ID NO: 137) contains 104 potential initiation codons including 8 ATG, including the authentic initiation codon, 15 ATC, 6 ACG, 13 GTG, 5 TTG, 27 CTG, 14 AGG, 10 AAG, 3 ATA, and 2 ATT codons. A modification was made in which the ATG codons were changed to ATC codons that resulted in a Methionine (M) to Isoleucine (I) amino acid substitution (SEQ ID NO: 138). A modification was also made in which the CTG codons were changed to CTC codons (SEQ ID NO: 139). Another modification was made in which the ATG codons were mutated to ATC codons that resulted in Methionine (M) to Isoleucine (I) amino acid substitution and CTG codons were changed to CTC codons (SEQ ID NO: 140). Another modification was made in which ATG codons were changed to ATC codons resulting in a Methionine (M) to Isoleucine (I) amino acid substitution, CTG codons were changed to CTC codons, and the context of initiator AUG was improved by changing the codon 3′ of it from CCC to GCT resulting in a Proline (P) to Argenine (R) amino acid substitution (SEQ ID NO: 141).

[0084] Signal peptides from other organisms were mutated as well (see Table 1). DNA sequences for signal peptides that function in yeast and mammalian cells were analyzed and mutated to create mutated versions (SEQ ID NOS: 145-156). It should be appreciated that in signal peptides, which are cleaved off of the protein, in-frame ATG codons can be mutated, e.g. to ATT or ATC, to encode Isoleucine, which is another hydrophobic amino acid. DNA constructs can be generated that contain these signal sequences fused in frame with a light chain from a human monoclonal antibody.

Upon expression in different organisms (such as yeast *Pichia pastoris* and mammalian cell lines), protein gel and Western assay can be used to check the expression level of human light chain antibody.

Table 1: DNA sequences for signal peptide that function in yeast and mammalian cells.

Organism/ signal sequence	DNA sequence	SEQ ID NO:
Pichia pastoris/ Kar2 Signal sequence	ATG/CTG/TCG/TTA/AAA/CCA/TCT/TGG/CTG/ ACT/TTG/GCG/GCA/TTA/ATG/TAT/GCC/ATG/ CTA/TTG/GTC/GTA/GTG/CCA/TTT/GCT/AAA/ CCT/GTT/AGA/GCT	145
Pichia pastoris/ Kar2 Signal sequence rescue version	ATG/CTC/TCG/TTA/AAA/CCA/TCT/TGG/CTC /ACT/TTG/GCG/GCA/TTA/ATT/TAC/GCC/AT C/CTA/TTG/GTC/GTA/GTG/CCA/TTT/GCT/A AA/CCC/GTT/AGA/GCT	146
chicken / lysozyme signal sequence	ATG/CTG/GGT/AAG/AAG/GAC/CCA/ATG/TG T/CTT/GTT/TTG/GTC/TTG/TTG/GGA/TTG/AC T/GCT/TTG/TTG/GGT/ATC/TGT/CAA/GGT	147
chicken / lysozyme signal sequence rescue version	ATG/CTC/GGT/AAG/AAC/GAC/CCA/ATT/TG T/CTT/GTT/TTG/GTC/TTG/TTG/GGA/TTG/AC C/GCT/TTG/TTG/GGT/ATT/TGT/CAA/GGT	148
Human / G-CSF-R signal sequence	ATG/AGG/CTG/GGA/AAC/TGC/AGC/CTG/AC T/TGG/GCT/GCC/CTG/ATC/ATC/CTG/CTG/CT C/CCC/GGA/AGT/CTG/GAG	149
Human / G-CSF-R signal sequence rescue version	ATG/AGG/CTT/GGA/AAT/TGT/AGC/CTC/AC T/TGG/GCC/GCC/CTC/ATC/ATC/CTC/CTT/C TC/CCC/GGA/AGT/CTC/GAG	150
Human / calcitonin receptor precursor signal sequence	ATG/AGG/ACA/TTT/ACA/AGC/CGG/TGC/TT G/GCA/CTG/TTT/CTT/CTT/CTA/AAT/CAC/CC A/ACC/CCA/ATT/CTT/CCT/G	151
Human / calcitonin receptor precursor signal sequence rescue version	ATG/AGG/ACA/TTT/ACA/AGC/CGT/TGC/TT G/GCA/CTC/TTT/CTT/CTT/CTA/AAT/CAC/CC A/ACC/CCA/ATT/CTT/CCC/G	152
Human / cell adhesion molecule 3 precursor (Immunoglobulin superfamily member,4B) signal sequence	ATG/GCC/CCA/GCC/GCC/TCG/CTC/CTG/CTC /CTG/CTC/CTG/CTG/TTC/GCC/TGC/TGC/TGG /GCG/CCC/GGC/GGG/GCC	153
Human / cell adhesion molecule 3 precursor (Immunoglobulin superfamily member,4B) signal sequence rescue	ATG/GCC/CCA/GCC/GCC/TCG/CTC/CTT/CTC /CTT/CTC/CTT/CTC/TTT/GCT/TGT/TGT/TGG /GCG/CCC/GGC/GGG/GCC	154

