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Stabilized nucleic acids encoding messenger ribonucleic acid (mRNA)

Abstract

This disclosure relates to the field of poly-adenylated (poly-A) tails. In some embodiments, a DNA encodes a poly-A tail located 3′ to nucleotides encoding a protein of interest, wherein the poly-A tail comprises one or more non-adenine nucleotide.

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Background/Summary

(1) This application is a Continuation of U.S. application Ser. No. 16/791,076, which was filed on Feb. 14, 2020, which is a Continuation of International Application No. PCT/US2018/046772, which was filed Aug. 14, 2018 and which claims the benefit of priority to U.S. Provisional

Application No. 62/545,883, which was filed on Aug. 15, 2017, all of which are incorporated by reference in their entirety. (2) The patent application is filed with a sequence listing in electronic format. The Sequence Listing is provided as a file entitled "2023-04-27_01155-0019-01US_ST26," which was created on Apr. 27, 2023, and which is 95,864 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety. (3) This disclosure relates to the field of stabilized messenger ribonucleic acid (mRNA) and DNA encoding the stabilized mRNA.

BACKGROUND

(1) Polyadenylation is the process of adding multiple adenine nucleotides to the 3′ end of a messenger RNA (mRNA), forming a poly-A tail. The poly-A tail consists of multiple repeated adenine nucleotides, such as adenosine monophosphates, without other bases interrupting the sequence. The poly-A tail is critical for the nuclear export, translation, and stability of mRNA. In nature, as mRNA is produced from DNA, a terminal transferase adds adenine nucleotides to the 3′ end of mRNA. This enzymatic process can be applied when producing mRNA ex vivo, but the process is difficult to control and results in poly-A tails of different lengths. By encoding a poly-A tail in the plasmid, it is possible to decrease the heterogeneity in the poly-A tail. However, it does not eliminate the heterogeneity, and has additional downsides such as potential instability of the plasmid.

(2) The poly-A tail acts as the binding site for poly-A-binding protein. Poly-A-binding protein assists in exporting mRNA from the nucleus, translation, and inhibiting degradation of the mRNA. In the absence of export from the nucleus, mRNAs are typically degraded by the exosome. The poly-A-binding protein recruits proteins necessary for translation.

(3) mRNA is now being used as a therapeutic molecule, for example, for the treatment of various diseases and disorders. mRNA is delivered to a subject in lieu of the protein so that the subject's cells produce the protein encoded by the mRNA within the cell. For these and other purposes, mRNA may be prepared via transcription from a DNA template, often contained in a plasmid. During mRNA production, the poly-A tail may be added to mRNA enzymatically after transcription from a plasmid or encoded on the plasmid itself. When the poly-A tail is encoded on a plasmid, the poly-A tail may become shorter (i.e., lose adenine nucleotides) over cycles of plasmid DNA replication, potentially leading to large variations in the resulting DNA and subsequent mRNA population. Thus, there exists a need in the art to design plasmids encoding poly-A tails that are stable and resistant to gradual loss of nucleotides encoding poly-A adenine nucleotides during DNA replication.

SUMMARY

(4) Disclosed herein are DNA encoding, and mRNA comprising, poly-adenylated (poly-A) tails comprising consecutive adenine nucleotides located 3′ to nucleotides encoding a protein of interest, wherein the poly-A tail is stabilized by inserting non-adenine nucleotide "anchors."

(5) As used herein, the term "poly-A tail" refers to a poly-A tail on an mRNA molecule, or a sequence encoding a poly-A tail within a DNA plasmid. A poly-A tail may be encoded by a complementary DNA sequence within a plasmid. A sequence of repeating thymine (T) nucleotides in a DNA sequence, e.g. a homopolymer T sequence, may encode a poly-A tail on an mRNA. Two or more consecutive adenosine (e.g. adenosine or deoxyadenosine), thymidine, or other nucleotides are called homopolymers. Naturally-occurring poly-A tails comprise long, uninterrupted homopolymer A sequences.

(6) The non-adenine nucleotide anchors disclosed herein interrupt the poly-A tail at regular or irregularly spaced intervals and stabilize the DNA encoding the poly-A tail as well as the mRNA produced from the DNA. Exemplary non-adenine nucleotide anchors are provided in Table 4. An anchor sequence, for example, is adjacent to two adenine nucleotide homopolymer sequences within the poly-A tail.

(7) In some embodiments, a DNA composition comprising nucleotides encoding a poly-adenylated (poly-A) tail located 3′ to nucleotides encoding a protein of interest, wherein the poly-A tail comprises at least 8 consecutive adenine (A) nucleotides and one or more non-adenine (A) nucleotides is encompassed.
(8) In some embodiments, the poly-A tail comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, or 90 consecutive adenine nucleotides.
(9) In some instances, the one or more non-adenine nucleotides prevent the loss of one or more adenine nucleotides during DNA replication as compared to the loss that occurs in a DNA comprising a 3′ tail of a similar or same length that contains only adenine nucleotides.
(10) In some embodiments, the one or more non-adenine nucleotides are positioned to interrupt the consecutive adenine nucleotides so that a poly(A) binding protein can bind to a stretch of consecutive adenine nucleotides.
(11) In some embodiments, the poly-A tail comprises at least 50 total adenine nucleotides.
(12) In some embodiments, the poly-A tail comprises 40-500 total adenine nucleotides.
(13) In some instances, the poly-A tail comprises 95-100 total adenine nucleotides.
(14) In some embodiments, the poly-A tail comprises or contains 90, 91, 92, 93, 94, 95, 96, or 97 total adenine nucleotides.
(15) In some embodiments, the poly-A tail comprises or contains 96 or 97 total adenine nucleotides.
(16) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 non-adenine nucleotides.
(17) In some embodiments, the non-adenine nucleotide(s) is located after at least 8, 9, 10, 11, or 12 consecutive adenine nucleotides.
(18) In some instances, the one or more non-adenine nucleotides are located after at least 8-50 consecutive adenine nucleotides.
(19) In some embodiments, the one or more non-adenine nucleotides are located after at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(20) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail has one or more non-adenine nucleotides or one or more consecutive stretches of 2-10 non-adenine nucleotides irregularly spaced anywhere along the length of the poly-A tail, wherein somewhere along the length of the poly-A tail there are at least 8 consecutive adenines. For example, a poly-A tail may be 70-1000 nucleotides in length, and have any number of non-adenines (either singly or grouped) irregularly spaced along the length, as long as there is one or more stretch of at least 8 consecutive adenines.
(21) In some instances, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(22) In some instances, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(23) In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 8-50 consecutive adenine nucleotides.
(24) In some instances, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27,

28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(25) In some embodiments, the poly-A tail comprises or contains more than one non-adenine nucleotide or more than one consecutive stretch of 2-10 nucleotides as interrupting sequences irregularly spaced within the poly-A tail.
(26) In some embodiments, the poly-A tail comprises or contains more than one non-adenine nucleotide or more than one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides irregularly spaced within the poly-A tail.
(27) In some instances, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 12 consecutive adenine nucleotides.
(28) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 16 consecutive adenine nucleotides.
(29) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 25 consecutive adenine nucleotides.
(30) In some instances, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 30 consecutive adenine nucleotides.
(31) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 39 consecutive adenine nucleotides.
(32) In some embodiments, the non-adenine nucleotide is guanine, cytosine, or thymine. In some instances, the non-adenine nucleotide is a guanine nucleotide. In some embodiments, the non-adenine nucleotide is a cytosine nucleotide. In some embodiments, the non-adenine nucleotide is a thymine nucleotide.
(33) In some instances, where more than one non-adenine nucleotide is present, the non-adenine nucleotide may be selected from: a) guanine and thymine nucleotides; b) guanine and cytosine nucleotides; c) thymine and cytosine nucleotides; or d) guanine, thymine and cytosine nucleotides.
(34) In some embodiments, the non-adenine nucleotide consists of one non-adenine nucleotide selected from guanine, cytosine, and thymine.
(35) In some instances, the non-adenine nucleotides comprise two non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine.
(36) In some embodiments, the non-adenine nucleotides comprise three non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine.
(37) The adenine nucleotides may be adenosine monophosphate.
(38) In some embodiments, the protein encoded by the mRNA is a therapeutic protein. In some instances, the protein a cytokine, chemokine, growth factor, Cas9 or modified Cas9.
(39) In some embodiments, mRNA encoded by any of the DNAs described herein is encompassed.
(40) In some embodiments, the DNA is within a vector. The vector may be within a host cell, including insect, bacterial, or mammalian (e.g., human) cells.
(41) In some embodiments, the one or more non-adenine nucleotide prevents loss of nucleotides encoding the poly-A tail within the vector during growth of the host cell as compared to the loss that occurs in a DNA comprising nucleotides encoding a poly-A tail of a similar or same length that contains only adenine nucleotides.
(42) Methods of producing mRNA from any of the DNA vectors described herein are encompassed comprising: linearizing the vector downstream of the poly-A tail; denaturing the linearized vector; and contacting the denaturized DNA with an RNA polymerase in the presence of guanine, cytosine, uracil, and adenine nucleotides.
(43) In some embodiments, this disclosure includes a DNA comprising nucleotides encoding a poly-adenylated (poly-A) tail located 3′ to nucleotides encoding a protein of interest, wherein the poly-A tail comprises a first homopolymer sequence of at least 8 consecutive adenine (A) nucleotides and an interrupting sequence comprising one or more non-adenine (A) nucleotides. In some such embodiments, the poly-A tail further comprises a second homopolymer sequence of at

