and (iii) a sequence encoding a payload. Also disclosed herein are methods of making and using the same to, e.g., induce an immune response in a subject.

In one aspect, the present disclosure provides a composition or medical preparation comprising an RNA polynucleotide comprising a sequence encoding a payload, wherein: the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and the 5’ terminus of the RNA polynucleotide comprises m7(3’OMeG)(5')ppp(5')(2'OMeA_1)pG_2pA_3pA_4pN_5, wherein N_5 is uridine or a modified uridine.

Provided herein is a composition or medical preparation comprising an RNA polynucleotide comprising:

a 5’ cap comprising a Cap1 structure; a cap proximal sequence comprising positions +1, +2, +3, +4, and +5 of the RNA polynucleotide; and a sequence encoding a payload, wherein:

(i) the Cap1 structure comprises m7G(5')ppp(5')(2'OMeN_1)pN_2, wherein N_1 is position +1 of the RNA polynucleotide, and N_2 is position +2 of the RNA polynucleotide, and wherein N_1 and N_2 are each independently chosen from: A, C, G, or U; and

(ii) the cap proximal sequence comprises N_1 and N_2 of the Cap1 structure, and:

(a) a sequence selected from the group consisting of: $A_3A_4X_5$ (SEQ ID NO: 1); $C_3A_4X_5$ (SEQ ID NO: 2); $A_3C_4A_5$ (SEQ ID NO: 3) and $A_3U_4G_5$ (SEQ ID NO: 4); or

(b) a sequence comprising: $X_3Y_4X_5$ (SEQ ID NO: 7);

wherein X_3 (nucleotide X at position +3 in SEQ ID NO: 7) or X_5 (nucleotide X at position +5 in SEQ ID NO: 1 or SEQ ID NO: 2) is each independently chosen from A, G, C, or U; and

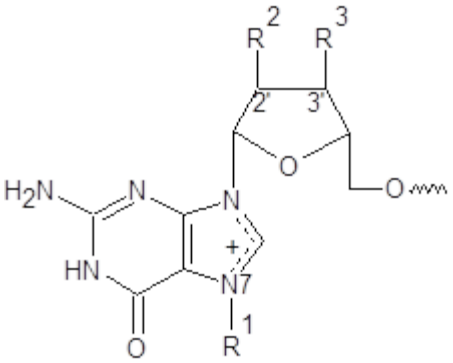
wherein Y_4 (nucleotide Y at position +4 in SEQ ID NO: 7) is not C.

This disclosure also provides a composition or medical preparation comprising an RNA polynucleotide comprising: a 5’ cap; a cap proximal sequence comprising positions +1, +2, +3, +4, and +5 of an RNA polynucleotide; and a sequence encoding a payload, wherein:

(i) the 5’ cap comprises a Cap1 structure comprising G*ppp($m_1^{2'\text{-}O}$)N_1pN_2, wherein:

N1 is position +1 of the RNA polynucleotide, and N_2 is position +2 of the RNA polynucleotide, and wherein N_1 and N_2 are each independently chosen from: A, C, G, or U; and

G* comprises the following structure:



wherein ~~~~ represents the bond by which G* is bound to the first phosphor atom of the ppp group, R^1 is CH_3, R^2 is OH or O-CH_3, and R^3 is O-CH_3; and

(ii) the cap proximal sequence comprises N_1 and N_2 of the Cap1 structure, and:

(a) a sequence selected from the group consisting of: $A_3A_4X_5$ (SEQ ID NO: 1); $C_3A_4X_5$ (SEQ ID NO: 2); $A_3C_4A_5$ (SEQ ID NO: 3) and $A_3U_4G_5$ (SEQ ID NO: 4); or

(b) a sequence comprising: $X_3Y_4X_5$ (SEQ ID NO: 7);

wherein X_3 (nucleotide X at position +3 in SEQ ID NO: 7) or X_5 (nucleotide X at position +5 in SEQ ID NO: 1 or SEQ ID NO: 2) is each independently chosen from A, G, C, or U; and

wherein Y_4 (nucleotide Y at position +4 in SEQ ID NO: 7) is not C.