version		
Human / HLA class I histocompatibility antigen signal sequence	ATG/GTC/GCG/CCC/CGA/ACC/CTC/CTC/CTG /CTA/CTC/TCG/GGG/GCC/CTG/GCC/CTG/AC C/CAG/ACC/TGG/GCG	155
Human / HLA class I histocompatibility antigen signal sequence rescue version	ATG/GTC/GCG/CCC/CGA/ACC/GTC/CTC/CTT /CTT/CTC/TCG/GCG/GCC/CTC/GCC/CTT/AC C/GAG/ACT/TGG/GCC	156

HcRed 1

[0085] HcRed1 encodes a far-red fluorescent protein whose excitation and emission maxima occur at 558 nm and 618 nm +/- 4nm, respectively. HcRed1 was generated by mutagenesis of a non-flourescent chromoptorein from the reef coral *Heteractis crispa*. The HcRed1 coding sequence was subsequently human codon-optimized for higher expression in mammalian cells. This ORF contains 99 potential initiation codons including 9 ATG, including the authentic initiation codon, 8 ATC, 12 ACG, 16 GTG, 21 CTG, 18 AGG, and 15 AAG codons (SEQ ID NO: 134). Full and partial modifications of HcRed1 ORF were generated (SEQ ID NOS: 135 and 136, respectively).

Erythropoietin (EPO)

[0086] Human erythropoietin (EPO) is a valuable therapeutic agent. Using methods described herein, the mRNA sequence that encodes for the human EPO this protein (provided below and available as GenBank Accession No. NM_000799) is optimized to eliminate multiple translation initiation sites within this mRNA transcript.

[0087] An exemplary human erythropoietin (EPO) protein is encoded by the following mRNA transcript, wherein the sequence encoding the mature peptide is underlined, all potential translation initiation start sites within all three reading frames are bolded, the canonical initiator codon corresponding to methionine is capitalized, and uracil (u) is substituted for thymidine (t) (SEQ ID NO: 111):

```
  1 cccggagccggaccggggccaccgcgcccgctctgctccgacaccgcgcccctggacag
 61 ccgccctctcctccaggcccgtggggctggccctgcaccgccgagcttcccgggATGagg
121 gcccccggtgtggtcacccggcgcgccccaggtcgctgagggaccccggccaggcgcgga
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 181 gATGggggtgcacgaATGtcctgcctggctgtggcttctcctgtccctgctgtcgctccc
 241 tctgggcctcccagtcctgggcgccccaccacgcctcatctgtgacagccgagtcctgga
 301 gaggtacctcttggaggccaaggaggccgagaatatcacgacgggctgtgctgaacactg
 361 cagcttgaATGagaatatcactgtcccagacaccaaagttaatttctATGcctggaagag
 421 gATGgaggtcgggcagcaggccgtagaagtctggcagggcctggccctgctgtcggaagc
 481 tgtcctgcggggccaggccctgttggtcaactcttcccagccgtgggagccccctgcagct
 541 gcATGtggataaagccgtcagtggccttcgcagcctcaccactctgcttcgggctctggg
 601 agcccagaaggaagccatctcccctccagATGcggcctcagctgctccactccgaacaat
 661 cactgctgacactttccgcaaactcttccgagtctactccaatttcctccggggaaagct
 721 gaagctgtacacaggggaggcctgcaggacaggggacagATGaccaggtgtgtccacctg
 781 ggcatatccaccacctccctcaccaacattgcttgtgccacaccctcccccgccactcct
 841 gaaccccgtcgaggggctctcagctcagcgccagcctgtcccATGgacactccagtgcca
 901 gcaATGacatctcaggggccagaggaactgtccagagagcaactctgagatctaaggATG
 961 tcacagggccaacttgagggcccagagcaggaagcattcagagagcagctttaaactcag
1021 ggacagagccATGctgggaagacgcctgagctcactcggcaccctgcaaaatttgATGcc
1081 aggacacgctttggaggcgatttacctgttttcgcacctaccatcagggacaggATGacc
1141 tggagaacttaggtggcaagctgtgacttctccaggtctcacgggcATGggcactccctt
1201 ggtggcaagagcccccttgacaccggggtggtgggaaccATGaagacaggATGggggctg
1261 gcctctggctctcATGgggtccaagttttgtgtattcttcaacctcattgacaagaactg
1321 aaaccaccaaaaaaaaaaaa
```