least consecutive adenine (A) nucleotides. In some embodiments, the poly-A tail comprises three or more homopolymer sequences of at least 8 consecutive adenine (A) nucleotides. In some embodiments, the first and/or subsequent homopolymer sequence comprises at least 10, 15, 20, 25, 30, 35, or 40 consecutive adenine nucleotides. In some embodiments, the one or more non-adenine nucleotide prevents the loss of one or more adenine nucleotide during DNA replication as compared to the loss that occurs in a DNA comprising a 3′ tail of a similar or same length that contains only adenine nucleotides. In some embodiments, the one or more non-adenine nucleotide is positioned to interrupt the consecutive adenine nucleotides so that a poly(A) binding protein can bind to a stretch of consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises at least 50 total adenine nucleotides. In some embodiments, the poly-A tail comprises 40-1000, 40-900, 40-800, 40-700, 40-600, 40-500, 40-400, 40-300, 40-200, or 40-100 total adenine nucleotides. In some embodiments, the poly-A tail comprises 95-100 total adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 90, 91, 92, 93, 94, 95, 96, or 97 total adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 96 or 97 total adenine nucleotides. In some embodiments, the one or more interrupting sequence comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 non-adenine nucleotides. In some embodiments, the one or more interrupting sequence comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides that includes two or more non-adenine nucleotides. In some embodiments, the non-adenine nucleotide(s) is located after at least 8, 9, 10, 11, or 12 consecutive adenine nucleotides. In some embodiments, the one or more non-adenine nucleotide is located after at least 8-50 consecutive adenine nucleotides. In some embodiments, the one or more non-adenine nucleotide is located after at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.

(44) In some embodiments, as described in the preceding paragraph, the interrupting sequence is a trinucleotide, dinucleotide or mononucleotide interrupting sequence. In some such embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 non-adenine nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains more than one non-adenine nucleotide or more than one consecutive stretch of 2-10 non-adenine nucleotides. In some embodiments, the more than one non-adenine nucleotide or more than one consecutive stretch of 2-10 non-adenine nucleotides are irregularly spaced within the poly-A tail. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 12 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 16 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 25 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 30 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or 2, 3, 4, or 5 consecutive non-adenine nucleotides every 39 consecutive adenine nucleotides. In some

embodiments, the non-adenine nucleotide is guanine, cytosine, or thymine. In some embodiments, the non-adenine nucleotide is a guanine nucleotide. In some embodiments, the non-adenine nucleotide is a cytosine nucleotide. In some embodiments, the non-adenine nucleotide is a thymine nucleotide. In some embodiments, the DNA comprises more than one non-adenine nucleotide selected from: (a) guanine and thymine nucleotides; (b) guanine and cytosine nucleotides; (c) thymine and cytosine nucleotides; or (d) guanine, thymine and cytosine nucleotides. In some embodiments described above, the non-adenine nucleotide consists of one non-adenine nucleotide selected from guanine, cytosine, and thymine. In some embodiments, non-adenine nucleotides comprise two non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine. In some embodiments, non-adenine nucleotides comprise three non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine. In some embodiments, adenine nucleotides are adenosine monophosphate. In some embodiments, the protein is a therapeutic protein. In some embodiments, the protein a cytokine or chemokine. In some embodiments, the protein a growth factor. In some embodiments, the protein is Cas9 or modified Cas9.

(45) This disclosure also encompasses an mRNA encoded by the DNA as described in the preceding paragraphs.

(46) In some embodiments, the DNA described in the preceding paragraphs may also be comprised within a vector. In some embodiments, the vector is comprised within a host cell. In some embodiments, where the DNA is within a vector, the one or more non-adenine nucleotide prevents loss of nucleotides encoding the poly-A tail within the vector during growth of the host cell as compared to the loss that occurs in a DNA comprising nucleotides encoding a poly-A tail of a similar or same length that contains only adenine nucleotides.

(47) This disclosure also encompasses methods of producing mRNA from the DNA vectors described herein, comprising: (a) linearizing the vector downstream of the poly-A tail; (b) denaturing the linearized vector; and (c) contacting the denaturized DNA with an RNA polymerase in the presence of guanine, cytosine, uracil, and adenine nucleotides.

Description

FIGURE LEGENDS

(1) FIG. **1** shows a sequence encoding a poly-A tail that contains only adenosines decreasing in length over rounds of growth. Each clone refers to a DNA generated by successive rounds of growth/purification of host cells expressing plasmid encoding the clones.

(2) FIG. **2** shows retention of size of a poly-A tail comprising non-adenine nucleotides over 2 growth passages.

(3) FIG. **3** shows secreted embryonic alkaline phosphatase (SEAP) levels measured in a Cas9 mRNA assay using Cas9 mRNA with a poly-A tail containing only adenosines or Cas9 mRNA with a poly-A tail comprising non-adenine nucleotides and single guide RNA targeting SEAP (SEQ ID NO: 8).

(4) FIG. **4** shows percent SEAP inhibition measured in a Cas9 mRNA assay using Cas9 mRNA with a poly-A tail containing only adenosines or Cas9 mRNA with a poly-A tail comprising non-adenine nucleotides and single guide RNA targeting SEAP (SEQ ID NO: 8) with a 24-hour incubation.

(5) FIG. **5** shows percent SEAP inhibition measured in a Cas9 mRNA assay using Cas9 mRNA with a poly-A tail containing only adenosines or Cas9 mRNA with a poly-A tail comprising non-adenine nucleotides and single guide RNA targeting SEAP (SEQ ID NO: 8) with a 48-hour incubation.

(6) FIG. **6** shows serum transthyretin (TTR) levels in mice 7 days after dosing of a control transformation and storage solution (TSS) buffer or dosing of liquid nanoparticles (LNP)

formulated with the single guide RNA of SEQ ID NO: 9 (targeting the mouse TTR gene) and either an mRNA encoded by SEQ ID NO: 6 (HiCas9 mRNA) or by SEQ ID NO: 7 (disrupted Poly-A mRNA).

(7) FIG. **7** shows percent SEAP inhibition measured in a Cas9 mRNA assay using Cas9 mRNA with a poly-A tails containing only adenosines or Cas9 mRNA with a poly-A tails comprising non-adenine nucleotides and single guide RNA targeting SEAP (SEQ ID NO: 8) with a 48-hour incubation.

DETAILED DESCRIPTION

(8) Disclosed herein are DNAs encoding a poly-adenylated tail located 3′ to nucleotides encoding a protein of interest, wherein the poly-A tail comprises one or more non-adenine nucleotides. During DNA replication, DNA encoding a poly-A tail comprising one or more non-adenine nucleotide may show less gradual loss of adenine nucleotides within the poly-A tail compared with poly-A tails consisting only of adenine nucleotides. Thus, plasmids comprising DNA encoding a poly-A tail comprising one or more non-adenine nucleotide are provided. mRNA encoded by such DNA is also encompassed. Both the DNA and RNA may exhibit greater stability against processive loss of adenine nucleotides than similar molecules comprising non-interrupted poly-A tails.

(9) The protein of interest may be any natural or non-natural protein. As used herein, “protein” refers to any sequence of consecutive amino acids. As such, a protein may refer to a protein that comprises the full amino acid sequence of a naturally occurring protein. In addition, a protein may refer to an amino acid sequence that comprises a fragment of a full-length protein. A protein may be a naturally-occurring sequence, a naturally-occurring sequence with one or more modifications, or an artificial sequence that does not occur in nature.

(10) The protein of interest may be of therapeutic use in a subject, or this protein may be of use in a biochemical reaction. Therapeutic proteins include, for example, growth factors, antigens for vaccines or immuno-oncology, and enzymes, among others. Therapeutic proteins may be naturally occurring or modified. In certain circumstances, a modified protein may be a fusion protein.

(11) In some embodiments, expression of a protein by an mRNA is for use as a treatment for a disease. In some embodiments, expression of a protein by an mRNA is for use as a cancer immunotherapy, vaccination against infectious disease, to induce tolerance to a type I allergy, as a replacement therapy, or as a regenerative medicine (see Sergeeva O V et al, *Biochemistry* (Moscow) 81(7):709-722 (2016)).

(12) In some embodiments, autologous dendritic cells are transfected ex vivo with an mRNA encoding for prostate-specific antigen (PSA) to modulate the T-cell immune response in subjects with metastatic prostate cancer.