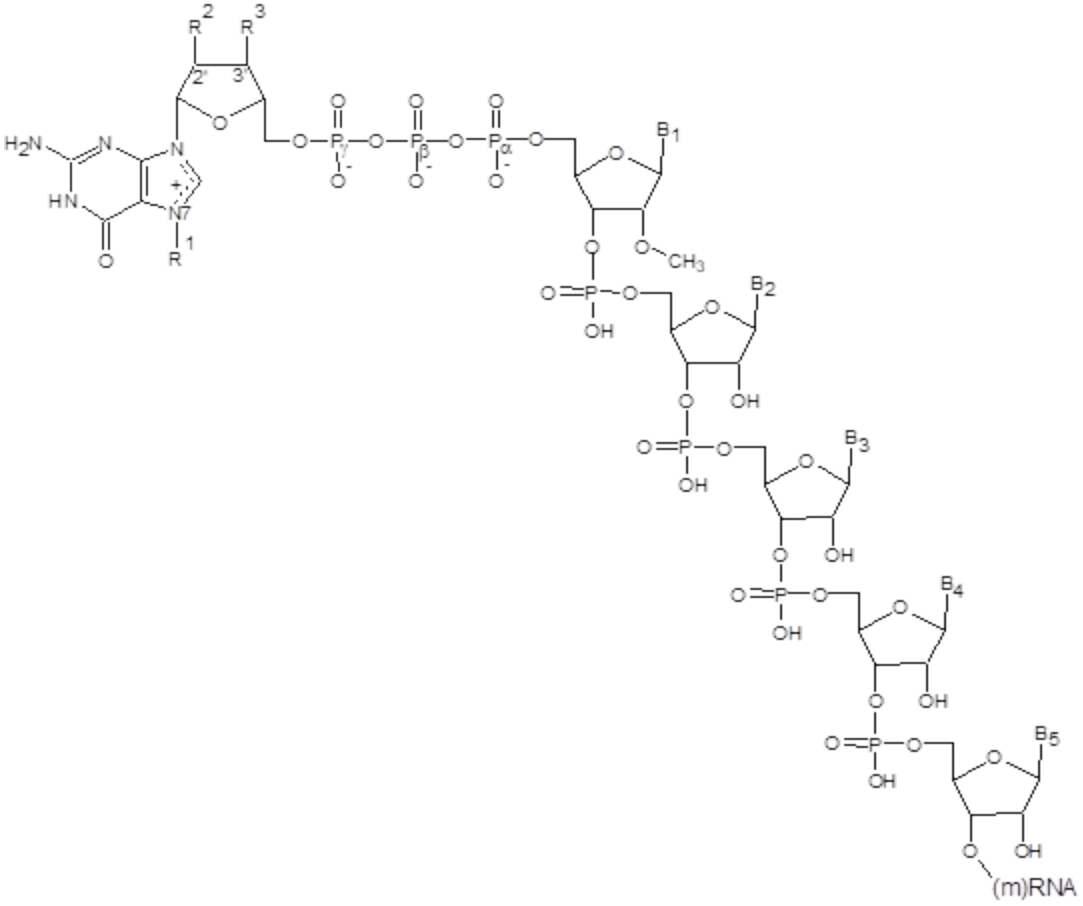
Also provided herein is a composition or medical preparation comprising an RNA polynucleotide comprising:

a 5’ cap comprising a Cap1 structure; a cap proximal sequence comprising positions +1, +2, +3, +4, and +5 of the RNA polynucleotide; and a sequence encoding a payload, wherein:

(i) the Cap1 structure comprises m7(3’OMeG)(5')ppp(5')(2'OMeA_1)pG_2, wherein A_1 is position +1 of the RNA polynucleotide, and G_2 is position +2 of the RNA polynucleotide; and

(ii) the cap proximal sequence comprises A_1 and G_2 of the Cap1 structure, and a sequence comprising: $A_3A_4U_5$ at positions +3, +4 and +5 respectively of the RNA polynucleotide.