[0088] To preserve the resultant amino acid sequence, silent or conserved substitutions are made wherever possible. In the case of Methionine and tryptophan, which are only encoded only by one codon (aug/atg) and (ugg/tgg), respectively,, a substitution replaces the sequence encoding methionine or tryptophan with a sequence encoding an amino acid of similar physical properties. Physical properties that are considered important when making conservative amino acid substitutions include, but are not limited to, side chain geometry, size, and branching; hydrophobicity; polarity; acidity; aromatic versus aliphatic structure; and Van der Waals volume. For instance, the amino acids leucine or isoleucine can be substituted for methionine because these amino acids are all similarly hydrophobic, non-polar, and occupy equivalent Van der Waals volumes. Thus, a substitution of leucine or isoleucine for methionine would not affect protein folding. Leucine is a preferred amino acid for methionine substitution. Alternatively, the amino acids tyrosine or phenylalanine can be substituted for tryptophan because these amino acids are all similarly aromatic, and occupy quivalent Van der Waals volumes.

[0089] The following sequence is an example of a modified mRNA transcript encoding human erythropoietin (EPO), wherein all potential translation initiation start sites upstream of the demonstrated initiator methionine (encoded by nucleotides182-184) and those potential translation initiation start sites downstream of

the demonstrated initiator methionine within the coding region, are mutated (mutations in italics) (SEQ ID NO: 113).