(13) In some embodiments, an mRNA is a prophylactic vaccine. In some embodiments, an mRNA encodes for one or more antigenic proteins. In some embodiments, the antigenic protein(s) is a viral protein. In some embodiments, the mRNA causes cells of the body to produce and express an antigenic protein. In some embodiments, the mRNA causes expression of antigenic proteins without a danger or disease or spread between individuals. In some embodiments, expression of antigenic proteins causes the immune system of a subject to produce antibodies. In some embodiments, these antibodies can neutralize a virus and prevent future infection after exposure to the virus. In some embodiments, the mRNA is a prophylactic vaccine for an infectious disease. In some embodiments, the mRNA is prophylactic vaccine against influenza, chikungunya, Zika, cytomegalovirus, human metapneumovirus (HMPV), or parainfluenza virus type 3 (PIV3). In some embodiments, the mRNA is a prophylactic vaccine against influenza H10 or H7 subtypes.

(14) In some embodiments, an mRNA is a personalized cancer vaccine. In some embodiments, an mRNA primes the immune system of a subject with cancer to recognize cancer cells and mount a response. In some embodiments, this response is tailored to the individual patient's cancer or tumor. In some embodiments, an mRNA encodes a patient's specific neoantigens (unique proteins with mutations present in the patient's cancer or tumor). In some embodiments, an mRNA causes

expression of a patient's specific neoantigens. In some embodiments, expression of neoantigens elicits a specific immune response in the patient to recognize and destroy cancer cells. In some embodiments, an mRNA is of use as a personalized cancer vaccine. In some embodiments, an mRNA is of use as a personalized cancer vaccine together with one or more checkpoint inhibitor antibodies, such as anti-PD-1 therapies.

(15) In some embodiments, an mRNA is of use for intratumoral immuno-oncology. In some embodiments, injection of an mRNA into a tumor reduces off-target effects and/or may be more potent compared to systemic administration. In some embodiments, the mRNA causes expression of OX40L (CD252), the ligand for CD134. In some embodiments, the mRNA causes expression of cytokines such as interleukin 12 (IL-12).

(16) In some embodiments, an mRNA causes expression of a protein for localized therapy. In some embodiments, an mRNA causes creation of more blood vessels and improved blood supply in a local tissue. In some embodiments, the mRNA causes expression of vascular endothelial growth factor A (VEGF-A). In some embodiments, expression of VEGF-A is local and transient. In some embodiments, local and transient expression of VEGF-A is of use for treatment of heart failure or after a heart attack, of diabetic wound healing, or of other ischemic vascular diseases.

(17) In some embodiments, an mRNA causes expression of a protein for replacement therapy. In some embodiments, the protein is surfactant protein-B.

(18) In some embodiments, an mRNA causes expression of an RNA-guided nuclease such as class 2 CRISPR-associated Cas endonuclease, e.g. Cas9/Csn1 (Cas9). An exemplary Cas9 sequence is UniProt Q99ZW2. In some embodiments, the protein is a modified Cas9 or a Cas9 protein fused to another functional protein or peptide. Modified versions of Cas9 having one catalytic domain, either RuvC or HNH, that is inactive are termed “nickases”. In some embodiments, the compositions and methods comprise nickases. In some embodiments, the compositions and methods comprise a nickase Cas9 that induces a nick rather than a double strand break in the target DNA.

(19) In some embodiments, the Cas protein may be modified to contain only one functional nuclease domain. For example, the Cas protein may be modified such that one of the nuclease domains is mutated or fully or partially deleted to reduce its nucleic acid cleavage activity. In some embodiments, a nickase Cas is used having a RuvC domain with reduced activity. In some embodiments, a nickase Cas is used having an inactive RuvC domain. In some embodiments, a nickase Cas is used having an HNH domain with reduced activity. In some embodiments, a nickase Cas is used having an inactive HNH domain.

(20) In some embodiments, chimeric Cas proteins are encoded by the DNA, where one domain or region of the protein is replaced by a portion of a different protein. In some embodiments, a Cas nuclease domain may be replaced with a domain from a different nuclease such as Fok1. In some embodiments, a Cas protein may be a modified nuclease.

(21) I. DNA Encoding Poly-A Tails Comprising Non-Adenine Nucleotides

(22) As used herein, a “poly-A tail” refers to a sequence comprising adenosines or other adenine nucleotides at the 3′ end of an mRNA. While natural poly-A tails may be comprised solely of adenine nucleotides, a “poly-A tail” of the present invention is stabilized by one or more non-adenine nucleotide “anchors”. In some embodiments, the poly-A tail comprises at least 8 consecutive adenine nucleotides and one or more interrupting sequence comprising a non-adenine nucleotide. In other words, the poly-A tails of the present invention comprise at least 8 consecutive adenines, but also comprise one or more non-adenine nucleotide within the interrupting or anchor sequences. The interrupting sequences disclosed herein interrupt the poly-A tail at regular or irregularly spaced intervals and stabilize the DNA encoding the poly-A tail as well as the mRNA produced from the DNA. Exemplary interrupting sequences are provided in Table 4.

(23) As used herein, “non-adenine nucleotides” refer to any natural or non-natural nucleotides that do not comprise adenine. Guanine, thymine, and cytosine nucleotides are exemplary non-adenine

nucleotides.
(24) Native poly-A tails are added in a process of polyadenylation that begins after transcription of a DNA into mRNA. In molecular biology methods, however, poly-A tails are often encoded by a section of DNA within a plasmid that encodes a protein of interest. In this instance, the size of the poly-A tail (i.e., the number of adenine nucleotides comprised in the poly-A tail) is directly dependent on the number of DNA nucleotides in the plasmid that encode for these consecutive adenine nucleotides.
(25) The number of DNA nucleotides encoding the poly-A tail may gradually decrease during DNA replication during, for example, growth of the plasmid in a host cell. When the number of consecutive adenine-encoding nucleotides in a plasmid reduces, the yield of plasmid encoding full-length poly-A tail is reduced, and the resulting mRNA having shorter poly-A tails may have decreased stability and/or increased degradation. For example, an mRNA with a poly-A tail of 40 consecutive adenine nucleotides might be expected to have lower stability than an mRNA with a poly-A tail of 90 or more nucleotides. By lower stability, it is meant that an mRNA may be degraded more quickly, and consequently expression of a target protein is decreased from an mRNA with a shorter poly-A tail. As such, maintaining the length of a poly-A tail within a DNA plasmid over multiple rounds of DNA replication within host cells is beneficial. In addition, the poly-A tail may be important for translation, and maintaining a longer poly-A tail may result in improved protein expression from the mRNA.
(26) Inclusion of one or more non-adenine nucleotides in a poly-A tail located 3′ to nucleotides encoding a protein of interest may prevent the loss of one or more adenine nucleotides during DNA replication as compared to the loss that occurs in a DNA comprising a 3′ poly-A tail of a similar or same length that contains only adenine nucleotides. The presence of a longer poly-A tail may also improve the efficiency of protein translation from an mRNA.
(27) A. Adenine Nucleotides
(28) The number of consecutive adenine nucleotides in a poly-A tail of this invention is designed to allow the poly-A-binding protein to bind to the consecutive adenosines. As used herein, “poly-A binding protein,” “poly A binding protein,” or “polyadenylate-binding protein” refers to a protein that binds to a poly-A tail of an mRNA. A poly-A binding protein may function to regulate translational initiation. By binding to poly-A tails, a poly-A binding protein may protect them from uridylation by ZCCHC6/ZCCHC11 and hence contribute to mRNA stability. A poly-A binding protein may be localized in cytoplasmic messenger ribonucleoprotein (mRNP) granules containing untranslated mRNAs that shuttle between the cytoplasm and the nucleus. An exemplary poly-A binding protein is PABPC1 (Uniprot Reference Number: P11940). DNA of the present invention may encode sufficient consecutive adenine nucleotides such that when transcribed into mRNA, one or more poly-A binding proteins retains ability to bind the poly-A tail. An interrupting non-adenine nucleotide anchor is placed after this functional number of consecutive adenine nucleotides.
(29) In some embodiments, the one or more non-adenine nucleotide is positioned to interrupt the consecutive adenine nucleotides so that a poly-A binding protein can bind to a stretch of consecutive adenine nucleotides (i.e. an adenine nucleotide homopolymer or “homopolymer A”. In some embodiments, the poly-A tail comprises at least 8 consecutive adenine nucleotides. In some embodiments, the at least 8 consecutive adenine nucleotides are 8, 9, 10, 11, and/or 12 consecutive nucleotides. In some embodiments, the poly-A tail comprises at least 10, 15, 20, 25, 30, 35, and/or 40 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, and/or 90 consecutive adenine nucleotides. A homopolymer, for example in a poly-A RNA sequence, may comprise at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, or 40 consecutive adenosine nucleotides. A homopolymer, for example in a plasmid sequence encoding the poly-A tail, may comprise at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, or 40 consecutive thymidine nucleotides. In some embodiments, the poly-A tail comprises two or more homopolymer