This disclosure provides a composition or medical preparation comprising a capped RNA polynucleotide encoding a gene product, which RNA polynucleotide comprises the formula:



wherein R^1 is CH_3, R^2 is OH or O-CH_3, and R^3 is O-CH_3,

wherein B_1 is any nucleobase, preferably A; B_2 is any nucleobase, preferably G; B_3 is any nucleobase, preferably A or C; B_4 is any nucleobase; and B_5 is any nucleobase, and

wherein, when the RNA polynucleotide is administered to a subject, the levels of expression of the encoded gene product at about 6 hours after administration and at about 48 hours after administration do not differ by more than 5-fold.

In another aspect, the present disclosure provides a pharmaceutical composition comprising an RNA polynucleotide as described herein, formulated as a particle, wherein the RNA polynucleotide comprises a sequence encoding a payload, wherein: the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and the 5’ terminus of the RNA polynucleotide comprises m7(3’OMeG)(5')ppp(5')(2'OMeA_1)pG_2pA_3pA_4pN_5, wherein N_5 is uridine or a modified uridine.

Provided herein is a pharmaceutical composition comprising an RNA polynucleotide disclosed herein. In some examples, a pharmaceutical composition comprises a composition or a medical preparation disclosed herein.

In yet another aspect, the present disclosure provides a composition or medical preparation comprising an RNA polynucleotide comprising a sequence encoding a payload, wherein: the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and the 5’ terminus of the RNA polynucleotide comprises m7(3’OMeG)(5')ppp(5')(2'OMeA$_1$)pG$_2$pA$_3$pN$_4$pN$_5$, wherein N$_4$ and N$_5$ are each independently selected from A, G, C, U, or a modified uridine.

In yet another aspect, the present disclosure provides a pharmaceutical composition comprising an RNA polynucleotide formulated as a particle, wherein the RNA polynucleotide comprises a sequence encoding a payload, wherein: the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and the 5’ terminus of the RNA polynucleotide comprises m7(3’OMeG)(5')ppp(5')(2'OMeA$_1$)pG$_2$pA$_3$pN$_4$pN$_5$, wherein N$_4$ and N$_5$ are each independently selected from A, G, C, U, or a modified uridine.

Also provided herein is a method of manufacturing a pharmaceutical composition, e.g., comprising an RNA polynucleotide disclosed herein, by combining an RNA polynucleotide with lipids to form lipid nanoparticles that encapsulate said RNA.

This disclosure provides a nucleic acid template suitable to produce a cap1-capped RNA, in which the first five nucleotides transcribed from the template strand of the nucleic acid template comprise the sequence $N_1pN_2pN_3pN_4pN_5$, wherein N_1 is any nucleotide, preferably T; N_2 is any nucleotide, preferably C; N_3 is any nucleotide, preferably T or G; N_4 is any nucleotide; and N_5 is any nucleotide. In some examples, a DNA template comprises: a 5' UTR, a sequence encoding a payload, a 3' UTR and a polyA sequence.

Provided herein is an vitro transcription reaction comprising:

(i) a template DNA comprising a polynucleotide sequence complementary to an RNA polynucleotide sequence disclosed herein;

(ii) a polymerase; and

(iii) an RNA polynucleotide.

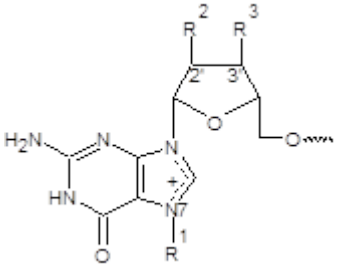
Also provided herein is an RNA polynucleotide isolated from an in vitro transcription reaction provided.

This disclosure provides, a method for producing a capped RNA comprising, transcribing a nucleic acid template in the presence of a cap structure, wherein the cap structure comprises $G^*ppp(m_1^{2'\text{-}O})N_1pN_2$,

wherein N_1 is complementary to position +1 of the nucleic acid template and N_2 is complementary to position +2 of the nucleic acid template, and N_1 and N_2 are independently chosen from A, C, G or U,

wherein position +3 of the nucleic acid template is any nucleotide, preferably T or G; position +4 of the nucleic acid template is any nucleotide; and position +5 of the nucleic acid template is any nucleotide,

wherein G* comprises the following structure:



wherein ^WWW^ represents the bond by which G* is bound to the first phosphor atom of the ppp group, R^1 is CH_3, R^2 is OH or O-CH_3, and R^3 is O-CH_3.