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   1 cccggagccggaccggggccaccgcgcccgctctactccgacaccgcgccccctagacag
  61 ccgccctctcctccaggcccgtagggctagccctacaccgccgagcttcccgggTTAagg
 121 gccccccgtctagtcacccggcgcgccccaggtcgctaagggaccccggccaggcgcgga
 181 gATGggggtacacaaTTAtcctacctagctctagcttctcctatccctactatcgctccc
 241 tctaggcctcccagtcctaggcgccccaccacacctcctctttaacagccgagtcctaga
 301 gaggtacctcttagaggccaaggaggccgagaatatcacgacgggctgtgctgaacactg
 361 cagcttgaTTAagattttaactatcccagacaccaaagttattatctTTAcctagaagag
 421 gTTAgaggtcgggcagcaggccgtagaagtctagcagggcctagccctactatcggaagc
 481 tgtcctacggggccaggccctattagtcaactcttcccagccgtaggagccccctacagct
 541 gcCTCtagttaaagccgtcagtagccttcgcagcctcaccactctacttcgggctctagg
 601 agcccagaaggaagccctctcccctccagTTAcggcctcagctactccactccgaacaat
 661 cactactaacactttccgcaaactcttccgagtctactccaatatcctccggggaaagct
 721 gaagctatacacaggggaggcctacaggacaggggacagTTAaccagttttatccaccta
 781 ggcttttacaccacctccctcaccaacttaccttttaccacaccctcccccgccactcct
 841 gaaccccgtcgaggggctctcagctcagcgccagcctatcccTTAgacactccagtacca
 901 gcaTTAacttatcaggggccagaggaactatccagagagcaactctaagttataaggTTA
 961 tcacagggccaacttaagggcccagagcaggaagcttacagagagcagctttaaactcag
1021 ggacagagccTTActaggaagacacctaagctcactcggcaccctacaaattttaTTAcc
1081 aggacacactttagaggcgttatacctattttcgcacctaccttaagggacaggTTAacc
1141 tggagaacttaggtagcaagctctcacttctccaggtctcacaggcTTAggcactcccctt
1201 ggtagcaagagccccctaacaccggggtagtaggaaccTTAaagacaggTTAggggcta
1261 gcctctagctctcTTAgggtccaagttctttattacttcaacctcttacacaagaacta
1321 aaaccaccaaaaaaaaaaaa
```

[0090] The unmodified open reading frame for erythropoietin contains 88 potential initiation codons (8 ATG, including the authentic initiation codon, 5 ATC, 4 ACG, 7 GTG, 3 TTG, 32 CTG, 14 AGG, 10 AAG, 3 ATA, and 2 ATT codons) (SEQ ID NO: 112). Modifications were made including a modified signal peptide by removal of most of the potential initiation codons (SEQ ID NO: 116), removal of ATG and CTGs of the signal peptide (SEQ ID NO: 211), removal of ATG, CTG and ACG codons resulting in a Glutamic acid (E) to Glutamine (Q) amino acid substitution (SEQ ID NO: 118) or a Histidine (H) to Argenine (R) amino acid substitution (SEQ ID NO: 119).

Example 2: Modification of miRNA binding sites within mRNA transcripts.

[0091] MicroRNA (miRNA) binding to target mRNA transcripts decreases translation efficiency by either inducing degradation of the target mRNA transcript, or by preventing translation of the target mRNA transcript. To improve translation efficiency of mRNA transcripts encoding commercially-valuable human proteins, all known or predicted miRNA binding sites within a target mRNA's 5' leader sequence, 5' untranslated region (UTR) sequence, coding sequence, and 3' untranslated region (UTR)

sequence are first identified, and secondly mutated or altered in order to inhibit miRNA binding.

[0092] In a preferred aspect of this method, the seed sequence, comprising the first eight 5'- nucleotides of the mature miRNA sequence is specifically targeted. Seed sequences either include 5' nucleotides 1-7 or 2-8 of the mature miRNA sequence. Thus, a seed sequence, for the purposes of this method, encompasses both alternatives. The miRNA seed sequence is functionally significant because it is the only portion of the miRNA which binds according to Watson-Crick base-pairing rules. Without absolute complementarity of binding within the seed sequence region of the miRNA, binding of the miRNA to its target mRNA does not occur. However, unlike most nucleotide pairings, the seed sequence of a miRNA is capable of pairing with a target mRNA such that a guanine nucleotide pairs with a uracil nucleotide, known as the G:U wobble.