A sequences of different lengths, e.g. the interrupting sequences in the poly-A tail are irregularly spaced. In some embodiments, the poly-A tail comprises regularly spaced interrupting sequences and two or more homopolymers of the same length.
(30) In some embodiments, the poly-A tail comprises a first homopolymer sequence of at least 8 consecutive adenine nucleotides, a second homopolymer sequence of at least 5 consecutive adenine nucleotides, and an anchor comprising one or more non-adenine nucleotides.
(31) In some embodiments, the poly-A tail comprises one or more sets of 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises one or more sets of 8-100 consecutive adenine nucleotides. For poly-A tails with multiple sets of consecutive adenine nucleotides, i.e. multiple homopolymer sequences, each set of adenine nucleotides does not need to be the same length.
(32) In addition to the number of consecutive adenine nucleotides, a poly-A tail may also be characterized by the number of total adenine nucleotides. The number of total adenine nucleotides is simply the sum of all adenine nucleotides in a poly-A tail. All adenine nucleotides in different groups of consecutive or non-consecutive groupings of adenine nucleotides would therefore be included in the number of total adenine nucleotides in a poly-A tail.
(33) In some embodiments, the poly-A tail comprises 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, 140-150, 150-160, 160-170, 170-180, 180-190, 190-200, 200-210, 210-220, 220-230, 230-240, 240-250, 250-260, 260-270, 270-280, 280-290, 290-300, 300-310, 310-320, 320-330, 330-340, 340-350, 350-360, 360-370, 370-380, 380-390, 390-400, 400-410, 410-420, 420-430, 430-440, 440-450, 450-460, 460-470, 470-480, 480-490, 490-500, 500-510, 510-520, 520-530, 530-540, 540-550, 550-560, 560-570, 570-580, 580-590, or 590-600 total adenine nucleotides. In some embodiments, the poly-A tail comprises one or more homopolymer A sequence of at least 8, 9, 10, 12, 25, 30, 50 nucleotides in length.
(34) In some embodiments, the poly-A tail comprises 40-1000, 40-900, 40-800, 40-700, 40-600, 40-500, 40-400, 40-300, 40-200, or 40-100 total adenine nucleotides.
(35) In some embodiments, the poly-A tail comprises at least 40 total adenine nucleotides. In some embodiments, the poly-A tail comprises at least 50 total adenine nucleotides. In some embodiments, the poly-A tail comprises at least 40, 50, 60, 70 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 220, 240, 260, 280, or 300 adenine nucleotides.
(36) In some embodiments, the poly-A tail comprises or contains 90, 91, 92, 93, 94, 95, 96, or 97 total adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 96 or 97 total adenine nucleotides.
(37) In some embodiments, the adenine nucleotides are adenosine monophosphate. The nucleotides may be modified.
(38) B. Interrupting Sequences Comprising Non-Adenine Nucleotides
(39) Non-adenine nucleotides of the present invention may comprise or consist of natural or non-natural nucleotides such as guanine, cytosine, or thymine. The nucleotides may be modified.
(40) In some embodiments, a poly-A tail comprises one non-adenine nucleotide in a poly-A tail that otherwise consists only of adenine nucleotides. The one non-adenine nucleotide may interrupt a sequence of adenine nucleotides. The one non-adenine nucleotide may be selected from guanine, cytosine, and thymine. In some embodiments, the one non-adenine nucleotide is a guanine nucleotide. In some embodiments, the one non-adenine nucleotide is a cytosine nucleotide. In some embodiments, the one non-adenine nucleotide is a thymine nucleotide. The interrupting sequence may be a mononucleotide, dinucleotide, trinucleotide sequence. The interrupting sequence may comprise 1, 2, 3, 4, 5, or more non-adenine nucleotides and it may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides in length.
(41) In some embodiments, a single non-adenine nucleotide may interrupt sets or groups of consecutive adenine nucleotides. The one non-adenine nucleotide may be positioned to interrupt consecutive adenine nucleotides in such a way that a poly-A binding protein can bind to a stretch of

consecutive adenine nucleotides.
(42) In some embodiments, there are more than one non-adenine nucleotides in a poly-A tail. The more than one non-adenine nucleotide may be positioned to interrupt consecutive adenine nucleotides in such a way that a poly-A binding protein can bind to a stretch of consecutive adenine nucleotides. In some embodiments, non-adenine nucleotides are interspersed between more than one set of consecutive adenine nucleotides, with the number of adenine nucleotides in each series of consecutive adenine nucleotides being sufficient to allow binding of a poly-A binding protein.
(43) The non-adenine nucleotides may be in stretches of more than one non-adenine nucleotide. The non-adenine nucleotides may be in stretches of 2-10 consecutive nucleotides that comprise one or more non-adenine nucleotides. The non-adenine nucleotides may be in interrupting sequences that are interspersed between more than one set of consecutive adenine nucleotides, e.g., more than one homopolymer A sequence. In some embodiments, the number of consecutive non-adenine nucleotides may be one, two, three, four, or five. In some embodiments, there are consecutive stretches of 2-10 non-adenine nucleotides. In some embodiments, there are consecutive stretches of 2-10 nucleotides comprising at least two non-adenine nucleotides.
(44) The consecutive non-adenine nucleotides may be more than one of the same nucleotide or the consecutive non-adenine nucleotides may be different from each other. For example, the non-adenine nucleotides may be more than one guanine, cytosine, or thymine nucleotides. The non-adenine nucleotides may also be guanine and thymine nucleotides; guanine and cytosine nucleotides; thymine and cytosine nucleotides; or guanine, thymine and cytosine nucleotides. The non-adenine nucleotides may comprise two non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine. The non-adenine nucleotide may comprise three non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine. The non-adenine nucleotide may comprise more than three non-adenine nucleotides selected from one or more of guanine, cytosine, and thymine. The poly-A tail may comprise adenine nucleotides between non-adenine nucleotides at regular or irregular intervals. For example, one may view the poly-A tail as having a pattern, where the pattern is regular or irregular. The key to the pattern is the presence of one or more non-adenine nucleotide anywhere in the poly-A tail so long as there are at least 8 consecutive adenines anywhere along the length. In some embodiments, a poly-A may comprise a stretch of at least 8 consecutive adenine nucleotides anywhere along the length, where the adenine nucleotides are “interrupted” anywhere after 8 or more adenines with one or more non-adenine nucleotide. The interrupting sequence may be one non-adenine nucleotide, or 2 to 10 consecutive nucleotides, optionally comprising at least two non-adenine nucleotides. Each one or consecutive stretch of nucleotides comprising at least two non-adenine nucleotides may be followed by one or more adenines, optionally followed by one or more non-adenine nucleotides, optionally followed by one or more than one adenine nucleotides and so on until the end of the poly-A tail. This pattern of adenine nucleotides/non-adenine nucleotides may repeat at regular or irregular intervals. Alternatively, there may be no pattern, such as where there is only one or one consecutive stretch of 2-10 nucleotides, optionally comprising at least two non-adenine nucleotides along the entire length of poly-A.
(45) II. Exemplary Patterns of Adenine and Non-Adenine Nucleotides in Poly-A Tails
(46) Poly-A tails of this invention may comprise or consist of a number of different patterns of interrupting sequences such as consecutive adenine nucleotides and one or more non-adenine nucleotide.
(47) A poly-A tail may begin with one or a series of consecutive adenine nucleotides followed by a non-adenine nucleotide. A poly-A tail that begins with a series of adenine nucleotides means that the 5′ end of the poly-A tail consists of one or a series of consecutive adenine nucleotides with one or more non-adenine nucleotide coming after the consecutive adenine nucleotides. “After,” means that the non-adenine nucleotides are 3′ to a series of consecutive adenine nucleotides.
(48) In some embodiments, the 5′ end of the poly-A tail may consist of a series of consecutive

adenine nucleotides followed by one or more non-adenine nucleotide(s). In some embodiments, one or more non-adenine nucleotide(s) is located after at least 8, 9, 10, 11, or 12 consecutive adenine nucleotides. In some embodiments, the one or more non-adenine nucleotide is located after at least 8-50 consecutive adenine nucleotides. In some embodiments, the one or more non-adenine nucleotide is located after at least 8-100 consecutive adenine nucleotides. In some embodiments, the non-adenine nucleotide is after one, two, three, four, five, six, or seven adenine nucleotides and is followed by at least 8 consecutive adenine nucleotides.

(49) In some embodiments, the 5′ end of the poly A tail consists of one to eight adenine nucleotides followed by one or more non-adenine nucleotide(s). In such embodiments, the non-adenine nucleotide(s) are followed by more adenine nucleotides. The adenine nucleotides that follow the one or more non-adenine nucleotide comprise at least 8 adenines nucleotides before another non-adenine nucleotide.

(50) The range of size of a group of consecutive adenine nucleotides that begins the poly-A tail may vary. In some embodiments, the 5′ end of the poly-A tail consists of 1, 2, 3 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 consecutive adenine nucleotides. Where the first non-adenine nucleotide falls after 1-7 adenine nucleotides, the poly-A tail further comprises a stretch of at least 8 adenine nucleotides after the non-adenine nucleotide.

(51) In some embodiments, the one or more non-adenine nucleotide is located after at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.

(52) The poly-A tail may end with a stretch of non-adenine nucleotides at the 3′ end. The number of non-adenine nucleotides at the 3′ end of the poly-A tail may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 non-adenine nucleotides. Alternatively, the 3′ end of the poly-A tail may consist of one or more adenine nucleotides.

(53) The poly-A tail of the present invention may comprise one sequence of consecutive adenine nucleotides followed by one or more non-adenine nucleotides, optionally followed by additional adenine nucleotides. The poly-A tail of the present invention may also comprise more than one sequence of consecutive adenine nucleotides interrupted by one or more non-adenine nucleotides. The sequence of consecutive adenine nucleotides may be at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides. The number of non-adenine nucleotides in an interrupting sequence may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 non-adenine nucleotides.