Also provided herein is a composition comprising a DNA polynucleotide comprising a sequence complementary to an RNA polynucleotide sequence provided. In some examples, a DNA polynucleotide disclosed herein can be used to transcribe an RNA polynucleotide disclosed herein.

This disclosure provides, a method comprising: administering to a subject, a pharmaceutical composition comprising an RNA polynucleotide disclosed herein formulated in a lipid nanoparticle (LNP) or a lipoplex (LPX) particle, e.g., as disclosed herein.

Also provided herein is a method of inducing an immune response in a subject, comprising administering to a subject, a pharmaceutical composition comprising an RNA polynucleotide disclosed herein formulated in a lipid nanoparticle (LNP) or a lipoplex (LPX) particle, e.g., as disclosed herein

Provided herein is a method of vaccination of a subject by administering a pharmaceutical composition comprising an RNA polynucleotide disclosed herein formulated in a lipid nanoparticle (LNP) or a lipoplex (LPX) particle, e.g., as disclosed herein.

This disclosure provides, a method of decreasing interaction with IFIT1 of an RNA polynucleotide that comprises a 5’ cap and a cap proximal sequence comprising positions +1, +2, +3, +4, and +5 of the RNA polynucleotide, the method comprising a step of:

providing a variant of an RNA polynucleotide that differs from a parental RNA polynucleotide by substitution of one or more residues within a cap proximal sequence, and

determining that interaction of a variant with IFIT1 is decreased relative to that of a parental RNA polynucleotide.

Also provided herein is a method of producing a polypeptide comprising a step of:

providing an RNA polynucleotide that comprises a 5’ cap, a cap proximal sequence that comprises positions +1, +2, +3, +4, and +5 of the RNA polynucleotide, and a sequence encoding a payload;

wherein an RNA polynucleotide is characterized in that when assessed in an organism administered an RNA polynucleotide or a composition comprising the same, elevated expression and/or increased duration of expression of a payload is observed relative to an appropriate reference comparator.

Provided herein is a method of increasing translatability of an RNA polynucleotide that comprises a 5' cap, a cap proximal sequence that comprises positions +1, +2, +3, +4, and +5 of the RNA polynucleotide and a sequence encoding a payload, the method comprising a step of:

providing a variant of an RNA polynucleotide that differs from a parental RNA polynucleotide by substitution of one or more residues within a cap proximal sequence; and

determining that expression of a variant is increased relative to that of a parental RNA polynucleotide.

Provided herein is a therapeutic RNA comprising a 5' cap that includes a Cap1 structure, a cap proximal sequence and a sequence encoding a payload, the improvement that comprises: including one or more of the following residues in a cap proximal sequence: X at position +1 of an RNA polynucleotide, X at position +2 of an RNA polynucleotide, A at position +3 of an RNA polynucleotide, A at position +4 of an RNA polynucleotide, and X at position +5 of an RNA polynucleotide, demonstrated to increase expression of an RNA when administered to a subject in an LNP formulation. In some examples, X is chosen from A, C, G or U.

This disclosed provides, a therapeutic RNA comprising a 5' cap that includes a Cap1 structure, a cap proximal sequence and a sequence encoding a payload, the improvement that comprises: including one or more of the following residues in a cap proximal sequence: X at position +1 of an RNA polynucleotide, X at position +2 of an RNA polynucleotide, C at

position +3 of an RNA polynucleotide, A at position +4 of an RNA polynucleotide, and X at position +5 of an RNA polynucleotide, demonstrated to increase expression of an RNA when administered to a subject in an LNP formulation. In some examples, X is chosen from A, C, G or U.

Also provided herein is therapeutic RNA comprising a 5’ cap that includes a Cap1 structure, a cap proximal sequence and a sequence encoding a payload, the improvement that comprises:including one or more of the following residues in a cap proximal sequence: A at position +1 of an RNA polynucleotide, G at position +2 of an RNA polynucleotide, A at position

+3 of an RNA polynucleotide, A at position +4 of an RNA polynucleotide, and U at position +5 of an RNA polynucleotide, demonstrated to increase expression of an RNA when administered to a subject in an LNP formulation.