[0093] For example, human erythropoietin (EPO) is a valuable therapeutic agent that has been difficult to produce in sufficient quantities. Using the instant methods, the sequence of the mRNA sequence that encodes this protein (GenBank Accession No. NM_000799) is optimized to inhibit miRNA down-regulation. The PicTar Web Interface (publicly available at pictar.mdc_berlin.de/cgi-bin/PicTar_vertebrate.cgi) predicted that human miRNAs hsa-miR-328 and hsa-miR-122a targeted the mRNA encoding for human EPO (the mature and seed sequences of these miRNAs are provided below in Table 2). Thus, in the case of hsa-miR-122a, for instance, having a seed sequence of uggagugu, one or more nucleotides are mutated such that hsa-miR-122a no longer binds, and the seed sequence of another known miRNA is not created. One possible mutated hsa-miR-122a seed sequence that should prevent binding is "uagagugu." It is unlikely that this mutated seed sequence belongs to another known miRNA because this sequence is not represented, for instance, within Table 2 below.

[0094] Similarly, the PicTar Web Interface predicted that human miRNAs hsa-miR-149, hsa-let7f, hsa-let7c, hsa-let7b, hsa-let7g, hsa-let7a, hsa-miR-98, hsa-let7i, hsa-let7e and hsa-miR-26b targeted the mRNA encoding for human interferon beta 2 (also known as IL-6, Genbank Accession No. NM_000600) (the mature and seed sequences of these miRNAs are provided below in Table 2).

[0095] *MiRNA binding sites can also be identified by entering any* sequence of less than 1000 base pairs into the Sanger Institute's MiRNA:Sequence database (publicly available at microrna.sanger.ac.uk/sequences/search.shtml).

Table 2: Known Human MiRNAs, mature sequences, and seed sequences.

MiRNA	Mature Sequence	SEQ ID NO:	Seed Sequence
hsa-let-7a	ugagguaguagguuguauaguu	1	ugagguag
hsa-let-7b	ugagguaguagguugugugguu	2	ugagguag
hsa-let-7c	ugagguaguagguuguaugguu	3	ugagguag
hsa-let-7d	agagguaguagguugcauaguu	4	agagguag
hsa-let-7e	ugagguaggagguuguauaguu	5	ugagguag
hsa-let-7f	ugagguaguagauuguauaguu	6	ugagguag
hsa-let-7g	ugagguaguaguuuguacaguu	7	ugagguag
hsa-let-7i	ugagguaguaguuugugcuguu	8	ugagguag
hsa-miR-1	uggaauguaaagaaguauguau	9	uggaaugu
hsa-miR-100	aacccguagauccgaacuugug	10	aacccgua
hsa-miR-101	uacaguacugugauaacugaa	11	uacaguac
hsa-miR-103	agcagcauuguacagggcuauga	12	agcagcau
hsa-miR-105	ucaaaugcucagacuccuguggu	13	ucaaaugc
hsa-miR-106a	aaaagugcuuacagugcagguag	14	aaaagugc
hsa-miR-106b	uaaagugcugacagugcagau	15	uaaagugc
hsa-miR-107	agcagcauuguacagggcuauca	16	agcagcau
hsa-miR-10a (mmu-miR-184)	uggacggagaacugauaagggu	17	uggacgga
hsa-miR-10b	uacccuguagaaccgaauuugug	18	uacccugu
hsa-miR-122a	uggagugugacaaugguguuug	19	uggagugu
hsa-miR-124a	uaaggcacgcggugaaugcc	20	uaaggcac
hsa-miR-125a	ucccugagacccuuuaaccuguga	21	ucccugag

hsa-miR-125b	ucccugagacccuaacuuguga	22	ucccugag
hsa-miR-126	Ucguaccgugaguaauaaugcg	23	ucguaccg
hsa-miR-127	cugaagcucagagggcucugau	24	cugaagcu
hsa-miR-128	ucacagugaaccggucucuuu	25	ucacagug
hsa-miR-129	cuuuuugcggucugggcuugc	26	cuuuuugc
hsa-miR-130a	cagugcaauguuaaaagggcau	27	cagugcaa
hsa-miR-130b	cagugcaaugaugaaagggcau	28	cagugcaa
hsa-miR-132	uaacagucuacagccauggucg	29	uaacaguc
hsa-miR-133a	uuuggucccuucaaccagcug	30	uuuggucc
hsa-miR-133b	uuuggucccuucaaccagcua	31	uuuggucc
hsa-miR-134	ugugacugguugaccagagggg	32	ugugacug
hsa-miR-135a	uauggcuuuuuauuccuauguga	33	uauggcuu
hsa-miR-135b	uauggcuuuucauuccuauguga	34	uauggcuu
hsa-miR-136	acuccauuuguuuugaugaugga	35	acuccauu
hsa-miR-137	uuauugcuuaagaauacgcguag	36	uuauugcu
hsa-miR-138	agcugguguugugaaucaggccg	37	agcuggug
hsa-miR-139	ucuacagugcacgugucuccag	38	ucuacagu
hsa-miR-140	cagugguuuuacccuaugguag	39	cagugguu
hsa-miR-141	uaacacugucugguaaagaugg	40	uaacacug
hsa-miR-142-5p	cauaaaguagaaagcacuacu	41	cauaaagu
hsa-miR-143	ugagaugaagcacuguagcuc	42	ugagauga
hsa-miR-144	uacaguauagaugauguacu	43	uacaguau
hsa-miR-145	guccaguuuucccaggaaucccu	44	guccaguu
hsa-miR-146	ugagaacugaauuccauggguu	45	ugagaacu
hsa-miR-147	guguguggaaaugcuucugc	46	gugugugg
hsa-miR-148a	ucagugcacuacagaacuuugu	47	ucagugca

hsa-miR-148b	ucagugcaucacagaacuuugu	48	ucagugca