(54) A poly-A tail of the invention may also comprise more than one series of consecutive adenine nucleotides that are interrupted or interspersed with non-adenine nucleotides. The length of the interrupting sequence may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides. The length of the interrupting sequence may be 1-3, 1-5, 1-10, 2-10, 2-8, 2-6, or 2-5 nucleotides. The poly-A tails of the invention may comprise more than one set of consecutive adenine nucleotides and an interrupting sequence comprising one non-adenine nucleotide or more than one consecutive stretch of 2-10 non-adenine nucleotides between each set of consecutive adenine nucleotides. The poly-A tails of the invention may comprise more than one set of consecutive adenine nucleotides and one non-adenine nucleotide or more than one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides between each set of consecutive adenine nucleotides. The poly-A tails of the invention may comprise more than one set of consecutive adenine nucleotides and one or more interrupting sequences, each comprising one or more non-adenine nucleotide. The sets may each comprise the same or different number of adenine nucleotides. In embodiments with multiple sets of consecutive adenine nucleotides, each set of consecutive adenine nucleotides may

be sufficient in length to allow binding of a poly-A binding protein.
(55) In some embodiments, one or more non-adenine nucleotide is an interrupting sequence located at regular intervals with the poly-A tail. By regular intervals, it is meant that a set number of consecutive adenine nucleotides is followed by non-adenine nucleotides in a repeated fashion.
(56) In some embodiments, one or more non-adenine nucleotide is located at irregular intervals with the poly-A tail. By irregular intervals, it is meant that a set number of consecutive adenine nucleotides is followed by non-adenine nucleotides followed by another set of consecutive adenine nucleotides that comprise a different number of adenines than the first set.
(57) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides non-adenine nucleotides every 8-100 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 non-adenine nucleotides every 8-100 consecutive adenine nucleotides.
(58) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8-50 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8-100 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8-100 consecutive adenine nucleotides.
(59) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising a non-adenine nucleotide every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(60) In some embodiments, the poly-A tail comprises or contains one non-adenine nucleotide or one consecutive stretch of 2-10 nucleotides comprising at least two non-adenine nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(61) In some embodiments, number of non-adenine nucleotides may be 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides. In some embodiments, the number of consecutive adenine nucleotides may be 8-50 adenine nucleotides. In some embodiment embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 consecutive adenine nucleotides.
(62) The numbers of consecutive adenine nucleotides in a poly-A tail may be 12, 16, 25, 30, or 39. The number of consecutive adenine nucleotides may also be greater than 39. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 12 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 16 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 25 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5 consecutive non-adenine nucleotides every 30 consecutive adenine nucleotides. In some embodiments, the poly-A tail comprises or contains 1, 2, 3, 4, or 5

consecutive non-adenine nucleotides every 39 consecutive adenine nucleotides. The number of consecutive non-adenine nucleotides may also be greater than 5.

(63) Exemplary trinucleotide interrupting sequences include GCG, CCG, GTG, TGG, CGG, GGT, TAT, CAT, CGT, CTC, GAT, CCT, TGT, CGC, CAC, TGC, TCG, TCT, CCC, GAC, TAG, GTT, CTG, and TTT. There are 63 possible trinucleotide interrupting sequences, and 36 trinucleotide interrupting sequences that omit a terminal A. In some embodiments, the poly-A tail comprises one or more trinucleotide interrupting sequences chosen from TGG, CGG, GGT, TAT, CAT, CGT, CTC, GAT, CCT, TGT, CGC, CAC, TGC, TCG, TCT, CCC, GAC, TAG, GTT, CTG, and TTT. In some embodiments, the poly-A tail comprises multiple interrupting sequences designed to minimize hybridization and annealing between 3 or more nucleotides within the sequence encoding the poly-A tail or within the poly-A tail. In certain embodiments, the interrupting sequences that minimize annealing between 3 or more nucleotides are chosen from the 34 trinucleotide interrupting sequences that omit a terminal A. In some embodiments, the interrupting sequences that minimize annealing between 3 or more nucleotides are chosen from TGG, CGG, GGT, TAT, CAT, CGT, CTC, GAT, CCT, TGT, CGC, CAC, TGC, TCG, TCT, CCC, GAC, TAG, GTT, CTG, and TTT. In some embodiments, e.g. SEQ ID NO: 18, the poly-A tail comprises di- and/or tri-nucleotide interrupting sequences chosen from TGG, CGG, GGT, TAT, CAT, CGT, CTC, GAT, CCT, TGT, CGC, CAC, TGC, TCG, TCT, CCC, GAC, TAG, GTT, CTG, TTT, and CG. In certain embodiments, the poly-A tail comprises trinucleotide interrupting sequences chosen from GCG, CCG, and GTG. Exemplary dinucleotide interrupting sequences include CG, GC, CC, GG, TT, CT, TC, GT, and TG. There are 15 possible dinucleotide interrupting sequences, and 9 dinucleotides that do not include a terminal A. Mononucleotide interrupting sequences can be C, G, and T. Note that, with respect to any nucleotide sequence above, when referring to an RNA sequence (such as an mRNA), as opposed to a DNA sequence, T is replaced by U.

(64) One skilled in the art would be able to design a number of different patterns of DNA encoding poly-A tails with consecutive adenine nucleotides and one or more non-adenine nucleotide. Some exemplary poly-A tails comprising at least 8 consecutive adenine nucleotides and one or more adenine-nucleotide are presented, for example, in SEQ ID Nos: 1-5, 10, 11, and 18.

(65) III. Methods of Use

(66) The DNA of this invention may be used for production of mRNA encoded by the DNA. In some embodiments, an mRNA is encoded by the DNA of the invention.

(67) In some embodiments, the DNA of the invention is prepared for production of mRNA. In some embodiments, the DNA is within a vector. In some embodiments, the vector is within a host cell. In some embodiments, an mRNA encoded by the DNA of this invention is used for translating the protein of interest encoded by the DNA.

(68) In some embodiments, the one or more non-adenine nucleotide prevents the loss of one or more adenine nucleotides during DNA replication as compared to the loss that occurs in a DNA comprising a 3′ tail of a similar or same length that contains only adenine nucleotides. DNA replication is a necessary step in growth of plasmid for DNA purification. As such, a plasmid comprising the DNA of this invention encoding a poly-A tail comprising at least 8 consecutive adenine nucleotides and one or more non-adenine nucleotide may show improved stability over one more rounds of growth and purification of the plasmid, as compared to a plasmid encoding a poly-A tail consisting only of adenine nucleotides.

(69) A plasmid comprising the DNA of this invention comprising a sequence encoding a poly-A tail comprising at least 8 consecutive adenine nucleotides and one or more non-adenine nucleotide may have greater stability when grown in a host cell compared to a plasmid comprising a DNA comprising a sequence encoding a poly-A tail consisting only of consecutive adenine nucleotides. During growth of the host cell expressing a plasmid with a DNA sequence, a DNA sequence encoding a poly-A tail that comprises consecutive adenine nucleotides and one or more non-adenine nucleotide may be resistant to a decrease in length of the DNA encoding the poly-A tail

compared to a poly-A tail consisting only of adenine nucleotides. In some embodiments, a plasmid comprising a DNA encoding a poly-A tail comprising one or more non-adenine nucleotide prevents loss of adenines during growth of a host cell as compared to a plasmid comprising a DNA encoding a poly-A tail comprising only adenine nucleotides.

(70) Any means of growing and purifying a vector known to one skilled in the art may be used for growth of a host cell encoding a plasmid. The process of growth and purification of a vector may also be referred to as plasmid preparation. Standard steps of plasmid purification include growth of a bacterial culture, harvesting and lysis of the bacteria, and purification of plasmid DNA. Many kits are available from various manufacturers to purify plasmid DNA. The step of plasmid preparation may be minipreparation (with expected yield of 20 to 40 μg or 50 to 100 μg of plasmid DNA), midipreparation (with expected yield of 100 to 350 μg of plasmid DNA), maxipreparation (with expected yield of 500-850 μg of plasmid DNA), megapreparation (with expected yield of 1.5-2.5 mg of plasmid DNA), or gigapreparation (with expected yield of 7.5-10 mg of plasmid DNA). For therapeutic mRNA production, plasmids may be produced at scales of 100 mg, 1 g, 10 g, or more. The increased stability and replication efficiency of plasmids encoding poly-A tails with non-adenine nucleotides as described herein may improve the consistency and efficiency of plasmids made at such scales.

(71) In some embodiments, a method of producing mRNA from a DNA vector of the present invention is encompassed. In some embodiments, the method of producing mRNA from the DNA vector comprises linearizing the vector downstream of the poly-A tail; denaturing the linearized vector; and contacting the denaturized DNA with an RNA polymerase in the presence of RNA nucleotides such as guanine, cytosine, uracil, adenine, or chemically modified version of such nucleotides such as pseudouridine, N-1-methyl pseudouridine, methoxyuridine, among others. Modified residues, such as base, sugar, and backbone modifications of nucleotide residues can be used in the mRNAs, polynucleotides, and methods described herein.