This discosure provides, a method of increasing translation of an RNA polynucleotide comprising a 5’ cap that includes a Cap1 structure, a cap proximal sequence and a sequence encoding a payload, the improvement that comprises: including one or more of the following residues in a cap proximal sequence: X at position +1 of an RNA polynucleotide, X at position +2 of an RNA polynucleotide, A at position +3 of an RNA polynucleotide, A at position +4 of an RNA polynucleotide, and X at position +5 of an RNA polynucleotide. In some examples, X is chosen from A, C, G or U.

Provided herein is a method of increasing translation of an RNA polynucleotide comprising a 5’ cap that includes a Cap1 structure, a cap proximal sequence and a sequence encoding a payload, the improvement that comprises: including one or more of the following residues in a cap proximal sequence: X at position +1 of an RNA polynucleotide, X at position +2 of an RNA polynucleotide, C at position +3 of an RNA polynucleotide, A at position +4 of an RNA polynucleotide, and X at position +5 of an RNA polynucleotide. In some examples, X is chosen from A, C, G or U.

Also provided herein is a method of increasing translation of an RNA polynucleotide comprising a 5’ cap that includes a Cap1 structure, a cap proximal sequence and a sequence encoding a payload, the improvement that comprises: including one or more of the following residues in a cap proximal sequence: A at position +1 of an RNA polynucleotide, G at position +2 of an RNA polynucleotide, A at position +3 of an RNA polynucleotide, A at position +4 of an RNA polynucleotide, and U at position +5 of an RNA polynucleotide.

Also provided herein is a method of providing a framework for an RNA polynucleotide that comprises a 5’ cap, a cap proximal sequence, and a payload sequence, the method comprising a step of:

assessing at least two variants of an RNA polynucleotide, wherein:

each variant includes a same 5’ cap and payload sequence; and

CLAIMS

What is claimed is:

1. A composition or medical preparation comprising an RNA polynucleotide comprising a sequence encoding a payload, wherein:

the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and

the 5’ terminus of the RNA polynucleotide comprises m7(3’OMeG)(5')ppp(5')(2'OMeA$_1$)pG$_2$pA$_3$pA$_4$pN$_5$, wherein N$_5$ is uridine or a modified uridine.

2. The composition or medical preparation of claim 1, wherein the RNA polynucleotide further comprises a 3’ UTR sequence optionally comprising a modified uridine in place of one or more uridines.

3. The composition or medical preparation of claim 2, wherein the 3’ UTR sequence comprises the nucleotide sequence of SEQ ID NO: 13 optionally comprising a modified uridine in place of one or more uridines.

4. The composition or medical preparation of any one of the preceding claims, wherein the RNA polynucleotide further comprises a polyA sequence.

5. The composition or medical preparation of claim 4, wherein the polyA sequence comprises at least 100 nucleotides.

6. The composition or medical preparation of claim 5, wherein:

(i) the polyA sequence is an interrupted sequence of adenine nucleotides; and/or

(ii) the polyA sequence comprises 30 adenine nucleotides followed by 70 adenine nucleotides, wherein the 30 adenine nucleotides and 70 adenine nucleotides are separated by a linker sequence.

7. The composition or medical preparation of claim 6, wherein the polyA sequence comprises the nucleotide sequence of SEQ ID NO: 14 optionally comprising a modified uridine in place of one or more uridines.

8. The composition or medical preparation of any one of the preceding claims, wherein the RNA polynucleotide comprises, in a 5’ to 3’ orientation, the m7(3’OMeG)(5')ppp(5')(2'OMeA$_1$)pG$_2$pA$_3$pA$_4$pN$_5$, other 5’ UTR sequences, the sequence encoding a payload, 3’ UTR sequences, and a polyA sequence.

9. The composition or medical preparation of any one of the preceding claims, wherein the RNA polynucleotide comprises a modified uridine in place of every uridine.

10. The composition or medical preparation of claim 9, wherein the modified uridine is N1-methyl-pseudouridine (m1ψ).

11. The composition or medical preparation of any one of the preceding claims, wherein the sequence encoding a payload comprises a sequence encoding a protein replacement polypeptide; a sequence encoding an antibody agent; a sequence encoding a cytokine; a sequence encoding an antigenic polypeptide; a sequence encoding a gene editing component; a sequence encoding a regenerative medicine component or combinations thereof.