hsa-miR-149	ucuggcuccgugucuucacuccc	49	ucuggcuc
hsa-miR-150	ucucccaacccuuguaccagug	50	ucucccaa
hsa-miR-151	ucgaggagcucacagucuagu	51	ucgaggag
hsa-miR-152	ucagugcaugacagaacuugg	52	ucagugca
hsa-miR-153	uugcauagucacaaaagugauc	53	uugcauag
hsa-miR-154	uagguuauccguguugccuucg	54	uagguuau
hsa-miR-155	uuaaugcuaaucgugauaggggu	55	uuaaugcu
hsa-miR-15a	uagcagcacauaaugguuugug	56	uagcagca
hsa-miR-15b	uagcagcacaucaugguuuaca	57	uagcagca
hsa-miR-16	uagcagcacguaaauauuggcg	58	uagcagca
hsa-miR-17	caaagugcuuacagugcagguag	60	caaagugc
hsa-miR-18	uaaggugcaucuagugcagauag	61	uaaggugc
hsa-miR-181a	aacauucaacgcugucggugagu	62	aacauuca
hsa-miR-181b	Aacauucauugcugucgguggguu	63	aacauuca
hsa-miR-181c	aaccaucgaccguugaguggac	64	aaccaucg
hsa-miR-182	uuuggcaaugguagaacucacacu	65	uuuggcaa
hsa-miR-183	uauggcacugguagaauucacu	66	uauggcac
hsa-miR-184	uggacggagaacugauaagggu	67	uggacgga
hsa-miR-185	uggagagaaaggcaguuccuga	68	uggagaga
hsa-miR-186	caaagaauucuccuuuugggcu	69	caaagaau
hsa-miR-187	ucgugucuuguguugcagccgg	70	ucgugucu
hsa-miR-188	caucccuugcaugguggaggg	71	caucccuu
hsa-miR-190	ugauauguuugauauauuaggu	72	ugauaugu
hsa-miR-191	caacggaaucccaaaagcagcug	73	caacggaa
hsa-miR-192	cugaccuaugaauugacagcc	74	cugaccua
hsa-miR-193	ugggucuuugcgggcgagauga	75	ugggucuu

hsa-miR-194	uguaacagcaacuccaugugga	76	uguaacag
hsa-miR-195	uagcagcacagaaauauuggc	77	uagcagca
hsa-miR-196a	uagguaguuucauguuguuggg	78	uagguagu
hsa-miR-196b	uagguaguuuccuguuguuggg	79	uagguagu
hsa-miR-197	uucaccaccuucuccacccagc	80	uucaccac
hsa-miR-198	gguccagaggggagauagguuc	81	gguccaga
hsa-miR-199a	cccaguguucagacuaccuguuc	82	cccagugu
hsa-miR-199b	cccaguguuuagacuaucuguuc	83	cccagugu
hsa-miR-19a	aguuuugcauaguugcacuaca	84	aguuuugc
hsa-miR-19b	ugugcaaauccaugcaaaacuga	85	ugugcaaa
hsa-miR-20	uaaagugcuuauagugcagguag	86	uaaagugc
hsa-miR-200a	uaacacugucugguaacgaugu	87	uaacacug
hsa-miR-200b	uaauacugccugguaaugauga	88	uaauacug
hsa-miR-200c	uaauacugccgggguaaugaugga	89	uaauacug
hsa-miR-203	gugaaauguuuaggaccacuag	90	gugaaaug
hsa-miR-204	uucccuuugucauccuaugccu	91	uucccuuu
hsa-miR-205	uccuucauuccaccggagucug	92	uccuucau
hsa-miR-206	uggaauguaaggaagugugugg	93	uggaaugu
hsa-miR-208	auaagacgagcaaaaagcuugu	94	auaagacg
hsa-miR-21	uagcuuaucagacugauguuga	95	uagcuuau
hsa-miR-210	cugugcgugugacagcggcuga	96	cugugcgu
hsa-miR-211	uucccuuugucauccuucgccu	97	uucccuuu
hsa-miR-212	uaacagucuccagucacggcc	98	uaacaguc
hsa-miR-213 (hsa-miR-181a)	aacauucaacgcugucggugagu	61	aacauuca
hsa-miR-214	acagcaggcacagacaggcagu	99	acagcagg
hsa-miR-215	augaccuaugaauugacagac	100	augaccua
hsa-miR-216	uaaucucagcuggcaacuguga	101	uaaucuca
hsa-miR-217	uacugcaucaggaacugauugga	102	uacugcau

hsa-miR-218	uugugcuugaucuaaccaugu	103	uugugcuu
hsa-miR-219	ugauuguccaaacgcaauucu	104	ugauuguc
hsa-miR-22	aagcugccaguugaagaacugu	105	aagcugcc
hsa-miR-220	ccacaccguaucugacacuuu	106	ccacaccg
hsa-miR-221	agcuacauugucugcuggguuuc	107	agcuacau
hsa-miR-222	agcuacaucuggcuacugggu	108	agcuacau
hsa-miR-223	ugucaguuugucaaauacccca	109	ugucaguu
hsa-miR-224	caagucacuagugguuccguu	110	caagucac
hsa-miR-26b	uucaaguaauucaggauaggu	114	uucaagua

[0096] The miR-183 binding sequence (SEQ ID NO: 59) was mutated (SEQ ID NO: 142) and embedded into the coding sequence of a reporter gene, such as in a CAT gene that also contains a FLAG Tag (SEQ ID NO: 143). This allows for the evaluation of expression in cells by Western blot analyses using an anti-FLAG Tag antibody in which mutations of the miR-183 binding sequence were made (SEQ ID NO: 144).

Claims:

1. A method of improving full-length protein expression efficiency comprising:

a) providing a polynucleotide comprising:

i) a coding sequence for the full-length protein;

ii) a primary initiation codon that is upstream of the coding sequence of the full-length protein, said primary initiation codon encoding the first amino acid of the coding sequence of the full-length protein; and