(72) This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about,” to the extent they are not already so modified. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

(73) It is noted that, as used in this specification and the appended claims, the singular forms “a,” “an,” and “the,” and any singular use of any word, include plural referents unless expressly and unequivocally limited to one referent. As used herein, the term “include” and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items.

Description of Sequences

(74) This table provides a listing of certain sequences referenced herein. Note again that, when referring to the RNA version of a DNA sequence in the table below, T is replaced by U. When referring to a DNA version of an RNA sequence in the table below, U is replaced by T.

(75) TABLE-US-00001 TABLE 1 SEQ ID Description Sequence No sequence of an exemplary AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1 poly-A tail comprising GCGAAAAAAA AAAAAAAAAA AAAAAAAAAA non-adenine nucleotides AAACCGAAAA AAAAAAAAAA AAAAAAAAAA with 30, 30, and 39 AAAAAAAAAA AAAAACCC consecutive adenosines and ending with non- adenine nucleotides 30PA-sequence of an AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 2

exemplary poly-A tail GCGAAAAAAA AAAAAAAAAA AAAAAAAAAA comprising non-adenine AAACCGAAAA AAAAAAAAAA AAAAAAAAAA nucleotides with 30, 30, AAAAAAAAAA AAAA and 39 consecutive adenosines 25PA-sequence of an AAAAAAAAAA AAAAAAAAAA AAAAAGCGAA 3 exemplary poly-A tail AAAAAAAAAA AAAAAAAAAA AAACCGAAAA comprising non-adenine AAAAAAAAAA AAAAAAAAAA AGTGAAAAAA nucleotides with four AAAAAAAAAA AAAAAAAAA sets of 25 consecutive adenosines 16PA-sequence of an AAAAAAAAAA AAAAAAGAAA AAAAAAAAAA 4 exemplary poly-A tail AAACAAAAAA AAAAAAAAAA TAAAAAAAAA comprising non-adenine AAAAAAATAA AAAAAAAAAA AAAACAAAAA nucleotides with six sets AAAAAAAAAA A of 16 consecutive adenosines 16PA long-sequence of AAAAAAAAAA AAAAAAGAAA AAAAAAAAAA 5 an exemplary poly-A tail AAACAAAAAA AAAAAAAAAA TAAAAAAAAA comprising non-adenine AAAAAAATAA AAAAAAAAAA AAAACAAAAA nucleotides with six sets AAAAAAAAAA ACAAAAAAAA AAAAAAAAAA of 16 consecutive AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA adenosines and 63 AAAAAAAAAA AAAAA consecutive adenosines Cas9 mRNA with a poly-A TAATACGACTCACTATAGGGTCCCGCAGTCGGCGTCCAGC 6 tail consisting of 97 GGCTCTGCTTGTTCGTGTGTGTGTCGTTGCAGGCCTTATT adenosines
CGGATCCATGGATAAGAAGTACTCAATCGGGCTGGATATC
GGAACTAATTCCGTGGGTTGGGCAGTGATCACGGATGAAT
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AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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CUGAUGGAGA UGACAGGUUU 8 targeting SEAP UAGAmGmCmU
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UmGmCmU* mUmUmU 12PA-sequence of an
AAAAAAAAAAAATAAAAAAAAAAAAATAAAAAAAAAAAACA 10 exemplary poly-A
tail AAAAAAAAAAATAAAAAAAAAAAACAAAAAAAAAAAAGAA comprising non-
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sets of 12 consecutive adenosines and mononucleotide interrupting sequences 8PA-
sequence of an AAAAAAAATAAAAAAAATAAAAAAAACAAAAAAAAAAAAA 11
exemplary poly-A tail AAAGAAAAAAAATAAAAAAAACAAAAAAAACAAAAAAAAT
comprising non-adenine AAAAAAAAGAAAAAAAACAAAAAAAATAAAAAAAA
nucleotides with twelve sets of 8 consecutive adenosines and mononucleotide
interrupting sequences PolyA-1
TCTTCCTTCAGTCTGTAAACCTCAGCTCGAGAAAAAAAAA 12 Bcllla primer annealing
AAATGGAAAAAAAAAAAACGGAAAAAAAAAAAAAGGTAAAA sites flanking sequence
AAAAAAAATATAAAAAAAAAAAACATAAAAAAAAAAAACG comprising five
TTCATATCGGTTCTAGACCACACTTCTTACTGAGGTCCC interrupting sequences
separating six repeats of 12 consecutive adenosines PolyA-2
TCTTCCTTCAGTCTGTAAACCTCAGAATTCATCTAGCTCG 13 Bcllla primer annealing
AGAAAAAATTCGAAAAAAAAAAAACGTAAAAAAAAAAAAC sites flanking sequence
TCAAAAAAAAAAAAGATAAAAAAAAAAAACCTAAAAAAAA comprising five
AAAATGTAAAAAAAAAAAAGGGAAAGTCTTCCATATCGGT interrupting sequences
TCTAGACCACACTTCTTACTGAGGTCCC separating six sets of 12 consecutive

adenosines PolyA-3 TCTTCCTTCAGTCTGTAAACCTCAGCTCGAGGAAGACAAG 14
Bcllla primer annealing GGAAAAAAAAAAAACGCAAAAAAAAAAAAACACAAAAAAAA
sites flanking sequence AAAATGCAAAAAAAAAAAATCGAAAAAAAAAAAAATCTAAA
comprising five AAAAAAAAACGTTCATATCGGTTCTAGACCACACTTCTTA interrupting
sequences CTGAGGTCCC separating six sets of 12 consecutive adenosines PolyA-4
TCTTCCTTCAGTCTGTAAACCTCAGCTCGAGAAAAAATTC 15 Blclla primer annealing
GAAAAAAAAAAAACCCAAAAAAAAAAAAGACAAAAAAAAA sites flanking sequence
AAATAGAAAAAAAAAAAAGTTAAAAAAAAAAAACTGAAAA comprising six
AAAAAAAATTTAAAAAAAAAAAATCTAGACCACACTTCTT interrupting sequences
ACTGAGGTCCC separating seven sets of 12 consecutive adenosines PolyA 1-2
TCTTCCTTCAGTCTGTAAACCTCAGAATTCATCTAGCTCG 16 Blclla primer annealing
AGAAAAAAAAAAAATGGAAAAAAAAAAAACGGAAAAAAAA sites flanking sequence
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AAAAAAAAACGAAAAAAAAAAAACGTAAAAAAAAAAAACT interrupting sequences
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12 AAATGTAAAAAAAAAAAAGGGAAAGTCTTCCATATCGGTT consecutive adenosines
CTAGACCACACTTCTTACTGAGGTCCC PolyA 3-4
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GGAAAAAAAAAAAACGCAAAAAAAAAAAACACAAAAAAAA sites flanking sequence
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CACACTTCTTACTGAGGTCCC 300PA
AAAAAAAAAAAATGGAAAAAAAAAAAACGGAAAAAAAAAA 18 sequence of an
exemplary AAGGTAAAAAAAAAAAATATAAAAAAAAAAAACATAAAAA poly-A tail
comprising 24 AAAAAAACGAAAAAAAAAAAACGTAAAAAAAAAAAACTCA
interrupting sequences AAAAAAAAAAAGATAAAAAAAAAAAACCTAAAAAAAAAAA
separating 13 repeats of
ATGTAAAAAAAAAAAAGGGAAAAAAAAAAACGCAAAAAA 12 consecutive
adenosines AAAAAACACAAAAAAAAAAAATGCAAAAAAAAAAAATCGA
AAAAAAAAAAATCTAAAAAAAAAAAAACGAAAAAAAAAAAA
CCCAAAAAAAAAAAAAGACAAAAAAAAAAAAATAGAAAAAAA
AAAAAGTTAAAAAAAAAAAACTGAAAAAAAAAAAATTTAA AAAAAAAAAA 100PA-
sequence of an AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 19 exemplary
poly-A tail AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA comprising 97 adenine
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAA nucleotide
homopolymer pUC-M seq2 forward primer GGGTTATTGTCTCATGAGCG 20 pUC-M
seq reverse primer TTTTGTGATGCTCGTCAGGG 21 RN-Ballla for
TCTTCCTTCAGTCTGTAAACCTCAG 22 RN-Bollla rev GGGACCTCAGTAAGAAGTGTGG
23 Liv-Udepleted: Cas9 mRNA
TCCCGCAGTCGGCGTCCAGCGGCTCTGCTTGTTCGTGTGT 24 with a poly-A tail
GTGTCGTTGCAGGCCTTATTCGGATCCGCCACCATGGACA consisting of 98
AGAAGTACAGCATCGGACTGGACATCGGAACAAACAGCGT consecutive adenosines
CGGATGGGCAGTCATCACAGACGAATACAAGGTCCCGAGC
AAGAAGTTCAAGGTCCTGGGAAACACAGACAGACACAGCA
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AAAAAAAAAAAAAAAAAA