12. The composition or medical preparation of claim 11, wherein the sequence encoding a payload comprises a sequence encoding an antigenic polypeptide.

13. The composition or medical preparation of claim 12, wherein the antigenic polypeptide comprises an epitope from an antigen.

14. The composition or medical preparation of claim 12, wherein the antigenic polypeptide comprises a plurality of distinct epitopes from one or more antigens.

15. The composition or medical preparation of any one of claims 12-14, wherein the antigenic polypeptide comprises: an antigenic polypeptide from an allergen, a viral antigenic polypeptide, a bacterial antigenic polypeptide, a fungal antigenic polypeptide, a parasitic antigenic

polypeptide, an antigenic polypeptide from an infectious agent, an antigenic polypeptide from a pathogen, a tumor antigenic polypeptide, or a self-antigenic polypeptide.

16. The composition or medical preparation of any one of the preceding claims, wherein the RNA polynucleotide is mRNA.

17. The composition or medical preparation of any one of the preceding claims, wherein the RNA polynucleotide comprises a human alpha globin (hAg) 5′ UTR or a fragment thereof, and wherein the hAg 5′ UTR or a fragment thereof optionally comprise a modified uridine in place of one or more uridines.

18. The composition or medical preparation of claim 17, wherein the RNA polynucleotide further comprises a Kozak sequence optionally comprising a modified uridine in place of one or more uridines.

19. The composition or medical preparation of claim 18, wherein the RNA polynucleotide comprises the nucleotide sequence of SEQ ID NO: 27 optionally comprising a modified uridine in place of one or more uridines.

20. A pharmaceutical composition comprising an RNA polynucleotide formulated as a particle, wherein the RNA polynucleotide comprises a sequence encoding a payload, wherein:
the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and
the 5’ terminus of the RNA polynucleotide comprises
m7(3’OMeG)(5')ppp(5')(2'OMeA_1)pG_2pA_3pA_4pN_5, wherein N_5 is uridine or a modified uridine.

21. The pharmaceutical composition of claim 20, wherein the particle is a lipid nanoparticle (LNP) or a lipoplex (LPX) particle.

22. The pharmaceutical composition of claim 21, wherein the particle is an LNP comprising a cationically ionizable lipid; a sterol; a phospholipid; and a pegylated lipid.

23. The pharmaceutical composition of claim 22, wherein the concentration of the cationically ionizable lipid in the LNP is within a range of 40 to 55 mole percent, the concentration of the sterol in the LNP is within a range of 30 to 50 mole percent, the concentration of the phospholipid in the LNP is within a range of 5 to 15 mole percent, and the concentration of the pegylated lipid in the LNP is within a range of 1 to 10 mole percent.

24. The pharmaceutical composition of any one of claims 20-23, which is formulated as a liquid.

25. The pharmaceutical composition of any one of claims 20-23, which is formulated as a solid.

26. The pharmaceutical composition of any one of claims 20-25, wherein the RNA polynucleotide comprises m7(3'OMeG)(5')ppp(5')(2'OMeA$_1$)pG$_2$pA$_3$pN$_4$pN$_5$, the 5' UTR, the sequence encoding a payload, the 3' UTR, and a polyA sequence.

27. The pharmaceutical composition of any one of claims 20-26, wherein the RNA polynucleotide comprises a modified uridine in place of at least one uridine.

28. The pharmaceutical composition of claim 27, wherein, the modified uridine is N1-methyl-pseudouridine (m1Ψ).

29. The pharmaceutical composition of any one of claims 20-26, wherein the RNA polynucleotide comprises a modified uridine in place of every uridine.

30. The pharmaceutical composition of claim 29, wherein the modified uridine is N1-methyl-pseudouridine (m1ψ).

31. A composition or medical preparation comprising an RNA polynucleotide comprising a sequence encoding a payload, wherein:
the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and

the 5′ terminus of the RNA polynucleotide comprises m7(3′OMeG)(5′)ppp(5′)(2′OMeA$_1$)pG$_2$pA$_3$pN$_4$pN$_5$, wherein N$_4$ and N$_5$ are each independently selected from A, G, C, U, or a modified uridine.