iii) one or more secondary initiation codons located within the coding sequence of the full-length protein downstream of the primary initiation codon; and

b) mutating the one or more secondary initiation codons, located within the coding sequence of the full-length protein downstream of the primary initiation codon, wherein the mutation results in a decrease in initiation of protein synthesis at the one or more secondary initiation codons resulting in a reduction of ribosomal diversion away from the primary initiation codon,

thereby increasing expression efficiency of the full-length protein initiated at the primary initiation codon,

wherein mutating the one or more secondary initiation codons located within the coding sequence of the full-length protein downstream of the primary initiation codon comprises mutating one or more nucleotides such that the amino acid sequence of the protein remains unaltered.

2. The method of claim 1, wherein the one or more secondary initiation codons is in the same reading frame as the coding sequence.

3. The method of claim 1, wherein the one or more secondary initiation codons is out-of-frame with the coding sequence.

4. The method of claim 1, wherein the one or more secondary initiation codons is located one or more nucleotides upstream or downstream from a ribosomal recruitment site.

5. The method of claim 4, wherein the ribosomal recruitment site comprises a cap or an IRES.

6. The method of claim 1, wherein the one or more secondary initiation codons is selected from the group consisting of AUG, ACG, GUG, UUG, CUG, AUA, AUC, and AUU.

7. The method of claim 1, wherein more than one secondary initiation codon within the coding sequence is mutated.

8. The method of claim 1, wherein all secondary initiation codons within the coding sequence are mutated.

9. The method of claim 1, wherein mutating the one or more secondary initiation codons comprises mutating one or more nucleotides flanking the one or more secondary initiation codons to diminish the efficiency of the one or more secondary initiation codons.

10. The method of claim 1, wherein mutating the one or more secondary initiation codons does not introduce new initiation codons.

11. The method of claim 1, wherein mutating the one or more secondary initiation codons does not alter usage bias of mutated codons.

12. The method of claim 1, wherein the generation of truncated proteins, polypeptide, or peptides other than the full-length encoded protein is decreased.

13. The method of claim 1, wherein mutating one or more secondary initiation codons does not introduce miRNA seed sequences, splice donor site, splice acceptor site, or mRNA destabilization elements.

14. The method of claim 1, wherein the polynucleotide sequence further comprises one or more miRNA binding sites located within the coding sequence, said method further

comprises the step of mutating the one or more miRNA binding sites, wherein the mutation results in a decrease in miRNA binding at the one or more miRNA binding sites resulting in a reduction of miRNA-mediated down regulation of protein translation.

15. The method of claim 14, wherein mutating the one or more miRNA binding sites comprises mutating one or more nucleotides in a miRNA seed sequence.

16. The method of claim 14, wherein mutating the one or more miRNA binding sites comprises mutating one or more nucleotides such that initiation codons are not introduced into the polynucleotide sequence.

17. The method of claim 14, wherein mutating the one or more miRNA binding sites comprises mutating one or more nucleotides such that rare codons are not introduced into the polynucleotide sequence.

18. The method of claim 14, wherein mutating the one or more miRNA binding sites mutating one or more nucleotides such that additional miRNA seed sequences are not introduced into the polynucleotide sequence.