(76) Phosphorothioate (PS) linkage or bond refers to a bond where a sulfur is substituted for one nonbridging phosphate oxygen in a phosphodiester linkage, for example in the bonds between nucleotides bases. When phosphorothioates are used to generate oligonucleotides, the modified

oligonucleotides may also be referred to as S-oligos.
(77) A "*" may be used to depict a PS modification. In this application, the terms A*, C*, U*, or G* may be used to denote a nucleotide that is linked to the next (e.g., 3′) nucleotide with a PS bond.
(78) In this application, the terms "mA*," "mC*," "mU*," or "mG*" may be used to denote a nucleotide that has been substituted with 2′-O-Me and that is linked to the next (e.g., 3′) nucleotide with a PS bond.
EXAMPLES
(79) The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.
Example 1—Design and Stability of Stable Plasmids for Poly-A Coding
(80) Poly-A tails were designed that comprised non-adenine nucleotides. The stability of plasmids encoding these poly-A tails with consecutive adenine nucleotides and non-adenine nucleotides (e.g., interrupting sequences) were compared to poly-A tails composed solely of adenine nucleotides.
(81) The issue of loss of the number of adenosines in an mRNA poly-A tail consisting of only adenosines is highlighted in Table 2. A sequence containing a poly-A tail of 96 adenosines was inserted into a pUC57 plasmid (Genscript) and transformed into *E. coli*. Cells were plated on LB-Amp plates, and incubated overnight at either 30° C. or 37° C. Eight colonies were picked and inoculated into 96-well plates with LB-Amp media and grown overnight at 30° C. or 37° C. (Day 1). Samples from the Day 1 cultures were added to fresh LB-Amp media and grown for two additional days at 30° C. or 37° C. (Day 2). DNA was purified from Day 1 and Day 2 cultures and sequenced to determine poly-A tail length in the plasmids. Exemplary results are shown in Table 2 below and in FIG. **1**.
(82) TABLE-US-00002 TABLE 2 Poly-A length after plasmid growth in *E. Coli* 37° C. 30° C. Initial colony Day 1 poly- Day 2 poly- Initial colony Day 1 poly- size A length A length size A length Sm 95 18 Reg 80 Reg 95 68 Sm 95 Reg 95 94 Reg 39 Sm 95 N/A Reg 48 Reg 96 N/A Sm 95 Sm 36-95 mix 18 Sm 95 Sm 62 61 Reg 47 Reg 69 68 Sm 95
(83) For a number of the colonies each round of growth was associated with a decrease in the number of adenosines within the poly-A tail, with only one colony maintaining over 90 adenosines through two rounds of replication. In addition, the size of bacterial colonies correlated with loss of poly-A tail length from the plasmid (i.e., larger colonies corresponded with loss of poly-A length), suggesting that sequences encoding longer poly-A tails may inhibit bacterial growth during plasmid production. DNA purified from colonies of *E. coli* represent a population of DNAs from individual *E. coli* harboring plasmid DNA. Thus, the values provided in Table 2 (and similar values described herein) represent average poly-A length of the population. Further, during PCR and sequencing of long repeats such as poly-A, the polymerase may slip, resulting in the appearance that the sequence is slightly shorter than the actual sequence. Thus, for results showing 95 adenosines, it is not certain whether the plasmid has lost one adenosine, or whether it is a PCR artifact. However, significant loss is not an artifact of polymerase slippage during PCR amplification and sequencing.
(84) In a separate experiment, *E. coli* were transformed with a pUC57 plasmid containing a poly-A tail of SEQ ID NO: 1 and plated on LB-Amp plates. Eight clones were cultured through two rounds of growth and tested for maintenance of the sequence encoding the poly-A tail. Representative data on one clone is shown in FIG. **2**, where no change in size of the tail was seen with the poly-A tail of SEQ ID NO: 1 over 2 rounds of growth of a plasmid encoding it. Miniprep 1 refers to the first round of growth, while Miniprep 2 refers to the second round of growth. Minipreps were performed using an Invitrogen Purelink Quick Plasmid Miniprep kit.
(85) A plasmid encoding a poly-A tail with an additional non-adenosine pattern (SEQ ID NO: 3) was tested for its ability to withstand replication in *E. coli*. A sequence containing a poly-A tail of SEQ ID NO: 3 was inserted into a pUC19 plasmid (Genscript) and transformed into *E. coli*. Cells

were plated on LB-Kan plates, and incubated overnight at either 30° C. or 37° C. Eight colonies were picked and inoculated into 96-well plates with LB-Kan media, and grown overnight at 30° C. or 37° C. (Day 1). Samples from the Day 1 cultures were added to fresh LB-Kan media and grown for two additional days at 30° C. or 37° C. (Day 2). DNA was purified from Day 1 and Day 2 cultures and sequenced to determine poly-A tail length in the plasmids. Of eight Day 1 cultures sequenced, six maintained stretches of 25, 24, 24, and 24 adenosines, and of twelve Day 2 cultures sequenced, nine maintained stretches of 25, 24, 24, and 24 adenosines, demonstrating an improvement of poly-A retention compared to adenosine-only sequences.

(86) These data indicate that DNAs encoding poly-A tails comprising non-adenine nucleotides have improved stability over multiple rounds of plasmid growth and purification in comparison to DNAs encoding poly-A tails containing only adenosines.

Example 2—Activity of Constructs with Poly-A Tails Comprising Non-Adenine Nucleotides

(87) Experiments were performed to determine whether there was a difference in efficacy of mRNA with poly-A tails comprising non-adenine nucleotides (interrupting sequences) versus those with poly-A tails containing only adenosines. A model system was used where mRNA encoding Cas9 protein was transfected by electroporation into HEK-293 cells with a reporter plasmid encoding secreted embryonic alkaline phosphatase (SEAP), as well as a guide RNA targeting SEAP. Successful expression of Cas9 protein from the mRNA results in cleavage of the SEAP target sequence, leading to a color change reflecting decreased production of SEAP. The SEAP HEK-Blue reporter reagents were obtained from Invivogen. A sequence containing a T7 promoter and encoding a Cas9 mRNA with adenosine-only poly-A tail (designed to have 100 adenosine nucleotides, but shown as having 97 adenosine nucleotides by sequencing) (SEQ ID NO: 6) or a sequence containing a T7 promoter and encoding a Cas9 mRNA with a poly-A tail of SEQ ID NO: 1 (SEQ ID NO: 7) were cloned into pUC57 plasmid (Genscript). mRNA was produced by in vitro transcription from the linearized plasmids encoding each mRNA.

(88) FIG. **3** shows titration of Cas9 mRNA with adenosine-only poly-A or the poly-A of SEQ ID NO: 1 in the HEK-Blue cell assay at concentrations from 0.005-50 nM, and 1 μM single guide RNA targeting SEAP (SEQ ID NO: 8).

(89) The HEK-Blue results show that the effect of mRNA with either poly-A tail was similar across the dose-response curve. Higher concentrations of mRNA led to a decrease in SEAP reporter gene expression as evidenced by the color change to pink, as the baseline blue color indicates SEAP expression. Thus, the poly-A tail comprising non-adenine nucleotides did not change the efficacy of expression and function of a Cas9 construct compared to a poly-A tail containing only adenosines.

(90) The efficacy of editing conferred by expression of a Cas 9 mRNA of SEQ ID NO: 6 was also compared to the Cas9 mRNA of SEQ ID NO: 7 (i.e., adenosine-only poly-A tail compared to poly-A tail of SEQ ID NO: 1). For these experiments, HEK-Blue cells were transfected with sgRNA (SEQ ID NO: 8) and the two different mRNAs by electroporation.

(91) FIG. **4** shows percent SEAP inhibition for both constructs after 24-hour incubation. The EC.sub.50 for SEAP editing for mRNA with a poly-A tailing containing only adenosine and a poly-A tail comprising non-adenine nucleotides were similar at 0.050 and 0.054, respectively.

(92) FIG. **5** shows percent SEAP inhibition for both constructs after a 48-hour incubation. The EC.sub.50 for SEAP editing for mRNA with a poly-A tailing containing only adenosine and a poly-A tail comprising non-adenine nucleotides were similar at 0.086 and 0.082, respectively.

(93) mRNA expression and activity were also confirmed in vivo. The Cas9 mRNAs of SEQ ID NO: 6 (HiCas9 mRNA) and SEQ ID NO: 7 (Disrupted PolyA mRNA) were formulated with single guide RNA of SEQ ID NO: 9 (targeting mouse TTR gene) at a 1:1 weight ratio into lipid nanoparticles (LNPs) and administered to CD-1 female mice (n=5) by intravenous dosing at 1 or 0.5 mg/kg of total RNA. Blood was collected from the animals at 7 days post-dose, and serum levels of TTR protein were measured by ELISA. In short, total TTR serum levels were determined using a Mouse Prealbumin (Transthyretin) ELISA Kit (Aviva Systems Biology, Cat. OKIA00111).

Kit reagents and standards were prepared according to the manufacture's protocol. The plate was read on a SpectraMax M5 plate reader at an absorbance of 450 nm. Serum TTR levels were calculated by SoftMax Pro software ver. 6.4.2 using a four parameter logistic curve fit off the standard curve. Final serum values were adjusted for the assay dilution.