32. The composition or medical preparation of claim 31, wherein the RNA polynucleotide comprises the nucleotide sequence of SEQ ID NO: 27 optionally comprising a modified uridine in place of one or more uridines.

33. The composition or medical preparation of claim 31 or claim 32, wherein the RNA polynucleotide further comprises a 3′ UTR sequence, wherein the 3′ UTR sequence comprises the nucleotide sequence of SEQ ID NO: 13 optionally comprising a modified uridine in place of one or more uridines.

34. The composition or medical preparation of any one of claims 31-33, wherein the RNA polynucleotide further comprises a polyA sequence.

35. The composition or medical preparation of claim 34, wherein the polyA sequence comprises the nucleotide sequence of SEQ ID NO: 14 optionally comprising a modified uridine in place of one or more uridines.

36. The composition or medical preparation of any one of claims 31-35, wherein the RNA polynucleotide comprises, in a 5′ to 3′ orientation, the m7(3′OMeG)(5′)ppp(5′)(2′OMeA1)pG2pA3pN4pN5, other 5′ UTR sequences, the sequence encoding a payload, 3′ UTR sequences, and a polyA sequence.

37. The composition or medical preparation of any one of claims 31-36, wherein the RNA polynucleotide comprises a modified uridine in place of every uridine.

38. The composition or medical preparation of claim 37, wherein the modified uridine is N1-methyl-pseudouridine (m1Ψ).

39. The composition or medical preparation of any one of claims 31-38, wherein the sequence encoding a payload comprises a sequence encoding a protein replacement polypeptide; a sequence encoding an antibody agent; a sequence encoding a cytokine; a sequence encoding an antigenic polypeptide; a sequence encoding a gene editing component; a sequence encoding a regenerative medicine component, or combinations thereof.

40. The composition or medical preparation of claim 39, wherein the sequence encoding a payload comprises a sequence encoding an antigenic polypeptide.

41. The composition or medical preparation of claim 40, wherein the antigenic polypeptide comprises a plurality of distinct epitopes from one or more antigens.

42. The composition or medical preparation of claim 39, wherein the antigenic polypeptide comprises: an antigenic polypeptide from an allergen, a viral antigenic polypeptide, a bacterial antigenic polypeptide, a fungal antigenic polypeptide, a parasitic antigenic polypeptide, an antigenic polypeptide from an infectious agent, an antigenic polypeptide from a pathogen, a tumor antigenic polypeptide, or a self-antigenic polypeptide.

43. A pharmaceutical composition comprising an RNA polynucleotide formulated as a particle, wherein the RNA polynucleotide comprises a sequence encoding a payload, wherein:
the RNA polynucleotide optionally comprises a modified uridine in place of one or more uridines; and
the 5′ terminus of the RNA polynucleotide comprises
m7(3′OMeG)(5′)ppp(5′)(2′OMeA$_1$)pG$_2$pA$_3$pN$_4$pN$_5$, wherein N$_4$ and N$_5$ are each independently selected from A, G, C, U, or a modified uridine.

44. The pharmaceutical composition of claim 43, wherein the particle is a lipid nanoparticle (LNP) comprising a cationically ionizable lipid; a sterol; a phospholipid; and a pegylated lipid.

45. The pharmaceutical composition of claim 43 or claim 44, wherein the RNA polynucleotide comprises, in a 5’ to 3’ orientation, m7(3’OMeG)(5')ppp(5')(2'OMeA_1)pG_2pA_3pN_4pN_5, other 5’ UTR sequences, the sequence encoding a payload, a 3’ UTR, and a polyA sequence.

46. The pharmaceutical composition of any one of claims 43-45, wherein the RNA polynucleotide comprises a modified uridine in place of at least one uridine.

47. The pharmaceutical composition of claim 46, wherein the modified uridine is N1-methyl-pseudouridine (m1ψ).

48. The pharmaceutical composition of any one of claims 43-45, wherein the RNA polynucleotide comprises a modified uridine in place of every uridine.

49. The pharmaceutical composition of claim 48, wherein the modified uridine is N1-methyl-pseudouridine (m1ψ).