(94) FIG. **6** shows comparable levels of serum TTR knockdown (representative of percentage editing of the TTR gene) for both poly-A constructs at 7 days post-dose. Serum TTR knockdown results were confirmed by sequencing of the TTR locus in livers of the mice harvested at 7 days. Mice receiving the adenosine-only poly-A mRNA showed 61.74% and 69.84% editing at 0.5 and 1 mg/kg total RNA, respectively, while mice receiving the poly-A mRNA containing non-adenosine nucleotides showed 63.14% and 70.82% editing at 0.5 and 1 mg/kg total RNA.

(95) Therefore, expression of a Cas9 mRNA with a poly-A tail comprising non-adenine nucleotides produced similar editing efficacy compared to a Cas9 mRNA with a poly-A tail containing only adenosines.

Example 3—Activity of Constructs with Poly-A Tails Comprising Additional Interrupting Sequences

(96) Experiments were performed to determine efficacy of mRNA with poly-A tails comprising non-adenine nucleotides versus those with poly-A tails containing only adenosine nucleotides as in Example 2. Sequences containing a T7 promoter and encoding a Cas9 mRNA with an interrupted poly-A tail comprising SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 10, or SEQ ID NO: 11 were made by PCR amplification using primers to incorporate the poly-A sequences. mRNA was produced by in vitro transcription from these PCR products. mRNA for SEQ ID NO: 18 was produced by in vitro transcription from a linearized plasmid encoding the mRNA.

(97) FIG. **7** shows titration of Cas9 mRNA with adenosine-only poly-A [100PA] or the poly-A of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 10, or SEQ ID NO: 11 in the HEK-Blue cell assay at concentrations from 0.02-6 nM, and 1 μM single guide RNA targeting SEAP (SEQ ID NO: 8). Specifically, FIG. **7** shows percent SEAP inhibition for the constructs after a 48-hour incubation, and EC50 values are provided in Table 3, below. All constructs are active.

(98) TABLE-US-00003 TABLE 3 EC50 values for SEAP inhibition Cas9 mRNA Standard PolyA Construct EC50 Error 98 consecutive Liv (U- depleted Cas9 0.0627 0.0118 adenosines N1Me pseudo U) 97 consecutive 100 PA 0.0956 0.0041 adenosines SEQ ID NO: 4 16 PA 0.0692 0.0087 SEQ ID NO: 5 16 PA long 0.0705 2.237 SEQ ID NO: 3 25 PA 0.0500 0.0213 SEQ ID NO: 2 30 PA 0.0591 0.0086 SEQ ID NO: 10 12 PA 0.0549 0.0296 SEQ ID NO: 11 8 PA 0.04233 0.0295

Example 4—Cloning of Long PolyA with Interrupting Sequences

(99) A 300 nucleotide long polyA tail, SEQ ID NO:18 [300 pa], was designed comprising twelve interrupting sequences from Table 4 (below) and 13 repeats of 12 consecutive adenosines. Anchor Sequences of SEQ ID NOT: 18 were designed to minimize hybridization and self-annealing between trinucleotide interrupting sequences within the ˜300 nt the poly-A tail. Table 4 below provides interrupting sequences that minimize annealing between interrupting sequences, and include the anchors used in this experiment.

(100) To clone SEQ ID NO: 18, each of sequences PolyA-1 (SEQ ID NO: 12), PolyA-2 (SEQ ID NO: 13), PolyA-3 (SEQ ID NO: 14), and PolyA-4 (SEQ ID NO: 15) are created in the pUC57 mini vector (Genscript). The pA1-2 plasmid is created by amplifying SEQ ID NO:12 with Bcl11a primers, digesting the PCR product with restriction enzymes XhoI and AclI and ligating the restriction fragment into the pA2 plasmid comprising SEQ ID NO: 13 digested with XhoI and BstBI. The pA3-4 plasmid is created in the same manner amplifying SEQ ID NO: 14 and ligating it into the same restriction sites on plasmid pA4. The pA1-4 plasmid (comprising SEQ ID NO:18) is assembled by amplifying the SEQ ID NO: 17 sequence from pA3-4, digesting the PCR fragment with BbsI and XbaI restriction enzymes and cloning the restriction fragment into the polyA 1-2

(SEQ ID NO: 16) construct digested with BbsI and XbaI restriction enzymes. The inserts into pA1-2 and pA3-4 are assessed by Sanger sequencing from both directions using [pUC-M seq2 forward primer and pUC-M seq reverse primer] as primers (SEQ ID Nos: 20 and 21).
(101) The resulting SEQ ID NO: 18 (300PA) polyA sequence is excised by digesting pA1-4 with XhoI and XbaI for cloning into the same sites in a protein encoding vector. All steps are carried out under standard conditions.
(102) TABLE-US-00004 TABLE 4 CGG CGT CGC CTG CTT CTC CAG CAT CAC CCC CCG CCT GGG GGT GGC GCG GCT GCC GAG GAT GAC GTG GTT GTC TGG TGT TGC TTG TTT TTC TAG TAT TAC TCG TTC TCC

Claims

1. A DNA comprising nucleotides encoding a poly-adenylated (poly-A) tail located 3′ to nucleotides encoding a protein of interest, wherein the poly-A tail comprises: (a) a plurality of homopolymer sequences of 8, 9, 10, 11, and/or 12 consecutive adenine (A) nucleotides; and (b) an interrupting sequence between each homopolymer sequence, wherein the interrupting sequence comprises: (i) a dinucleotide comprising two consecutive non-adenine nucleotides; or (ii) a trinucleotide that does not include a terminal adenine (A).
2. The DNA of claim 1, wherein the interrupting sequence prevents the loss of one or more adenine nucleotides during DNA replication as compared to the loss that occurs in a DNA comprising a 3′ tail of a similar or same length that contains only adenine nucleotides.
3. The DNA of claim 1, wherein the interrupting sequence is positioned to interrupt the consecutive adenine nucleotides so that a poly (A) binding protein can bind to a stretch of consecutive adenine nucleotides.
4. The DNA of claim 1, wherein the poly-A tail comprises twenty-five homopolymer sequences of 11 or 12 consecutive adenine (A) nucleotides.
5. The DNA of claim 1, wherein the poly-A tail comprises at least 50 total adenine nucleotides.
6. The DNA of claim 1, wherein the poly-A tail comprises 40-1000, 40-900, 40-800, 40-700, 40-600, 40-500, 40-400, 40-300, 40-200, or 40-100 total adenine nucleotides.
7. The DNA of claim 1, wherein the poly-A tail comprises 300-310 total adenine nucleotides.
8. The DNA of claim 1, wherein the interrupting sequence is located after every 11 or 12 consecutive adenine nucleotides.
9. The DNA of claim 1, wherein the non-adenine nucleotide is guanine, cytosine, or thymine.
10. The DNA of claim 1, wherein the adenine nucleotides are adenosine monophosphate.
11. The DNA of claim 1, wherein the interrupting sequence comprises a trinucleotide chosen from TGG, CGG, GGT, TAT, CAT, CGT, CTC, GAT, CCT, TGT, CGC, CAC, TGC, TCG, TCT, CCC, GAC, TAG, GTT, CTG, and TTT.
12. The DNA of claim 1, wherein the interrupting sequence comprises a dinucleotide chosen from CG, GC, CC, GG, TT, CT, TC, GT, and TG.
13. The DNA of claim 1, wherein the dinucleotide interrupting sequence is CG.
14. The DNA of claim 1, wherein the interrupting sequence is chosen from TGG, CGG, GGT, TAT, CAT, CGT, CTC, GAT, CCT, TGT, CGC, CAC, TGC, TCG, TCT, CCC, GAC, TAG, GTT, CTG, TTT, and CG.
15. The DNA of claim 1, wherein the poly-A tail comprises a sequence of SEQ ID NO: **18**.
16. The DNA of claim 1, wherein the protein is a therapeutic protein.
17. The DNA of claim 16, wherein the protein is a cytokine, chemokine, growth factor, RNA-guided nuclease, class 2 CRISPR-associated Cas endonuclease, chimeric Cas protein, Cas9, or modified Cas9.
18. An mRNA encoded by the DNA of claim 1.
19. An mRNA comprising a poly-adenylated (poly-A) tail located 3′ to nucleotides encoding a

protein of interest, wherein the poly-A tail comprises: (a) a plurality of homopolymer sequences of 11 or 12 consecutive adenine (A) nucleotides; and (b) an interrupting sequence between each homopolymer sequence, wherein the interrupting sequence comprises: (i) a dinucleotide comprising two consecutive non-adenine nucleotides; or (ii) a trinucleotide that does not include a terminal adenine (A).

20. A host cell comprising the DNA of claim 1.

21. The DNA of claim 1, wherein the DNA is within a vector.

22. The DNA of claim 21, wherein the interrupting sequence prevents loss of nucleotides encoding the poly-A tail within the vector during growth of the host cell as compared to the loss that occurs in a DNA comprising nucleotides encoding a poly-A tail of a similar or same length that contains only adenine nucleotides.

23. A method of producing mRNA from the DNA vector of claim 21, comprising: a. linearizing the vector downstream of the poly-A tail; b. denaturing the linearized vector; and c. contacting the denaturized DNA with an RNA polymerase in the presence of guanine, cytosine, uracil, and adenine nucleotides.